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Euphorbia Peroxidase Catalyzes Thiocyanate Oxidation in Two Different Ways, the Distal Calcium Ion Playing an Essential Role†

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ABSTRACT: The oxidation of the pseudohalide thiocyanate (SCN^-) by *Euphorbia* peroxidase, in the presence or absence of added calcium, is investigated. After incubation of the native enzyme with hydrogen peroxide, the formation of Compound I occurs and serves to catalyze the thiocyanate oxidation pathways. The addition of a stoichiometric amount of SCN^- to Compound I leads to the native enzyme spectrum; this process clearly occurs via two electron transfers from pseudohalide to Compound I. In the presence of 10 mM calcium ions, the addition of a stoichiometric amount of SCN^- to Compound I leads to the formation of Compound II that returns to the native enzyme after addition of a successive stoichiometric amount of SCN^-, indicating that the oxidation occurs via two consecutive one-electron transfer steps. Moreover, different reaction products can be detected when the enzyme−hydrogen peroxide−thiocyanate reaction is performed in the absence or presence of 10 mM Ca^{2+} ions. The formation of hypothiocyanous acid is easy demonstrated in the absence of added calcium, whereas in the presence of this ion, CN^- is formed as a reaction product that leads to the formation of an inactive species identified as the peroxidase−CN^- complex. Thus, although monomeric, *Euphorbia* peroxidase is an allosteric enzyme, finely tuned by Ca^{2+} ions. These ions either can enhance the catalytic efficiency of the enzyme toward some substrates or can regulate the ability of the enzyme to exploit different metabolic pathways toward the same substrate.

Peroxidases (EC 1.11.1.x; donor, hydrogen peroxide oxidoreductase) are enzymes utilizing hydrogen peroxide or other peroxides to oxidize a second reducing substrate. Heme-containing peroxidases (EC 1.11.1.7) are involved in a variety of defense mechanisms toward pathogens based on the so-called oxidative burst, in which the levels of H_2O_2 and other reactive oxygen species (mainly superoxide) rapidly increase (*1*−*3*). These enzyme are grouped in two major superfamilies: "animal" and "non-animal" peroxidases (*4*).

Animal peroxidases are secretory proteins playing essential roles in the biological defense mechanism. This group comprises lactoperoxidase (LPO),[1] myeloperoxidase, eosinophil peroxidase, and thyroid peroxidase. LPO, found in milk, saliva, and tears, is the most widely studied enzyme of this superfamily (*5*−*8*).

Non-animal peroxidases are subdivided into three classes (I, II, and III) on the basis of sequence similarity. Class III includes the secretory plant peroxidases, monomeric glycosylated proteins distributed throughout the plant kingdom (*4*, *9*). Plant peroxidases are considered to act as antioxidant enzymes, protecting cells, tissues, and organs against the toxic effects of peroxides. In addition, these enzymes are also directly involved in the synthesis of important metabolites in plants (*10*). Well-studied examples of class III peroxidases are the enzymes extracted from horseradish (HRP) and from the latex of the perennial Mediterranean shrub *Euphorbia characias* [*Euphorbia* latex peroxidase (ELP)] (*11*).

There are some differences between the two enzymes. The native HRP is generally thought to contain high-spin Fe^{3+} in a protoporphyrin IX pentacoordinated to a "proximal" histidine ligand. This histidine functions to stabilize the higher oxidation states of the iron atom (*12*), while another histidine, known as the "distal" ligand, functions as an acid−base catalyst to accept one proton from the peroxide. Native HRP-C also contains 2 mol of Ca^{2+}/mol of enzyme, and the metal binding sites are known as proximal and distal according to their location relative to the porphyrin plane (*13*, *14*).

ELP contains a Fe^{3+}-PPIX in a quantum mechanically mixed-spin state resulting from an admixture of high-spin and intermediate-spin species, pentacoordinated with a histidine proximal ligand (*15*). Although ELP has two calcium binding sites, proximal and distal to the heme, the purified protein contains 1 mol of endogenous calcium/mol of enzyme. This proximal Ca^{2+} ion is strongly bound and plays a critical role in retaining the active site geometry. A second Ca^{2+} ion appears to be located at the distal low-affinity binding site and is necessary for expression of the full activity of the enzyme (*11*).

The pseudohalide thiocyanate (SCN^-) is an attractive substrate for LPO and other mammalian heme peroxidases (*7*, *16*, *17*),

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[1]Abbreviations: ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; ELP, *Euphorbia* latex peroxidase; HRP, horseradish peroxidase; LPO, lactoperoxidase; NaOAc, Na^+ acetate buffer.

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and for HRP and other plant peroxidases (*18*−*21*). SCN^- is oxidized by mammalian and plant peroxidases in a different way. The oxidation of SCN^- by LPO results in the formation of hypothiocyanous acid and its anion ($OSCN^-$), products known to have antibacterial activity (*5*, *8*, *20*). The level of catalytic oxidation of SCN^- by HRP is 100-fold lower than that of LPO, and the product formed during the oxidation is dithiocyanogen [$(SCN)_2$] that is hydrolyzed to yield CN^- (*20*). Moreover, the $HRP/H_2O_2/SCN^-$ system is not a bacteriocidal system.

In this study, we investigated the oxidation of thiocyanate by *Euphorbia* latex peroxidase both in the presence and in the absence of excess Ca^{2+} ions. We show that native ELP uses a mammalian-like catalytic mechanism on the thiocyanate oxidation generating $OSCN^-$, and in the presence of an excess of calcium ions, the enzyme becomes a typical plant peroxidase forming CN^- as a product of SCN^- oxidation. Thus, Ca^{2+} seems to be the key for SCN^- catalytic pathways in *Euphorbia* peroxidase.

EXPERIMENTAL PROCEDURES

Materials. 2,2′-Azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), *o*-dianisidine, ascorbic acid, and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) were purchased from Sigma (St. Louis, MO). Hydrogen peroxide was from Merck (Darmstadt, Germany), and an ε_{240} of 43.6 M^{-1} cm^{-1} was used to determine its concentration. Deuterium oxide [99.9 at. % D (glass distilled), minimum isotopic purity of 99.96 at. % D_2O], potassium cyanide, potassium thiocyanate, and enriched potassium [^{15}N]thiocyanate were purchased from Aldrich (St. Louis, MO). All reagents were obtained as pure commercial products and used without further purification.

Enzyme. Peroxidase from *E. characias* latex [Reinheitzahl (RZ) value A_{399}/A_{278} = 2.7 in 100 mM NaOAc buffer (pH 5.75)] was purified as previously described (*11*). The enzyme concentration was determined spectrophotometrically using an ε_{399} of 130.7 mM^{-1} cm^{-1}.

Spectrophotometry. Absorption spectra and data from all activity assays were recorded with an Ultrospec 2100 spectrophotometer (Biochrom Ltd., Cambridge, England) using cells with a 1 or 5 cm path length.

Peroxidase Activity. Activity measurements were performed in 100 mM NaOAc buffer (pH 4−6), in the absence and presence of 10 mM Ca^{2+} ions, using hydrogen peroxide and the reducing substrates by the following procedures: (i) ABTS oxidation 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}), (ii) *o*-dianisidine oxidation following the increase in absorbance at 460 nm (ε_{460} = 11.3 mM^{-1} cm^{-1}), and (iii) SCN^- oxidation, which was followed by two methods.

(a) ELP was incubated with hydrogen peroxide and SCN^- in the absence or presence of 10 mM Ca^{2+} ions. At intervals, the reaction was stopped by the addition of catalase. To 0.2 mL of the reaction mixture were added 1.6 mL of 100 mM HCl and 0.2 mL of 100 mM $FeCl_3$. The absorbance of the $Fe-SCN^{2+}$ complex was measured at 450 nm, and the residual concentration of SCN^- was calculated from a standard curve. Because SCN^- might bind divalent metal ions, the concentration of SCN^- was calculated in the presence and absence of 10 mM calcium ions, and no differences were observed.

(b) The other method followed the increase in absorbance at 235 and 376 nm resulting in the formation of $OSCN^-$ (ε_{235} = 1.29 mM^{-1} cm^{-1}; ε_{376} = 26.5 M^{-1} cm^{-1}) (*8*, *22*).

Catalytic center activity (k_{cat}) was defined as the moles of substrate consumed per mole of active sites per second. Kinetic parameters were calculated as the mean of at least five different measurements.

SCN^- Binding Studies at Varying pH Values. Titrations of ELP with SCN^- were performed in 1 mL of 100 mM NaOAc buffer, over the pH range of 4.0−5.75, and at 25 °C. Enzyme spectra were recorded before and after addition of SCN^- aliquots to the solution in the absence and presence of 10 mM Ca^{2+} ions. The absorbance changes were measured 20 min after each addition.

Determination of the K_i and K_d Values of Cyanide Binding. The inhibition constants of cyanide for ferric ELP were determined from Dixon's plot (*23*). Titrations of ELP with cyanide were made at 25 °C in 100 mM NaOAc buffer (pH 4.0−5.75) in the presence or absence of 10 mM Ca^{2+} ions, by stepwise addition of freshly prepared cyanide to the ELP solution, and the absorbance changes were measured at the appropriate wavelength (see Results) 20 min after each addition. The mass law yields $[E-CN]/[E]_t = [CN^-]/K_d + [CN^-]$. Taking into account the total concentrations of cyanide ($[CN^-]_t$) and ELP ($[E]_t$), and the relationships $[CN^-] = [CN^-]_t - [E-CN]$ and $[E] = [E]_t - [E-CN]$, we obtain the following equation, whose solution was fed to the minimization routine:

$$[E-CN]^2 - [E-CN]([CN^-]_t + [E]_t + K_d) + [CN^-]_t[E]_t = 0$$

Stopped-Flow Experiments. Stopped-flow experiments were conducted using an Applied Photophysics (Leatherhead, U.K.) MV 17 apparatus, equipped with an observation chamber with a 1 cm path length and either a monochromator and a photomultiplier tube (for single-wavelength measurements) or a spectrometer and a photodiode array detector (for rapid acquisition of absorbance spectra over the range of 250−800 nm). Single-wavelength measurements were analyzed by a least-squares minimization routine (developed using the Borland Pascal 7.0 compiler) capable of fitting any desired theoretical model to the experimental data, using either analytical or numerical integration (*24*).

Acquisition of NMR Spectra. ^{15}N NMR spectra of $SC^{15}N^-$ (20 mM) in a solution containing ELP (10 μM) in 100 mM NaOAc buffer (pH 5.75) in the presence and absence of 10 mM Ca^{2+} ions were recorded on a Bruker Avance 600 MHz spectrometer at a resonance frequency of 60.815 MHz before and after addition of H_2O_2 (20 mM). A 5 mm BBO probe was used to acquire ^{15}N NMR spectra. Measurements were taken in a 5 mm NMR tube with D_2O for frequency lock, using a 90° (12 μs) RF pulse and 60 s relaxation delay. Spectra were recorded by accumulation of 128 transients at 9.7K data points and a spectral width of 20000 Hz. The temperature was held at 298 K. A sample of 100 mM $SC^{15}N^-$ in the same buffer was examined under identical experimental conditions to calibrate the chemical shift scale and to compare the line widths. Resonance due to $SC^{15}N^-$ was set at −170.37 ppm according to the literature (*19*, *25*, *26*).

RESULTS

pH Dependence of the Reaction of ELP with Hydrogen Peroxide and the Reducing Substrates ABTS, o-Dianisidine, and SCN^-. The pH effect on ELP enzymatic activity was tested in 100 mM NaOAc buffer (pH 4−6) in the absence and presence of 10 mM Ca^{2+} ions, using hydrogen peroxide and the reducing substrates ABTS, *o*-dianisidine, and SCN^- as reported in Peroxidase Activity.

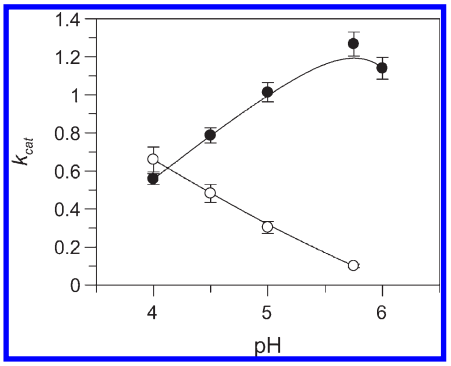

FIGURE 1: Effect of pH on *Euphorbia* peroxidase activity using SCN^- as a substrate. Experimental conditions: 0.5 μM ELP, 2 mM SCN^-, 2 mM H_2O_2, and 100 mM NaOAc buffer (pH 4−6). k_{cat} is the number of moles of substrate consumed per mole of enzyme per second. The ELP native enzyme in the absence (●) and presence (○) of 10 mM calcium ions. Data reported are calculated as the means of at least five different measurements.

When native ELP was incubated for 10 min in the presence of Ca^{2+}, a drastic activation was observed toward the reducing substrate ABTS or *o*-dianisidine (the pH dependence of k_{cat} values for both ABTS and *o*-dianisidine is reported in Figure S1 of the Supporting Information). The activation by Ca^{2+} ions was strongly pH-dependent; 100- and 27-fold activation were observed in the presence of 10 mM Ca^{2+} in NaOAc buffer (pH 5.75) with ABTS and *o*-dianisidine, respectively. At pH 4.0, very little activation was measured.

The pH dependence of k_{cat} values for SCN^- is plotted in Figure 1. Native ELP exhibited, at pH 5.75, the highest value of k_{cat}, i.e., 1.3 s^{-1}. Unexpectedly, in the presence of 10 mM calcium ions, the activity of the enzyme decreased as the pH value increased. This surprising result spurred us to make a detailed study of thiocyanate oxidation processed by ELP.

Spectrophotometric Features of Native ELP and ELP−Ca^{2+}−Complex. The catalytic cycle of ELP is well-established (*11*) and is briefly summarized here to facilitate the description of the results obtained. The reaction of hydrogen peroxide with ELP ($PrIXFe^{III}$) generates the green enzyme intermediate Compound I ($PrIX^{\bullet+}Fe^{IV}$=O), with both of the oxidizing equivalents of H_2O_2 transferred to the enzyme. One of the two oxidizing equivalents of peroxide is ascribed to the loss of an electron from the iron atom that is oxidized to a ferryl complex in Compound I (Fe^{IV}=O^{2-}), whereas the second electron is donated by the porphyrin ring, oxidized to a π-cation radical. Compound I then reverts to the resting state by two successive one-electron reactions with reducing substrate molecules (DH_2). The red Compound II ($PrIXFe^{IV}$=O), a second enzyme intermediate, is produced by the first electron transfer from DH_2 to Compound I. With a large excess of H_2O_2 and in the absence of reducing substrates, ELP decomposes hydrogen peroxide to oxygen and water in a catalase-like reaction (*27*), and Compound I, Compound II, and Compound III, the oxyperoxidase, are obtained.

The electronic absorption spectrum of native ELP, in the presence or absence of 10 mM Ca^{2+} ions, showed maxima at 278, 399, 498, and 637 nm in 100 mM NaOAc buffer (pH 5.75) (Figure 2A,B). At this pH value, in the absence of added calcium, SCN^- induced an increase and a red shift of the Soret band from 399 to 401 nm with no modification of the band's width. A K_d value of 28 mM was calculated (Figure S2 of the Supporting Information). When the same experiment was performed at pH 5.75 in the presence of 10 mM Ca^{2+} ions, no modification in the ELP

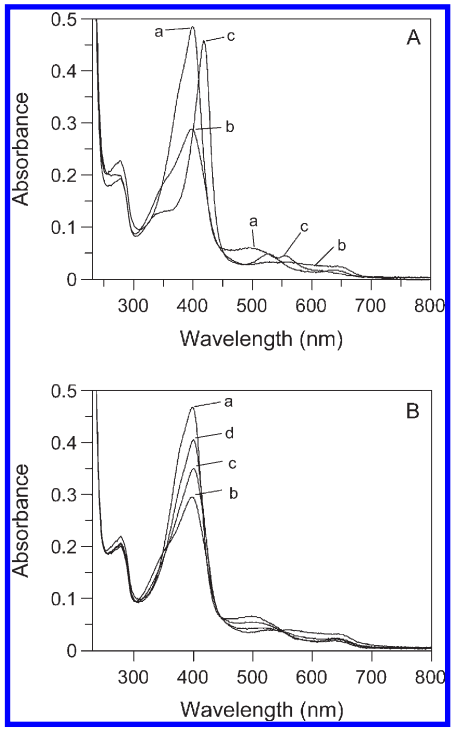

FIGURE 2: Absorption spectra of *Euphorbia* peroxidase at pH 5.75. (A) (a) ELP (3.7 μM) in the presence of 10 mM calcium ions. (b) Compound I is formed after addition of an equimolar amount of hydrogen peroxide to the native enzyme. (c) Compound II is formed after addition of 3.7 nmol of SCN^-. Addition of another amount of SCN^- (3.7 nmol) reduced Compound II to the native enzyme. The buffer was 100 mM NaOAc (pH 5.75). (B) (a) Native enzyme (3.5 μM). (b) Compound I is formed after addition of an equimolar amount of hydrogen peroxide to the native enzyme. (c and d) After two additions of SCN^- (1.2 μM). The buffer was 100 mM NaOAc (pH 5.75).

spectrum was seen after addition of SCN^- up to 400 mM (not shown).

Compound I, with characteristic absorption maxima at 278, 398, and 651 nm, was produced by addition of an equimolar amount of hydrogen peroxide to native ELP, in the absence and presence of 10 mM Ca^{2+} ions. In the presence of these ions, the addition of 1.0 mol of SCN^-/mol enzyme to the reaction mixture reduced Compound I to Compound II with characteristic absorption maxima at 278, 417, 522, and 555 nm (Figure 2A). This absorption spectrum was identical to that generated from Compound I by the addition of 1 equiv of ascorbic acid (*11*). Compound II was then reduced to the native enzyme via addition of a successive stoichiometric amount of SCN^-.

In the absence of added calcium, the addition of SCN^- to Compound I did not lead to the formation of Compound II, but a progressive disappearance of the Compound I absorption spectrum, leading to the native spectrum, was observed (Figure 2B). In this process, clear isosbestic points at 550, 448, 358, and 303 nm were seen. As shown in panel B, 1.0 mol of SCN^-/mol of enzyme was used to completely reverse the reaction of Compound I to the native ELP.

Formation of the Cyanide Derivative, Spectrophotometric Features of ELP in SCN^- Oxidation, and Effect of a Spin Trap. As previously reported (*20*), free radicals derived by the oxidation of SCN^-, in the presence of HRP and hydrogen peroxide, form dithiocyanogen $(SCN)_2$ due to two successive one-electron transfers from SCN^-. $(SCN)_2$ is hydrolyzed to yield CN^-,

Table 1: Apparent K_i and K_d Values of CN^- for the Native ELP Enzyme in the Absence and Presence of 10 mM Ca^{2+} Ions in 100 mM NaOAc Buffer (pH 4−5.75)[a]

pH value	K_i (μM)	K_d (μM)
4.0 without Ca^{2+}	184 (9×10^{-4})	188 (9×10^{-4})
4.0 with Ca^{2+}	106 (5.1×10^{-4})	103 (5.1×10^{-4})
4.5 without Ca^{2+}	125 (2×10^{-3})	117 (1.8×10^{-3})
4.5 with Ca^{2+}	73 (1.1×10^{-3})	64 (1.0×10^{-3})
5.0 without Ca^{2+}	89 (4.5×10^{-3})	33.4 (1.7×10^{-3})
5.0 with Ca^{2+}	38 (1.9×10^{-3})	9.2 (4.6×10^{-4})
5.75 without Ca^{2+}	6.0 (1.7×10^{-3})	6.0 (1.7×10^{-3})
5.75 with Ca^{2+}	1.0 (2.9×10^{-4})	2.0 (5.7×10^{-4})

[a]In parentheses are the intrinsic K_i and K_d values recalculated for the actual concentration of the CN^- anion, given that the fraction of the substance present in the form of hydrocyanic acid does not bind to the heme iron.

the well-known inactivating agent for all the heme peroxidases. The reaction proceeds through the following mechanism:

$$HRP + H_2O_2 \rightarrow \text{Compound I}$$

$$\text{Compound I} + SCN^- \rightarrow \text{Compound II} + {}^{\bullet}SCN$$

$$\text{Compound II} + SCN^- \rightarrow HRP + {}^{\bullet}SCN$$

$${}^{\bullet}SCN + {}^{\bullet}SCN = (SCN)_2$$

$$3(SCN)_2 + 4H_2O \rightarrow CN^- + 5SCN^- + 8H^+ + SO_4{}^{2-}$$

CN^- behaved as a competitive inhibitor of ELP. The apparent K_i values, determined by Dixon's plot, decreased from pH 4.0 to 5.75 (Table 1). In the presence of 10 mM Ca^{2+} ions, the K_i value decreased as the pH values increased, reaching the lowest value at pH 5.75 (Table 1). Overall, this behavior was compatible with the expected competition between the ferric heme iron and the hydrogen ion for combination with the cyanide anion, but other contributions from ionizable residues on the surface of the protein or within the heme pocket could not be excluded.

To analyze the influence of Ca^{2+} ions on the binding of cyanide, we titrated ELP with CN^- in the absence or presence of 10 mM Ca^{2+}. When CN^- was added to 3.8 μM ELP, at pH 5.75, the absorption band at 399 nm disappeared in parallel with formation of a band at 418 nm, and a new peak at 540 nm appeared (Figure S3 of the Supporting Information). In this process, isosbestic points at 466, 525, and 590 nm were observed. The extinction coefficient of the ELP−CN^- adduct (ε_{418}) was calculated to be 138 mM^{-1} cm^{-1}. Similar spectral changes were seen in the titration of ELP with cyanide in the presence of 10 mM Ca^{2+} ions, whereas the K_d values for cyanide were markedly changed when 10 mM Ca^{2+} ions were present (Table 1). As expected, the K_i values for CN^- were equal to or slightly higher than the corresponding K_d values.

Titration of 3 μM ELP in the presence of 10 mM Ca^{2+} ions, at a fixed concentration of SCN^- (200 μM) and at varying hydrogen peroxide concentrations (from 3 to 30 μM), is shown in Figure 3. A gradual increase in hydrogen peroxide concentration caused a gradual decrease in the magnitude of the Soret peak at 399 nm with an increase at 418 nm. Moreover, the formation of a new peak at 540 nm was observed. These spectral features were very similar to those of the ELP−CN^- complex. From the ε_{418}, we calculated that most if not all the native enzyme was

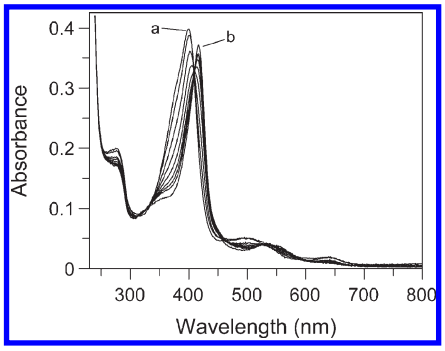

FIGURE 3: *Euphorbia* peroxidase spectra in the presence of SCN^- and hydrogen peroxide. Modification of the ELP optical spectrum (3.0 μM) in the presence of 10 mM calcium ions and 200 μM SCN^- before (a) and after (b) 10 additions of 5 μL aliquots of H_2O_2 (3 μM). The buffer was 100 mM NaOAc (pH 5.75).

transformed in the ELP−CN^- complex. The enzyme so treated was largely inactivated. Given the measured K_d and K_i values (Table 1) and the stoichiometric H_2O_2:SCN:CN ratio of 3:6:1 (see Discussion), one can easily calculate the number of turnovers and the substrate concentrations required to achieve 50% inhibition (e.g., 3 μM ELP at pH 5.75 would be 50% inhibited by a total CN^- concentration of ~7.5 μM, corresponding to 22.5 μM H_2O_2 and 2.5 turnovers under our experimental conditions).

The addition of excess SCN^- and H_2O_2 (enzyme:SCN^-:H_2O_2 ratio of 1:60:10) to ELP in the presence of 10 mM Ca^{2+} generated Compound I and Compound II. This last species exhibited peculiar characteristics. (i) It was inactive as observed via addition of *o*-dianisidine to the reaction mixture. (ii) It was not reduced to the native spectrum after addition of ascorbic acid. (iii) It was not stable, and its spectrum slowly changed (~20 min), accompanied by the formation of peaks at 418 and 540 nm (Figure S4A of the Supporting Information). Again, this inactivated species had features very similar, if not identical, to those obtained after the reaction of the ELP−Ca^{2+} adduct with CN^-. Moreover, no significant further changes in absorbance were seen when CN^- was added to this inactivated species.

When the same experiment was performed in the absence of calcium ions, the formation of Compound II and the inactive species was never observed (Figure S4B of the Supporting Information).

The kinetics of ELP-catalyzed SCN^- oxidation was further studied, and then in the presence of Ca^{2+} ions, in the presence of a DMPO spin trap. Although the initial rate of SCN^- oxidation was not increased in the presence of DMPO, after incubation for 20−30 min the activity of the enzyme was fully recovered. Moreover, when SCN^- was added to the reaction mixture containing ELP, hydrogen peroxide, calcium ions, and the DMPO spin trap, we observed that Compound II was slowly reduced to native ELP (~20 min), and obviously, the spectrum of the inactive species did not appear (not shown).

Reaction Products. The results reported above allowed us to hypothesize the formation of different reaction products when ELP was incubated with SCN^- and H_2O_2 in the presence or absence of added calcium ions. In the native ELP-catalyzed oxidation of thiocyanate by hydrogen peroxide, an increase in the absorbance at 235 nm was observed, and it is well-known that the absorbance increase at 235 nm is due to the formation of hypothiocyanite ion, a typical product of thiocyanate oxidation by mammalian peroxidases (*8*, *22*). For native ELP, the K_M value for SCN^- at pH 5.75 and at saturating concentrations of

hydrogen peroxide (2 mM) was shown to be 0.23 mM, whereas the K_M for hydrogen peroxide, at saturating concentrations of SCN^- (2 mM), was calculated to be 0.12 mM. Moreover, we found that the k_{cat} value was the highest with both substrates at 2 mM, thus when $[H_2O_2]/[SCN^-] = 1$, in analogy with other peroxidases reacting with SCN^- (*19*).

Moreover, we also observed the formation of a new absorption at 376 nm typical of hypothiocyanite. Because of the low molar extinction coefficient at 376 nm (26.5 M^{-1} cm^{-1}), we had to use a spectrophotometric cell with a path length of 5 cm to quantify the production of $OSCN^-$ (*8*).

In contrast, in the ELP-catalyzed reaction, in the presence of 10 mM Ca^{2+} ions, at pH 5.75, A_{235} and A_{376} were almost zero, indicating no formation of hypothiocyanite ion.

^{15}N NMR Studies of the SCN^-/H_2O_2/ELP System. In 100 mM NaOAc buffer (pH 5.75), 20 mM $SC^{15}N^-$ exhibited a resonance at −170.37 ppm. We monitored NMR spectra at different times, and neither the ^{15}N NMR resonance at −80.6 ppm, typical of $OSCN^-$ (*8*), nor new ^{15}N NMR resonances were seen after addition of 10 μM ELP and 20 mM hydrogen peroxide (Figure S5 of the Supporting Information). It may be explained by the slow rate of oxidation of SCN^- by ELP and by the fact that, as reported by Nagy et al. (*8*), hypothiocyanite is a transient species that is not stable.

Stopped-Flow Determinations. Because of the poor catalytic efficiency of ELP versus thiocyanate, it was unpractical to reproduce the steady state in the stopped-flow apparatus. Therefore, two types of experiments were conducted to investigate the fundamental steps of the catalytic cycle of ELP. First, the resting ferric enzyme was mixed with hydrogen peroxide in the absence of SCN^-. Second, the enzyme was mixed with hydrogen peroxide and SCN^-. By repeating this experiment several times over some minutes, we checked that H_2O_2 was not consumed by SCN^- while being kept in the instrument. Both experiments were conducted at several concentrations of each substrate in the presence and/or absence of calcium ions.

When ferric ELP was mixed with hydrogen peroxide, at pH 4.0 and in the absence of a reducing substrate, a second-order reaction was observed in which the ferric enzyme was converted to Compound I. Over longer time regimes (tens of seconds), a small fraction of the enzyme was converted to Compound II (Figure 4A) presumably because of the catalase-like reaction of ELP (*27*). At pH 4.0 and 25 °C, the rate constant of the first reaction was 2300 M^{-1} s^{-1}, whereas at pH 5.75, it was 22000 M^{-1} s^{-1}. This last value was somewhat lower than the one we reported previously (77000 M^{-1} s^{-1}) (*11*). This discrepancy was explained by the fact that our estimate was obtained from direct measurement, whereas the older one was obtained from the fit of a steady state obtained using ascorbate as the reducing substrate.

When native ferric ELP was mixed with hydrogen peroxide and thiocyanate, few spectral changes were observed over the same time regime (milliseconds to seconds), pointing to the appearance of Compound I (and possibly Compound II) in minute amounts (Figure 4B at pH 4.0 and Figure 5 at pH 5.75). Because in the absence of thiocyanate the formation of Compound I occurred at a rate faster than that of the spectral changes observed in its presence, we assumed that in the presence of both substrates the enzyme either (i) entered into a steady state condition in which the ferric form was highly prevalent or (ii) reacted with thiocyanate forming a slowly reactive derivative with a spectrum similar to that of the ferric species. Although the last hypothesis could be prevalent because we demonstrated that

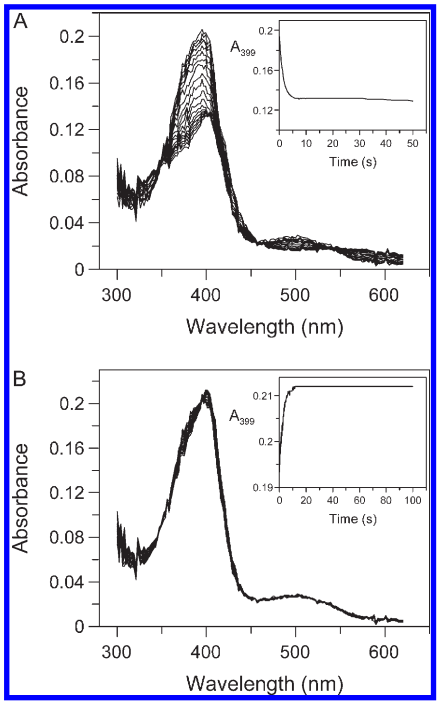

FIGURE 4: (A) Time course of the reaction of ELP with hydrogen peroxide. Experimental conditions: NaOAc buffer (pH 4.0) in the absence of added calcium ions, 25 °C, 1.5 μM ELP, and 250 μM hydrogen peroxide (concentrations after mixing). Selection of the spectra collected between 1 ms and 50 s (only 25 spectra of the original 500 are shown for the sake of clarity). The inset shows the time course recorded at 399 nm, as obtained from the same data plotted in the figure but using the information from all 500 absorbance spectra collected over 50 s. (B) Time course of the reaction of ELP with hydrogen peroxide and thiocyanate. Experimental conditions as described for panel A, with 0.5 mM thiocyanate (concentration after mixing). Selection of the spectra collected between 1 ms and 100 s (only 25 spectra of the original 500 are shown for the sake of clarity). The inset shows the time course recorded at 399 nm, as obtained from the same data plotted in the figure but using the information from all 500 absorbance spectra collected over 100 s.

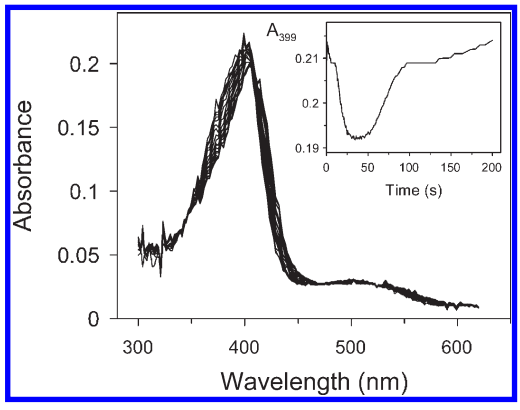

FIGURE 5: Time course of the reaction of ELP with thiocyanate and hydrogen peroxide. Experimental conditions: NaOAc buffer (pH 5.75) in the absence of added calcium ions, 25 °C, 1.5 μM ELP, 1 mM thiocyanate, and 0.5 mM hydrogen peroxide (concentrations after mixing). Selection of the spectra collected between 5 ms and 150 s (only 19 spectra of the original 500 are shown for the sake of clarity). The inset shows the time course recorded at 399 nm, as obtained from the same data plotted in the figure but using the information from all 500 absorbance spectra collected over 200 s.

SCN induced a significant modification of the ELP native optical spectrum (i.e., in the absence of calcium added), distinguishing between these two possibilities was not easy. The former hypothesis,

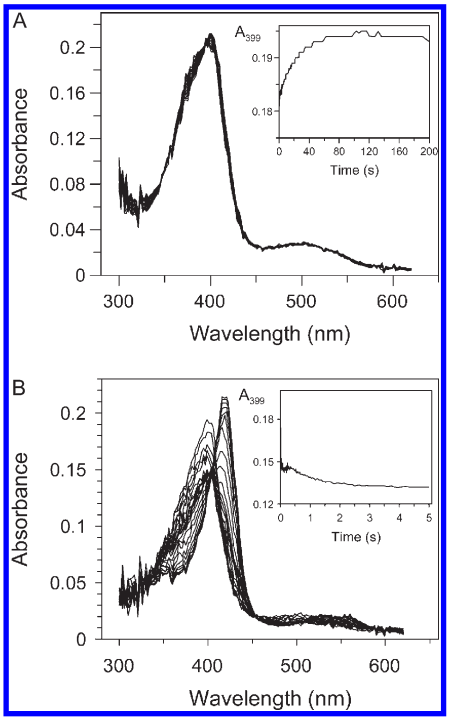

FIGURE 6: (A) Time course of the reaction of ELP with hydrogen peroxide and thiocyanate. Experimental conditions: NaOAc buffer (pH 4) with 10 mM $CaCl_2$, 25 °C, 1.5 μM ELP, 10 μM hydrogen peroxide, and 500 μM thiocyanate (concentrations after mixing). Selection of the spectra collected between 1 ms and 200 s (only 25 spectra of the original 500 are shown for the sake of clarity). The inset shows the time course recorded at 399 nm, as obtained from the same data plotted in the figure but using the information from all 500 absorbance spectra collected over 200 s. (B) Time course of the reaction of ELP with thiocyanate and hydrogen peroxide. Experimental conditions: NaOAc buffer (pH 5.75) with 10 mM $CaCl_2$, 25 °C, 1.5 μM ELP, 1 mM thiocyanate, and 10 μM hydrogen peroxide (concentrations after mixing). Selection of the spectra collected between 1 ms and 5 s (only 25 spectra of the original 500 are shown for the sake of clarity). The inset shows the time course recorded at 399 nm, as obtained from the same data plotted in the figure but using the information from all 500 absorbance spectra collected over 200 s.

which was simpler and more likely, did not yield a good fit of the experimental data; i.e., it required a catalytic cycle more complex than the commonly accepted one (with its three intermediates, ferric, Compound I, and Compound II). The latter hypothesis would fit the experimental data but had poor independent support.

When the experiments described above were conducted in a buffer containing 10 mM calcium ions, some similarities and some differences were apparent. When ferric ELP was mixed with hydrogen peroxide in the absence of any reducing substrate, the reaction was faster by a factor of $\geq$1000, and at pH 4.0 and 5 μM H_2O_2, Compound I was 100% populated within the dead time of the instrument (2 ms). The second-order rate constant was therefore estimated to exceed 10^7 M^{-1} s^{-1}, consistent with previous determinations (*27*).

In the presence of thiocyanate, the behavior of the enzyme at pH 4.0 was strikingly similar to that recorded in the absence of calcium ions; i.e., a species or a mixture of species with a spectrum similar to that of the resting ferric enzyme was slowly formed (Figure 6A). At pH 5.75, even in the presence of calcium ions, ELP behaved very differently and two well-separated kinetic processes were recorded, the faster one leading to a mixture of species in which a clear spectroscopic contribution of Compound II was apparent, with a half-time of 68 ms (at 10 μM H_2O_2). The slower process led to a species whose spectrum was reminiscent of that of Compound II, with a half-time of 600−800 ms (at 0.5−1 mM SCN^-). No recovery of the resting ferric state of the enzyme was observed under the experimental conditions described (Figure 6B). From these data, it was unclear whether some cycling occurred in the time window of 50−200 ms, but as this would be incompatible with the steady state determinations, we were inclined to rule out this possibility.

There are two difficulties in the interpretation of the kinetic data, namely (i) why and how the presence of thiocyanate slows by a factor of 100 the reaction between ferric ELP and H_2O_2 that leads to Compound I (in the presence and absence of added calcium) and (ii) the nature of the species (or mixture of species) obtained at the end of the time course (5 s). Both difficulties would be remedied if one accepts the hypothesis that SCN^- binds noncovalently to the heme group, thus modifying its reactivity and leading to a slow oxidation of the ferric derivative and to a modified and almost unreactive Compound II. We shall consider this hypothesis in Discussion, together with the other evidence obtained (see above).

DISCUSSION

The pseudohalide thiocyanate is an attractive substrate for peroxidases because it is oxidized by mammalian and plant peroxidases in different ways. The oxidation of SCN^- by the mammalian lactoperoxidase results in two-electron transfer from SCN^- to the heme iron with the formation of hypothiocyanous acid as follows:

$$\text{LPO} + \text{H}_2\text{O}_2 \rightarrow \text{Compound I}$$

$$\text{Compound I} + \text{SCN}^- \rightarrow \text{LPO} + \text{OSCN}^-$$

The product formed during SCN^- oxidation by the plant horseradish peroxidase is dithiocyanogen (*20*) because of two successive one-electron transfers from SCN^- as reported above.

The binding of SCN^- to LPO is facilitated by protonation of an ionizable group, presumably the distal histidine with a pK_a near 6.0. On the other hand, SCN^- binds to HRP away from the distal histidine, near the heme methyl $C1H_3$ and $C18H_3$, and the binding is facilitated by protonation of an acid group with a pK_a near 4.0 (*18*).

A cationic peroxidase extracted from the latex of the perennial Mediterranean shrub *E. characias* has peculiar characteristics. The native enzyme has one strongly bound endogenous Ca^{2+} ion that represents an essential factor in maintaining the protein structure around the heme environment. However, addition of a second Ca^{2+} ion to native ELP increases the enzyme activity several times with a drastic increase in k_{cat} and a decrease in the K_M for hydrogen peroxide (*11*), and thus, Ca^{2+} ion acts as a potent activator.

Upon examination of the extent of SCN^- oxidation by *Euphorbia* peroxidase, three main conclusions are immediately obvious from the experimental findings. First, SCN^- is both a substrate and a reversible inactivator of ELP. Second, the inhibitory effect is observed, in the presence of calcium ions, in all three species observed, i.e., the native ferric enzyme, Compound I, and Compound II. Third, the binding site of SCN^- as a substrate and as an inactivator needs to be different, and indeed, the pseudohalide seems not to directly interact with the heme iron. Thus, basically two different reaction mechanisms can be

hypothesized when the experiments are performed in the absence or presence of added Ca^{2+} ions.

(i) The native ELP-catalyzed oxidation of thiocyanate by hydrogen peroxide, in the absence of added calcium ions, is a reaction similar to the one catalyzed by mammalian peroxidases. Spectrophotometric features indicate that the addition of an equimolar amount of hydrogen peroxide to the native enzyme produces Compound I. The addition of SCN^- to Compound I does not lead to the formation of Compound II, but a progressive disappearance of the Compound I absorption spectrum, leading to the native spectrum, is observed. Because 1.0 mol of SCN^-/mol of enzyme is used to completely reverse the reaction of Compound I to the native ELP, it is clearly indicative of a two-electron transition from SCN^- to Compound I generating the native state. Moreover, an increase in the absorbance at 235 and 376 nm is observed, typical of the formation of hypothiocyanite ion, and the rate of oxidation is maximal at pH 5.75 and minimal at pH 4.0. Because of the slow rate of oxidation of SCN^- by ELP and the fact that hypothiocyanite is a transient species that is not stable, we are unable to detect $OSCN^-$ as a reaction product by NMR studies.

(ii) The ELP-catalyzed oxidation of thiocyanate by hydrogen peroxide, in the presence of added calcium ions, is the typical reaction catalyzed by plant peroxidases. The absorbance spectrum of ELP, in the presence of 10 mM Ca^{2+} ions and at pH 5.75, is very similar to that of the native enzyme, and the addition of an equimolar amount of hydrogen peroxide to the Ca^{2+}−ELP complex produces Compound I. The addition of 1.0 mol of SCN^-/mol of enzyme to the reaction mixture reduces Compound I to Compound II, which is then reduced to the native enzyme after a successive addition of 1.0 mol of SCN^-/mol of enzyme. Thus, in the presence of calcium ions, a one-electron transition from SCN^- to Compound I generating Compound II and a successive one-electron transition from SCN^- to Compound II generating again the native state are clearly demonstrated. Finally, CN^- is formed as a product of thiocyanate oxidation, whereas the increase in absorbance at 235 and 376 nm is almost zero, indicating no formation of hypothiocyanite.

Thiocyanate is the only substrate known to us that is processed by ELP faster, albeit differently, in the absence of calcium ions than in their presence. Indeed, 10 mM Ca^{2+} at pH 5.75 increases the k_{cat} values of ABTS and of *o*-dianisidine by factors of 100 and 27, respectively, whereas it decreases the k_{cat} of thiocyanate by 13-fold. Part of this effect is ascribed to the inhibitory effect of the anion on the reaction of the ferric enzyme with H_2O_2, leading to Compound I. As demonstrated in Results, the formation of Compound I is slowed by a factor of at least 10-fold by 1 mM thiocyanate. However, this effect, by itself, would not provide a sufficient explanation because the formation of Compound I remains much slower in the absence of Ca^{2+} than in its presence.

Three pieces of evidence point to a direct inhibitory effect of thiocyanate on Compound II (obtained in the presence of calcium ions with an excess of SCN^-), namely, (i) the inhibition of oxidation of *o*-dianisidine, (ii) the observation that Compound II is not reduced to the ferric derivative by addition of ascorbic acid, and (iii) the observation that SCN^- does not reduce Compound II to the ferric derivative in the time regime explored by the stopped-flow method. Although kinetic data never provide reliable structural information, it is obvious that a complex of thiocyanate with both the ferric and Compound I derivatives of ELP must be postulated to account for the experimental results. It is unlikely that this complex may be formed with the heme iron, because it does not alter the absorption spectrum of the ferric ELP and does not prevent its oxidation; it is more plausible that a noncovalent complex is formed with the porphyrin or with amino acid residues in the heme pocket. An important question therefore deals with the role of the calcium ions and their interaction with thiocyanate as a second substrate. We remark that thiocyanate is not so poor as a substrate in the absence of calcium as it is in the presence of this ion; e.g., its k_{cat} is 100 times slower than that of ABTS in the absence of calcium and 10000 times slower in its presence. Therefore, our data are compatible with the hypothesis that calcium ions, when present, participate in the (inactive) complex of ELP and thiocyanate.

Data previously reported indicated that HRP was reversibly inactivated when incubated with hydrogen peroxide and thiocyanate (*20*). The inactivating species has been identified as CN^- formed during SCN^- oxidation. The mechanism of inhibition, in our experiments, is clearly demonstrated to be different from the previously considered ones, and given that cyanide is produced only slowly, it is unclear how long an incubation must be to make it relevant.

CN^- behaves as a competitive inhibitor of ELP. At pH 5.75, in the presence of Ca^{2+}, the K_i value (1 μM) is shown to be lower than that in the native enzyme (6 μM), i.e., in the absence of added calcium, although on the same order of magnitude. In the absence or presence of Ca^{2+} ions, the K_i value increases as the pH decreases, showing the highest value at pH 4.0. Moreover, the K_d values for cyanide, in the presence and absence of calcium ions, suggest a particularly strong interaction between this anion and the heme iron, higher at pH 5.75 than at pH 4.0. The effect of pH is mainly due to the hydrogen ion competing with iron for cyanide ion, and therefore, it is not a property of ELP.

It is well-known that the cyanide adduct may be compared to the initial binding of hydrogen peroxide to peroxidases (*28*). The somewhat higher concentration of CN^- observed via measurement of the inhibitory effect of cyanide on the steady state parameters (K_i) with respect to the dissociation constant (K_d) may be explained as being due to the competition between CN^- and H_2O_2 for the reaction with the heme iron. Moreover, the effect of Ca^{2+} ions on the dissociation constants of cyanide reflected the activating effect, which was greater at pH 5.75 than at pH 4.0. At the end of the titration and at both pH values, the final spectrum obtained is typical of a low-spin derivative six-coordinate inactive complex.

The formation of inactive species absorbing at 418 and 540 nm, achieved at the end of the titration of ELP with hydrogen peroxide at fixed concentration of thiocyanate or with an excess of both the substrates, hydrogen peroxide and thiocyanate, even in the presence of calcium ions, is thus interpreted as a change from a pentacoordinated mixed-spin state of the heme iron native enzyme to a low-spin, six-coordinate inactive complex. This finding implies that thiocyanate and its derivatives combine with the enzyme at different sites and with different bonds; e.g., the derivative described here must be qualitatively different from that formed with the ferric enzyme and responsible for lowering the rate constant of the reaction with hydrogen peroxide, as described above.

Data reported for the catalytic activity of HRP and SCN^- indicate that free radicals derived from the oxidation on SCN^- are involved in limiting the catalytic turnover of the enzyme. Thus, the kinetic and spectroscopic changes of the ELP-catalyzed SCN^- oxidation, in the presence of Ca^{2+} ions, are further studied in the presence of the DMPO spin trap. We observe that the

initial rate of SCN^- oxidation is not increased in the presence of DMPO, but after incubation with this spin trap for 30 min, the activity of the enzyme is fully recovered. This can be indicative that the thiocyanate radical is not responsible for the fast inactivation of ELP but is certainly implicated in the formation of the stable reactive product CN^-.

From all the obtained results, we can hypothesize that the distal calcium ion is the key for two different reaction mechanisms between SCN^- and ELP. We believe that, in the absence of calcium, the distal site is "open" and the binding of SCN^- is facilitated by protonation of an ionizable group, probably of the distal histidine. Because both N and S of SCN^- can be electron donors, it may interact with iron and the imidazole ring of the distal histidine through the nitrogen and sulfur atom, respectively, as reported with lactoperoxidase (*29*, *30*). In the presence of hydrogen peroxide, Compound I and SCN^- can form an $NCS\cdots H^+-N{=}C{=}N-H\cdots O{=}Fe^{IV+}$ structure by conjugated double bonds and hydrogen bonds as described for the interaction between I^- and lactoperoxidase (*31*). It may facilitate the transfer of two electrons from SCN^- to the heme iron. A similar mechanism has been hypothesized in human myeloperoxidase reacting with thiocyanate (*32*).

The reorientation of the distal histidine after addition of calcium ions and the consequent rearrangement of the distal cavity induce the "closed" form of the distal site. Thus, under these conditions, SCN^- can bind ELP away from the ferric center, as reported for HRP (*21*). Thus, thiocyanate, bound in the porphyrin ring, can reduce Compound I to Compound II, releasing SCN radical that can be trapped by DMPO or can form the inactivating species CN^-. As reported, 1 mol of CN^- has to be formed from 6 mol of SCN^-, and 3 mol of hydrogen peroxide is required. Because 30 μM hydrogen peroxide is used in our experiments to completely change 3 μM native ELP to 3 μM ELP$-CN^-$ complex and 10 μM CN^- could be formed in the system, it is compatible with the dissociation constant of the ELP$-CN^-$ complex (K_d = 2 μM) and with the competition between CN^- and hydrogen peroxide for the heme iron.

In plants, as in all eukaryotes, calcium plays a central role as a second messenger in the regulation of a number of physiological processes. Decodification of Ca^{2+} signals is generally devoted to a group of specialized cytosolic proteins that transduce these messages into molecular and cellular responses and integrate calcium signaling into virtually all aspects of plant functioning (*33*−*35*).

SCN^- is one of the components of the *E. characias* latex that also contains free calcium ions as determined by atomic absorption, and the Ca^{2+} content varies from 2.1 ± 0.1 mM in winter to 3.5 ± 0.15 mM in summer (*36*). To take into account under in vivo conditions for the in vitro observed modulation of ELP activity by Ca^{2+}, we could hypothesize that the oxidation of SCN^- by ELP reversibly blocks the peroxidase activity via the formation of CN^-. The formation of CN^- could be one of the deterrents for herbivores and insects. Following plant injury or environmental stresses, the content of calcium ions could decrease, so that SCN^- can interact with ELP and promote the formation of hypothiocyanite with associated defense responses against invading pathogens.

SUPPORTING INFORMATION AVAILABLE

Additional observations. This material is available free of charge via the Internet at http://pubs.acs.org.

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