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Halides inhibition of multicopper oxidases studied by FTIR spectroelectrochemistry using azide as an active infrared probe

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Abstract An infrared spectroelectrochemical study of *Trametes hirsuta* laccase and *Magnaporthe oryzae* bilirubin oxidase has been performed using azide, an inhibitor of multicopper oxidases, as an active infrared probe incorporated into the T2/T3 copper cluster of the enzymes. The redox potential-controlled measurements indicate that N_3^- stretching IR bands of azide ion bound to the T2/T3 cluster are only detected for the oxidized enzymes, confirming that azide only binds to Cu^{2+} . Moreover, the process of binding/dissociation of azide ion is shown to be reversible. The interaction of halide anions, which also inhibit multicopper oxidases, with the active site of the enzymes was studied by measuring the changes in the azide FTIR bands. Enzymes inhibited by azide respond differently upon addition of fluoride or chloride ions to the sample solution inhibited by azide. Fluoride ions compete with azide for binding at one of the T2/T3 Cu ions, whereas competition from chloride ions is much less evident.

Keywords Multicopper oxidase · Infrared · Halide inhibition · Spectroelectrochemistry

Introduction

Multicopper oxidases (MCOs), such as laccase (Lc) or bilirubin oxidase (BOx), are proteins typically incorporating four redox-active Cu ions, which are classified by optical and magnetic properties as either a type 1 (T1) copper ion, or a trinuclear copper cluster (TNC) formed by one type 2 (T2) copper and two type 3 (T3) copper ions. While accepting electrons one-by-one from the substrate, the T1 site gives rise to a distinct EPR signal, and electrons are subsequently transferred to the TNC, where dioxygen reduction finally takes place. An intramolecular electron pathway formed by one cysteine and two histidine residues connects the T1 site and TNC. There are also channels for O_2 and H_2O transport between the protein surface and the TNC. The T2 copper ion is EPR active, as opposite to the two T3 Cu ions [1, 2]. MCOs are inhibited in the presence of inorganic anions, such as cyanide, azide, halides, and hydroxyls, among others [3, 4]. Many different studies have been conducted to understand the different mechanisms behind the inhibition processes. In particular, thanks to the magnetic properties of the copper cations at the active site, numerous electron paramagnetic resonance (EPR) studies have been carried out. These studies have shown how N_3^- binds to the TNC, blocking the catalytic process by the formation of a bridging bond between the T2 Cu ion and one of the T3 ions. It has also been suggested that a second N_3^- may also bind the TNC, leading to a di-azide bound form [5, 6]. These EPR results have been corroborated by other spectroscopy techniques, such as infrared spectroscopy (FTIR) among others, where asymmetric intra-azide stretching bands were

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observed at 2035 or 2056 cm^{-1} after incubation of Lc with the inhibitor [7, 8].

The inhibition mechanism by halide anions has been intensely studied [9–12], as it is relevant for applications of MCO-based systems such as biosensors or biofuel cells operating in physiological media [13], or for bioremediation [14, 15]. Early EPR investigations showed that fluoride interacts mainly with the TNC, most likely by bridging the type 2 and one of the type 3 Cu^{2+} [16]. Moreover, N_3^-/F^- competition EPR experimental reports have shown that F^- probably does not bind to the T2 Cu^{2+} in the presence of a reduced T3 Cu^{1+} , suggesting that F^- interacts only with an oxidized T3 Cu^{2+} [1]. It is assumed that binding of the F^- to the TNC interrupts intramolecular electron transfer (IET) from the T1 site, thus abolishing completely the oxygen reduction ability of Lcs [9–12].

More controversial is the inhibition mechanism by chloride. Xu proposed that halides larger than fluoride were unable to reach the TNC site due to the size of Lcs' putative access channel for the substrate, thus explaining the decreasing inhibition power in the order $\text{F}^- > \text{Cl}^- > \text{I}^-$ [11]. Naki et al. reported a kinetic study of *Polyporus versicolor* Lc showing that Cl^- was a competitive inhibitor with respect to the electron donor substrate (ferrocyanide), thus suggesting that the Cl^- was binding to or near the T1 site [10]. Furthermore, bioelectrocatalytic studies of Lcs have shown that direct electron transfer (DET) from the electrode to the enzyme is much less affected by Cl^- than the mediated electron transfer (MET) using different substrates, whereas F^- blocks both electron transfer modes [17–20]. This result was explained by considering that Cl^- binds near the substrate binding site, precluding T1 reduction by the substrate but not electron transfer from the electrode. Instead, F^- binds at a site of the enzyme where IET in the Lc is impeded, thus affecting both DET and MET electrocatalysis [17]. On the other hand, in the crystal structure of *Melanocarpus*

albomyces laccase a putative chloride is coordinated to the T2 copper [21]. More recently, from a steady-state kinetic analysis of *Trametes versicolor* Lc with different substrates, a two-site model of Lc inhibition by Cl^- has been proposed instead of a single Cl^- binding to each enzyme molecule [12].

Additional spectroscopic studies are required for clarifying the inhibition modes of MCOs by halides. FTIR is a powerful analytical tool for studying structure–function relationships in metalloenzymes [22, 23]. However, a direct FTIR study of inhibition by halides is not possible as the stretching modes of Cu-halides are not detectable. Alternatively, the aim of this work is to verify the hypothesis of halides coordination to the MCO active site by spectroelectrochemistry using N_3^- as active FTIR probe. As mentioned before, FTIR studies of the inhibition of MCOs by N_3^- have been done previously, although not under in situ redox potential control nor in the presence of halide inhibitors. Two different MCOs were employed in the present study: *Magnaporthe oryzae* BOx and *T. hirsuta* Lc, to provide a broader view of the different inhibition events when azide, F^- or Cl^- ions interact with these types of metalloenzymes. Scheme 1 shows the proposed binding sites of these inhibitors to MCOs based on the literature cited above and on the results obtained in the present work.

Materials and methods

Chemicals

All chemicals used were analytical grade and were used as purchased. 2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), sodium fluoride, 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO), quinhydrone, 2,6-dimethoxyphenol (DMP) and sodium azide were

Scheme 1 Scheme of proposed binding sites of azide and halide inhibitors to MCOs active site

purchased from Sigma. Sodium chloride, potassium chloride, di-sodium hydrogen phosphate and sodium di-hydrogen phosphate were purchased from Panreac. All aqueous solutions were prepared in deionized water (Milli-Q grade, Millipore).

Enzymes

Trametes hirsuta Lc from basidiomycete (strain *T. hirsuta* 56) was obtained from the laboratory collection of the Moscow State University of Engineering Ecology following the purification procedure previously reported [24].

Magnaporthe oryzae BOx from the Centre de Recherche Paul Pascal (Université de Bordeaux) was produced and purified as reported in the literature [25].

FTIR spectroelectrochemistry

Fourier transform infrared (FTIR) spectroscopy was performed with a Bruker Tensor 27 spectrometer equipped with a global IR source, a MCT detector cooled by liquid N₂ and a Whatman purge gas generator for removal of CO₂ and H₂O. The FTIR spectroelectrochemical cell was designed by Moss et al. [26]. For every measurement, 1024 scans were recorded with a resolution of 2 cm⁻¹. Background subtraction and baseline correction of the recorded spectra were done using OPUS software from Bruker. For FTIR measurements, an aliquot of purified Lc (5.8 mg mL⁻¹) was concentrated to 29.0 mg mL⁻¹ by ultrafiltration using a Centricon-30 (Amicon), while the BOx aliquot was used as received, i.e., 68.7 mg mL⁻¹.

Previous to the FTIR measurements, the enzymes were incubated overnight with a solution of NaN₃ (1:2 enzyme:inhibitor ratio) in phosphate buffer of 20 mM, pH 6.0 at 4 °C. When required, NaF or NaCl at a final concentration of 30 mM were added to the sample solution for 15 min at 4 °C before the FTIR measurements. 10 µL of the enzyme solution and 1 µL of a mixture of redox mediators, each 0.5 mM, were placed between the CaF₂ windows of the spectroelectrochemical cell, forming a thin layer film of 10 µm [26]. The redox mediators employed were ABTS, quinhydrone, DMP, and TEMPO with redox potentials equal to 690, 345, 640, and 735 mV vs. NHE at pH 6, respectively [27–29]. 0.2 M KCl was injected as electrolyte in the space that surrounded the CaF₂ windows of the spectroelectrochemical cell until it was filled, yielding electrical contact between the working, counter and reference electrodes. The strong capillary forces within the thin layer enzyme sample sandwiched between the CaF₂ windows and the small contact surface with the surrounding electrolyte prevent bulk mixing; therefore, the mass transfer between the sample and the external electrolyte during the experiments is negligible [26]. Different FTIR spectra were recorded in the range

of 0–650 mV vs. Ag/AgCl (3 M Cl⁻); the redox potential of the cell was controlled by a BAS CV-27 potentiostat and measured with a Fluke 77 serie II multimeter. Before each spectrum acquisition, the sample was biased with the selected potential for at least 15 min to ensure that redox equilibrium was achieved. For better comparison with the literature values, all redox potentials mentioned in the text and in the figures are set vs. NHE. The temperature of the cell was maintained at 25 °C with a Julabo CC 230 thermostat. A spectrum of phosphate buffer sample was recorded as the background.

Peak fitting and deconvolution was carried out using the OriginPro 8.0 software with a Gaussian fit in the 2100–2000 cm⁻¹ region. The peak wavenumber of the deconvoluted bands were not fixed nor their width, although negative bands were precluded. The fittings converged when a χ^2 tolerance value of 10⁻⁶ was reached.

Results and discussion

FTIR measurements of azido-inhibited MCOs required a careful addition of N₃⁻ since an excess of free ions in solution influences the spectrum by masking the band of bound azide [7, 8]. Removal of the excess azide in solution by ultrafiltration before the FTIR measurement caused the loss of all azide bands (free or bound to MCO) and 84% recovery of the enzymatic activity (Fig. S1), confirming that azide-binding to MCOs is a reversible process. Best FTIR results were obtained with samples that had a 1:2 enzyme:azide ratio. The FTIR spectra for the MCOs after overnight azide incubation samples were investigated under redox potential control. The spectra recorded from higher to lower potential for a BOx-N₃ sample in the range between 2100 and 2000 cm⁻¹ indicated the presence of at least two peaks centered at 2056 and 2049 cm⁻¹ (Fig. 1). Previously published FTIR reports assign these two bands to N₃⁻ bound to the TNC in a bridging mode between two Cu ions and to free azide, respectively [7, 8]. When the enzyme was in its resting state, corresponding to the open circuit potential (OCP) measurement at + 510 mV, these two bands overlap. Upon reduction, the potential to + 310 mV the band at 2056 cm⁻¹ decreases intensity and the one at 2049 cm⁻¹ predominates. After reoxidizing the sample at potentials above + 600 mV, the 2049 cm⁻¹ peak decreases in intensity, while the peak at 2056 cm⁻¹ reappears. At + 860 mV, the band corresponding to free azide has almost disappeared and the main band is the 2056 cm⁻¹ one. At this potential, all Cu atoms of the enzyme should be oxidized [1, 2]. The redox potential of the T1 Cu of BOxs is within the + 650 to 720 mV range [30, 31], whereas that of the T2 Cu is around 400 mV [30]. Based on the redox titrations of T3 Cu sites of different MCOs [32], it is reasonable to assume that the redox potential of

Fig. 1 FTIR spectra of BOx- N_3 sample recorded at different redox potentials vs. NHE. The intensity of each spectrum has been normalized

the T3 Cu site of BOx is close to 700 mV. Therefore, the FTIR spectroelectrochemical results clearly indicate that azide only binds to the oxidized Cu^{2+} of the TNC, in agreement with previous results reported in the literature regarding Cu complexes with azide ligands [33, 34]. The process of binding/dissociation of N_3^- was reversible, although the kinetics became more sluggish with time because when the sample was reduced again at +410 mV the amount of bound azide detected was higher than in the initial resting state at +510 mV.

It was observed, in this experiment and all others done, that the overall intensity of the N_3^- infrared bands decreased with time and independently of the applied potential. This effect could be due to the lack of stability of the enzyme or the N_3^- under the experimental conditions. Therefore, all spectra shown have been normalized so that the intensity

Fig. 2 FTIR spectra of NaN_3 sample recorded at different redox potentials vs. NHE. The intensity of each spectrum has been normalized

of the most intense peak of each spectrum is equal. As a control, FTIR spectra at different potential were recorded for a sample of 2.6 mM NaN_3 in 100 mM of phosphate buffer pH 6.0, without enzyme. The presence of a single peak at 2049 cm^{-1} , corresponding to free N_3^- , at every potential is evident (Fig. 2). The only effect noticed was the peak intensity reduction with time, confirming the degradation of N_3^- under the measurement conditions. A small peak at ca. 2030 cm^{-1} was detected, which was also observed in some of the spectra recorded with BOx (Fig. 1).

FTIR measurements were performed also for a Lc- N_3^- sample, under the same conditions. In that case, free N_3^- and azide-bound signals are still appreciable, although not as distinct as in the case of BOx due to bands broadening and overlapping (Supporting Information Fig. S2A). This effect suggests a higher conformational freedom of the bound azide in the case of laccases. By deconvolution of the overlapped bands using a fitting model based on a Gaussian function it was possible to determine that the 2056 cm^{-1} band is only detected clearly as a shoulder at +860 mV. (Supporting Information Fig. S6). The band at 2049 cm^{-1}

was predominant over the entire potential range, suggesting that the affinity of N_3^- for the TNC of Lc is lower than for the BOx one. On the one hand, although Lc has a higher T1 Cu (+780 mV) redox potential than BOx, the redox potential of the T2 Cu is around +400 mV for both enzyme [30, 35]. Thus, one cannot attribute the lower azide affinity to a difference in the redox potentials of the T2 Cu site of these oxidoreductases. On the other hand, because of ca., 100 mV differences between redox potentials of the T1 (and possibly T3 as well) Cu sites, the redox potential of the TNC might be higher in the case of Lc compared to BOx. Thus, the apparent lower affinity of azide for Lc could also be attributed to a high redox potential of the enzyme, resulting in an incomplete protein oxidation at 860 mV.

There are three possible N_3^- binding geometries to Cu–Cu structures: (i) terminal coordination [34], (ii) μ -1,3-bridge between the two Cu ions [33] or (iii) μ -1-1 bridged coordination. (i) and (ii) gave $\nu_{(\text{N}_3)}^{\text{asym}}$ frequencies $< 2060 \text{ cm}^{-1}$, whereas $\nu_{(\text{N}_3)}^{\text{asym}} > 2060 \text{ cm}^{-1}$ were measured for (iii) [2]. Our results are in agreement with previous FTIR studies [7, 8], indicating that the possible binding geometries are the first two. Moreover, from EPR and UV–Vis spectral studies, it has been proposed that N_3^- binds in a bridging mode between T2 Cu ion and one of the T3 Cu ions [6, 36].

The effect of NaF or NaCl addition to enzyme- N_3^- samples before introduction to the spectroelectrochemical cell was studied to (1) evaluate if halide ions could displace the

Fig. 3 FTIR spectra for **a** BOx- N_3 -F and **b** BOx- N_3 -Cl sample recorded at different redox potentials vs. NHE. The intensity of each spectrum has been normalized. The chronological order of spectra measurements in **(b)** was: 460, 610, 310, 560, 710, 410, 660, 860 and 360 mV

Fig. 4 FTIR spectra recorded at + 860 mV vs. NHE for **a** BOx and **b** Lc samples when incubated with only azide (solid line) or with azide–fluoride ions (dashed line) or with azide–chloride ions (dotted line)

binding of N_3^- to the TNC and if so, (2) infer the binding sites of halide inhibitors to MCOs. Figure 3a shows the spectra recorded at different potentials for the BOx- N_3 sample

after the addition of F^- . The predominant peak is centered at 2049 cm^{-1} , which is attributed to free azide. The spectra were practically insensitive to the applied potential and the peak at 2056 cm^{-1} barely appears as a shoulder. Therefore, in the case of F^- , we can conclude that the ions bind to the TNC or very close to it, mostly displacing the bound N_3^- and thus only the free azide band is clearly observed in the recorded spectra. There are two possible explanations for this result, viz. either the F^- binds to one of the TNC Cu ions to which N_3^- binds or there is electrostatic repulsion of the negatively charged N_3^- due to the F^- bound near the cluster. The weak intensity of the 2056 cm^{-1} band present in all the spectra suggests that a minor proportion of the BOx molecules still retain the azide bound to the TNC. However, the fact that this band is insensitive to the applied potential suggests that F^- is also bound to the TNC, as this inhibitor blocks the intramolecular electron transport pathway of MCOs [9–12].

Interestingly, measurement of BOx- N_3 sample incubated in the presence of Cl^- ions yielded FTIR results markedly different from the F^- incubation (Fig. 3b). The peak at 2056 cm^{-1} clearly appeared at an applied potential above + 660 mV; the peak at 2049 cm^{-1} almost disappeared at + 860 mV, whereas it increases at reductive potentials. The exception observed for the measurement at + 360 mV could be attributed to a decrease of the reversibility over time or to the low stability of free azide, as this was the last spectrum recorded for this experiment. Based on these results it could be concluded that F^- competes with N_3^- for the same BOx-binding site, while for Cl^- it is much less evident.

The same experiments in the presence of halides have been performed for the Lc- N_3 samples. It could be again observed that F^- ions compete with N_3^- to bind to the TNC as the only peak observed is the one of free azide at 2049 cm^{-1} (Supporting Information Fig. S2B). The addition of Cl^- ions to Lc- N_3 sample resulted in the detection of the 2056 cm^{-1} band only at + 860 and + 710 mV, which was drastically reduced or even disappeared after applying reductive potentials (Supporting Fig. S2C).

Fig. 5 FTIR spectra for Lc- N_3 , Lc- N_3 -F and Lc- N_3 -Cl samples recorded at + 860 mV vs. NHE. In each spectrum the black line represents the experimental data, the hatched gray lines are the deconvoluted bands, and the red ones are the total curve obtained by the sum of each component

luted bands, and the red ones are the total curve obtained by the sum of each component

To have a clearer picture of the results obtained for both enzymes with or without halides, in Fig. 4 the spectra recorded at oxidizing conditions for BOx (A) and Lc (B) are presented. A clear difference in each spectrum is observed depending on the presence/absence of either F^- or Cl^- besides N_3^- . For BOx, the peak at 2056 cm^{-1} of bound azide is clearly predominant when N_3^- ions only are present, but when F^- ions are added only the 2049 cm^{-1} peak of free azide appears. When Cl^- ions were included, the predominant band is the 2056 cm^{-1} band, although the 2049 cm^{-1} can be still detected with much lower intensity. In the case of Lc, when only N_3^- was present or when Cl^- was also present, it is difficult to discern the presence of two distinct bands without further data elaboration.

For this reason, it was necessary to carry out deconvolution using a fitting model based on a Gaussian function. The results obtained after deconvolution for the spectra reported in Fig. 4b are shown in Fig. 5. The deconvolution allows visualizing the peak at 2056 cm^{-1} for the Lc- N_3 sample and its absence when F^- was added. In the case of Lc- N_3 -Cl sample, the $2056/2049$ peak intensity ratio hardly changes compared to the Lc- N_3 . The deconvoluted band observed at 2040 cm^{-1} could correspond to azide attached to the T2/T3 site with a different binding mode [8].

In all the spectra recorded, the resolution of the peaks was not optimal and for this reason deconvolution of the peaks was made for each spectrum for both enzymes (Supporting Information Figs. S3–S8). From this data elaboration, the presence of only two bands for the BOx- N_3 sample could be confirmed (Supporting Information Fig. S3). A third weak band around 2035 cm^{-1} is also observed for the BOx- N_3 -F sample (Supporting Information Fig. S4), that has also been attributed to bound azide [8]. The presence of this third peak was also observed for the Lc- N_3 sample (Supporting Information Fig. S6). The deconvolution process suggests that BOx is less inhibited by Cl^- than Lc, confirming previous work [4]. Indeed, for the BOx- N_3 -Cl sample (Supporting Information Fig. S5) the contribution of the band attributed to free azide (2049 cm^{-1}) to the FTIR spectrum is lower when compared to that for the Lc- N_3 -Cl sample (Supporting Information Fig. S8), suggesting that Cl^- interaction is more pronounced with the TNC of Lc than with the TNC of BOx. Therefore, a double site binding process cannot be excluded in laccases [12], in which Cl^- may bind in some enzyme molecules to or near the TNC site in addition to the T1 pocket binding without displacing completely the bound azide (Scheme 1).

Conclusions

Different behavior is observed upon addition of fluoride or chloride ions to the sample solution for both MCOs tested

using azide as an infrared probe. Fluoride ions compete with azide for binding at one of the T2/T3 Cu ions, whereas chloride ions hardly do (Scheme 1). Our results are in agreement with electrocatalytical data reported in the literature [17–20]. Indeed, it has been shown that in all likelihood, F^- ions interact with the TNC of MCO blocking IET from the T1 site to the Cu cluster [9–12], which could explain why in Lc- or BOx-based biocathodes both DET and MET processes are inhibited after F^- addition [17–20]. Addition of Cl^- , however, predominantly suppresses MET but not DET, suggesting that chloride anions, being larger than fluoride ions, block the access of an electron donor to the T1 site, rather than binding to the TNC (Scheme 1) [9, 11, 17]. However, in the case of laccases a double site binding process cannot be excluded from the FTIR results of the present work, in which the chloride would be placed near the T2 site without displacing completely the bound azide.

Supporting information Figures S1–S8 containing additional FTIR spectra are presented as Supporting Information.

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References

- Quintanar L, Yoon J, Aznar CP, Palmer AE, Andersson KK, Britt RD, Solomon EI (2005) *J Am Chem Soc* 127:13832–13845
- Quintanar L, Stoj C, Taylor AB, Hart PJ, Kosman DJ, Solomon EI (2007) *Acc Chem Res* 40:445–452
- Baldrian P (2006) *FEMS Microbiol Rev* 30:215–242
- Mano N (2012) *Appl Microbiol Biotechnol* 96:301–307
- Cole JL, Clark PA, Solomon EI (1990) *J Am Chem Soc* 112:9534–9548
- Peyratout CS, Severns JC, Holm SR, McMillin DR (1994) *Arch Biochem Biophys* 314:405–411
- Sakurai T, Takahashi J, Huang HW (1996) *Chem Lett* 25:651–652
- Hirota S, Matsumoto H, Huang HW, Sakurai T, Kitagawa T, Yamauchi O (1998) *Biochem Biophys Res Commun* 243:435–437
- Naki A, Varfolomeev SD (1980) *FEBS Lett* 113:157–160
- Naki A, Varfolomeev SD (1981) *Biochemistry (Moscow)* 46:1344–1350
- Xu F (1996) *Biochemistry* 35:7608–7614
- Champagne PP, Nesheim ME, Ramsay JA (2013) *Appl Microbiol Biotechnol* 97:6263–6269
- Falk M, Alcalde M, Bartlett PN, De Lacey AL, Gorton L, Gutierrez-Sanchez C, Haddad R, Kilburn J, Leech D, Ludwig R, Magner E, Mate DM, O'Conghaile P, Ortiz R, Pita M, Poller S, Ruzgas T, Salaj-Kosla U, Schuhmann W, Sebelius F, Shao M, Stoica L, Sygmund C, Tilly J, Toscano MD, Vivekananthan J, Wright E, Shleev S (2014) *PLoS One* 9:e109104
- Liu Y, Dong S (2008) *Electroanalysis* 20:827–832
- Enaud E, Trovaslet M, Naveau F, Decristoforo A, Bizet S, Vanhulle S, Jolivald C (2011) *Enzyme Microb Technol* 49:517–525
- Malkin R, Malmström RG, Vänngård T (1968) *FEBS Lett* 1:50–54

17. Vaz-Domínguez C, Campuzano S, Rüdiger O, Pita M, Gorbacheva M, Shleev S, Fernández VM, De Lacey AL (2008) *Biosens Bioelectron* 24:531–537
18. Beyl Y, Guschin DA, Shleev S, Schuhmann W (2011) *Electrochem Commun* 13:474–476
19. Salaj-Kosla U, Pöller S, Schuhmann W, Shleev S, Magner E (2013) *Bioelectrochemistry* 91:15–20
20. Tominaga M, Sasaki A, Togami M (2016) *Electrochemistry* 84:315–318
21. Hakulinen N, Andberg M, Kallio J, Koivula A, Kruus K, Rouvinen J (2008) *J Struct Biol* 162:29–39
22. Wharton CW (1986) *Biochem J* 233:26–36
23. Gutiérrez-Sanz O, Rüdiger O, De Lacey AL (2014) In: Fontecilla-Camps JC, Nicolet Y (eds) *Metalloproteins: methods and protocols. Methods in Molecular Biology*, 1st edn. Springer, New York
24. Shleev SV, Morozova OV, Nikitina OV, Gorshina ES, Rusinova TV, Serezhenkov VA, Burbaev DS, Gazaryan IG, Yaropolov AI (2004) *Biochimie* 86:693–703
25. Durand F, Gounel S, Kjaergaard CH, Solomon EI, Mano N (2012) *Appl Microbiol Biotechnol* 96:1489–1498
26. Moss D, Nabedryk E, Breton J, Mantale W (1990) *Eur J Biochem* 187:565–572
27. Fabbri M, Galli C, Gentili P (2002) *J Mol Catal B Enzym* 16:231–240
28. Jovanovic SV, Tosic M, Simic MG (1991) *J Phys Chem* 95:10824–10827
29. Kulys J, Vidziunaite R (2005) *J Mol Catal B Enzyme* 37:79–83
30. Christenson A, Shleev S, Mano N, Heller A, Gorton L (2006) *Biochim Biophys Acta* 1757:1634–1641
31. Tsujimura S, Kuriyama A, Fujieda N, Kano K, Ikeda T (2005) *Anal Biochem* 337:325–331
32. Reinhammar B (1972) *Biochim Biophys Acta* 275:245–259
33. Sorrell TN, O'Connor CJ, Anderson OP, Reibenspies JH (1985) *J Am Chem Soc* 107:4199–4206
34. Karlin KD, Cohen BI, Hayes JC, Farooq A, Zubieta J (1987) *Inorg Chem* 26:147–153
35. Shleev S, Christenson A, Serezhenkov V, Burbaev D, Yaropolov A, Gorton L, Ruzgas T (2005) *Biochem J* 385:745–754
36. Cole JL, Avigliano L, Morpurgo L, Solomon EI (1991) *J Am Chem Soc* 113:9080–9089