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Co-dependency of calcium and porphyrin for an integrated molecular structure of peanut peroxidase: A circular dichroism analysis

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The contribution of calcium to the structure of cationic peanut peroxidase was examined using ultraviolet/visible and circular dichroism spectroscopies under conditions in which the 2 moles of Ca^{2+} bound per mole of enzyme were removed. Cadmium and terbium ions were used as substitutes for calcium in the calcium depleted peroxidase and their influence on the protein structure was examined spectroscopically and compared to native and heme depleted enzymes. A role for the calcium ions in maintaining the active conformation of the peroxidase is proposed.

Peroxidase (EC 1.11.17) is a hemeprotein that contains glycan chains and calcium (1). CPRx has 2 moles Ca^{2+}/mol protein (2). Calcium has recently been recognised as a key component in the peroxidatic cycle in plant peroxidases (3). It has been observed that calcium levels as high as 10 mM are required in solutions to conserve enzymatic activity (4), as well as to stabilize the tertiary structure of the peroxidase (5). The variations in Reinheitzahl index (Rz) when Ca^{2+} was removed from CPRx suggested that Ca^{2+} ions were essential in maintaining the protein structure in the vicinity of the heme group. Substitution of the Ca^{2+} by Cd^{2+} does not affect enzymatic activity (5), however, like Ca^{2+}, Cd^{2+} is not a spectroscopically useful probe. Replacement of Ca^{2+} by lanthanide ions (Ln^{3+}) can provide a useful spectroscopic tool in the study of the Ca^{2+} binding site in proteins because the chemical and physical properties of Ln^{3+} ions are similar to those of Ca^{2+} (3). In the present study we examine the conformation of cationic peanut peroxidase using UV/visible

Abbreviations:
CPRx: cationic peanut peroxidase; CaD-CPRx: Calcium depleted CPRx; Apo-CPRx: Heme and calcium depleted CPRx; CaR-CPRx: calcium dialyzed CaD-CPRx; CdR-CPRx: cadmium dialyzed CaD-CPRx; TbR-CPRx: terbium dialyzed CaD-CPRx; Rz: Reinheitzahl index, defined as A_{407nm}/A_{280nm}, SA: specific activity; CD: circular dichroism spectroscopy; EDTA: N,N'-bisethylenediamine-tetraacetic acid, disodium salt; HRP: horse radish peroxidase.

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absorption and CD spectroscopies in presence of Ca^{2+} ions, after their removal, and with subsequent reconstitution with Ca^{2+}, Cd^{2+} or Tb^{3+}.

METHODS

Plant materials Cell suspension cultures of peanut (*Arachis hypogaea* L.) were maintained in a modified Linsmaier and Skoog medium as described (2).

Purification of cationic peanut peroxidase The cationic peanut peroxidase (CPRx) was purified to a Reinheitzahl index [A407/A280] = 3.0 (6). The purified samples were stored in lyophilized state at 4^0C (4). 20 μg purified CPRx showed a single 40 kDa protein band in SDS/PAGE.

Preparation of calcium depleted cationic peanut peroxidase Calcium depleted cationic peanut peroxidase (CaD-CPRx) was prepared by treating CPRx either (i) with 6 M guanidine hydrochloride-10 mM EDTA, pH 7.0 for 4 h at room temperature, followed by dialysis against 5 mM EDTA, pH 7.0 overnight and subsequent exhaustive dialysis against double distilled water (7), or (ii) by exhaustive dialysis against 50 mM EDTA pH 7.0, or (iii) by exhaustive dialysis against double distilled water for 72 hours.

Reconstitution of CaD-CPRx Ca^{2+}, Cd^{2+} or Tb^{3+} were incorporated into CaD-CPRx by overnight dialysis against 10 mM of either calcium chloride at pH 6.2, or cadmium acetate at pH 6.6, or terbium nitrate at pH 5.8. Excess free Ca^{2+}, Cd^{2+} or Tb^{3+} was removed by filtration through a 5 mL Amberlite MB-3 or DP-1 column.

Peroxidase activity Enzymatic activity was determined using guaiacol as substrate (5). The change in absorbance at 470 nm was monitored on a Shimadzu UV-160 spectrophotometer.

Preparation of the heme depleted CPRx The heme was removed from the purified CPRx according to the cold acidic acetone method (8).

Atomic absorption spectroscopy The calcium content of the holoenzyme and the CaD-CPRx was determined by atomic absorption spectroscopy using a Varian Spectra AA 30. Protein samples were diluted in 0.1% $CsCl_2$ as an ionization suppressant.

UV/Visible spectroscopy Absorption spectra were digitized from a Cary 219 spectrometer at room temperature. Graphs derived from digital data are shown in the figures.

Circular Dichroism spectroscopy CD spectra were recorded on a Jasco J-500C spectropolarimeter controlled by the program CDSCAN operating on an IBM 9001 computer (9). The data were manipulated with the program Spectra Manager (10).

RESULTS AND DISCUSSION

We describe the variations in the structure of CPRx in the presence and absence of Ca^{2+} from analysis of UV/visible and circular dichroism spectra.

The calcium depleted enzyme, CaD-CPRx, made by each of the three methods exhibited similar Ca^{2+} content measured by atomic absorption spectroscopy (Table I). CaD-CPRx made by dialysis against water exhibits a reduced specific activity (SA) of 45% of the value measured for the native enzyme, while the Rz value decreases by 10% (Table II). Both dialysis against 50 mM EDTA (pH 7.0) or 6 M guanidine hydrochloride/10 mM EDTA (pH 7.0) for 72 h, (7) caused a decrease of 60% in the SA and a 40% decrease in the Rz value. Dialysis against 50 mM EDTA at pH 7.0 was chosen for all following experiments. Figure 1 shows the absorption and CD spectra of CPRx, CaD-CPRx and Apo-CPRx. The absorption maxima for the native CPRx at 635.0 (not shown), 499.5 and 405.5 nm in the

Table I. Calcium contents of native and modified cationic peanut peroxidase

FRACTION	μmol Ca^{2+}/μmolCPRx
CPRx (native)	**2.2 ± 0.1**
CaD-CPRx (ddH_2O)	**0.6 ± 0.1**
CaD-CPRx (EDTA)	**0.5 ± 0.1**
CaD-CPRx (Guanidine HCl/EDTA)	**0.7 ± 0.1**
Apo-CPRx	**0.3 ± 0.1**

The different proceedings used for calcium removal from CPRx were noted between brackets. CaD-CPRx reconstituted by dialysis against Cd2+ or Tb3+ had no calcium content. The Ca2+ reconstituted CaD-CPRx had slightly higher calcium level when compared to those of the native CPRx.

visible, and 278.0 and 217.0 nm (not shown) in the UV regions are characteristic of high spin ferric heme proteins (Figure 1a) (11). The native CPRx exhibits a CD spectrum characterized by negative bands at 540, 489, 458, 375, 340, 280 and 245 nm, and positive bands at 594, 507, 470, 428, 407, 306 and 258 nm (Figure 1b). Removal of Ca^{2+} (CaD-CPRx) changes the UV/visible absorption spectrum, increasing the absorbance between 250-280 nm while $A_{407\ nm}$ decreases only slightly (Figure 1a). Its CD spectrum shows a dramatic reduction in the intensity of the negative band located at 245 nm in CPRx, which is shifted to 252 nm in CaD-CPRx. The increase and broadening of the CD positive band at 407 nm and the appearance of two negative bands at 528 and 557 nm reflect changes in the spin state of the porphyrin iron (Figure 1b). CD bands near 286 nm arise from the heme, while

Table II. Reinheitzahl index and Specific Activity of native cationic peanut peroxidase, calcium depleted enzyme, and Ca^{2+}, Cd^{2+}, and Tb^{3+} reconstituted samples

FRACTION	Rz	Rz*	SA	SA*
CPRx (native)	3.1	3.0	1028	1007
CaD-CPRx (ddH_2O)	2.9	2.2	553	459
CaD-CPRx (EDTA)	2.1	1.8	414	409
CaD-CPRx (Guanidine HCl/EDTA)	1.8	1.7	432	415
CaD-CPRx (Ca_{2+})	2.6	2.6	774	770
CaD-CPRx (Cd_{2+})	2.8	2.7	998	990
CaD-CPRx (Tb_{3+})	1.9	0.9	528	173

The samples were prepared as described in the Methods section. The specific activity is expressed in Δ 470 nm.mg $CPRx^{-1}.min^{-1}$.
* Values were taken a week after Ca^{2+} removal or replacement with different cations. The proceedings used to remove Ca^{2+} form CPRx and the cations employed to replace calcium are listed between brackets.

bands located at 278 nm are due to tyrosyl residues. Tryptophanyl and phenylalanyl residues exhibit CD bands at 283 and 291 nm for the former and 262 and 268 nm for the latter (11). Therefore, the increase in absorbance and coupled decrease in CD intensity at 245, 262 and 278 nm reflects a change in the orientation of tyrosyl or phenylalanyl residues within the protein, which points to a role for Ca^{2+} in maintaining the conformation of the peroxidase, that in turn controls the environment of the heme. The CD spectral intensity under heme transitions, depends directly on the dissymmetric environment of the heme within the peptide structure. The CD intensity provides an additional marker for both the presence of the heme group and the environment of the heme pocket formed by the peptide chain. Apo-CPRx exhibits a positive CD band at 256 nm and a series of small negative CD bands with maxima at 268, 276 and 283 nm which are due to the transitions of the aromatic aminoacids. The decrease in $A_{286\ nm}$ in the apoenzyme confirms that this band is due to the heme; consequently, there is no CD intensity above 330 nm because of the loss of the heme chromophore.

Overnight dialysis of CaD-CPRx against 10 mM calcium chloride at pH 6.4 or against cadmium acetate at pH 6.6 restored the SA and Rz values to near those of the native (Table II). The Ca^{2+} and Cd^{2+} reconstituted CaD-CPRx exhibited absorption spectra similar to the native CPRx, with maxima at 636.0, 502.5, 407.0, 278.0 and 215.0 nm (Figure 2a). The CD spectrum of the Cd^{2+} reconstituted enzyme is in good agreement with the one of CPRx; a small shoulder located at 276 nm could be attributed to changes in the orientation of tyrosyl residues within the protein. The CD spectrum of the Ca^{2+} reconstituted enzyme exhibits major differences in comparison to CPRx: the 376 nm negative CD band is shifted to 394 nm in CaR-CPRx, and the heme peak located at 407 nm is displaced to 424 nm.

The CaD-CPRx was dialyzed against 10 mM $Tb(NO_3)_3$ at pH 5.4 for 24 h and both UV/visible and CD spectra were recorded. Major differences between the native CPRx and Tb^{3+} dialyzed CaD-CPRx can be seen in the UV/visible absorption spectrum (Figure 2a). Introduction of terbium reduced absorption of the Soret band by 70%: clearly, the heme was displaced as the absorbance at 407 nm decreased. The Rz and the SA of the Tb^{3+}-replaced enzyme were reduced to 50% and 60% of the native values, and decreased further to 30% and 17% of initial ones a week after the dialysis as anticipated from interpretation of the spectral data (Table II). These changes are also reflected by the CD spectrum (Figure 2b). Treatment of the peroxidase with terbium led to the denaturation of the protein in a time dependent process. When the dialysis was extended to 72 h the CD intensity was quenched throughout the spectral region indicating that not only did the lanthanide ion not replace Ca^{2+}, but it caused major alterations in the vicinity of the heme group (data not shown). The decrease in the Rz and SA values is attributable to the terbium insertion reaction within

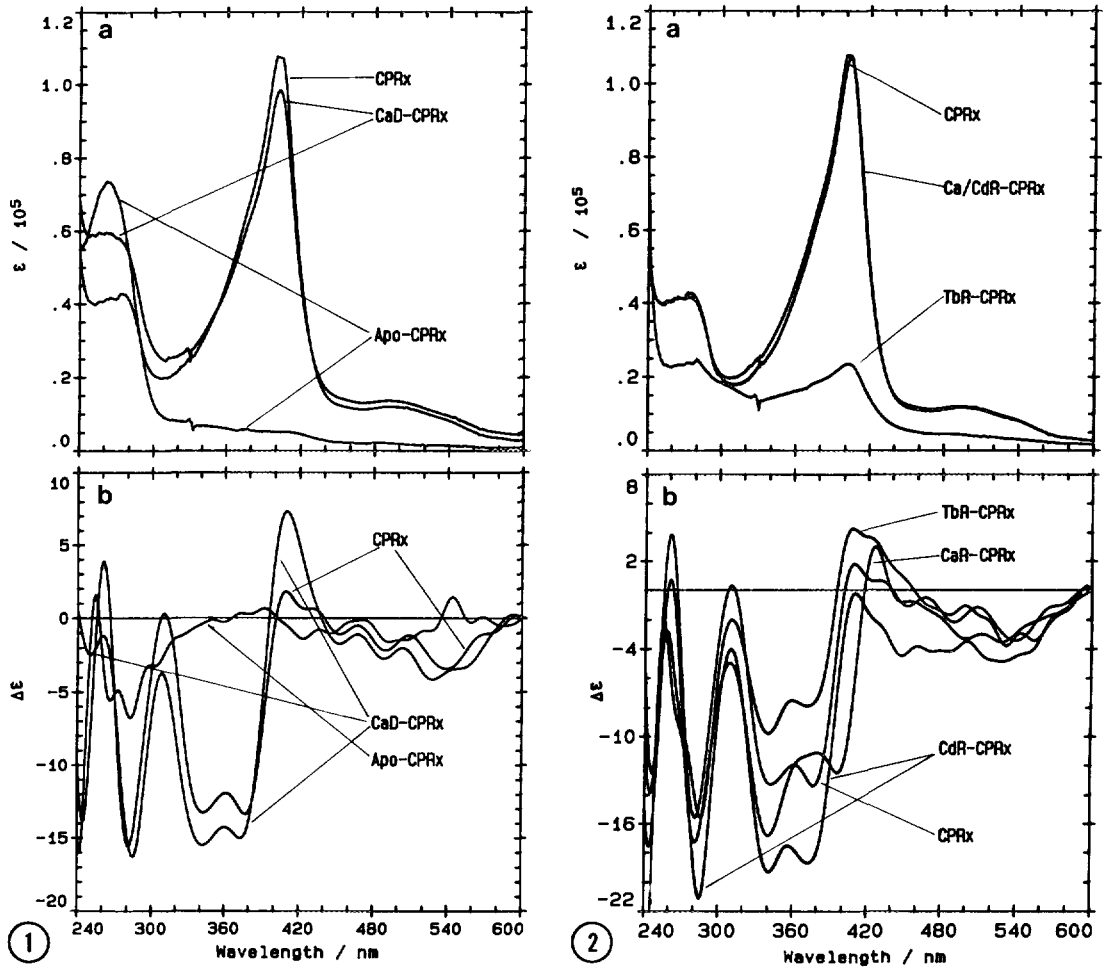

Figure 1. UV/visible absorption (**1a**) and circular dichroism (**1b**) spectra of cationic peanut peroxidase (CPRx), calcium depleted CPRx (CaD-CPRx) and heme and calcium depleted CPRx (Apo-CPRx).

Figure 2. UV/visible absorption (**2a**) and circular dichroism (**2b**) spectra of cationic peanut peroxidase (CPRx), and CaD-CPRx dialyzed against either 10 mM $CaCl_2$ (CaR-CPRx), $Cd(C_2H_3O_2)_2$ (CdR-CPRx), or $Tb(NO_3)_3$ (TbR-CPRx). Ca/CdR-CPRx identifies either the Ca^{2+} or the Cd^{2+} replaced CaD-CPRx.

the peroxidase and is not due to the removal of Ca^{2+} alone, since the CaD-CPRx maintained comparable Rz and SA values after the same period of time.

The above results confirm our earlier hypothesis (4) that endogenous Ca^{2+} binding is essential for maintenance of the environmental structure in the region of the heme and that the catalytic property of CPRx is regulated by the presence of 2 moles of bound Ca^{2+} (12). Reconstitution with Tb^{3+} results in major conformational changes which affect both the CPRx heme group and the polypeptide chain structures. This is in sharp contrast to the effect of substitution of Ca^{2+} with Tb^{3+} ions in proteins such as calmodulin or troponin C

(13,14). Our finding that substitution of Ca^{2+} by Tb^{3+} is not possible without disruption of the structural integrity of the peroxidase is in good agreement with data for HRP which suggested that the first metal-binding site in the peroxidase is structurally different from that of typical Ca^{2+}-binding proteins (3). Previous work (5,12) showed that Ca^{2+} ions could not be replaced by divalent cations with different ionic radii (Mg^{2+} 0.65 Å, Hg^{2+} 1.10 Å, and Pb^{2+} 1.20 Å did not bind) and we may assume that the metal ion binding in CPRx is quite specific. CPRx Ca^{2+} ions, on the other hand, can be replaced successfully by Cd^{2+} ions, probably due to the close similarity of their ionic radii (0.97 Å for Cd^{2+} and 0.99 Å for Ca^{2+}). In contrast to HRP where the endogenous 2 moles of Ca^{2+} can be removed from the enzyme only by incubation in the presence of metal chelator and denaturant (7), removal of endogenous Ca^{2+} from CPRx is easily achieved in the presence only of the metal chelator. This suggest that Ca^{2+} ions are not deeply buried within the protein but located on the CPRx periphery. The relative ease of removal of Ca^{2+} from CPRx indicates also that this cation is weakly bound, and may suggest the participation of tyrosyl phenoxide rather than carboxylate of glutamic and/or aspartic acids, which are expected to form stronger bonds with divalent cations such as calcium (1).

It has been suggested that calcium plays a key role in the peroxidatic cycle, since Ca^{2+}-free HRP is in a thermal spin mixing between ferric high spin and low spin states (15). The broadening of the peak at 407 nm in CaD-CPRx could be explained in terms of this hypothesis. In contrast to HRP, which has only a peak at 404 nm, a CPRx solution soon shows, in addition to the peak at 407 nm, the appearance of a second peak located at 428 nm. This second peak could be assigned to low spin iron by comparison with HRP (16), and its presence may be attributed to the conversion between iron spin states which is increased after calcium removal. Ca^{2+} removal and subsequent reconstitution either with Ca^{2+} or Cd^{2+} seem to displace the equilibrium towards one form or the other.

In summary, our results indicate that while the optical data for native CPRx are quite similar to those of HRP isozyme C (11), which indicates that these two peroxidases are closely related structurally, the major differences in the intensities of the CD bands at 340 and 370 nm suggest that heme environments in these two peroxidases are not the same. Further comparisons are needed to clarify the structural differences between CPRx and HRP isozyme C. The successful replacement of Ca^{2+} by Cd^{2+} make the $^{113}Cd^{2+}$ enriched CPRx provide an ideal subject for ^{113}Cd NMR and ^{1}H NMR studies, which will permit us to understand the position and role calcium ions have in peroxidases.

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