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Reversible thermal inactivation and conformational states in denaturant guanidinium of a calcium-dependent peroxidase from *Euphorbia characias*

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This paper is dedicated to Prof. Augusto Rinaldi, Faculty of Medicine and Surgery, University of Cagliari, who passed away on October 7, 2005.

Abstract

The changes in the heme environment and overall structure occurring during reversible thermal inactivation and in denaturant guanidinium of *Euphorbia characias* latex peroxidase (ELP) were investigated in the presence and absence of calcium ions. Native active enzyme had an absorption spectrum typical of a quantum-mixed spin ferric heme protein. After 40 min at 60 °C ELP was fully inactivated showing the spectroscopic behavior of a pure hexacoordinate low-spin protein. The addition of Ca^{2+} to the thermally inactivated enzyme restored its native activity and its spectroscopic features, but did not increase the stability of the protein in guanidinium. It is concluded that, in *Euphorbia* peroxidase, Ca^{2+} ion play a key role in conferring structural stability to the heme environment and in retaining active site geometry.
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Keywords: Peroxidase; *Euphorbia characias*; Thermal inactivation

1. Introduction

Heme-containing peroxidases (EC 1.11.1.7) are a widely distributed group on enzymes found in bacteria, fungi, plants, and animals, that utilize hydrogen peroxide, or other peroxides, to oxidize a second reducing substrate belonging to a large array of organic and inorganic compounds [1]. These enzymes can be divided into three classes, on the basis of amino acid sequence comparisons: class I contains bacterial, fungal and plant intracellular enzymes from the mitochondria and chloroplasts; class II consists of secretory fungal peroxidases such as manganese peroxidase and lignin-degrading peroxidases; class III is made of secreted plant peroxidases. In plants, heme peroxidases are found in the cytosol, vacuole, apoplast or cell wall, and are involved in the regulation of cell growth and differentiation, cell wall lignification, metabolism of hormones and alkaloids, wound healing and defense against pathogen infection [2–4]. Typically, class III peroxidases may exist under an extremely high number of isoforms within the same species, potentially implicated in different functions [5]. For example, the peroxidase isoenzyme family of *Arabidopsis thaliana* comprises over 70 full-length genes, most of which predicted to encode for stable enzymes [6,7].

Recently we isolated a class III cationic peroxidase from the latex of the Mediterranean shrub *Euphorbia characias* and characterized its main biochemical features [8]. The purified enzyme contains a ferric iron-protoporphyrin IX in a quantum mechanically mixed-spin state, pentacoordinated with a histidine "proximal" ligand. This unusual heme spin state, resulting from an admixture of high-spin and intermediate spin, is apparently limited to class III plant peroxidase, and its generation seems to be correlated to the heme out-of-plane distortions observed in these proteins [9,10]. Alike horseradish peroxidase and other secretory plant peroxidases, *Euphorbia* latex peroxidase (ELP)

Abbreviations: ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); 1,8-ANS, 1-anilinonaphthalene-8-sulfonic acid; CD, circular dichroism spectroscopy; EGTA, ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ELP, *Euphorbia* latex peroxidase; GdHCl, guanidinium chloride; PPIX, protoporphyrin IX; Tris, tris-(hydroxymethyl)aminomethane

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has two calcium binding sites, respectively proximal and distal to the heme. Whereas, the proximal Ca^{2+} ion is strongly bound and believed to play a critical role in retaining the active site geometry, the distal Ca^{2+} is in a low affinity-binding site but is necessary for expression of full enzyme activity, which is at variance with other known plant peroxidases [8]. Interestingly, the *Euphorbia* enzyme has low specific activity for classical peroxidase substrates as compared to horseradish and other reference peroxidases, and its activity seems to be more strictly dependent on the concentration of Ca^{2+} ions in the reaction medium.

In order to get more insights into the structural versus catalytic role of calcium ions in the peculiar ELP, we characterized the changes in the heme environment and at the whole enzyme level that occurred during protein reversible thermal inactivation, both in the presence and absence of calcium ions. The conformational stability of *Euphorbia* peroxidase in guanidinium chloride (GdHCl) as a denaturant was also investigated to probe the effects of Ca^{2+} as additional stabilizing element.

2. Experimental

2.1. Enzyme purification and chemicals

E. characias latex drawn from cut branches was collected at several locations in southern Sardinia (Italy), immediately frozen in liquid N_2 and stored at −80 °C until use. ELP (RZ value = 2.6; M_r = 47 kDa) was purified from crude latex as previously described [8]. 1-Anilinonaphthalene-8-sulfonic acid (1,8-ANS), 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and guanidinium chloride were purchased from Sigma (Chemical Co., St. Louis, USA). Hydrogen peroxide was from Merck (Darmstadt, Germany), and an ε_{240} = 43.6 M^{-1} cm^{-1} was used to estimate its concentration. All chemicals were obtained as pure commercial products and used without further purification.

2.2. Metal and heme content

The presence of metals was measured by atomic absorption using a Unicam 969 AA Solar spectrometer (Bournemouth, Dorset, UK). Heme content was determined by measuring the oxidized and reduced forms of the pyridine hemochromogen derivative, assuming an absorption coefficient of $\Delta\varepsilon_{541}$ (for the dithionite-reduced spectrum) − $\Delta\varepsilon_{557}$ (for the ferricyanide-oxidized spectrum) = 20.7 mM^{-1} cm^{-1}.

2.3. Peroxidase activity

ELP activity was measured in 100 mM Na-acetate buffer, pH 5.75, at 25 °C, in the presence of hydrogen peroxide (25 mM) and of the reducing-substrate ABTS (10 mM), following the increase in absorbance at 415 nm resulting from the formation of the ABTS cation radical product (ε_{415} = 36 mM^{-1} cm^{-1}). Activity was calculated in standard enzyme units (μmol min^{-1} mg^{-1}), and catalytic-centre activity (k_c) was defined as mol of substrate consumed per mol of active sites in 1 s. The effects of Ca^{2+} ions on ELP activity were examined performing the reaction in buffers with and without given amounts of $CaCl_2$ (0.5–10 mM).

2.4. Thermal inactivation

Temperature-induced inactivation of ELP was monitored as follows: stock protein solutions (3 μM) were incubated in 100 mM Tris/HCl buffer, pH 7.25, or in Na-acetate buffer, pH 5.75, in the presence of the metal chelator EGTA (0.2 mM) in a water bath at the desired temperatures. At given time intervals, aliquots of enzyme mixture were taken and assayed for peroxidase activity at 25 °C as described above. The activity was expressed as a percentage of remaining activity. Thermal inactivation of ELP was also monitored both in the presence and absence of Ca^{2+} ions (from 0 to 10 mM). Reactivation of the enzyme was obtained by adding $CaCl_2$ (0.5–10 mM) to the thermally inactivated enzyme solution at 25 °C. The temperature-induced conformational changes of ELP were monitored using a wide range of spectroscopic techniques. Protein solutions (3 μM) in 100 mM Tris/HCl buffer, pH 7.25, or in Na-acetate buffer, pH 5.75, were prepared in vials and incubated in a water bath at the desired temperatures, in the presence of 0.2 mM EGTA. At given time intervals, some vials were brought to 25 °C and the absorption and fluorescence spectra recorded using 1 cm path length cells with a Ultrospec 2100 spectrophotometer (Biochrom Ltd., Cambridge, England) and with a Perkin-Elmer LS-3 spectrofluorimeter (Perkin-Elmer Ltd., Buckinghanshire, England), respectively. To test the reversibility of inactivation, solutions of thermally inactivated ELP were incubated at 25 °C for 12 h in Tris/HCl and Na-acetate buffers and in the presence of 10 mM Ca^{2+} ions, and the spectra recorded as above. Aliquots of native and thermally inactivated ELP (0.1–0.5 μM) in Tris/HCl buffer were also incubated with 1,8-ANS (50 μM) for 15 min, and fluorescence emission spectra (excitation 374 nm) recorded.

In another set of experiments, circular dichroism (CD) spectra were measured with a Jasco J-715 spectropolarimeter (Jasco Ltd., Japan) equipped with an ETC thermal control unit. ELP solutions (0.8 mg mL^{-1}) in 100 mM Tris/HCl buffer, pH 7.25, were placed in a strain free 0.1 mm quartz cuvette and spectra recorded between 260 and 190 nm with the following set up: bandwidth 2 nm, time constant 2 s, scan rate 20 nm min^{-1}, N_2 purging rate 25 L min^{-1}. Near-UV spectra were also measured between 260 and 350 nm on the same protein solutions using 1 cm path length cuvettes. Four spectra corrected for background (buffer and salt) were averaged and successively smoothed with eight points Savisky Golay smoothing procedure. Spectra were measured at 20 and 60 °C.

2.5. Chemical denaturation

ELP conformational changes induced by chemical denaturation were also analyzed by absorption and fluorescence spectroscopy. Stock protein solutions (2 μM) were prepared in 100 mM Tris/HCl buffer, pH 7.25, or in Na-acetate buffer, pH 5.75. GdHCl was added to the protein solution to obtain a concentration in guanidinium ranging from 0 to 6 M, incubating for 12 h at room temperature prior to recording the Soret absorption

spectra and the intrinsic tryptophan fluorescence (excitation 295 nm, emission 342–348 nm). The emission fluorescence of the GdHCl in buffer was subtracted before integration. To test the reversibility of denaturation, ELP solutions (30 μM) were first incubated for 24 h in 2.5 and 6 M GdHCl, in the presence or absence of 10 mM Ca^{2+} in 100 mM Tris/HCl buffer, pH 7.25, or in Na-acetate buffer, pH 5.75. Samples were then diluted stepwise to give the desired final concentrations of denaturant (from 2.5 to 0 M and from 6 to 0 M), and incubated overnight at 25 °C to ensure the reaching of equilibrium prior to recording the absorption spectra and the Trp fluorescence as above.

3. Results

3.1. Thermal inactivation of Euphorbia peroxidase: kinetic parameters and reactivation by Ca^{2+}

The effects of temperature on the rate of the reaction of ELP on ABTS as reducing substrate was studied over the range 20–65 °C. A straight line was obtained in the relevant Arrhenius plot, with an activation energy of 14 kcal mol^{-1} (Fig. 1(A)). In

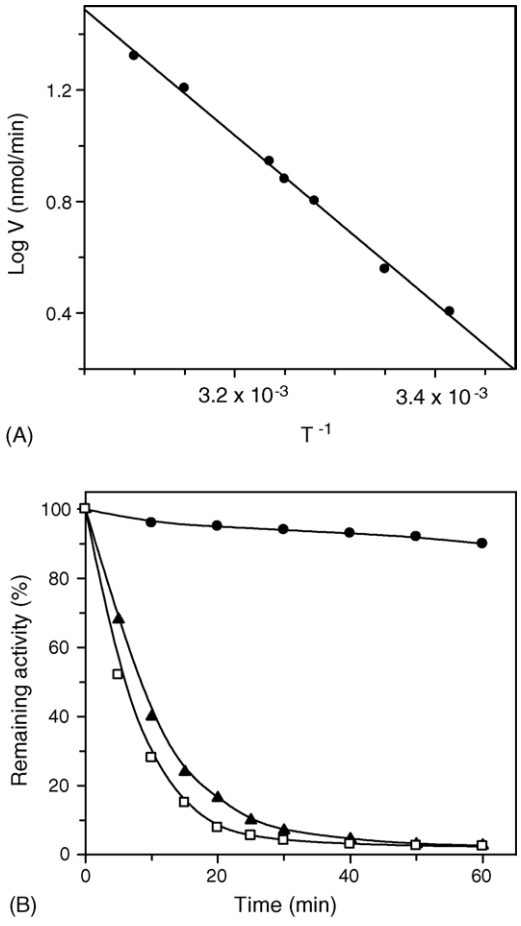

Fig. 1. Thermal inactivation of *Euphorbia* peroxidase. (A) Arrhenius plot for the reaction catalyzed by *Euphorbia* peroxidase. Reactions were assayed under standard assay conditions over the range 20–50 °C. (B) Kinetics of inactivation. The enzyme (3 μM) was incubated at 50 °C (●) and 60 °C (□) in 100 mM Tris/HCl buffer, pH 7.25 or at 60 °C (▲) in 100 mM Na acetate buffer, pH 5.75. At given times, aliquots of the enzyme mixture were taken and assayed for activity on ABTS as reported in Section 2.

Table 1
Activity recovery of fully thermal-inactivated *Euphorbia* peroxidase as a function of Ca^{2+} concentration in the reaction medium

Ca^{2+} (mM)	Activity recovered (%)[a]
0.5	8 ± 0.2
1	21 ± 1
2	50 ± 4
5	90 ± 2
10	92 ± 2
10[b]	98 ± 1

[a] Reactions were assayed in 100 mM Tris/HCl, pH 7.25 under standard conditions using ABTS (10 mM) as reducing substrate. Reactivation of the thermally inactivated enzyme (40 min at 60 °C) was obtained by adding $CaCl_2$ to the enzyme solution at 25 °C for 12 h. Values are means of three independent measurements ± S.D.

[b] Reactivation of the enzyme thermally inactivated for 20 min at 60 °C).

100 mM Tris/HCl buffer, pH 7.25, the enzyme was quite stable at 50 °C for up to 2 h, but the inactivation process accelerated sharply at 60 °C, when a thermal inactivation occurred with a half-life for enzyme inactivation of 6 min (Fig. 1(B)). Similar results were obtained in 100 mM Na-acetate buffer, pH 5.75 (Fig. 1(B)).

As previously reported [8], ELP contains 1 mol of endogenous calcium per mol of enzyme strongly bound at the proximal site, and this proximal calcium ion is known to play a critical role in retaining the heme active site geometry. Therefore, the involvement of calcium in the thermal inactivation process of ELP was investigated. The addition of increasing amounts of $CaCl_2$ to the thermally inactivated ELP, either in Tris/HCl or in Na-acetate buffer, resulted in a progressive recovery of activity reaching the maximum value when the enzyme was incubated in 10 mM Ca^{2+} for 12 h at 25 °C (Table 1). The amount of recovered activity decreased when the time of thermal incubation at 60 °C increased, and about 92% of the original activity was detected after 40 min at 60 °C (Table 1). This was probably due to a slow heme loss during the thermally induced denaturation of ELP, in analogy with what reported for manganese peroxidase [11]. Furthermore, Ca^{2+} ions prevented the thermal inactivation of ELP in a manner directly dependent on the metal ion concentration, and at 60 °C the enzyme retained 92% of the original activity when in the presence of 10 mM Ca^{2+} (Fig. 2). On the contrary, ELP thermal inactivation was accelerated by the efficient Ca^{2+} chelator EGTA (Fig. 2).

3.2. Temperature-dependent changes in the heme environment of Euphorbia peroxidase

Native ELP contains a quantum mixed spin state ferric iron [8] and, in 100 mM Tris/HCl buffer, pH 7.25, shows electronic absorption maxima at 278, 401, 498, and 637 nm (Fig. 3). Incubation of the enzyme at 60 °C caused significant modifications in the absorption spectrum. In particular, the heme environment was affected by the temperature increase, as showed by the marked change in absorbance at 401 nm, the Soret band. Indeed, absorbance at this wavelength decreased with time and the peak red-shifted to 410 nm in the thermally inactivated form, while a weak shoulder at 360 nm appeared (Fig. 3). The original

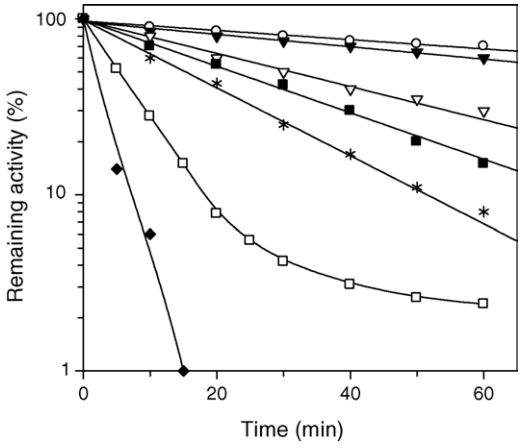

Fig. 2. Effects of calcium and EGTA on the thermal inactivation of *Euphorbia* peroxidase. Mixtures containing 3 μM ELP in 100 mM Tris/HCl buffer, pH 7.25, were incubated in a water bath at 60 °C in the absence of both calcium and EGTA (□), in the presence of increasing amounts of added calcium: 0.5 mM (*), 1 mM (■), 2 mM (▽), 5 mM (▼) and 10 mM (◯) or in the presence or 1 mM EGTA (♦). At given times, aliquots were assayed for activity as reported in Section 2.

absorption maxima at 498 and 637 nm were also bleached, and new bands emerged around 550 nm. In this process, clear isosbestic points at 460, 545 and 600 nm were observed suggesting that structural changes occurred in the enzyme scaffold with no obvious intermediates. After 40 min incubation at 60 °C, the final absorbance of the Soret band was 71% of that of the native protein and remained almost unchanged for at least the following 2 h. After exhaustive dialysis calcium ion was not detected by atomic absorption in the inactivated enzyme.

We attempted to further investigate the thermally induced inactivation of ELP by analyzing the formation of reaction intermediates and the action of specific inhibitors. Compound I (CI) is an intermediate in the reaction mechanism of plant heme peroxidases that stores two oxidizing equivalents from hydrogen peroxide as an oxyferryl iron center and a radical [12]. Characteristic absorption spectrum of CI with maxima in the visible

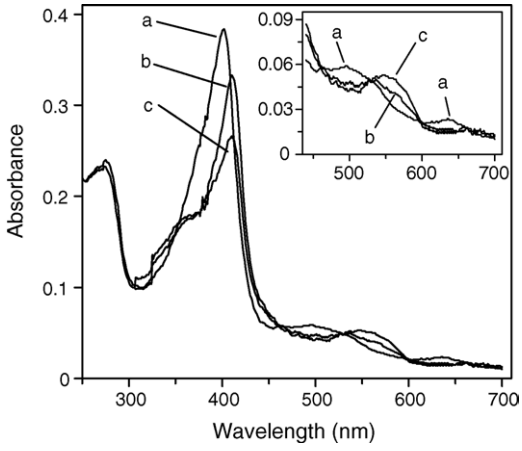

Fig. 3. Temperature-induced modifications of the absorption spectrum of *Euphorbia* peroxidase. Native ELP (3 μM) in 100 mM Tris/HCl buffer, pH 7.25 (a); ELP after 20 min incubation at 60 °C (b); ELP after 40 min incubation at 60 °C (c). The last spectrum did not change after addition of hydrogen peroxide or CN^-. Inset shows the expanded visible region.

range at 398, 544, 577, and 651 nm was observed by pre-treating the native enzyme with equimolar amounts of hydrogen peroxide [8]. No visible change was observed in the spectrum of the thermally inactivated enzyme upon addition of hydrogen peroxide (Fig. 3), indicative that CI was not formed. Cyanide is a classic competitive inhibitor of class III peroxidases, whose adduct to these enzymes may be compared to the initial binding of hydrogen peroxide [13]. When CN^- was added to native ELP, the absorption band at 401 nm disappeared in parallel with the formation of a band at 418 nm, and two additional maxima at 359 and 544 nm were observed [8]. Again, the absorption spectrum of thermally inactivated ELP was not prone to reaction with CN^- (Fig. 3). Finally the addition of 10 mM imidazole did not affect the absorption spectrum of thermally inactivated ELP (not shown). Together, these results suggest that the heme iron of thermally inactivated ELP was in a pure hexacoordinate low-spin state with a very strong His–Fe bond.

To investigate the Ca^{2+}-dependent reversibility of inactivation from the spectroscopic point of view, solutions of fully thermally inactivated ELP were incubated for 12 h in the presence of 10 mM Ca^{2+} ions. The Soret band gradually blue-shifted from 410 to 401 nm and the absorption bands at 498 and 637 reappeared suggesting that enzyme inactivation and reactivation follow identical pathways. After 12 h the absorption spectrum of reactivated ELP was identical to that of the native enzyme, with the Soret band displaying 92% of its original intensity. This observation is thus consistent with the enzyme activity recovery obtained by adding equal amounts of calcium to the reaction mixture, as reported in Table 1.

To get further insights into the conformational changes induced in ELP by exposure to relatively high temperatures, fluorescence and CD spectroscopy were also used. As indicated by the nucleotide sequence ([14]; GenBank accession number AY586601) ELP contains a single tryptophan residue (Trp_{12}), which simplifies the interpretation of fluorescence studies. ELP showed a Trp fluorescence emission spectrum with a maximum at 342 nm when excited at 295 nm, and this spectrum remained unaltered even in the thermally inactivated enzyme (not shown). In order to notice microenvironment changes in thermally inactivated ELP, we, therefore, recurred to the fluorescent hydrophobic probe 1,8-ANS, an amphiphilic compound, which displays a strong fluorescent enhancement when its exposure to water is lowered. 1,8-ANS binding studies conducted with native and thermally inactivated ELP revealed that thermal inactivation of ELP caused a two-fold increase in the intensity of 1,8-ANS fluorescence with respect to that recorded with the native enzyme, concomitantly to a shift of the emission maximum of the protein-bound probe from 505 to 487 nm (Fig. 4). This observation indicates that thermal inactivation causes the exposure of ELP hydrophobic regions that were inaccessible to the dye in the native structure.

CD experiments in the far-UV region indicated that only negligible changes occurred upon heating ELP solutions up to 60 °C for 40 min, suggesting that the protein secondary structure is substantially unaffected by heat stress (Fig. 5). In turn, heat induced dichroic changes were clearly observed in the near-UV region (Fig. 5). In this case, the broadness of the spectral transition

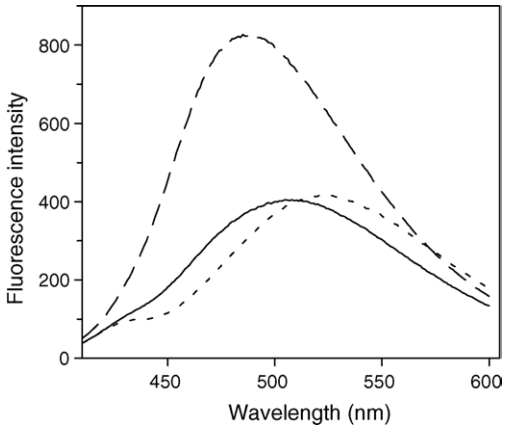

Fig. 4. Fluorescence emission spectra of 1,8-ANS bound to native and thermally inactivated *Euphorbia* peroxidase. 50 μM 1,8-ANS (- - -); 50 μM 1,8-ANS in the presence of native ELP (—); 50 μM 1,8-ANS in the presence of thermally inactivated ELP (– –). Buffer 100 mM Tris/HCl, pH 7.25. Native and thermally inactivated ELP concentration 0.5 μM. 1,8-ANS excitation wavelength, 374 nm.

and the lack of distinct temperature dependent changes in the peak positions of the Trp, suggest that the difference between the near-UV spectra at 60 °C and at 20 °C is mainly accounted for by the heme iron coordination and spin state changes. In particular, the relevant decrease in ellipticity that occurs within the 280–350 nm region parallels the spectral changes observed in the UV–vis absorption spectra in correspondence of the high energy shoulder of the Soret peak (vibronic component of the Soret band).

3.3. Denaturation of Euphorbia peroxidase by guanidinium chloride

The use of denaturants is a useful tool for examining the conformational states and related stabilities of heme peroxidases [15]. To determine the changes occurring in the heme environment following exposure to the widely used denaturant GdHCl, the denaturation of ELP was studied by recording the heme Soret absorbance (at 401 nm) and intrinsic Trp fluorescence, both in the presence and absence of Ca^{2+} ions. At GdHCl concentrations in the range 0.5–2.5 M, with or without 10 mM calcium ions, a regular decrease in the heme absorbance was observed,

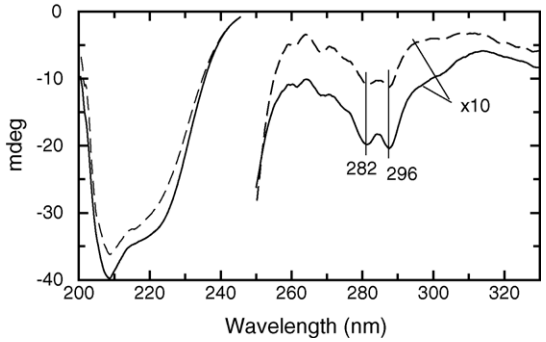

Fig. 5. Far- and near-UV CD spectra of *Euphorbia* peroxidase. Native ELP (0.8 mg mL^{-1}) in 100 mM Tris/HCl buffer, pH 7.25 at 20 °C (continuous line); ELP after 40 min incubation at 60 °C (dashed line). Spectra are the average of four measurements and are not normalized.

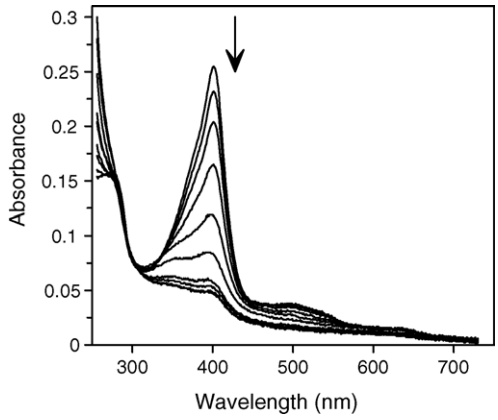

Fig. 6. Absorption spectra of *Euphorbia* peroxidase in GdHCl. Effect of stepwise addition of 0.5 M GdHCl on the absorption spectrum of ELP (2 μM in 100 mM Tris/HCl, pH 7.25).

indicative of the disruption of the heme active site and release of heme from the protein scaffold (Figs. 6 and 7(A)). The change in the 401 nm absorption band of ELP was also recorded during renaturation. In this case, the final absorbance of the Soret band was only 80% of that of the native protein, probably due to

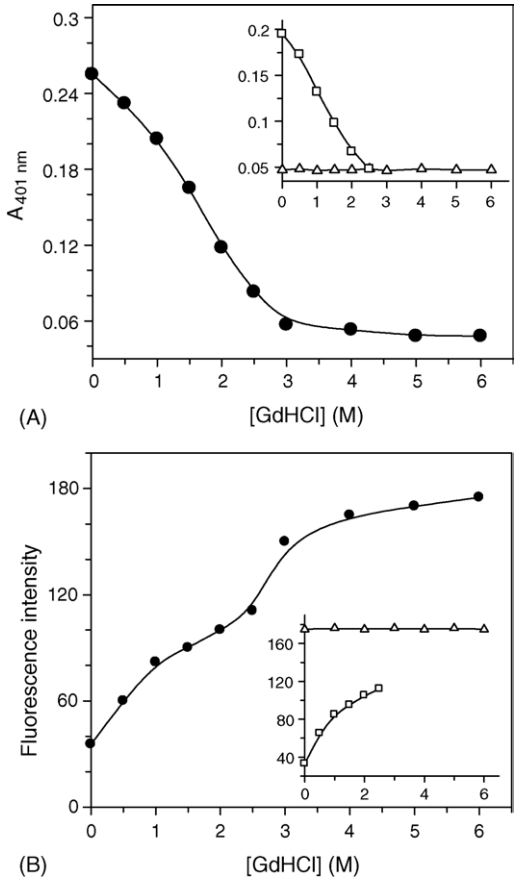

Fig. 7. GdHCl-induced denaturation of *Euphorbia* peroxidase. (A) Effects of GdHCl on the heme Soret absorbance of ELP (2 μM in 100 mM Tris/HCl, pH 7.25). Inset: ELP stepwise renaturation from 2.5 to 0 M (□) and from 6 to 0 M (△) GdHCl. (B) Effects of GdHCL on ELP Trp fluorescence. Inset: ELP stepwise renaturation from 2.5 to 0 M (□) and from 6 to 0 M (△) GdHCl. Trp excitation 295 nm, emission 348 nm.

incomplete reincorporation of dissociated heme during refolding of the enzyme (Fig. 7(A), inset). Indeed, the Soret absorption in 3 M GdHCl resembles that of free hemin in the presence of 3 M GdHCl (Fig. 6). Also the renaturation process, as monitored by reading the intensity of the Soret band, was unaffected by the presence or absence of 10 mM calcium in the mixture. As for fluorescence spectroscopy studies, we observed that GdHCl red shifted the tryptophan emission maximum of ELP from 342 to 348 nm, and the Trp fluorescence was progressively enhanced in the presence of increasing amounts of the denaturant (Fig. 7(B)), possibly indicating a relief of Trp fluorescence quenching from excitation energy transfer to the heme due to an increase in the Trp–heme distance, that reflects protein unfolding. Renaturation of ELP probably occurred by the same mechanism as denaturation since the curves were similar (Fig. 7(B), inset). At concentrations of GdHCl greater that 3 M, a more pronounced increase in Trp fluorescence was observed that reached its maximum (five folds) at 6 M GdHCl (Fig. 7(B)). Denaturation of ELP by GdHCl caused the removal of Ca^{2+} from the enzyme, as confirmed by atomic absorption measurements, and the activity of Ca^{2+}/heme-free enzyme was approximately 0.2% that of native enzyme. The GdHCl-denatured enzyme did not recover its activity neither after addition of 10 mM Ca^{2+} ions nor in the presence of a two-fold excess of free protoporphyrin IX (PPIX) or in the contemporary presence of Ca^{2+} ions and PPIX.

4. Discussion

The role of calcium has been investigated in several peroxidases. Whereas, class I peroxidase, such as cytochrome *c* peroxidase, do not possess bound Ca^{2+}, 2 mol of Ca^{2+}/mol of enzyme are normally present in peroxidases of classes II and III, with metal binding sites proximal and distal to the porphyrin plane, respectively. In horseradish peroxidase isozyme C, the proximal Ca^{2+} has been shown to play an important role in maintaining the protein structure around the heme microenvironment [16,17], and removal of the metal ion from the enzyme causes a two-fold decrease in its activity [16,18–20]. The loss of the distal calcium ion during reversible thermal inactivation was previously described in lignin peroxidase and manganese peroxidase, and reported to be associated to slight changes in the overall protein structure and alterations in the heme environment, with the formation of a hexacoordinate low-spin heme iron [11,21,22]. Conformational states of an anionic form of horseradish peroxidase, HRPA1, during thermal inactivation were reported and the role of calcium ions during refolding of HRPA1 were also discussed [23,24].

The peroxidase object of this study, isolated from the latex of the perennial Mediterranean plant *Euphorbia characias*, contains ferric iron in protoporphyrin IX in a quantum mixed spin state pentacoordinated with a proximal histidine ligand, and 1 mol of proximal calcium per mol of enzyme [8]. Removal of this calcium ion results in an almost complete loss of enzyme activity. Based on these characteristics, ELP can thus be used as a suitable model to investigate the role of the proximal calcium ion in maintaining the heme environment and spin state of Fe^{3+} during thermal inactivation and in the presence of denaturants. Our kinetics and spectroscopic results show that thermal inactivation of ELP is due to loss of the proximal calcium from the enzyme, which causes distinct modifications in the heme environment, without gross changes in the protein secondary and tertiary structure, as observed by CD and fluorescence spectroscopy. In particular, the spectrophotometric measurements of the thermally inactivated form, showing the red shift and decreased intensity of Soret absorbance and the appearance of the absorption maxima at 540 and 565 nm, were indicative of a conversion of a quantum mixed-spin state pentacoordinated active heme iron form to a pure hexacoordinate low-spin inactive complex, as shown in other peroxidases ([22] and references therein). Consistent with disruption of the structural integrity of the heme environment and with the consequently hexacoordination of the heme iron, thermally inactivated ELP did not react with hydrogen peroxide to form compound I – whose rate of formation normally depends on the reactivity of the distal histidine as a general acid–base catalyst [12] – nor with the heme iron-directed inhibitor CN^-. Moreover, the presence of imidazole did not change the visible spectrum of thermally inactivated ELP, indicating the existence of an additional His–Fe bond probably formed with the distal His_{50} as the sixth iron ligand, caused by loss of the proximal calcium ion. Finally, addition of calcium to the thermally inactivated enzyme solutions resulted in recovery of the initial activity together with the spectroscopic properties of the native enzyme. Although the ELP inactivation mechanism might involve a minor loss of heme after prolonged incubation time, all experiments reported clearly indicated the role played by Ca^{2+} in both the decrease in heme absorbance and in the thermal inactivation of ELP.

While calcium seems to play a key role in the thermal inactivation of ELP, it appeared to be unimportant as for what concerns the stability of the protein in denaturants. It is well known that the normal quenching of peroxidase Trp fluorescence emission is caused by the vicinity of the heme prosthetic group [25,26]. At 1–2.5 M GdHCl ELP unfolded, as shown the red shift and increase in intensity of Trp fluorescence due to relief of heme quenching that followed a stretching of the Trp–heme distance [27]. At this point, however, the heme was still bound to the protein, as shown by heme Soret 401 absorption band, and renaturation of ELP occurred following the same pathway as denaturation. At GdHCl concentrations above 3 M the protein was fully denaturated and inactive, and lost the heme and calcium ions. Upon addition of 10 mM Ca^{2+} ions, or in the contemporary presence of both Ca^{2+} ions and PPIX, renaturation of ELP did not occur and the enzyme did not recover its activity. Thus, following exposure to molar amounts of GdHCl, the protein irreversibly loses its secondary and tertiary structure, and the refolding of ELP is not achieved irrespectively of the presence or not of Ca^{2+} ions.

In conclusion, the data herein collected provide a viable explanation that the Ca^{2+} ion strongly bound to the proximal histidine in *Euphorbia* latex peroxidase plays a critical role in conferring structural stability to the heme environment and in retaining the enzyme active site geometry. The loss of proximal Ca^{2+} ion induced by temperature results in an enhanced flexibility of heme surroundings, with the formation of an additional

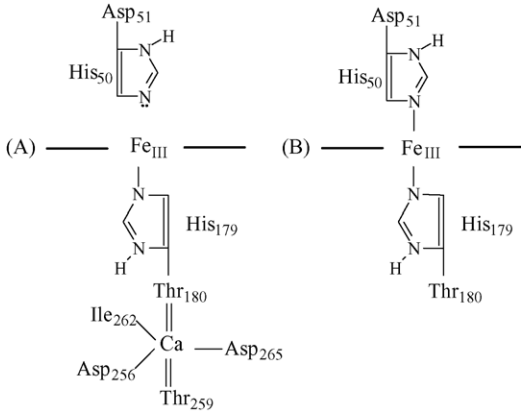

Fig. 8. Schematic representation of the proximal calcium ion site and heme pocket residues in *Euphorbia* latex peroxidase before (A) and after (B) thermal inactivation. The amino acid residues are numbered on the basis of the known ELP nucleotide sequence (Reference [14]; GenBank accession number AY586601).

coordination of heme Fe^{3+} with the distal His_{50} (for a schematic view of the structural changes proposed, see Fig. 8) and the conversion of the spin state of the heme iron from a quantum mixed-spin state to a pure low-spin, essentially causing the reversible inactivation of ELP.

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