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Does diphenylene iodonium chloride have any effect on the $O_2^{\bullet-}$-generating step of plant peroxidases?

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Abstract The $O_2^{\bullet-}$-generating step of plant peroxidases during their catalytic cycle is represented by the decay of compound III (CoIII) into ferriperoxidase, which most likely involves the dissociation of a ferric-$O_2^{\bullet-}$ complex to yield the ferric form of the enzyme and $O_2^{\bullet-}$. Diphenylene iodonium chloride (DPI), at 50–100 μM, does not significantly enhance the stability of CoIII of peroxidase, as judged by the values of k_{decay}, and therefore, DPI appears to have no effect on the $O_2^{\bullet-}$-generating step of plant peroxidases. From these results, it is concluded that caution should be exercized when considering peroxidase as a possible enzyme target of $O_2^{\bullet-}$-mediated plant physiological processes sensitive to DPI inhibition.

Key words: Compound III stability; Peroxidase; Diphenylene iodonium chloride; $O_2^{\bullet-}$-generating activity

1. Introduction

H_2O_2 is produced by plant cells at the plasma membrane surface either as a defence mechanism during plant-pathogen interaction, in the so-called 'oxidative burst' [1,2], or constitutively in specific lignifying tissues [3,4]. In both cases, H_2O_2 is needed by plant cell wall peroxidases for the biosynthesis of lignins and the cross-linking of both extensin and feruloylpectins, a key process in the developmental program that determines the final cell wall architecture of differentiated plant cells and which is the basis of plant cell reinforcement. However, the real molecular mechanism of H_2O_2 production at the plant cell surface is still unknown. Thus, and although during recent years, great efforts have been made by plant biologists to resolve the paradigm of the mechanism of H_2O_2 production at the plasma membrane/cell wall interface, the actual knowledge on this enzymatic mechanism is fragmentary and, in most cases, not easily to understand.

Among other [5], two possible mechanisms have been proposed to explain how this H_2O_2 is produced at the plant cell surface. One involves the action of a peroxidase [6], whereas the other involves the action of a NADPH oxidase [1], similarly to that observed in mammalian neutrophil cells. Although each hypothesis is supported by the corresponding body of evidence [2], discrimination between one and the other mechanism is generally based on the results obtained using several metabolic inhibitors. Thus, the participation of a putative NADPH oxidase in H_2O_2 production is supported by the sensitivity of H_2O_2 production to inhibitors of neutrophil NADPH oxidase, such as diphenylene iodonium chloride (DPI), imidazole, pyridine, quinacrine and α-naphthol, five known inhibitors of mammalian neutrophil NADPH oxidase, but which have no effect on peroxidase during some reactions in which this enzyme generates the $O_2^{\bullet-}/H_2O_2$ redox pair [7].

However, the specificity of iodonium compounds, including DPI, of inhibiting NADPH oxidases activities is actually a matter of debate. In this way, iodonium compounds are capable of inhibiting a variety of flavoproteins, including mitochondrial NADH dehydrogenase [8], bacterial nicotine oxidase [9], macrophage NO synthase [10] and xanthine oxidase [8], as well as of interacting with hemoproteins, such as cytochrome *P*-450 [11] and neutrophil cytochrome b_{558} [12]. Recently, DPI has also been reported to be an inhibitor of the $O_2^{\bullet-}$-generating activity of horseradish peroxidase [13,14]. In this last case, the $O_2^{\bullet-}$-generating activity of horseradish peroxidase has been evaluated during the aerobic oxidation of NADH, a reaction which includes several chemical and enzymatic steps, and it is therefore constituted by a plethora of both enzymatic and non-enzymatic (radical free-propagated) bimolecular reactions [15], which make it difficult to understand at which level (enzymatic or not) DPI exerts its inhibitory effect.

In fact, during the peroxidase-catalyzed aerobic oxidation of NADH, $O_2^{\bullet-}$ may be generated either chemically, by the reaction of NAD radicals with dioxygen:

$$O_2 + NAD^{\bullet} \rightarrow O_2^{\bullet-} + NAD^+ \ (k = 2.0\times10^9\ M^{-1}\ s^{-1})$$

or enzymatically during the decay of compound III (CoIII) of peroxidase [16]:

$$CoIII \rightarrow ferriperoxidase + O_2^{\bullet-} \ (k = 8.2\times10^{-3}\ s^{-1})$$

During the aerobic oxidation of NADH, CoIII may be formed by reduction of the native ferric enzyme to ferroperoxidase by the NAD radical [15]:

$$ferriperoxidase + NAD^{\bullet} \rightarrow ferroperoxidase + NAD^+$$

$$(k = 8.0\times10^8\ M^{-1}\ s^{-1})$$

followed by the addition of oxygen:

$$ferroperoxidase + O_2 \rightarrow CoIII \ (k = 5.8\times10^4\ M^{-1}\ s^{-1})$$

Although other active intermediate forms of peroxidase, such as compound I and compound II, are also involved in the aerobic oxidation of NADH [15], they do not appear to be the targets of DPI inhibition [13]. Since decay of CoIII ap-

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pears to be the rate limiting step ($k_{decay} = 8.2 \times 10^{-3}$ s^{-1}) in the NADH-dependent $O_2^{\bullet -}$-generating activity of horseradish peroxidase, we studied the effect of DPI on the stability of CoIII, in order to ascertain if CoIII is really the target of DPI inhibition. Because ferroperoxidase is the direct precursor of CoIII and since it shows electronic properties similar to cytochrome *P*-450, which is capable of reacting with aryl-iodonium salts [11], we also studied the possibility that ferroperoxidase may act as a target of DPI inhibition.

2. Materials and methods

2.1. Chemicals and peroxidase source

Horseradish peroxidase C (type IX) was purchased from Sigma (Madrid, Spain) and used without further purification. DPI was purchased from ICN Iberica (Barcelona, Spain). It was dissolved in dimethyl sulfoxide and the solution was standardized at 20 mM ($\lambda_{max} = 264$ nm) by using $\varepsilon_{264} = 15.1$ mM^{-1} cm^{-1} [12]. All the other chemicals were of analytical grade and supplied by Sigma/Merck.

The purity of horseradish peroxidase C was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% acrylamide gels (Fig. 1). For this, the protein sample was prepared in 10% glycerol, 2% SDS, 5% mercaptoethanol in 62.5 mM Tris-HCl buffer (pH 6.8). Acrylamide gels were cast and electrophoresed using a Mini Protean II electrophoresis system (Bio-Rad) according to the manufacturer's specifications. The molecular markers used were from the LMW Electrophoresis calibration kit (Pharmacia Biotech). Proteins were stained with Coomassie brilliant blue R (Sigma) according to the manufacturer's specifications.

2.2. Preparation of ferroperoxidase

Reduced horseradish peroxidase C (ferroperoxidase) was prepared by incubation of enzyme (1.0 μM) with an excess of sodium dithionite for 1–3 min in 20 mM potassium phosphate (pH 7.0) until no further change in the reduced Soret band ($\lambda_{max} = 437$ nm) could be detected. Since ferroperoxidase reacts rapidly with oxygen, the reaction of ferroperoxidase with DPI was performed in evacuated buffer.

2.3. Preparation of CoIII of peroxidase and determination of k_{decay}

CoIII of horseradish peroxidase C was prepared from ferroperoxidase by passing the enzyme through a pre-packed Sephadex G-25 PD-10 column (Pharmacia Biotech) that was equilibrated with deoxygenated 20 mM potassium phosphate (pH 7.0) buffer to remove the excess of sodium dithionite. The disappearance of peroxidase CoIII and the appearance of the native ferric enzyme was followed at their respective Soret bands ($\lambda_{max} = 417$ nm and $\lambda_{max} = 403$ nm) by repeated rapid scans of the reaction media in a Uvikon 940 spectrophotometer. The rate constant of decay (k_{decay}) of CoIII was determined at 25°C by fitting the absorbance values at 417 nm versus time to a single exponential function [16].

3. Results and discussion

3.1. Effect of DPI on ferroperoxidase

Aryl-iodonium salts are known to react with Fe(II)-containing hemeproteins to give σ-aryl ferric complexes characterized by a Soret peak at 480 nm [11]. To study the possibility that ferroperoxidase may act as a target of DPI, ferroperoxidase was prepared from ferriperoxidase by reducing it with an excess of sodium dithionite. Under these conditions, ferroperoxidase is moderately stable and showed a Soret peak at 437 nm, characteristic of this reduced form of the enzyme. Incubation of ferroperoxidase with 50 μM DPI does not provoke the shift of the Soret peak at 480 nm (Fig. 2), which is characteristic of σ-aryl ferric complexes, the minor spectral changes observed corresponding to the transition of ferroperoxidase to CoIII. From these results, it can be concluded that the Fe(II)-reduced form of ferriperoxidase, ferroperoxidase, is not the apparent target of DPI, which is one of the mechanisms [13] that would explain the inhibition of the $O_2^{\bullet -}$-generating activity of peroxidase by DPI, since ferroperoxidase does not react with DPI to give σ-aryl ferric complexes.

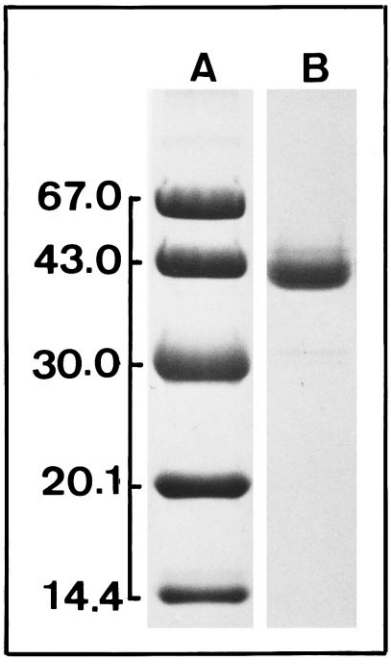

Fig. 1. Analysis of the purity of horseradish peroxidase C by SDS-PAGE. A: Coomassie staining of molecular mass markers (kDa). B: Commercial available horseradish peroxidase C.

3.2. Effect of DPI on the stability of CoIII

In order to study the effect of DPI on the transition of CoIII to ferriperoxidase, which is the $O_2^{\bullet -}$-generating enzymatic step during the peroxidase-mediated aerobic oxidation of NADH, CoIII was prepared from ferriperoxidase by reducing it with an excess of sodium dithionite and further removing the dithionite excess under aerobic conditions. The CoIII thus obtained showed λ_{max} at 417, 543 and 577 nm and decayed to ferriperoxidase (λ_{max} at 403, 499 and 643 nm) with a k_{decay} of $8.6 \pm 3.9 \times 10^{-3}$ s^{-1} (mean ± S.E.M., $n = 5$). That is, CoIII totally decayed to ferriperoxidase in 5–10 min at 25°C. That the spectral transition observed under these conditions

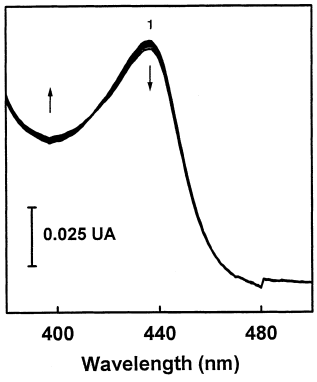

Fig. 2. Consecutive spectra of a reaction medium containing 1.0 μM ferroperoxidase and 50 μM DPI left to react at 25°C for 10 min. Spectrum 1 was obtained immediately after mixing. The arrows show the direction of absorbance change with time.

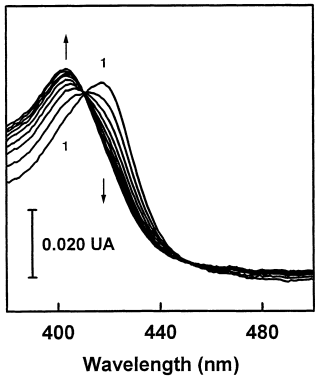

Fig. 3. Consecutive spectra of a reaction medium containing 1.0 μM CoIII of peroxidase and 50 μM DPI left to react at 25°C for 10 min. Spectrum 1 was obtained immediately after mixing. The arrows show the direction of absorbance change with time.

corresponds exclusively to the transition CoIII → ferriperoxidase was deduced from the spectrum of the initial species (λ_{max} at 417, 543 and 577 nm), the spectrum of the final species (λ_{max} at 403, 499 and 643 nm) and the k_{decay} ($=8.6 \pm 3.9\times10^{-3}$ s^{-1}), all of which were in accordance with the literature [16].

The addition of DPI at 50 μM does not affect the VIS properties of CoIII (λ_{max} at 417, 543 and 577 nm) or the transition rate of CoIII to ferriperoxidase when the spectral changes of the reaction medium with time are monitored (Fig. 3). In fact, in the presence of 50 μM DPI, CoIII decayed to ferriperoxidase with a $k_{decay} = 6.6 \pm 2.0\times10^{-3}$ s^{-1} (mean ± S.E.M., $n=6$), which is not statistically different at $P=0.05$ from the value observed in the absence of DPI ($8.6 \pm 3.9\times10^{-3}$ s^{-1}). Similarly, higher DPI concentrations (100 μM) had no statistically significant effect ($k_{decay} = 5.4 \pm 3.3\times10^{-3}$ s^{-1}, mean ± S.E.M., $n=3$) on the stability of CoIII.

The mechanism of CoIII decay to ferriperoxidase has been studied previously in detail [16] and it was thought to most likely involve the dissociation of a ferric-$O_2^{\bullet-}$ complex to yield the ferric form of the enzyme and $O_2^{\bullet-}$. The present results clearly demonstrate that DPI does not significantly enhance the stability of CoIII of peroxidase and raise several doubts on the fate of DPI as inhibitor of the $O_2^{\bullet-}$-generating activity of peroxidase. These results further suggest that DPI does not act on the enzymatic-controlled processes, but probably on the plethora of non-enzymatic (radical free-propagated) bimolecular reactions [15], which may also be propagated by a vast array of biologically available non-enzymatic catalysts. In fact, Frahry and Schopfer [13] have previously reported that DPI itself may act as a $O_2^{\bullet-}$-scavenging compound and also probably as a scavenger of NAD radicals, since it stimulates NADH consumption in the same way that it inhibits $O_2^{\bullet-}$ production. From these results, it can be concluded that care should be taken when considering peroxidase as a possible enzyme target of $O_2^{\bullet-}$-mediated plant physiological processes sensitive to DPI inhibition.

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