The Critical Role of the Proximal Calcium Ion in the Structural Properties of Horseradish Peroxidase*

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Peroxidases of the plant peroxidase superfamily are heme-containing enzymes that oxidize a variety of aromatic molecules in the presence of hydrogen peroxide. They include peroxidases of plant, fungal, and prokaryotic origins that can be divided into three classes based on sequence alignment (1). Additional support for such a classification was gained as the crystal structures of representative enzymes of each class became available. Class I contains bacterial peroxidases and peroxidases from plant mitochondria and chloroplasts, for example most ascorbate peroxidases and cytochrome e peroxidase. Class II contains extracellular fungal peroxidases such as Coprinus cinereus peroxidase (a peroxidase essentially identical to Arthromyces ramosus peroxidase) and lignin-degrading peroxidases. Class III contains secretary plant peroxidases, typified by the classical horseradish peroxidase isoenzyme C (HRPC).1

The peroxidases of classes II and III share a number of structural elements considered to be of importance for maintaining protein stability and activity. These include calcium-binding sites proximal and distal to the heme and four disulfide bridges (1–5); the latter was in different locations in class II with respect to class III peroxidases. These features are absent in class I peroxidases. Class III peroxidases also have a loop insertion in the sequence, composed of the D’, F’, and F” helices, not found in the other classes. The relationship between calcium binding and enzyme inactivation has been treated primarily by studies on lignin peroxidase (LIP) (6, 7), manganese peroxidase (MNP) (8, 9), and HRPC (10, 11). Thermal (6) and alkaline (7) inactivation of LIP has been correlated with loss of calcium ions and the formation of an inactive bis-histidyl hexacoordinate low-spin (6-c LS) heme state. In apparent contrast with alkaline inactivation, which results in the loss of both calcium ions, thermal inactivation causes the loss of only the more weakly bound distal calcium ion. Likewise, thermal inactivation of MNP has been correlated with loss of calcium and the formation of an inactive 6-c LS state proposed to result from the dissociation of the distal calcium only (8, 9).

The x-ray crystal structure of HRPC (4) confirmed previous analyses of calcium content indicating 2 mol of calcium per mol of enzyme. A schematic representation of the locations of the two calcium ions and heme pocket residues is shown in Fig. 1. ApoHRPC has been found not to refold properly in the absence of calcium ions (12), and removal of the bound calcium ions from native HRPC results in a considerable decrease in activity and thermal stability (10, 11, 13, 14). These observations demonstrate the essential role played by the structural calcium ions in HRPC in maintaining a heme pocket architecture conducive to high activity. The present work extends previous spectroscopic studies of Ca2+-depleted HRPC to obtain further insight into the consequences on the heme environment of removing calcium from the enzyme. On the basis of the present results, the relative importance of the proximal and distal calcium ions in determining the heme pocket structural properties is discussed.

EXPERIMENTAL PROCEDURES

HRPC Ca2⁺ Ion Depletion and Reconstitution—In a manner similar to the procedure reported by Haschke and Friedhoff (10), the Ca2⁺-
were prepared by adding aliquots of 0.2M benzohydroxamic acid and flushing with CO (Rivoira, Italy), and reducing the complexes were prepared by first flushing the ferric protein solution with nitrogen, then flushing with CO (Rivoira, Italy), and reducing the protein fraction of the elute from that of free 45Ca2⁺ showed the distal and proximal Ca²⁺ ions. Reconstitution with 45Ca2⁺ ions (see below).

Reconstitution of the Ca²⁺-depleted protein was achieved by incubation for 18 h at 4 °C with 30 mM CaCl₂ and a heme solution, prepared fresh before use, of final concentration 50% that of the protein. After incubation the protein was passed through a Sephacryl S100 column (Amersham Pharmacia Biotech), equilibrated and eluted with 50 mM Tris/HCl in 50 mM EDTA in 50 mM Tris/HCl, pH 7.8. The protein concentration of eluted fractions (0.8 ml) was determined from the electronic absorption spectrum and the corresponding slope of native HRPC which is assigned a value of 100%. Each value was determined three or more times to obtain the standard deviation.

**Determination of Enzyme Activity**—Peroxidase activity was measured in 60 mM phosphate at pH 7.0, using the substrate ABTS (12). Initial reaction rates were measured by following the increase in absorbance at 405 nm resulting from the formation of the ABTS cation radical product. A final concentration of 0.3 mM ABTS and a saturating concentration of 1 mM H₂O₂ were used for the assays, which were initiated by addition of enzyme to a final concentration in the range of 0.05–0.5 mM. For each enzyme (native, Ca²⁺-depleted, and reconstituted HRPC), the value of Vₘₐₓ was determined for three different concentrations, and the values were plotted to determine the resultant slope by regression analysis. The percentage of activities reported in Table I has been obtained by such a measurement of the slope of Vₘₐₓ against enzyme concentration for each enzyme, taking as reference the corresponding slope of native HRPC which is assigned a value of 100%.

**Electronic Absorption and Resonance Raman Spectroscopy**—Electronic absorption spectra were measured with a Cary 5 spectrophotometer using a cuvette of 1- or 10-mm path length. RR spectra were obtained at room temperature with excitation from the 406.7, 530.9, and 568.2 nm lines of a Kr⁺ laser (Coherent, Innova 90/K), 496.5 and 514.5 nm lines of an Ar⁺ laser (Coherent, Innova 90/5), and from the 441.6 nm line of a HeCd laser (Liconix). The back-scattered light from a slowly rotating NMR tube was collected and focused into a computer-controlled double monochromator (Jobin-Yvon HGS2) equipped with a charge-coupled device (CCD) and photon-counting electronics. To minimize local heating of the protein by the laser beam, the sample was cooled by a gentle flow of N₂ gas passed through liquid N₂. RR spectra were calibrated to an accuracy of 1 cm⁻¹ for intense isolated bands, with indene as the standard for the high frequency region and with indene and CCl₄ for the low frequency region. Polarized spectra were obtained by inserting a polaroid analyzer between the sample and the entrance slit of the monochromator. The depolarization ratios of the bands at 314 and 460 cm⁻¹ were measured to check the reliability of the polarization measurements. The values obtained, 0.73 and 0.00, compared well with the theoretical values of 0.75 and 0.00, respectively. Peak intensities were determined using a curve-fitting program to simulate experimental spectra with Lorentzian line shapes.

**RESULTS**

**Specific Activity and Calcium Content**—The specific activity at pH 7.0 of Ca²⁺-depleted HRPC is reported in Table I. The loss of 50% activity compared with native protein noted for the Ca²⁺-depleted form is reversed upon reconstitution by incubation with heme and excess CaCl₂ (see “Experimental Procedures”). Reconstitution leads to a rise in the specific activity, achieving 85% that of native HRPC.

When Ca²⁺-depleted HRPC was reconstituted with heme and ⁴⁵Ca-labeled CaCl₂ radioactivity equivalent to 1.18 g atom of ⁴⁵Ca²⁺ ions per mol of enzyme was found to co-chromato-

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**Table I**

| HRPC enzyme form | Specific activity⁶ | s | Kᵢ⁹₂₅a⁷ |
|------------------|--------------------|---|---------|
| Native           | 100                | 112 ± 6 | 21 ± 0.1 |
| Ca²⁺-depleted    | 50 ± 5             | 107 ± 3 | 6.0 ± 0.6 |
| Reconstituted    | 85 ± 2             |        |         |

⁶ The percentages quoted for the activity (measured with ABTS) are with respect to native protein. The procedure used to determine the values is described under "Experimental Procedures."

⁷ The dissociation constant for BHA: native HRPC (16), Ca²⁺-depleted HRPC, this work.
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A comparison between the high frequency RR spectra for Soret excitation (406.7 nm) of Ca\(^{2+}\)-depleted and native HRPC at pH 7.8 is presented in Fig. 3B. The spectrum of the native has been reported previously and found to be a mixture of 5-coordinate (5-c) and 6-c heme forms, both of which display anomalously high core size marker band frequencies (19). On the basis of this observation and other data available for HRPC, it has been proposed that resting HRPC at room temperature cannot be adequately described by pure HS heme states and that the two heme forms should be considered as QS states having a very low level of IS state admixed with the HS states and that the two heme forms should be considered as QS states resulting from the quantum mechanical mixing of high- and intermediate-spin states (\(S = 5/2\) and 3/2, respectively). The extinction coefficient of the Soret maximum of Ca\(^{2+}\)-depleted HRPC is unchanged with respect to that of the native (Table I). The electronic absorption spectra of Ca\(^{2+}\)-depleted HRPC and native enzyme at pH 7.8 are shown in Fig. 3A. The most striking difference between the spectra of native and Ca\(^{2+}\)-depleted HRPC is the higher frequencies of the core size marker bands of the Ca\(^{2+}\)-depleted form. This immediately indicates the presence of a QS heme state, which is more fully expressed compared with that of the native protein in which only a low level of IS is apparently present. It is noteworthy that reconstitution of the Ca\(^{2+}\)-depleted protein gave rise to electronic absorption and RR spectra which were identical to those of native HRPC (data not shown). This clearly demonstrates that the Ca\(^{2+}\) depletion process is reversible, in agreement with the activity measurements noted above, and hence that the observed variations in enzyme activity and spectral features are a direct consequence of conformational changes induced by calcium removal.

RR excitation at wavelengths in the visible region (Fig. 4) and the use of polarized light has enabled the band frequencies of all the core size marker bands to be determined. A comparison of these frequencies with those noted for model heme
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**Fig. 4.** Resonance Raman spectra of Ca<sup>2+</sup>-depleted HRPC at pH 7.8 in 5 mM EDTA, 50 mM Tris/HCl recorded with excitation at 406.7, 496.5, 514.5, 530.9, and 568.2 nm. Experimental conditions are as follows: 5 cm<sup>-1</sup> resolution, 406.7 nm, concentration 25 μM, 39 s/0.5 cm<sup>-1</sup> collection interval, 15-mW laser power at the sample; 496.5 nm, concentration 90 μM, 77 s/0.5 cm<sup>-1</sup> collection interval, 50-mW laser power at the sample; 514.5 nm, concentration 250 μM, 68 s/0.5 cm<sup>-1</sup> collection interval, 50-mW laser power at the sample; 530.9 nm, concentration 300 μM, 19 s/0.5 cm<sup>-1</sup> collection interval, 40-mW laser power at the sample; 568.2 nm, concentration 300 μM, 90 s/0.5 cm<sup>-1</sup> collection interval, 50-mW laser power at the sample.

| Mode | 6-e HS | 5-e HS | QS | LS |
|------|--------|--------|----|----|
| ν<sub>10</sub> | 1622 | 1631 | 1638 | 1640 |
| μ(C=C) | F | 1631 | 1631 | F |
| μ(C=C) | F | 1622 | 1622 | F |
| ν<sub>19</sub> | 1574<sup>a</sup> | 1574<sup>a</sup> | 1574<sup>a</sup> | 1586 |
| ν<sub>11</sub> | 1553 | 1553 | 1548 | 1548 |
| ν<sub>3</sub> | 1485 | 1493 | 1503 | 1503 |

<sup>a</sup> The population of the spin species is too small to be able to assign with precision its vinyl mode frequencies.

<sup>b</sup> Only the mean frequency can be determined due to overlap of the bands.

compounds (25), native HRPC (26), and a number of other class III peroxidases, which display a QS state (22–24), has resulted in a detailed assignment of the RR bands of Ca<sup>2+</sup>-depleted HRPC (Table I). RR spectra recorded in polarized light (data not shown) reveal that while the band at 1638 cm<sup>-1</sup> (Fig. 4) is depolarized the bands at 1622 and 1631 cm<sup>-1</sup> have intermediate polarization (0.4 and 0.5, respectively). The difference in polarization ratios between polarized modes (0.125) and depolarized modes (0.75) suggests that each of the two latter bands results from the overlap of a polarized and a depolarized mode. Therefore, the band at 1638 cm<sup>-1</sup> is assigned to a ν<sub>10</sub> mode, whereas the bands at 1622 and 1631 cm<sup>-1</sup> are considered to be each a superposition of a vinyl (polarized) and ν<sub>10</sub> (depolarized) mode. The band at 1576 cm<sup>-1</sup> is inversely polarized and is, thus, assigned to a ν<sub>19</sub> mode. The bands at 1548 and 1553 cm<sup>-1</sup> are depolarized and can be assigned to ν<sub>11</sub> modes. The vinyl bands are evident at 1622 and 1631 cm<sup>-1</sup> for all the visible excitation wavelengths shown in Fig. 4. This effect has been observed previously for other heme proteins (18). It is noted that the frequencies of the vinyl stretching modes at 1622 and 1631 cm<sup>-1</sup> are not influenced by Ca<sup>2+</sup> depleting the deoxyvinyl group indicating that the orientation of the vinyl groups is unchanged.

The ν<sub>3</sub> band at 1503 cm<sup>-1</sup> together with the ν<sub>2</sub> band at 1574 cm<sup>-1</sup> (only the mean frequency can be given, due to overlap with the ν<sub>5</sub> modes of other heme species), ν<sub>19</sub> at 1576 cm<sup>-1</sup>, and ν<sub>10</sub> at 1638 cm<sup>-1</sup> are consistent with the QS heme state being the dominant heme form in Ca<sup>2+</sup>-depleted HRPC. The low frequency of the ν<sub>11</sub> mode at 1548 cm<sup>-1</sup> compared with the high frequencies observed for the other modes is a characteristic feature of QS states that had been noted previously (22–24) and is a further demonstration that the QS state is the major heme species. The predominant QS species co-exists with a 5-c HS (ν<sub>3</sub> at 1493 cm<sup>-1</sup>, ν<sub>11</sub> at 1553 cm<sup>-1</sup>, and ν<sub>10</sub> at 1631 cm<sup>-1</sup>) and a 6-c HS form (ν<sub>3</sub> at 1485 cm<sup>-1</sup> and ν<sub>10</sub> at 1622 cm<sup>-1</sup>). Moreover, excitation at 568.2 nm, in resonance with the α-band of a 6-c LS heme state, gives rise to spectral changes consistent with the presence of a LS species (ν<sub>11</sub> at 1560 cm<sup>-1</sup>, ν<sub>19</sub> at 1586 cm<sup>-1</sup>, and ν<sub>10</sub> at 1640 cm<sup>-1</sup>) with band frequencies higher than those of the QS state. However, the LS species is present in such a small amount that it cannot be detected in the electronic absorption spectrum. Additional evidence of a QS state in Ca<sup>2+</sup>-depleted HRPC is revealed by examination of previous NMR measurements (11). On the basis of recent reports (20, 27), the reduction in the mean hyperfine shift of the heme methyl resonances, noted in the previous NMR study for Ca<sup>2+</sup>-depleted HRPC compared with the native protein, can be correlated with the presence of a QS state.

It is worthwhile noting that the low frequency RR spectrum of Ca<sup>2+</sup>-depleted HRPC, obtained with excitation at 406.7 nm, is essentially unchanged with respect to that of native HRPC (data not shown). This spectral region is characterized by out-of-plane heme modes and the bending modes of the peripheral substituents, vinyl and propionate groups (18), indicating that removal of calcium does not have a significant effect on heme deformation. Furthermore, the close similarity of the electronic absorption spectra of native and Ca<sup>2+</sup>-depleted HRPC at pH 10.0 (data not shown) indicates that the behavior of the Ca<sup>2+</sup>-depleted protein at alkaline pH remains invariant compared with the native. At alkaline pH native HRPC binds a hydroxyl group giving rise to a 6-c LS heme species (28, 29), which has a pK<sub>a</sub> for the alkaline transition of 11.1 (30). Hence, the present data demonstrate that the pK<sub>a</sub> of the alkaline transition is effectively unchanged in the Ca<sup>2+</sup>-depleted protein.

**Carbon Monoxide and BHA Binding to Ca<sup>2+</sup>-depleted HRPC**—A study of ligand binding at the sixth coordination site of the heme iron can be an effective means of identifying differences between the distal heme cavities of native and Ca<sup>2+</sup>-depleted HRPC. The electronic absorption and RR spectra of the Fe(II)-CO complex of Ca<sup>2+</sup>-depleted HRPC at pH 7.0 (data not shown) show close similarities with the corresponding spectra of the native protein. This indicates that, as in the native protein, two conformers are formed in the CO complex of Ca<sup>2+</sup>-depleted HRPC corresponding to hydrogen bonding between CO and either the distal Arg or distal His residue (31).

The BHA molecule is a useful probe of the distal heme cavity as it enters into a number of hydrogen bond interactions with Arg<sup>436</sup>, His<sup>42</sup>, Pro<sup>339</sup> (32), and a distal water molecule coordinated to the heme iron, resulting in a 6-c QS complex (33, 34). The electronic absorption spectra of the native and Ca<sup>2+</sup>-depleted HRPC in the presence of saturating amounts of BHA are very similar (data not shown). The only difference of note is a slight blue shift of the spectrum of the Ca<sup>2+</sup>-depleted BHA complex compared with that of the native, particularly with respect to the CT1 band (4 nm). The high frequency RR spectra of the Ca<sup>2+</sup>-depleted and native BHA complexes are also very similar (data not shown); the minor differences evident can be explained in terms of a slightly different relative proportion of
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the two hexacoordinate states, 6-c HS and 6-c QS, which characterize the native BHA complex (19, 34). In fact, subtraction of the spectrum of the native complex from that of the Ca\(^{2+}\)-depleted form (data not shown) indicates an increased proportion of the 6-c HS state (\(v_3\) at 1486 cm\(^{-1}\), \(v_2\) at 1562 cm\(^{-1}\), \(v_9\) at 1579 cm\(^{-1}\), and \(v_{37}\) at 1615 cm\(^{-1}\) ) in the Ca\(^{2+}\)-depleted complex. An increased proportion of the 6-c HS species with respect to 6-c QS in the Ca\(^{2+}\)-depleted BHA complex compared with the native complex is also supported by the blue shift of the CT1 band. It has been reported previously (26) that the 6-c HS form of HRPC has a CT1 band significantly blue-shifted compared with the 6-c QS form. Furthermore, the low frequency RR spectra of the native and Ca\(^{2+}\)-depleted BHA complexes were found to be identical (data not shown). The finding of only small spectroscopic differences between the native and Ca\(^{2+}\)-depleted BHA complexes is reflected in the relatively small difference between the \(K_4\) values for the complexes (Table I).

**Electronic Absorption and RR Spectra of Ferrous Enzymes**—The electronic absorption spectra of the reduced Ca\(^{2+}\)-depleted HRPC at pH 7.8 and 9.6 are shown in Fig. 5, together with those of native ferrous HRPC (pH 6.7) and its imidazole (Im) complex (pH 10.1). The spectrum of Ca\(^{2+}\)-depleted HRPC at pH 7.8 has marked similarities with that of the HRPC-Im complex and is considerably different from that of the native which, between pH 4 and 10, is characterized by a 5-c HS heme species (35). Consequently, Ca\(^{2+}\)-depleted HRPC at pH 7.8 is mainly characterized by a bis-histidyl LS heme with a small amount of HS, as indicated by a shoulder at 586 nm. The transition to the LS species in Ca\(^{2+}\)-depleted HRPC is even more complete at pH 9.6. At pH values higher than 9.6 the protein began to undergo important changes upon excitation at 413.1 nm and the Fe-Im stretching mode can be derived from the near-absence of the band for excitation at 413.1 nm. This wavelength is out of resonance for the HS heme species (Soret maximum at 437 nm); hence, the Fe-Im stretching mode, which is strongly coupled to the Soret resonance (40), is considerably weakened. This effect is also evident from the reduced intensity of the 244 cm\(^{-1}\) \(\tau(\text{Fe-Im})\) mode of the native protein upon 413.1 nm excitation and has been observed in a recent study of Fe(II) catalase-peroxidase (41).

**DISCUSSION**

The two calcium ions and four disulfide bridges present in class II and III peroxidases are structural elements that have attracted much interest. The determination of their precise roles in maintaining the heme pocket structure, with consequent important implications for enzyme activity, offers significant scope for resolving questions of peroxidase structure-
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function properties and identifying potential biotechnological applications. The essential nature of these features is, in fact, clearly revealed by Ca$^{2+}$ depletion of HRPC. Both the present and previous (10, 11) work on Ca$^{2+}$- depleted HRPC demonstrate that removal of calcium leads to profound changes in enzyme activity and electronic structure of the heme iron. In all cases the specific activity is reduced to ~50% that of native after calcium removal despite that, in the present study, only one Ca$^{2+}$ ion is lost upon Ca$^{2+}$ depletion, whereas both Ca$^{2+}$ ions were reported to be lost in the previous work. Nevertheless, it is clear that Ca$^{2+}$ ions are necessary for optimum catalysis but are not essential for low levels of activity. Similar behavior was found for another class III peroxidase upon loss of both Ca$^{2+}$ ions, peanut peroxidase (42), whereas the class II peroxidases MNP and LIP were rendered inactive following loss of either one or two Ca$^{2+}$ ions after a less drastic thermal or alkaline pH treatment, respectively (6–8). Such contrasting behavior between proteins of the two peroxidase classes, in which the Ca$^{2+}$ ions appear indispensable for activity in class II peroxidases, may be due to the different locations of the disulfide bridges in the two classes. In the specific cases of HRPC and LIP, it has been noted that the cysteine present in distal helix B of HRPC can form a disulfide bridge and limit flexibility of the helix. Formation of such a disulfide bridge is not possible in LIP, which thus favors binding of the distal His residue in LIP to the heme iron after calcium release forming an inactive LS species (7). Furthermore, the close proximity of this disulfide bridge (Cys$^{44}$-Cys$^{49}$) to the distal calcium site may have a stabilizing influence on the local structure, absent in LIP, hindering loss of the distal Ca$^{2+}$ ion in HRPC. The foregoing considerations find support in a recent study of MNP (43). The insertion of an additional disulfide bond close to the distal Ca$^{2+}$ ion-binding site resulted in an apparent increase in the stability of the heme environment and active state of the protein. The particular significance of the Ca$^{2+}$ ions and disulfide bridges to protein stability has been underlined by a comparative study of HRPC and the class I peroxidase cytochrome c peroxidase in the presence of denaturants (44). The considerably lower kinetic stability found for cytochrome c peroxidase was attributed to the absence of these two stabilizing structural elements.

The details of the local structure in the proximity of the distal and proximal Ca$^{2+}$ ion-binding sites are now available from the x-ray structure determination of native HRPC (Fig. 1) (4). Both Ca$^{2+}$ ion sites are seven-coordinate with ligands composed of side chain oxygens and carbonyl groups and one water molecule in the case of the distal site. The distal and proximal Ca$^{2+}$ ions are structurally coupled to the active site (His$^{42}$ and His$^{170}$, respectively) through an intermediate residue adjacent in sequence (Asp$^{43}$ and Thr$^{171}$, respectively). Each of these intermediate residues provides two bonds to the corresponding Ca$^{2+}$ ion; therefore, the loss of either Ca$^{2+}$ ion has considerable potential to perturb the active site structural and catalytic properties. Nevertheless, there have been some indications from previous work that one of the two Ca$^{2+}$ ions may play a more essential role in maintaining the heme pocket structural characteristics necessary for high catalytic activities (11, 45). The observation of NMR spectral changes upon titration of Ca$^{2+}$ ions to Ca$^{2+}$-depleted HRPC, where both Ca$^{2+}$ ions were reported lost, indicated that one Ca$^{2+}$ ion was determinant for the protein structure (11, 45). A similar Ca$^{2+}$ ion titration NMR study of the Ca$^{2+}$-depleted (with loss of both Ca$^{2+}$ ions) class III peanut peroxidase, was inconclusive concerning the relative contribution of each Ca$^{2+}$ ion site to the structural properties and activity of the enzyme (46). Reconstitution of Ca$^{2+}$-depleted HRPC with $^{45}$Ca demonstrates that in the present study only one Ca$^{2+}$ ion is lost upon Ca$^{2+}$ depletion. However, as will become particularly evident from the following discussion, the structural properties of both the distal and proximal sides of the heme are modified by Ca$^{2+}$ depletion of HRPC. To shed further light on the question of which Ca$^{2+}$ ion is lost from HRPC upon Ca$^{2+}$ depletion, the evidence that favors either the distal or proximal Ca$^{2+}$ ion is now discussed on the basis of the body of currently available data for HRPC.

The present study clearly reveals that, contrary to the native protein, a bis-histidyl LS heme is formed in reduced Ca$^{2+}$-depleted HRPC. This indicates, as suggested by a previous study (11), that loss of calcium has induced a conformational change in the heme pocket. This leads to alterations of the distal side heme structure enabling the imidazole group of the distal histidine to bind to the sixth coordination position of the reduced heme iron. It is noteworthy that such effects are not
noted in the ferric Ca\textsuperscript{2+}-depleted form. In fact, the behavior of the ferric enzyme at alkaline pH appears to follow closely that of native HRPC, indicating that hydroxyl binding to the heme iron is favored over coordination of the distal His and, hence, that the ferric distal pocket is not extensively perturbed by Ca\textsuperscript{2+} depletion. The formation of a bis-histidyl LS heme following Ca\textsuperscript{2+} depletion has been noted for LIP (with loss of either only the distal Ca\textsuperscript{2+} ion or both Ca\textsuperscript{2+} ions) (6, 7) and MNP (upon loss of the distal Ca\textsuperscript{2+} ion) (8, 9); however, in these cases the LS species was observed in both the ferric and ferrous states. The presence of a bis-histidyl LS heme in Fe(II) Ca\textsuperscript{2+}-depleted HRPC apparently favors the case of loss of the distal Ca\textsuperscript{2+} ion, as proposed for MNP (8). Conversely, the similarities of the Fe(III) Ca\textsuperscript{2+}-depleted properties at alkaline pH to those of the native protein suggest a relatively minor disturbance of the distal cavity occurs upon Ca\textsuperscript{2+} depletion. If one recalls that LS heme is formed in MNP (47) and Fe(II) HRPC (27) following mutation of the proximal Phe residue, which is stacked approximately parallel to the proximal histidine ligand, it is evident that proximal effects can be transmitted to the distal cavity inducing some flexibility in the position of the distal histidine. It is therefore suggested that the contrasting observations noted for the ferric and ferrous states can be reconciled if the proximal Ca\textsuperscript{2+} ion is lost from HRPC upon Ca\textsuperscript{2+} depletion. The example of the proximal Phe\textsuperscript{221}–Met HRPC mutant is particularly appropriate as it mimics in many ways the behavior of the Ca\textsuperscript{2+}-depleted enzyme. In common with Ca\textsuperscript{2+}-depleted HRPC, the ferric mutant protein at neutral pH is characterized by a dominant QS heme state (see below) and the ferrous state by the presence of a significant proportion of LS heme. Nevertheless, the spectral characteristics of the ferrous CO and ferric BHA adducts closely resemble those of the native. Analogous behavior is observed for Ca\textsuperscript{2+}-depleted HRPC in the presence of CO and BHA, suggesting that modification of the distal cavity by Ca\textsuperscript{2+} depletion is not particularly extensive and/or can be easily reversed. Furthermore, it provides evidence that strongly favors loss of the proximal Ca\textsuperscript{2+} ion as the origin of these distal effects.

A particularly relevant study in the context of the present discussion is that of the Glu\textsuperscript{64} HRPC mutants (48). The Glu\textsuperscript{64} residue is linked to the distal Ca\textsuperscript{2+} ion through a hydrogen bond with a water molecule. Changes in the position of the distal His and Arg residues in the mutants, leading to a substantial depression of the catalytic activity, was attributed to dissociation of the distal Ca\textsuperscript{2+} ion. Interestingly, despite loss of the distal Ca\textsuperscript{2+} ion, the geometry of the heme in the Glu\textsuperscript{64} mutants was found to be very similar to the native protein, and there was only minor perturbation around the proximal His. Hence, the structural properties of the Glu\textsuperscript{64} mutants have little in common with those displayed upon Ca\textsuperscript{2+} depletion, where completely contrasting behavior is observed, the distal cavity suffering only minor changes whereas the proximal cavity is considerably perturbed (see below). Consequently, this supports loss of the proximal Ca\textsuperscript{2+} ion in Ca\textsuperscript{2+}-depleted HRPC. Moreover, it suggests that the distal Ca\textsuperscript{2+} ion is of limited importance and that the proximal Ca\textsuperscript{2+} ion is responsible for retaining the heme geometry and coordination structure of the proximal ligand.

The marked downshift (27 cm\textsuperscript{−1}) of the Fe-Im stretching frequency observed in reduced Ca\textsuperscript{2+}-depleted HRPC compared with the native indicates a significant weakening of the bond between the iron atom and the proximal histidine residue. This reflects a marked change in the strength of the hydrogen bond between the conserved proximal His and Asp residues characteristic of peroxidases, which imparts a pronounced imidazolate character to the proximal His and results in higher v(Fe-Im) frequencies than found for other heme proteins (39). Furthermore, it demonstrates that substantial structural changes of the heme environment in the vicinity of the proximal histidine are induced by Ca\textsuperscript{2+} depletion. In fact, the Fe-Im stretching frequency occurs at 220 cm\textsuperscript{−1} in myoglobin, where the proximal His is hydrogen-bonded with a neutral peptide carbonyl group (49), and at 196 cm\textsuperscript{−1} in the Fe\textsuperscript{III} protoporphyrin IX histidine complex, where the proximal His is not involved in hydrogen bonding interactions (50). In this context, it should be recalled that the residue adjacent to the proximal His (Thr\textsuperscript{171}) provides two coordination bonds to the proximal Ca\textsuperscript{2+} ion (Fig. 1) (4); hence one might reasonably expect that loss of this Ca\textsuperscript{2+} ion would cause a change in the disposition of the histidine leading to a weakening of the His–Asp hydrogen bond and, therefore, of the heme iron-His\textsuperscript{770} interaction.

Native Fe(III) HRPC is characterized at room temperature by a QS state having a very low level of intermediate-spin admixed with the high-spin state (19–21). In ferric Ca\textsuperscript{2+}-depleted HRPC at pH 7.8, however, the pentacoordinate QS state becomes more fully expressed compared with the native protein, having a greater relative proportion of IS compared with HS in the quantum mechanical admixture, as demonstrated by the higher frequencies of the core size marker bands of the Ca\textsuperscript{2+}-depleted form. It constitutes, together with a 5-c HS form, one of the two major heme states of Ca\textsuperscript{2+}-depleted HRPC; 6-c HS and 6-c LS states are also present at very low levels. The appearance of the QS state is particularly interesting. This heme species is uncommon in biological systems. Known examples include the neutral pH form of cytochrome c‘ (51, 52) and class III peroxidases (18, 23). Unfortunately, the biological significance and structural properties of the heme that give rise to the QS state remain obscure (24). Hence, the presence of this unusual heme state cannot be exploited as a means to uncover subtle modifications to the heme environment associated with Ca\textsuperscript{2+} depletion. In this regard, however, a recent report (27) revealed that the proximal Phe\textsuperscript{221} residue of HRPC also produces a QS heme state, whereas the many distal mutants of HRPC that have been characterized lack such a state (18). Therefore, the appearance of a QS heme state in Ca\textsuperscript{2+}-depleted HRPC in concomitance with a significant weakening of the Fe-Im bond suggests that disturbance of the proximal, rather than distal, heme domain may be more important in the generation of this heme state. It is noted that, in contrast to the Ca\textsuperscript{2+}-depleted protein, in the case of the Phe\textsuperscript{221} mutant a strengthening of the Fe-Im bond was observed. This was attributed to the loss of the π-π interaction between the approximately parallel stacked aromatic rings of the His\textsuperscript{770} and Phe\textsuperscript{221} residues, reduction in the steric constraints imposed on the proximal His residue and, consequently, a modification of the His disposition.

In summary, evaluation of the experimental data available for HRPC, both from this study and previous work, firmly favors the proximal Ca\textsuperscript{2+} ion as that which is lost upon Ca\textsuperscript{2+} depletion and which likely plays the more critical role in regulating the heme pocket structural and catalytic properties.

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