Pergamon

PII: S0031–9422(98)00004–1

Phytochemistry, Vol. 48, No. 2, pp. 223–227, 1998

Printed in Great Britain
0031-9422/98 $19.00+0.00

INHIBITION OF O_2-REDUCING ACTIVITY OF HORSERADISH PEROXIDASE BY DIPHENYLENEIODONIUM

GITTA FRAHRY and PETER SCHOPFER*

Albert-Ludwigs-Universität Freiburg, Institut für Biologie II, Schänzlestrasse 1, D-79104 Freiburg, Germany

(*Received in revised form* 8 *December* 1997)

Key Word Index—horseradish peroxidase; H_2O_2 production; active oxygen species; NAD(P)H oxidase; diphenyleneiodonium.

Abstract—Plant cells respond to pathogen attack with a burst of H_2O_2 secretion. The question whether this defense reaction is catalysed by a NAD(P)H oxidase similar to the NADPH oxidase of phagocytic leukocytes in mammals or by an extracellular peroxidase is presently a matter of controversial debate. The observation that H_2O_2 production by plant cells can be inhibited by diphenyleneiodonium (DPI), a potent inhibitor of the mammalian NADPH oxidase, has fostered the view that a mammalian-type enzyme is responsible for the H_2O_2 production by plant cells. Here we show that DPI inhibits the NADH-dependent H_2O_2 production by horseradish peroxidase in the same concentration range as previously used for the inhibition of putative NADPH oxidase activity in plants. The peroxidative activity normally used for assaying peroxidase is not affected by DPI, indicating that the inhibitor specifically interferes with a partial reaction that is exclusively involved in the O_2 reducing activity of the enzyme. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Mammalian cells such as phagocytic leukocytes possess an inducible NADPH oxidase in their plasma membrane producing toxic active oxygen species (O_2^-, H_2O_2; AOS) as a defense reaction against pathogens [1, 2]. Evidence is accumulating that also plant cells can rapidly secrete AOS in response to pathogen attack ("oxidative burst", see [3] for review). The striking analogy between animal and plant cells prompted the question of whether this reaction is mediated in the plasma membrane of plant cells by an enzyme similar to the animal NADPH oxidase. Potential alternative sources of O_2^- and H_2O_2 in plant cells are the flavoprotein oxidases localized in peroxisomes [4] or cell walls [5] and cell-wall- or plasma-membrane-bound peroxidases that catalyse the univalent reduction of O_2 to O_2^-, and of O_2^- to H_2O_2, using NAD(P)H as electron donor [6–10]. It has been proposed that peroxidase-generated H_2O_2 may function as an antifungal agent in the disease resistance of plants [10]. Bolwell *et al.* [11, 12] concluded that the elicitor-induced H_2O_2 production in French bean cells is mediated by peroxidase.

Discrimination between the different enzymic mechanisms of AOS production by plant cells has been attempted by using diphenyleneiodonium (DPI), a potent inhibitor of the mammalian NADPH oxidase. DPI binds to the flavoprotein moiety as well as to the cytochrome b_{558} moiety of the reduced NADPH oxidase complex, inhibiting electron transfer from NADPH to O_2 both in living leukocytes and isolated leukocyte membranes [13–15]. The observation that DPI, in the range of 1–100 μM, inhibits the elicitor-induced production of AOS by cell suspension cultures of Arabidopsis [16], soybean [17–19], chickpea [20] and rose [21], as well as in Arabidopsis leaves [22], has been used as evidence that an enzyme homologous to the NADPH oxidase of leukocytes is present in the plasma membrane of plant cells and might be responsible for the oxidative burst by pathogens or chemical elicitors. This idea has been supported by the finding that DPI does not inhibit the activity of peroxidases in the standard assay reaction (oxidation of a phenolic substrate by H_2O_2), apparently excluding peroxidases as mediators of DPI-inhibitable AOS production in plant cells [19, 21].

In this communication we show that the O_2-reducing activity of peroxidases producing O_2^- and H_2O_2 in the presence of NADH is strongly inhibited by DPI. Thus, this inhibitor cannot be used to discriminate between a mammalian-type NAD(P)H oxidase and peroxidases in mediating the pathogen-induced oxidative burst in plants.

---

* Author to whom correspondence should be addressed.

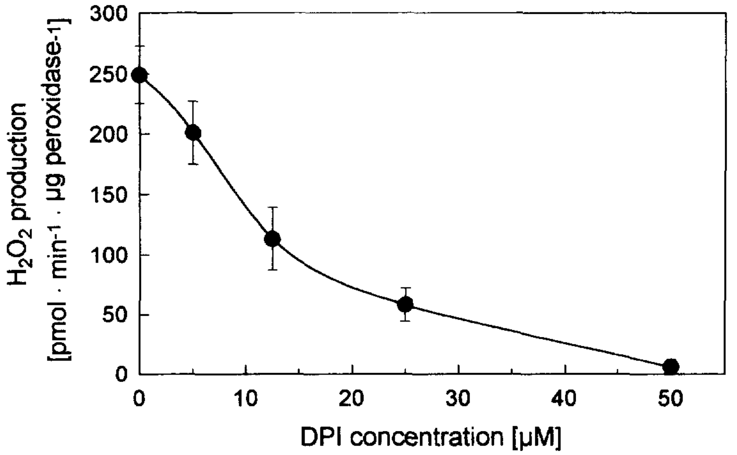

Fig. 1. Effect of DPI on NADH-dependent H_2O_2 production by peroxidase. Control experiments showed that DPI did not directly react with NADH or H_2O_2 and that the peroxidase-catalysed oxidation of scopoletin used in the H_2O_2 assay was not affected by the inhibitor.

RESULTS AND DISCUSSION

The oxidative burst catalysed by NADPH oxidase of leukocytes can be inhibited by DPI concentrations in the range of 0.1–10 μM [23]. Ten-fold higher DPI concentrations have been found to be necessary to inhibit the oxidative burst in plants [16–21]. The effect of DPI on H_2O_2 production in the presence of NADH by horseradish peroxidase is shown in Fig. 1. The reaction can be inhibited by DPI concentrations of about 5–50 μM, i.e. in the same range as reported for the inhibition of AOS production by plant cells [16–21]. In contrast, the oxidative activity of peroxidase assayed by the oxidation of guaiacol with H_2O_2 is not affected by the inhibitor (Table 1). Also the oxidation of scopoletin with H_2O_2 used as the assay reaction for H_2O_2 was not inhibited by DPI (data not shown). These results demonstrate that DPI can interfere with a step that is specifically involved in the reduction of O_2 by electron donors such as NADH, whereas the steps involved in the binding of H_2O_2 (formation of compound I) and the subsequent steps leading to electron transfer from a phenolic substrate to H_2O_2 are unaffected.

Interestingly, the inhibition by DPI of peroxidase-catalysed O_2 reduction is not accompanied by a corresponding decrease in the consumption of NADH. Table 2 shows that about 2 molecules of NADH are oxidized for each H_2O_2 molecule formed and that the oxidation of NADH is even slightly increased when H_2O_2 formation is inhibited by DPI. This indicates that there is no strict stoichiometric interdependence between these two reactions. Obviously NADH can be oxidized by peroxidase at a reaction site not affected by DPI and the reducing equivalents produced can be utilized in a reaction other than H_2O_2 net production from O_2. Inactivation of the iron in the haem moiety of peroxidase by cyanide inhibits both H_2O_2 production as well as the oxidation of NADH (Table 2). This result clearly shows that the haem moiety is also necessary for the O_2-reducing activity of peroxidase. However, DPI and cyanide obviously affect different partial reactions catalysed by the enzyme.

Table 1. Effect of DPI on the oxidative activity of peroxidase assayed with guaiacol and H_2O_2 as substrates

Inhibitor treatment	Peroxidase activity [nmol tetraguaiacol · 3 min^{-1} · μg peroxidase^{-1}]
—	51.1±0.4
12.5 μM DPI	54.5±0.8
50 μM DPI	57.5±2.7

The inhibitory effect of DPI on the O_2-reducing activity of peroxidase can also be demonstrated at the level of the intermediate O_2^-. Table 3 shows that NADH-dependent O_2^- production by peroxidase determined with the cytochrome c reduction assay at pH 6 is reduced by about 90% in the presence of 50 μM DPI. The kinetics of this inhibition (Fig. 2a) reveal that DPI reduces the reaction rate after a delay of about 1 min, similar to the mammalian NADH oxidase that can be inhibited only after reduction by NADPH [24]. Assayed under similar conditions, the oxidation by peroxidase of the substrate diaminobenzidine with H_2O_2 is not affected by DPI (Fig. 2b).

It has been shown previously that DPI and related iodonium compounds can interact with haemoproteins such as cytochromes [14, 15, 25] and peroxidase [18] under particular conditions. In order to check whether the inhibitor also has an effect on cytochrome c reduction used as an assay reaction for the determination of O_2^- formation, peroxidase was replaced by KO_2 at slightly alkaline pH [26] as a source of O_2^- in the assay mixture. Table 3 shows that 50 μM

Table 2. Effect of DPI on H_2O_2 production and NADH consumption by peroxidase, measured in the same experiments. The specificity of the scopoletin oxidation assay reaction for H_2O_2 is demonstrated by inhibitory effect of catalase (CAT)

Inhibitor treatment	H_2O_2 production [pmol·min^{-1}·μg peroxidase^{-1}]	NADH consumption [pmol·min^{-1}·μg peroxidase^{-1}]
—	249±24	531±7
30 μg ml CAT	22±6	—
5 μM DPI	201±26	678±39
12.5 μM DPI	113±26	651±33
25 μM DPI	58±14	672±42
50 μM DPI	6±6	746±24
1 mM KCN	17±8	156±7

Table 3. Effect of DPI on O_2^- production by peroxidase. (a) peroxidase reaction at pH 6.0; (b) peroxidase reaction at pH 7.8; (c) assay reaction with KO_2 in the absence of peroxidase. O_2^- production within 1 min by 6 μg ml^{-1} peroxidase was measured 2 min after the start of the reaction. The KO_2 concentration was adjusted such as to produce a similar amount of reduced cytochrome c. The specificity of the cytochrome c reduction assay reaction for O_2^- is demonstrated by the inhibitory effect of superoxide dismutase (SOD)

Inhibitor treatment	O_2^- production [nmol]
a. Peroxidase, pH 6.0	
—	0.56±0.10
10 μg ml SOD	0.00±0.00
12.5 μM DPI	0.29±0.08
50 μM DPI	0.05±0.01
b. Peroxidase, pH 7.8	
—	1.05±0.02
50 μM DPI	0.13±0.01
c. KO_2, pH 7.8	
—	0.96±0.09
50 μM DPI	0.49±0.05

DPI inhibits the reduction of cytochrome c by ca 50%. However, the peroxidase-catalysed O_2^- production is inhibited by DPI under the same conditions by ca 90%. Thus, the inhibitory effect of DPI on O_2^- production by peroxidase shown in Fig. 2 is only partly due to an interference with the assay reaction; the O_2^--producing reaction of peroxidase appears to be also subject to inhibition by DPI.

In conclusion, our results demonstrate that DPI is an effective inhibitor of the H_2O_2-producing catalytic activity of peroxidase whereas the classical peroxidase activity determined by the H_2O_2-mediated oxidation of aromatic substrates such as guaiacol or diaminobenzidine is not affected. Thus, this inhibitor is unsuitable as a tool for differentiating between the mammalian-type NAD(P)H oxidase and peroxidase in the enzymic mechanism of the oxidative burst of plant cells, and the related experiments of previous investigators are inconclusive. Although evidence for the existence of proteins with sequence homologies to components of the animal enzyme has recently been found also in plants [3], there is presently no reliable functional assay for the identification of mammalian-type NAD(P)H oxidase in H_2O_2 production by plant cells. The molecular target of DPI action on peroxidase is not yet clear. It has been suggested that diaryliodonium compounds such as DPI can react specifically with the Fe^{2+} form of haemoproteins to give aryl Fe^{3+} porphyrin complexes [14, 15, 25]. If this also applies to peroxidase, it appears possible that DPI arrests the O_2-reducing activity by reacting with the enzyme in the Fe^{2+} state (Compound III) formed in the presence of a reductant such as NADH [9, 27]. This would provide an explanation for the delay in the inhibitory action of DPI (Fig. 2a). However, alternative mechanisms of inhibition, e.g. by interaction with the formation of intermediates of the O_2-reducing pathway, not required in the phenol-oxidizing pathway, such as O_2^- and NAD radical cannot be excluded.

EXPERIMENTAL

Chemicals

Peroxidase (EC 1.11.1.7) from horseradish, ferricytochrome c from horse heart, catalase (EC 1.11.1.6) from bovine liver, superoxide dismutase (EC 1.15.1.1) from bovine erythrocytes and NADH were from Boehringer; diphenyleneiodoniumHCl (DPI) from Biomol, Hamburg, Germany; 3,3-diaminobenzidine-4 HCl from Serva, Heidelberg, Germany; guaiacol and KO_2 from Merck. H_2O_2 from Fluka, and scopoletin from Sigma. Acetylated cytochrome c was prepared according to Ref. [28] and purified by gel chromatography on Sephadex G-25. DPI was used as a stock soln of 10 μM in DMSO. At the concns used, DMSO had no effect on the enzyme assays.

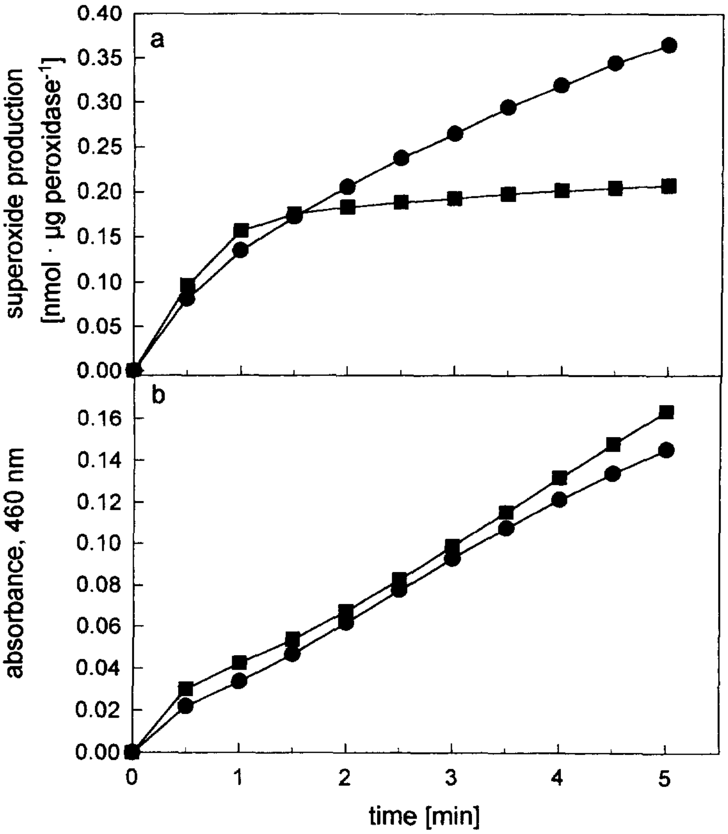

Fig. 2. Effect of DPI on NADH-dependent O_2^- production (a) and H_2O_2-dependent diaminobenzidine oxidation (b). (●), −DPI; (■), +50 μM DPI.

Enzyme assays (25°)

Citrate buffer (10 mM, pH 6) was used except where indicated otherwise. The phenol-oxidizing activity of peroxidase was determined by mixing 21 ng ml^{-1} enzyme, 13 mM guaiacol and 33 μM H_2O_2 in buffer and reading the *A* increase at 436 nm after 3 min [29]. Alternatively, the assay was performed with 14 ng ml^{-1} enzyme, 1 mM diaminobenzidine and 0.1 mM H_2O_2 (measurement at 460 nm). The O_2-reducing activity of peroxidase was determined by mixing 2 μg ml^{-1} enzyme and 0.2 mM NADH in aerated buffer. NADH consumption was measured by monitoring the *A* decrease at 340 nm. Aliquots were removed from the reaction mixture after 2 and 5 min for H_2O_2 determination. After destroying NADH (and removing CN^-, if present) with 0.1 M HCl, H_2O_2 was assayed in these samples by measuring the decrease of fluorescence of 5 μM scopoletin (excitation at 346 nm, emission at 455 nm), in the presence of 3 μg ml^{-1} peroxidase, in 100 mM citrate buffer (pH 6) in a spectrofluorimeter [30]. The calibration curve was linear in the range of 0–5 μM H_2O_2. The H_2O_2 assay was not affected by 50 μM DPI or 1 mM KCN in the sample. Superoxide production by peroxidase was determined by mixing 6 μg ml^{-1} enzyme, 0.6 mM NADH and 50 μM acetylated ferricytochrome c in buffer and measuring the *A* increase at 550 nm [31]. Experiments with KO_2 were performed in 50 mM Kpi buffer (pH 7.8). The data represent means of 4–6 measurements ± s.e.

Acknowledgement—This work was supported by Deutsche Forschungsgemeinschaft.

REFERENCES

1. Cross, A. R. and Jones, O. T. G., *Biochimica et Biophysica Acta*, 1991, **1057**, 281.
2. Segal, A. W. and Abo, A., *Trends in Biochemical Sciences*, 1993, **18**, 43.
3. Lamb, C. and Dixon, R. A., *Annual Reviews of Plant Physiology and Plant Molecular Biology*, 1997, **48**, 251.
4. Del Rio, L. A. and Donaldson, R. P., *Journal of Plant Physiology*, 1995, **146**, 283.
5. Angelini, R. and Federico, R., *Journal of Plant Physiology*, 1989, **135**, 212.
6. Elstner, E. F. and Heupel, A., *Planta*, 1976, **130**, 175.
7. Gross, G. G., Janse, C. and Elstner, E. F. *Planta*, 1977, **136**, 271.
8. Askerlund, P., Larsson, C., Widell, S. and Møller, I. M., *Physiologia Plantarum*, 1987, **71**, 9.
9. Halliwell, B., *Planta*, 1978, **140**, 81.

10. Peng, M. and Kuc, J., *Phytopathology*, 1992, **82**, 696.
11. Bolwell, G. P., *Biochemical Society Transactions*, 1996, **24**, 438.
12. Bolwell, G. P., Butt, V. S., Davies, D. R. and Zimmerlin, A., *Free Radical Research*, 1995, **23**, 517.
13. Cross, A. R. and Jones, O. T. G., *Biochemical Journal*, 1986, **237**, 111.
14. Doussiere, J. and Vignais, P. V., *European Journal of Biochemistry*, 1992, **208**, 61.
15. Dème, D., Doussiere, J., De Sandro, V., Dupuy, C., Pommier, J. and Virion, A., *Biochemical Journal*, 1994, **301**, 75.
16. Desikan, R., Hancock, J. T., Coffey, M. J. and Neill, S. J., *FEBS Letters*, 1996, **382**, 213.
17. Levine, A., Tenhaken, R., Dixon, R. and Lamb, C., *Cell*, 1994, **79**, 583.
18. Dwyer, S. C., Legendre, L., Low, P. S. and Leto, T. L., *Biochimica et Biophysica Acta*, 1996, **1289**, 231.
19. Mithöfer, A., Daxberger, A., Fromhold-Treu, D. and Ebel, J., *Phytochemistry*, 1997, **45**, 1101.
20. Otte, O. and Barz, W., *Planta*, 1996, **200**, 238.
21. Auh, C.-K. and Murphy, T. M., *Plant Physiology*, 1995, **107**, 1241.
22. Jabs, T., Dietrich, R. A. and Dangl, J. L., *Science*, 1996, **273**, 1853.
23. Ellis, J. A., Mayer, S. J. and Jones, O. T. G., *Biochemical Journal*, 1988, **251**, 887.
24. O'Donnell, V. B., Tew, D. G., Jones, O. T. G. and England, P. J., *Biochemical Journal*, 1993, **290**, 41.
25. Battioni, J.-P., Dupré, D., Delaforge, M., Jaouen, M. and Mansuy, D., *Journal of Organometallic Chemistry*, 1988, **358**, 389.
26. Lokesh, B. R. and Cunningham, M. L., *Toxicology Letters*, 1986, **34**, 75.
27. Halliwell, B. and Guttridge, J. M. C., *Free Radicals in Biology and Medicine*, 2nd edn, Clarendon Press, Oxford, 1989.
28. Azzi, A., Montecucco, C. and Richter, C., *Biochemical and Biophysical Research Communications*, 1975, **65**, 597.
29. Chance, B. and Maehly, A. C., In *Methods of Enzymology*, Vol. II, Academic Press, New York, 1955, p. 764.
30. Andreae, W. A., *Nature*, 1955, **175**, 859.
31. Kakinuma, K. and Minakami, S., *Biochimica et Biophysica Acta*, 1978, **538**, 50.