Reversible alkaline inactivation of lignin peroxidase involves the release of both the distal and proximal site calcium ions and bishistidine co-ordination of the haem

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Phanerochaete chrysosporium lignin peroxidase isoenzyme H2 (LiP H2) exhibits a transition to a stable, inactive form at pH 9.0 with concomitant spectroscopic changes. The Soret peak intensity decreases some 55% with a red shift from 408 to 412 nm; the bands at 502 nm and 638 nm disappear and the peak at 536 nm increases. The EPR spectrum changes from a signal typical of high spin ferric haem to an exclusively low spin spectrum with $g = 2.92, 2.27, 1.50$. These data indicate that the active pentacoordinated haem is converted into a hexacoordinated species at alkaline pH. Room temperature near-IR MCD data coupled with the EPR spectrum allow us to assign the haem co-ordination of alkali-inactivated enzyme as bishistidine. Re-acidification of the alkali-inactivated enzyme to pH 6 induces further spectroscopic changes and generates an irreversibly inactivated species. By contrast, a pH shift from 9.0 to 6.0 with simultaneous addition of 50 mM CaCl₂ results in the recovery of the initial

activity together with the spectroscopic characteristics of the native ferric enzyme. Incubating with 50 mM CaCl₂ at a pH between 6.0 and 9.0 can also re-activate the enzyme. Divalent metals other than Ca²⁺ do not result in restoration of activity. Experiments with ⁴⁵Ca indicate that two tightly bound calcium ions per enzyme monomer are lost during inactivation and reincorporated during subsequent re-activation, consistent with the presence of two structural Ca²⁺ ions in LiP H2. It is concluded that both the structural Ca²⁺ ions play key roles in the reversible alkaline inactivation of LiP H2.

Key words: basidiomycete *Phanerochaete chrysosporium*, calcium release, electron paramagnetic resonance (EPR) spectroscopy, magnetic circular dichroism (MCD) spectroscopy, pH dependence.

INTRODUCTION

Lignin peroxidases (LiPs) are largely responsible for lignin degradation by the white rot fungus *Phanerochaete chrysosporium* [1–4]. These extracellular enzymes are secreted by the fungus during secondary metabolism under conditions of nitrogen, carbon, sulphur or phosphorus starvation [5]. The LiPs are monomeric haemglycoproteins that exist in at least eight isoforms with pI values in the range 3.2 to 4.7 and M_r in the range 38 000–46 000 [2,6–8]. LiPs, together with other fungal peroxidases (manganese peroxidase from *P. chrysosporium*, peroxidases from *Coprinus cinereus*, *Coprinus macrorrhizus* and *Arthromyces ramosus*) belong to the class II peroxidases superfamily [9]. Crystal structures are available for both the LiP H2 [10] and LiP H8 [11–13] isoenzymes (see the end of the Introduction for isoenzyme nomenclature). Both have very similar structures with the same basic conserved fold as horseradish peroxidase (HRP), an archetypal class III plant peroxidase. However, LiP H2 has four disulphide bridges (between cysteine residues 3–15, 14–285, 34–120 and 249–317) that are located differently with respect to the four disulphides in HRP. Importantly for the present paper, LiP possesses both proximal and distal calcium sites, but lacks additional structural elements between helices F and G that occur in the class III peroxidases [12–15] (see also [16] for a review).

The catalytic cycle of LiP involves the oxidation of ferric peroxidase (Fe³⁺) by two electrons from hydrogen peroxide, resulting in the formation of a ferryl iron (Fe^{IV}=O) and

porphyrin cation radical intermediate, compound (I). Reduction of compound (I) by a single electron transfer, usually from an organic substrate (such as veratryl alcohol), yields a second spectroscopically detectable intermediate, compound (II), that lacks the porphyrin cation radical, but retains the ferryl haem. Compound (II) can also oxidize substrates by one electron, thereby completing the catalytic cycle of LiP [17–20]. Many electron donating substrates oxidized by LiP have redox potentials beyond the reach of plant peroxidases [21,22]. This unique feature of the enzyme has raised considerable commercial interest in *P. chrysosporium* and its extracellular LiPs for use in the textile, paper and pulp bleaching industries. One of the key factors in evaluating the technological feasibility of these environmentally acceptable bio-bleaching systems is the stability of the peroxidases, particularly at high pH. Unfortunately, LiP has an extremely narrow pH optimum (pH 2.7–3.0) with respect to the majority of its substrates, and rapidly converts into a denatured species at pH > 7.5 [23], particularly at elevated temperatures [24,25].

Using stopped-flow rapid-scan techniques, we have recently found that LiP H2 is able to oxidize textile azo dyes (Direct Blue 98 and Direct Red 48) with a pH optimum of approx. 9.0 with initial rates two orders of magnitude higher than those with HRP isoenzyme C (HRPC) (M. Kvaratskhelia, S. J. George and R. N. F. Thorneley, unpublished work). However, during LiP dependent dye bleaching, the rate decreases significantly after 2 min as a result of inactivation of the enzyme; hence our current interest in the alkaline transition of LiP. In the present paper we

Abbreviations used: LiP, lignin peroxidase; MCD, magnetic circular dichroism; HRP(C), horseradish peroxidase (isoenzyme C).

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report that the rapid inactivation of LiP H2 at pH 9.0 is associated with the release of both structural calcium ions from the enzyme. The inactive form of LiP H2 can be reactivated by the addition of an excess of calcium ions with or without a pH shift from 9.0 to 6.0.

Our studies complement the thermal inactivation studies on LiP H8 [24,25] and manganese peroxidase [26] when only the distal and adventitious calcium ions were lost (the proximal Ca^{2+} being retained) and show that thermal and alkali treatments yield structurally different inactive species. Recent studies with barley peroxidase have suggested that the activity of plant peroxidases may be reversibly controlled by pH and Ca^{2+} -induced conformation changes [27]. Our data show how isotope labelling with $^{45}\text{Ca}^{2+}$ has potential for kinetic studies of protein folding, such as those with *C. cinereus* peroxidase [28], which are relevant to the solubilization from inclusion bodies and refolding of variant recombinant peroxidases for academic research and commercial purposes [29,30].

The naming of LiP isoenzymes in the literature is confused. Some authors follow the nomenclature of Tien and Kirk [31] naming the isoenzymes H1, H2, H3, etc., based on their order of elution from a Pharmacia MonoQ ion-exchange column at pH 6.0. Other authors [18,32] refer to the isoenzyme's pI; LiP H8, with a pI of 4.15, is known as LIP415. Others use their own numbering scheme; Ritch et al. [33], for example, refer to LiP H8 (LIP415) as LiP2. This issue is further confused by reports that some isoenzymes are genetically distinct [34,35] while others differ solely in the degree of glycosylation or phosphorylation and may be interconverted [36]. Here we have adopted Tien and Kirk's nomenclature. The isoenzyme used in this study is by far the majority isoenzyme in our tartrate carbon-limited cultures which we refer to as LiP H2. Our criterion for this assignment is that a 20 residue N-terminal sequence analysis corresponds with the published gene sequence GLG4 attributed to LiP H2 [34,35]. This gene sequence in turn is virtually identical with the crystallographically defined sequence for LIP465 [Brookhaven Protein Data Bank (PDB) entry 1QPA] [10]. We are aware that LiP H1 is reported to be a dephosphorylated form of LiP H2 [36] and cannot at this stage rigorously exclude the possibility that we are in fact working with this isoenzyme.

EXPERIMENTAL

Enzyme preparation, assay and general handling

For all experiments, the reagents used were of at least ANALAR or equivalent purity. LiP H2 was isolated from the media of 7-day old, agitated, carbon-limited cultures of *P. chrysosporium* I.M.I.174727 (BKM-F-1767, A.T.C.C. 24725) and purified using a Pharmacia Mono-Q FPLC column as described by Tien and Kirk [31]. Identity of the isoenzyme was confirmed by analysis of the first 20 N-terminal residues and comparison with the published gene sequence [34,35]. LiP H2 concentrations were routinely determined from the visible absorption spectrum using $\epsilon_{409} = 133 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ [37].

LiP activity was determined by following the formation of veratraldehyde from veratryl alcohol. Enzyme solution (1–50 μl) was added to 1 ml of 1 mM veratryl alcohol in 100 mM sodium phosphate buffer (pH 2.75) and the reaction was started by the addition of 10 μl of 20 mM hydrogen peroxide and followed at 310 nm over 2 min.

Enzyme inactivation and re-activation

'Stock' LiP H2 was prepared by dialysis into 5 mM acetate buffer (pH 6.0) and concentrated to approx. 0.5 mM using a

Centricon concentrator (10000 Da cut-off; Amicon Ltd., Stonehouse, Gloucestershire, U.K.). Samples for the time-dependent activity and UV-visible experiments were typically prepared by adding 1–5 μl of stock enzyme to 0.5 ml of the appropriately buffered solution. For the EPR and MCD measurements the pH of 200 μl of approx. 0.2 mM enzyme was adjusted by stirred additions of microlitre quantities of 100 mM borate buffer (pH 10.5) or 100 mM acetate buffer (pH 4.5), while monitoring the pH with a small volume pH electrode.

Spectroscopy

UV-visible spectra were typically recorded at 20 °C on a Shimadzu UV-210 PC spectrophotometer using 1 cm path-length quartz cuvettes. EPR spectra were recorded on a Bruker 200D spectrometer (Bruker U.K. Ltd., Coventry, U.K.) fitted with an Oxford Instruments ER900 liquid helium cooled flow cryostat (Oxford Instrument Company Ltd., Osney Mead, Oxford, U.K.). Stopped-flow kinetic measurements used a Hi-Tech SF-61 DX2 double mixing stopped-flow system (Hi-Tech Ltd., Salisbury, U.K.) fitted with a diode array detector and a xenon arc source. A 320 nm low-pass filter was used to minimize enzyme photolysis. Room temperature magnetic circular dichroism (MCD) spectra were recorded using either a JASCO J500D spectrometer (400–1000 nm) or a JASCO J700C spectrometer (800–2000 nm) fitted with an antimony arsenide detector. Samples were contained in a 2 mm path-length quartz cuvette mounted inside an Oxford Instruments SM1 super-conducting magnet running at 6 T.

$^{45}\text{Ca}^{2+}$ labelling

LiP H2 ($\approx 1 \text{ mg}$) in 5 mM acetate buffer (pH 6.0) was adjusted to pH 9.0 with borate buffer to a final concentration of 50 mM borate and a volume of 180 μl . After incubation for 60 min at 20 °C the remaining activity was $< 1\%$ of the initial activity. To this mixture were added 11 μmol of CaCl_2 , 22 μmol of sodium acetate buffer (pH 5.5) and 185 kBq of $^{45}\text{CaCl}_2$ (39.9 MBq $\cdot \mu\text{mol}^{-1}$; Amersham Life Science, Amersham, Bucks., U.K.). After incubation for 90 min, when 80 % of the activity had been recovered, the total mixture (220 μl) was loaded on to a 28 cm $\times$ 1 cm column of Sephacryl S100 High Resolution gel (Pharmacia Biotech, Uppsala, Sweden). The column was developed with 200 mM sodium acetate buffer (pH 5.5); enzyme concentration in fractions of measured volumes was determined from the absorbance spectrum and radioactivity by liquid scintillation counting. Enzyme from peak fractions (550 μl) was diluted with a further 550 μl of 200 mM sodium acetate buffer (pH 5.5) and centrifuged through a Centricon concentrator at 5000 g for 60 min until only 200 μl of sample remained in the upper chamber. A further 800 μl of 200 mM borate buffer (pH 9.0) was added and the mixture was incubated for 60 min, at which time $< 1\%$ of activity remained. The mixture was transferred to a fresh concentrator and centrifuged for 60 min at 5000 g; the enzyme-free filtrate was assayed for radioactivity.

A control experiment used $\approx 1 \text{ mg}$ of active LiP incubated with $^{45}\text{CaCl}_2$ in sodium acetate buffer (pH 5.5) containing 50 mM CaCl_2 as above, but without the preceding inactivation step at pH 9.0. Gel filtration, enzyme assays and the determination of radioactivity were carried out as described above.

RESULTS

Spectroscopic and activity changes occurring with LiP H2 at pH 9.0

Incubation of LiP H2 in 50 mM borate buffer (pH 9.0) caused significant changes in the absorption spectrum (Figure 1). The

Figure 1 Changes in the UV-visible spectrum of LiP H2 during inactivation at alkaline pH

A 25 μ l aliquot of a stock solution LiP H2 in 10 mM acetate buffer, pH 6.0, was added to 425 μ l of 20 mM borate buffer, pH 9.0, in a 1-cm-pathlength quartz cuvette. Spectra were recorded at 30 s, 2 min, 4 min and 30 min. Each scan took approx. 1 min to record. A spectrum recorded at 60 min virtually superimposes on to a spectrum recorded at 30 min. The inset shows loss of activity in a parallel experiment. Activity was measured as described in the Experimental section.

Soret band shifted from 408 to 412 nm, and its intensity decreased to 55% of that of the native ferric enzyme within 30 min. At higher wavelengths, the peaks at 638 and 502 nm disappeared and absorption at 536 nm increased. After 30 min the product spectrum remained unchanged for at least 2 h. In all, five isosbestic points (at 357, 420, 481, 520 and 602 nm) were observed during this alkali-induced change in LiP H2 (Figure 1), indicating that there is no significant accumulation of any intermediate species. Measurements of the remaining activity showed that the Soret peak bleaching was associated with the complete loss of activity (Figure 1, inset). The half-time for LiP inactivation at pH 9.0 was approx. 2 min.

It should be noted that the details of these spectral changes vary with the buffer conditions. For example, incubation in 100 mM Tris/HCl, pH 9.0, produces no shift in the Soret band and different, less well defined isosbestic.

At more alkaline conditions than pH 9.0 the enzyme appears to undergo further, irreversible changes. At pH 10–10.5 the Soret band intensity decreased to 30% of that for active enzyme and shifted to 406 nm. However, this did not result in haem dissociation, since centrifugation of a sample treated at pH 10–10.5 through a 10 kDa cut-off Centricon concentrator fully retained the Soret peak absorbance in the concentrate and spectrophotometry showed no free haem in the filtrate.

We have attempted to quantify the rate of inactivation as a function of buffer and pH. Stopped-flow data (results not shown) indicate a complex kinetic profile for pH adjustment and subsequent inactivation. Typically there is a rapid initial change in Soret intensity which is completed within 10 s and is probably associated with the change in buffer or ionic strength. This is followed by an apparently biphasic exponential decay associated with inactivation. As an illustration, Figure 2 shows the rates of haem Soret band (408 nm) bleaching for 1.5 μ M enzyme measured 10 s after mixing (after the initial rapid phase) plotted as a

Figure 2 Dependence of the rate of inactivation of LiP H2 with pH monitored through bleaching of the Soret band at 408 nm

The rate of absorbance loss was measured 10 s after adjustment of pH. Data were measured in a stopped-flow apparatus, mixing enzyme in 5 mM acetate buffer, pH 6.0, with an equal volume of concentrated buffer. Alternatively, a UV-visible spectrophotometer was used mixing 18 μ l of enzyme with 700 μ l of buffer in a quartz cuvette. In either case the final LiP H2 concentration was 1.5 μ M. Buffers used: 100 mM sodium borate (●), 100 mM Tris/HCl (○), 200 mM Bis-Tris propane/HCl (▽) and 50 mM acetate/Na (◇).

function of pH in a variety of buffers. At pH 6.0 no intensity change is observed. At pH 8.0 in 100 mM Tris buffer there is an observable bleaching of the haem (0.0006 A/min). Figure 2 shows that the rate increases dramatically with pH. The rates of inactivation of LiP H2 below pH 9.0 in different buffers, while buffer dependent, are comparable, indicating that the major factor inducing changes in the protein is the alkaline pH shift and not the nature of the buffer. As noted above, inactivation of LiP H2 at pH > 9.0 tends to cause irreversible damage, with a rate that appears to be significantly buffer dependent.

Stabilization of LiP activity at alkaline pH by Ca²⁺

The addition of 50 mM CaCl₂ to 1.5 μ M LiP H2 in 50 mM borate buffer, pH 9.0, slowed the rate of haem bleaching measured 10 s after mixing to 0.0036 A/min, 8% of the value with no Ca²⁺ present (0.047 A/min). This accompanied a corresponding retention of activity. In a similar solution at pH 8.6 the enzyme retained its initial activity and the Soret band remained unchanged for at least 30 min. This effect, however, was buffer dependent. For example, in 100 mM Tris/HCl, pH 9.0, addition of 100 mM CaCl₂ slowed the rate to 35%.

Our interest in the alkaline inactivation of LiP was stimulated by its high reactivity compared with HRP in bleaching textile azo dyes (Direct Blue 98 and Reactive Red 48), with a pH optimum of approx. pH 9.0, although this activity was transient because of enzyme inactivation. (M. Kvaratskhelia, S. J. George and R. N. F. Thorneley, unpublished work). In this context, we note that similar experiments in the presence of 50 mM CaCl₂ showed that the presence of Ca²⁺ ions significantly reduced the loss of activity. However, detailed interpretation of these data has been complicated by changes in the product absorption spectrum which may arise from the chemistry of the dye oxidation intermediates and products in the presence of excess Ca²⁺ ions (M. Kvaratskhelia, S. J. George and R. N. F. Thorneley, unpublished work).

Figure 3 Changes in the UV-visible spectrum of LiP H2 during re-activation with calcium at pH 6.0

Inactivated LiP H2 prepared as described in Figure 1 (total volume 450 μ l) was mixed with 50 μ l of CaCl_2 in acetate buffer, pH 6.0, to give a final concentration of 50 mM CaCl_2 and 100 mM acetate. The inset shows recovery of activity in a parallel experiment. The activity was measured as described in the Experimental section. Trace A: comparison of the inactivated LiP H2 in 20 mM borate, pH 9.0 (—) with the spectrum at pH 6.0 (---) immediately after mixing. Data have been offset by 0.4 A. Trace B: spectra recorded after 30 s, 4 min, 12 min and 30 min after mixing. Trace C: a control experiment incubating alkaline-inactivated LiP H2 at pH 6.0 in acetate buffer in the absence of calcium: (—) spectra recorded 1 min and 15 min after mixing; (---) spectrum at pH 6.0 in the presence of calcium from (trace A) for comparison. Data have been offset —0.4 A.

Re-activation of alkali-treated LiP H2

The addition of 50 mM CaCl_2 to the fully inactivated alkali-treated LiP H2 at pH 9.0 with a simultaneous pH shift to acidic pH (6–8) resulted in the recovery of 70–100% activity. Addition of 50 mM CaCl_2 without a pH shift restored some activity but usually more slowly and with much less efficacy. In the example illustrated in Figure 3, the addition of the calcium and a pH shift from 9.0 to 6.0 resulted in the recovery of approx. 75% of the initial activity. In this case, recovery was associated with a rapid Soret shift from 412 to 408 nm (Figure 3, part A), with a subsequent increase in its intensity (Figure 3, part B). Five isosbestic points (384, 438, 474, 528 and 605 nm) were maintained during this phase of the re-activation (Figure 3, part B). While the spectrum of the reactivated enzyme always closely resembled that of native LiP H2, the detailed spectroscopic changes during re-activation apparently depend on sample buffer and the pH. For example, on adding 50 mM CaCl_2 at pH 8.5 no initial rapid Soret shift was seen. Instead there were rapid changes in the bands at approx. 500 nm followed by a slow shift in the Soret band accompanying its increase in intensity. The detailed kinetics

Figure 4 Gel-filtration chromatograms showing the binding of $^{45}\text{Ca}^{2+}$ on re-activation of alkali-inactivated LiP H2

(a) Enzyme at pH 6.0 with $^{45}\text{Ca}^{2+}$. Clearly the radioactive label co-chromatographs with the active enzyme. Details are described in the Experimental section. $\blacktriangle$, ^{45}Ca ; $\bullet$, [LiP]. (b) Control experiment with native enzyme at pH 6.0 mixed with $^{45}\text{Ca}^{2+}$ showing that no radioactive label is associated with the enzyme.

of calcium loss and uptake is proving to be a significant study and beyond the scope of this paper.

A pH adjustment from 9.0 to 6.0 with alkali-inactivated enzyme, without the addition of CaCl_2 , did not result in any re-activation. Instead, the Soret band shifted from 412 to 409 nm, with a significant loss of intensity (Figure 3, part C). This decrease does not seem to be a result of haem dissociation from the protein since no free haem was detected in the filtrate after centrifugation of the enzyme sample on a Centricon concentrator, and the Soret band intensity was also fully retained in the concentrate. After 15 min incubation at pH 6.0, this yielded an irreversibly inactivated species that could not be activated by the addition of 50 mM CaCl_2 . A longer (> 60 min) incubation of the inactive enzyme at pH 6.0 resulted in formation of a precipitate.

Any net loss of activity after an alkali inactivation followed by re-activation with calcium at pH 6.0 is apparently due to the formation of this irreversibly inactivated enzyme. This species could be easily separated from active enzyme by gel filtration suggesting that the irreversibly inactivated enzyme has aggregated to form a high molecular mass complex.

MgCl_2 , MnCl_2 , CoCl_2 , NiCl_2 and BaCl_2 did not substitute for CaCl_2 in restoring the LiP activity. These data provide further evidence for the specificity of LiP H2 for Ca^{2+} with respect to the two structurally important metal-binding sites.

Addition and removal of $^{45}\text{Ca}^{2+}$

When fully inactivated alkali-treated LiP H2 was reactivated at pH 5.5 with CaCl_2 labelled with $^{45}\text{Ca}^{2+}$, radioactivity equivalent to 2.03 g-atom of $^{45}\text{Ca}^{2+}$ per mol of enzyme co-chromatographed with it on gel filtration (Figure 4a). The gel-filtration column

Figure 5 X-Band EPR spectra recorded during alkaline inactivation and subsequent re-activation of LiP H2

(A) Native enzyme in 10 mM acetate buffer, pH 6.0. (B) Enzyme inactivated by incubation in borate buffer, pH 9.0 (see the Experimental section for details). (C) Enzyme reactivated by the addition of 50 mM CaCl₂ and adjustment to pH 6.0 with acetate buffer (see the Experimental section for details). Experimental conditions: temperature, 17 K; modulation amplitude, 12 G [$1 \text{ G} = 10^{-4} \text{ T}$ (tesla)] at 100 kHz; microwave frequency 9.33 GHz; microwave power 20 mW; gain for (A) and (C), 2×10^4 ; gain for sample (B) 8×10^5 .

separated the fully reactivated LiP H2 from irreversibly inactivated enzyme. None of the radio-labelled calcium could be removed by filtration through the semi-permeable membrane of a Centricon concentrator, indicating that two Ca²⁺ ions were firmly bound to the active protein. Subsequent exposure of ⁴⁵Ca²⁺-labelled LiP H2 to a pH of 9.0 resulted in the release of 1.94 g-atom of ⁴⁵Ca²⁺ per mol of enzyme.

To determine whether adventitiously-bound, easily exchangeable Ca²⁺ was present in our LiP H2 preparations as suggested by the data of Nie and Aust [24], active ferric enzyme was incubated with ⁴⁵CaCl₂ without prior treatment at pH 9.0. Following gel filtration, no radioactivity was recovered with the enzyme (Figure 4b), indicating that structural Ca²⁺ did not exchange with exogenous ⁴⁵Ca²⁺ and that any adventitiously bound ⁴⁵Ca²⁺ could be removed from LiP H2 by gel filtration.

EPR spectroscopy

Ferric LiP H2 at pH 6.0 gives an EPR signal with $g = 6.09, 5.76, 2.00$, typical of a high spin ($S = 5/2$) ferric haem (Figure 5, sample A). By contrast, the EPR spectrum of alkali-treated enzyme shows that the iron in the inactivated enzyme is low spin, with g values 2.92, 2.27 and 1.50 (Figure 5, sample B). The signal at $g = 6.00$ is a residual, native high-spin signal, with an intensity approx. 1% that of the initial ferric enzyme and consistent with the 1% activity of the sample. The signal at $g = 4.27$ arises from a low level of adventitious non-haem iron and is present unchanged in all the spectra in Figure 5. Upon the addition of 50 mM CaCl₂ to the enzyme sample with simultaneous adjustment of the pH to 6.0, the low-spin signal essentially disappeared (Figure 5, sample C) and the high-spin signal was largely restored. After correction for sample dilution, the integrated intensity of this signal was 75% that of the initial ferric haem signal, consistent with the recovery of 75% of the activity. The changed line shape of this signal compared with the starting enzyme can be ascribed to ionic strength and buffer effects.

Figure 6 Room temperature MCD spectroscopy of alkali-inactivated LiP H2 in borate buffer, pH 9.0

(A) UV-visible absorption spectrum. (B) UV-visible MCD spectrum. (C) Near-IR MCD spectrum. Sample preparation and MCD conditions are as described in the Experimental section. Absorption coefficients are estimated from the native LiP H2 concentration of 220 μM prior to inactivation and corrected for subsequent dilution. Spectra were measured in either a 1 mm or 2 mm quartz cuvette.

Quantitative comparison of the EPR signals with LiP H2 activity measurements indicated that active LiP H2 and its alkali-treated form are exclusively high- and low-spin species respectively. The pH adjustment of alkali-treated LiP H2 to pH 6.0 without addition of CaCl₂ resulted in an approx. 10-fold decrease in the low-spin signal and a slight (1.5-fold) increase in the high-spin signal. At the same time, the EPR signal at $g = 2.05$ increased compared with that of alkali-treated or active enzyme.

MCD spectroscopy

Room temperature UV-visible absorption, UV-visible MCD and near infra-red MCD spectra from the alkali-inactivated enzyme at pH 9.0 are presented in Figure 6. The absorption and MCD spectra are typical of low-spin ferric haem [38]. Of particular interest are the near infra-red MCD bands at 1300 nm and 1550 nm. These arise from porphyrin $\pi(a_{1u}) \rightarrow \text{Fe(III)}d_{xz,yz}(e_g)$ transitions. Their wavelength and intensity are well known to be diagnostic of the state of haem co-ordination [38,39]. This is discussed further below.

DISCUSSION

This discussion draws on the similar high resolution crystal structures published for LiP H8 [13] (Brookhaven Protein Data Bank entry 1LGA) and LiP H2 (LIP 465; Brookhaven Protein

Scheme 1 Proposed haem structural changes in LiP H2 consequent to reversible alkaline inactivation and Ca^{2+} dissociation

Figure produced using the program O [48].

Data Bank entry 1QPA) [10]. As LiP H2 has one more N-terminal residue than LiP H8, the numbering of the conserved residues in LiP H2 (e.g., His-48, His-177) is one greater than the equivalent residue in LiP H8 (His-47, His-176). The H8 numbering has been used here to facilitate comparison with the crystallographic description of the haem and calcium sites by Poulos et al. [13].

Implications for Ca^{2+} dissociation from alkali-treated LiP H2

Our data clearly show that alkaline inactivation of LiP H2 can yield a stable species with the dissociation of two Ca^{2+} ions. The crystal structures of LiP H2 and H8 show two Ca^{2+} ions at sites distal and proximal to the haem pocket [10,13], both of which are regarded as essential for active site integrity [13]. Folding and activation of recombinant LiP H8 overexpressed in *E. coli* requires Ca^{2+} [40]. The distal calcium has as one ligand Asp-48; its neighbour, His-47, lies at the C-terminal end of the distal helix but is normally not co-ordinated to the haem. The proximal calcium is liganded by Ser-177, located at the C-terminus of the proximal helix and next to His-176, the fifth haem ligand [13] (Scheme 1). The affinities of these sites for Ca^{2+} are predicted to be significantly different [13] with the distal calcium less tightly bound than the proximal because of its near-surface location and

heptaco-ordinate status (two water molecules, Asp-48-carboxyl and -carbonyl-O, Gly-66-carbonyl-O, Ser-70-hydroxyl-O and Asp-68-carboxyl). By contrast, the proximal calcium predicted to be more tightly bound is inaccessible to bulk solvent and has no bound water. Its ligands are Ser-177-hydroxyl-O and -carbonyl-O, Asp-194-carboxyl, Thr-196-hydroxyl-O and -carbonyl-O, Ile-199-carbonyl-O and Asp-201-carboxyl.

During thermal inactivation of LiP [24,25] and manganese peroxidase [26,41] only the more weakly-bound distal Ca^{2+} is thought to dissociate. The LiP H8 preparation used by Nie and Aust [24] contained 5 g-atom of Ca^{2+} per mol, of which four were lost on thermal inactivation at 50 °C, the authors proposing that their enzyme contained four weakly-bound, adventitious calcium ions that are not detectable in the X-ray crystal structure. It is clear from our control experiment with $^{45}\text{Ca}^{2+}$ that our preparation of LiP H2 carried no adventitious Ca^{2+} resistant to gel filtration (Figure 4b). It is also clear that alkaline inactivation is associated with dissociation of two Ca^{2+} ions, presumably the structural ones, and that their re-introduction results in re-activation. Our data therefore support the assignment [13] of an essential structural role to the two Ca^{2+} ions identified by X-ray crystallography. Whether it is possible to associate the changes to the UV-visible, EPR and MCD spectra with the release of the distal or proximal Ca^{2+} ions will depend on whether the kinetics

of their removal are sufficiently different to be resolved by simultaneous fluorimetric determination of Ca²⁺ and recording of other spectra.

Spectroscopic characterization and haem co-ordination of alkali-treated LiP

The UV-visible and EPR data indicate that the haem in native LiP H2 is high-spin ferric ($S = 5/2$), either pentaco-ordinate, or hexaco-ordinate with a weak ligand such as water at the distal position. The proximal haem ligand in LiP is histidine. Alkaline treatment markedly changes the spectroscopic nature of the haem. The UV-visible, EPR and MCD spectra show the haem iron to now be low-spin ferric ($S = 1/2$), with the distal sixth co-ordination site occupied by either a small molecule such as the hydroxide ion or by a protein ligand such as His-47. Taken together, the EPR and near-IR region of the MCD spectra unambiguously identify the haem co-ordination as bishistidine. For LiP H2 the wavelength of the major $a_{1u} \rightarrow d_{xz,yz}$ transition is 1550 nm, indicating either bishistidine or histidine-lysine co-ordination (see Figure 9 in Cheesman et al. [38]). The EPR values for alkali-treated LiP H2 are typical of those for many bishistidine complexes [39] with the g_z value of 2.92 much lower than that (3.51) for histidine-lysine co-ordinated haem. Indeed the EPR spectrum for the complex Fe(III)-octaethylporphyrine-(imidazole)₂ [38] is almost identical with that in Figure 5 (sample B). The haem iron in alkali-treated LiP H2 can therefore be regarded as hexaco-ordinate with two histidine ligands.

From where then, does the second histidine ligand come? We propose that release of the distal Ca²⁺ from Asp-48 allows the adjacent His-47 to become the sixth iron ligand (Scheme 1). A similar suggestion, without MCD data, was made by Nie and Aust [24] to explain UV-visible and EPR spectra for thermally inactivated LiP465. Movement of His-47, which is normally located 5.3 Å (1 Å = 0.1 nm) from the haem iron, would be easier if helix B, which carries His-47-Asp-48 at its C-terminus, becomes more flexible with the loss of the distal Ca²⁺; tight binding of this deprotonated imidazole to haem iron as its sixth ligand would render the enzyme inactive.

Since alkali treatment of LiP H2 releases both structural Ca²⁺ ions, changes resulting from loss of the proximal Ca²⁺ need also to be considered; such changes may be minor, since the proximal His-176-Fe bond should be stabilized at pH 9.0, anchoring the proximal helix against movement resulting from release of the proximal Ca²⁺ from its C-terminal residue, Ser-177. Indeed, alkali-inactivated LiP showed no spectrophotometric or EPR changes after a month's storage at 4 °C, and was reactivated by addition of acid and Ca²⁺ at a similar rate to the freshly-inactivated enzyme. Dual co-ordination of the haem iron to His-47 and to His-176 must play a key role in the stability of the alkaline conformer of LiP H2 (Scheme 1).

In other peroxidases, alkali treatment also results in formation of hexaco-ordinate low-spin iron, the sixth ligand varying-hydroxyl in HRP [42] and *C. cinereus* peroxidase [43], and histidine in cytochrome *c* peroxidase [44]. However, the formation of these species is not associated with calcium release. In fact, cytochrome *c* peroxidase contains no structural calcium ions. These enzymes differ significantly in the distal haem pocket, since Cys-49 in helix B in HRP may form a disulphide with Cys-44 and restrict the flexibility of the distal helix at alkaline pH. Limiting movement of the distal His-42 would therefore favour hydroxyl binding in HRP; binding of the distal histidine is not so constrained in cytochrome *c* peroxidase which lacks the disulphide bonds. In LiP the four disulphide bridges are in different locations to those in classical plant peroxidases such as HRP

[9]. Significantly, there is no cysteine in helix B in LiP, and therefore no constraint on imidazole from His-47 binding to haem iron after release of Ca²⁺ at alkaline pH.

Comparison of alkali and thermal inactivation of LiP H2

Thermal inactivation of LiP H8 and alkali treatment of LiP H2 are clearly related phenomena, inasmuch as both release Ca²⁺ [24], and the thermal stability of LiP H8 is reported to be decreased at more alkaline pH. Indeed, the thermal inactivation experiments reported by Nie and Aust [24] are performed at pH 7.9 in Tris buffer, conditions where we see measurable haem bleaching in LiP H2 at room temperature. Nevertheless, the two processes appear to be distinct. The primary difference is that thermal inactivation is reported to release only the distal Ca²⁺ ion, whereas our data for alkali treatment clearly show that both Ca²⁺ ions are released. In addition, there are significant differences in UV-visible spectra of the inactivated forms; for example the extent of bleaching and red shift of the Soret band [63% loss and a 2 nm shift by thermal treatment, and 45% loss with a 4 nm shift by alkali (Figure 1)]. While these changes may be ascribed to buffer differences, the EPR data clearly show that thermal inactivation produces a mixture of spin-states whereas alkali inactivation produces a pure low-spin system. The reported g -values of the low-spin state in thermally-inactivated enzyme ($g = 2.71, 2.26, 1.76$) are not consistent with those in Figure 5. Furthermore, the addition of 15 mM imidazole to alkali-treated LiP H2 (results not shown) does not change the UV-visible or EPR spectra, suggesting a strong His-47-Fe bond. The ready displacement of the distal histidine by imidazole in thermally-inactivated LiP H8 [24] suggests much weaker interaction between haem iron and distal histidine, consistent with the observed mixture of spin states.

It is difficult, with the current data, to speculate on the nature of the pK_a that must induce the alkaline change, and presumably may also thermally destabilize the enzyme. We note that the onset of the alkaline transition of LiP is at $\approx$ pH 8.0 (Figure 2), which suggests that any inducing pK_a is within a pH unit alkaline to this.

Re-activation and stabilization of LiP H2 with Ca²⁺

Alkali-treated LiP H2 can be reactivated by the addition of a substantial excess of Ca²⁺ over protein. The rate and efficacy of the re-activation is significantly enhanced by adjusting the pH to a more acidic value, presumably because this slows the inactivation chemistry and thus minimizes competition. Similarly the presence of excess Ca²⁺ tends to stabilize LiP H2 at alkaline pH. These results imply that the effect of alkaline pH is, in the first instance, to reduce the affinity of one or both Ca²⁺ binding sites rather than cause conformational changes in the protein that extrude the metal ions. Re-activation is accompanied by a gain of two Ca²⁺ ions, presumably inserted into the distal and proximal sites detected by crystallography [13]; the failure of Mn²⁺, Mg²⁺, Co²⁺, Ni²⁺ or Ba²⁺ to similarly reactivate LiP indicates a specificity for Ca²⁺ supporting this presumption. The detailed UV-visible spectroscopic changes depend on the buffer and pH used; however we typically see a biphasic reaction. Whether this is a consequence of the pH shift causing a spectroscopic change followed by Ca²⁺ binding, or simply differential binding of two Ca²⁺ ions is currently unclear.

The pH shift to 6.0 without added Ca²⁺ further bleached the Soret peak to 35% of normal, and gave no recovery of activity. We suggest that structural calcium may be essential to prevent weakening of the proximal His-176-Fe link which would be

assisted by protonation of the unco-ordinated imidazole at pH 6.0. The residue adjacent to His-176 (Ser-177) provides two of the links to the proximal Ca^{2+} , making it reasonable that loss of this calcium could weaken the interaction between His-176 and haem iron.

LiP exhibits a high reactivity in bleaching of textile azo dyes at alkaline pH, although the activity is transient due to inactivation associated with loss of Ca^{2+} . Genetic modifications to produce active, alkali-stable LiP variants are likely to require changes in sequence which stabilize the binding of both structural Ca^{2+} ions. Since thermal inactivation of LiP [24] may involve only the release of the distal Ca^{2+} ion, mutations giving stability against alkali-induced release of Ca^{2+} ions may also give thermal stability. Many peroxidases such as HRP and peanut peroxidase are stable and active at high pH [42]. Of special interest are the fungal peroxidases from *C. cinereus* and *A. ramosus* (these fungal enzymes are essentially identical [45]) which have a 40–45% identity in amino-acid sequence to the LiP isoenzymes [46] and a particularly similar tertiary structure to LiP [47]. However, these enzymes are also stable at high pH, exhibiting an alkaline transition involving the binding of a hydroxy group with a pK_a of approx. 11 [43]. Hence, a detailed comparison of the crystal structures of LiP [10,12,13] with that of *C. cinereus* peroxidase/*A. ramosus* [47] as well as those from the class III peroxidases HRP [15] and peanut peroxidase [14] may well suggest mutational strategies to achieve an alkali-stable LiP variant.

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REFERENCES

- Buswell, J. A. and Odier, E. (1987) *CRC Crit. Rev. Biotechnol.* **6**, 1–60
- Kirk, T. K. and Farrell, R. L. (1987) *Annu. Rev. Microbiol.* **41**, 465–505
- Barr, D. P. and Aust, S. D. (1994) *Environ. Sci. Technol.* **28**, 78A–87A
- Hammel, K. E., Mozuch, M. D., Jensen, Jr., K. A. and Kersten, P. J. (1994) *Biochemistry* **33**, 13349–13354
- Tien, M. and Kirk, T. K. (1983) *Science* **221**, 661–663
- Renganathan, V., Miki, K. and Gold, M. H. (1985) *Arch. Biochem. Biophys.* **241**, 304–314
- Leisola, M. S. A., Kozulic, B., Meussdoerffer, F. and Fiechter, A. (1987) *J. Biol. Chem.* **262**, 419–424
- Gold, M. H., Wariishi, H. and Valli, K. (1989) in *Biocatalysis in Agricultural Biotechnology* (Whitaker, J. R. and Sonnet, P., eds.) (ACS Symposium Series 389), pp. 127–140, American Chemical Society, Washington DC
- Welinder, K. G. (1992) *Curr. Opin. Struct. Biol.* **2**, 388–393
- Choinowski, T. (1996) Ph.D. Thesis, No. 11859, ETH, Zürich, Switzerland
- Edwards, S. L., Raag, R., Wariishi, H., Gold, M. H. and Poulos, T. L. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 750–754
- Piontek, K., Glumoff, T. and Winterhalter, K. (1993) *FEBS Lett.* **315**, 119–124
- Poulos, T. L., Edwards, S. L., Wariishi, H. and Gold, M. H. (1993) *J. Biol. Chem.* **268**, 4429–4440
- Schuller, D. J., Nenad, B., van Huystee, R. B., McPherson, A. and Poulos, T. L. (1996) *Structure* **4**, 311–321
- Gajhede, M., Sculler, D. J., Henriksen, A., Smith, A. T. and Poulos, T. L. (1997) *Nat. Struct. Biol.* **4**, 1032–1038
- Smith, A. T. and Veitch, N. C. (1998) *Curr. Opin. Chem. Biol.* **2**, 269–278
- Tien, M. (1987) *CRC Crit. Rev. Microbiol.* **15**, 141–168
- Harvey, P. J., Palmer, J. M., Schoemaker, H. E., Dekker, H. L. and Wever, R. (1989) *Biochim. Biophys. Acta* **994**, 59–63
- Wariishi, H., Huang, J., Dunford, H. B. and Gold, M. H. (1991) *J. Biol. Chem.* **266**, 20694–20699
- Khindaria, A., Yamazaki, I. and Aust, S. D. (1996) *Biochemistry* **35**, 6418–6424
- Hammel, K. E., Kalyanaraman, B. and Kirk, T. K. (1986) *J. Biol. Chem.* **261**, 16948–16952
- Kersten, P. J., Kalyanaraman, B., Hammel, K. E., Reinhammer, B. and Kirk, T. K. (1990) *Biochem. J.* **268**, 475–480
- Millis, C. D., Cai, D., Stankovich, M. T. and Tien, M. (1989) *Biochemistry* **28**, 8484–8489
- Nie, G. and Aust, S. D. (1997) *Biochemistry* **36**, 5113–5119
- Nie, G. and Aust, S. D. (1997) *Arch. Biochem. Biophys.* **337**, 225–231
- Sutherland, G. R. J., Zapanta, L. S., Tien, M. and Aust, S. D. (1997) *Biochemistry* **36**, 3654–3662
- Rasmussen, C. B., Hiner, A. N. P., Smith, A. T. and Welinder, K. G. (1998) *J. Biol. Chem.* **273**, 2232–2240
- Tams, J. W. and Welinder, K. G. (1996) *Biochemistry* **35**, 7573–7579
- Smith, A. T., Santama, N., Dacey, S., Edwards, M., Bray, R. C., Thorneley, R. N. F. and Burke, J. F. (1990) *J. Biol. Chem.* **265**, 13335–13343
- Gazaryan, I. G., Doseeva, V. V., Galkin, A. G. and Tishkov, V. I. (1994) *FEBS Lett.* **354**, 248–250
- Tien, M. and Kirk, T. K. (1988) *Methods Enzymol.* **161**, 238–249
- Blodig, W., Doyle, W. A., Smith, A. T., Winterhalter, K., Choinowski, T. and Piontek, K. (1998) *Biochemistry* **37**, 8832–8838
- Ritch, Jr., T. G., Nipper, V. J., Akileswaran, L., Smith, A. J., Pribnow, D. G. and Gold, M. H. (1991) *Gene* **107**, 119–126
- de Boer, H. A., Zhang, Y. Z., Collins, C. and Reddy, C. A. (1987) *Gene* **60**, 93–102
- de Boer, H. A., Zhang, Y. Z., Collins, C. and Reddy, C. A. (1988) *Gene* **69**, 369
- Kuan, I.-C. and Tien, M. (1989) *J. Biol. Chem.* **264**, 20350–20355
- Tuisel, H., Sinclair, R., Bumpus, J. A., Ashbaugh, W., Brock, B. J. and Aust, S. D. (1990) *Arch. Biochem. Biophys.* **279**, 158–166
- Cheesman, M. R., Greenwood, C. and Thomson, A. J. (1991) *Adv. Inorg. Chem.* **36**, 201–255
- Gadsby, P. M. A. and Thomson, A. J. (1990) *J. Am. Chem. Soc.* **112**, 5003–5011
- Doyle, W. A. and Smith, A. T. (1996) *Biochem. J.* **315**, 15–19
- Sutherland, G. R. J. and Aust, S. D. (1997) *Biochemistry* **36**, 8567–8573
- Feis, A., Marzocchi, M. P., Paoli, M. and Smulevich, G. (1994) *Biochemistry* **33**, 4577–4583
- Smulevich, G., Neri, F., Marzocchi, M. P. and Welinder, K. G. (1996) *Biochemistry*, **35**, 10576–10585
- Smulevich, G., Miller, M. A., Kraut, J. and Spiro, T. G. (1991) *Biochemistry* **30**, 9546–9558
- Dunford, H. B. (1999) *Haem Peroxidases*, pp. 299–300, John Wiley and Sons Inc., New York
- Baunsgaard, L., Dalboge, H., Houen, G., Rasmussen, E. M. and Welinder, K. G. (1993) *Eur. J. Biochem.* **213**, 605–611
- Kunishima, N., Fukuyama, K. and Matsubara, H. (1994) *J. Mol. Biol.* **235**, 331–344
- Jones, T. A., Zou, J. Y., Cowan, S. W. and Kjeldgaard, M. (1991) *Acta Crystallogr.* **D47**, 110–119

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