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ON THE REACTION OF PLANT FERREDOXINS WITH HYDROGEN PEROXIDE. WHAT REACTIVE OXIDANTS ARE GENERATED?

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Key Word Index—*Spinacia oleracea*; Chenopodiaceae; *Porphyra umbilicalis*; Verbenaceae; ferredoxin; chloroplast; hydrogen peroxide; hydroxyl radical; iron (in ferredoxin); deoxyribose assay; ferredoxin-NADP oxidoreductase.

Abstract—Oxidized ferredoxins from *Spinacia oleracea* or *Porphyra umbilicalis* react with hydrogen peroxide to form a reactive species that can degrade the sugar deoxyribose. This species is not the hydroxyl radical and its formation is partially inhibited by superoxide dismutase. No hydroxyl radical could be detected when ferredoxins were incubated with hydrogen peroxide, NADPH and ferredoxin reductase at ambient oxygen concentrations, unless chelating agents were added to the reaction mixture. It is concluded that ferredoxin is unlikely to catalyse hydroxyl radical formation in illuminated plant tissues, but that it may form a different highly-oxidizing radical species.

INTRODUCTION

The superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) are formed in illuminated plant tissues and have many damaging effects (reviewed in [1]). Much of the toxicity of O_2^- and H_2O_2 in mammalian and bacterial systems appears to arise by their conversion into highly-reactive oxidizing radical species, of which the best-characterized is the hydroxyl radical, ·OH (reviewed in [2]). Formation of ·OH from H_2O_2 requires the presence of transition metal ions; iron ions are the most likely catalysts of ·OH formation *in vivo* [2, 3]. Reaction of iron complexes with O_2^- and H_2O_2 may generate not only ·OH, but also iron-oxygen radical complexes such as ferryl and perferryl [2–4]. For example, when human oxyhaemoglobin is incubated with H_2O_2, a reactive oxidizing species not identical to ·OH is produced. In addition, some of the haemoglobin is degraded to release iron ions that react with H_2O_2 to form ·OH outside the protein [5]. By contrast, ·OH was the only reactive oxidant detected when horse-heart myoglobin, or leg-haemoglobin from *Glycine max* root nodules, was incubated with H_2O_2: again, the H_2O_2 degraded the proteins and iron was released, which then reacted with H_2O_2 to form ·OH [6, 7].

The iron (II) forms of plant ferredoxins are known to reduce O_2, producing O_2^- and H_2O_2 [8, 9]. There has therefore been interest in the possibility that chloroplasts contain iron ions or iron proteins that can mediate formation of ·OH from O_2^- and H_2O_2 (reviewed in [1]). Elstner *et al.* [10] reported that reaction of reduced spinach ferredoxin with H_2O_2 generates a 'Fenton type oxidant' similar to ·OH but not necessarily identical to it. Hosein and Palmer [11] claimed that reduced spinach ferredoxin does react with H_2O_2 to form ·OH; Bowyer and Camilleri [12] used the technique of spin-trapping to measure ·OH and reached a similar conclusion. By contrast Morehouse and Mason [13], also using spin-trapping, concluded that ferredoxin does not react directly with H_2O_2 to yield ·OH, but that ·OH is produced by reaction of H_2O_2 with metal ions contaminating the reaction mixture. Indeed, previous studies have shown that production of ·OH upon incubation of several iron proteins (lactoferrin, transferrin, leghaemoglobin, ferritin, haemosiderin, haemoglobin, myoglobin) with H_2O_2 (plus or minus O_2^-) is due to oxidant-mediated release of iron ions from the proteins, followed by reaction of the released iron ions with H_2O_2 [5–7, 14–16]. By contrast, iron-containing purple acid phosphatase enzymes do appear to form ·OH on direct reaction with H_2O_2, so production of this radical by reaction of an intact protein is not impossible [17].

Because of the importance, to our understanding of oxidative damage in chloroplasts, of establishing whether ferredoxin does, or does not, form ·OH on reaction with H_2O_2, we have thoroughly reinvestigated this problem. We used the sugar 2-deoxy-D-ribose as a detector for ·OH. Deoxyribose is degraded by ·OH to form a range of products that can be measured by a simple colorimetric procedure [18, 19]. Deoxyribose can also be degraded to such products by reactive oxidants other than ·OH [5], but this can be distinguished from ·OH-mediated degradation by the use of appropriate 'scavengers' of ·OH [19, 20].

RESULTS

Action of H_2O_2 *upon spinach ferredoxin*

Several iron proteins are degraded upon exposure to a molar excess of H_2O_2, with release of iron ions (see Introduction). Incubation of 20 μM of the oxidized form of spinach (*Spinacia oleracea*) ferredoxin with 200 μM H_2O_2 for up to 2 hr at pH 7.4 produced no significant changes in its UV and visible absorption spectrum, or in its circular dichroism spectrum. An example of the circular dichroism spectrum of spinach ferredoxin before and after treatment with H_2O_2 is shown in Fig. 1. Similarly,

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the spectra of ferredoxin from the red marine alga *Porphyra umbilicalis* were unaffected by treatment for up to 2 hr with a 10-fold molar excess of H_2O_2.

Deoxyribose degradation by oxidized ferredoxin and H_2O_2

Attack of hydroxyl radicals, or similar highly-oxidizing species, upon the sugar 2-deoxy-D-ribose produces fragments that, when heated with thiobarbituric acid at low pH, generate a chromogen that can be measured at 532 nm. Incubation of oxidized spinach or *Porphyra* ferredoxins with H_2O_2 at pH 7.4 led to deoxyribose degradation: Table 1 shows a typical experimental result for the spinach protein. This deoxyribose degradation increased with concentration of H_2O_2 and of ferredoxin (Table 2) and was not inhibited by scavengers of ·OH (mannitol, Hepes, arginine, dimethylsulphoxide, phenylalanine: see [5–7]), as shown in Table 1. Deoxyribose degradation was also not inhibited by two iron-binding agents, the chelator desferrioxamine and the protein apotransferrin (Table 1). Both of these are known to bind iron ions in ways that prevent such ions from participating in ·OH generation [14, 21–23]. Control experiments showed that neither apotransferrin nor desferrioxamine were able to remove iron from ferredoxin under our reaction conditions.

Superoxide dismutase partially inhibited the deoxyribose degradation, and catalase inhibited almost completely. These were not non-specific effects of protein, since bovine serum albumin had no action.

Deoxyribose degradation by reduced ferredoxin and H_2O_2

Incubation of oxidized spinach or algal ferredoxin with NADPH and ferredoxin reductase caused deoxyribose

Table 1. Deoxyribose degradation by oxidized spinach ferredoxin and H_2O_2 (pH 7.4)

Additions to reaction mixture (final concentration)	Extent of deoxyribose degradation (A_{532})
None (complete reaction mixture)	0.212
None (omit H_2O_2)	0.035
None (omit ferredoxin)	0.000
100 μM Desferrioxamine	0.210
10 μM Apotransferrin	0.218
20 mM Mannitol	0.215
100 mM Mannitol	0.220
50 mM Arginine	0.242
20 mM Dimethylsulphoxide	0.216
5 mM Phenylalanine	0.236
20 mM Hepes	0.224
Bovine serum albumin (80 μg)	0.215
Superoxide dismutase 200 units	0.118
50 units	0.143
20 units	0.150
Catalase 1000 units	0.067

Reaction mixtures contained, in a total volume of 1 ml, the following reagents at the final concentrations stated: ferredoxin (20 μM), H_2O_2 (200 μM), KH_2PO_4–KOH buffer pH 7.4 (25 mM), deoxyribose (6.7 mM). Tubes were incubated at 25° for 1 hr. Then 1 ml of 1% (w/v) thiobarbituric acid in 0.05 M NaOH, and 1 ml of 2.8% (w/v) trichloroacetic acid were added. Tubes were heated at 100° for 15 min on a heating block, cooled and A_{532} measured. Centrifugation at 1000 *g* for 10 min was carried out, if necessary, to remove any turbidity before reading A_{532}. Superoxide dismutase was the bovine copper-zinc enzyme.

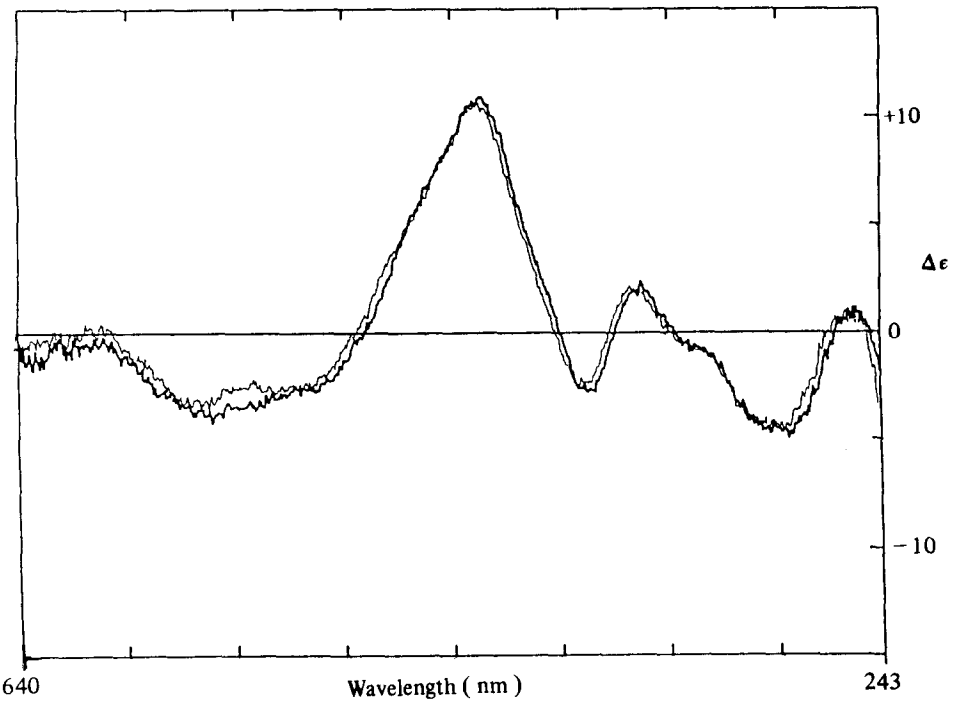

Fig. 1. CD spectrum of spinach-leaf ferredoxin. The CD spectrum of 20 μM spinach ferredoxin was determined as described in the Experimental section both before (dense line) and after (light line) incubation with 200 μM H_2O_2 at pH 7.4 for 2 hr.

Table 2. Deoxyribose degradation by oxidized spinach ferredoxin and H_2O_2, effects of concentration

[Ferredoxin] (μM)	[H_2O_2] (μM)	Extent of deoxyribose degradation
0	0	0
20	10	0.011
20	20	0.043
20	50	0.064
20	100	0.103
20	200	0.175
20	500	0.258
2	200	0.036
5	200	0.097
10	200	0.142
40	200	0.200
60	200	0.204

Reaction mixtures were as described in the legend to Table 1, except that they contained spinach ferredoxin and H_2O_2 at the final concentrations stated.

degradation, which was further stimulated by adding H_2O_2. However, the amount of deoxyribose degradation seen in the NADPH/reductase/ferredoxin system + H_2O_2 was not significantly greater than that observed on incubation of oxidized ferredoxin with H_2O_2 alone, Table 3 shows a typical experimental result. Experiments conducted under anaerobic conditions (described in the legend to Table 4) showed that the reductase enzyme was indeed reducing the ferredoxin, which was quickly re-oxidized on readmission of air. Desferrioxamine had only a small inhibitory effect on deoxyribose degradation in the presence of H_2O_2, ferredoxin, NADPH and the reductase enzyme (Table 3) and the hydroxyl radical scavengers mannitol (100 mM) or dimethylsulphoxide (20 mM) had no inhibitory action. Indeed, mannitol stimulated slightly; we do not know the reason for this. However, the chelating agents EDTA and diethylene triaminepenta-acetic acid (DTPA) had a striking stimulatory effect, even in the absence of added H_2O_2.

The results of further investigations of the rapid deoxyribose degradation seen in the presence of ferredoxin, NADPH, reductase and EDTA or DTPA are shown in Table 4. All components of the reaction mixture were required. Catalase, or incubation under anaerobic conditions, almost completely prevented deoxyribose degradation, although addition of H_2O_2 had only slight stimulatory effects (when allowance is made for the fact that H_2O_2 reacts with ferredoxin to generate a deoxyribose-degrading species; Table 1). The hydroxyl radical scavengers mannitol, arginine, phenylalanine and Hepes significantly inhibited the deoxyribose degradation (Table 4), but superoxide dismutase did not. As a control, urea was used, which has little reactivity with ·OH [24]. In the absence of ferredoxin, addition of ferric chloride (which will chelate to EDTA or to DTPA in the reaction mixture) restored some deoxyribose degradation (Table 4, last 2 lines), which was dependent on the presence of the reductase enzyme.

Table 3. Deoxyribose degradation by spinach ferredoxin and H_2O_2 (pH 7.4 under aerobic conditions: addition of a reducing system)

Additions to reaction mixture	Extent of deoxyribose degradation (A_{532})
Fd + H_2O_2	0.186*
Fd + H_2O_2 + catalase (10^3 units/ml)	0.000
Reductase only	0.000
NADPH only	0.000
Reductase + NADPH	0.000
Fd + reductase	0.000
Reductase + H_2O_2	0.000
Reductase + NADPH + H_2O_2	0.000
Fd + NADPH + reductase	0.150
Fd + NADPH + reductase + catalase (10^3 units/ml)	0.000
Fd + NADPH + reductase + desferrioxamine (100 μM)	0.110
Fd + NADPH + reductase + mannitol (100 mM)	0.185
Fd + NADPH + reductase + DMSO (20 mM)	0.174
Fd + NADPH + reductase + EDTA (100 μM)	0.900
Fd + NADPH + reductase + DTPA (100 μM)	0.672
Fd + NADPH + reductase + H_2O_2	0.193*
Fd + NADPH + reductase + H_2O_2 + desferrioxamine (100 μM)	0.149
Fd + NADPH + reductase + H_2O_2 + EDTA (100 μM)	1.304
Fd + NADPH + reductase + H_2O_2 + DTPA (100 μM)	1.006

Assays were as described in the legend to Table 1, except that they also contained, where indicated, ferredoxin–$NADP^+$ oxidoreductase (0.1 units/ml) and NADPH (2 mM). Incubations were for 1 hr at 25°. Reaction mixtures contained deoxyribose, buffer and the reagents listed below.

Abbreviations—Fd, ferredoxin; DTPA diethylenetriaminepenta-acetic acid: reductase, ferredoxin–NADP oxidoreductase.

*Results not significantly different (5 experiments)

Table 4. Deoxyribose degradation by ferredoxin/ferredoxin reductase/NADPH/chelator systems

Reaction mixture	Extent of deoxyribose degradation: EDTA present		DTPA present	
	A	inhibition of degradation (%)	*A*	inhibition of degradation (%)
Complete (as above)	0.835	0*	0.729	0†
Omit ferredoxin	0.043	95	0.035	95
Omit reductase	0.041	95	0.031	96
Omit NADPH	0.037	96	0.035	95
Plus superoxide dismutase (220 units/ml)	0.813	3	0.702	4
Plus catalase (10^3 units/ml)	0.062	93	0.060	92
Complete, with anaerobiosis	0.142	83	0.095	87
Plus mannitol (20 mM)	0.526	37	0.439	40
Plus arginine (50 mM)	0.468	44	0.366	50
Plus phenylalanine (5 mM)	0.534	36	0.494	32
Plus Hepes (20 mM)	0.326	61	0.319	56
Plus urea (10 mM)	0.827	1	0.744	0
Plus $FeCl_3$ (5 μM)	0.983	—	0.932	—
Plus H_2O_2 (200 μM)	1.185	—	1.187	—
Plus $FeCl_3$ (5 μM) and H_2O_2 (200 μM)	1.345	—	1.286	—
Plus $FeCl_3$ (5 μM), omit ferredoxin	0.363	—	0.397	—
Plus $FeCl_3$ (5 μM) and H_2O_2 (200 μM), omit ferredoxin	0.374	—	0.439	—
Plus $FeCl_3$ (5 μM) and H_2O_2 omit reductase, omit ferredoxin	0.034	—	0.027	—

Reaction mixtures contained, unless otherwise stated, spinach ferredoxin (20 μM), deoxyribose (6.7 mM), KH_2PO_4–KOH buffer pH 7.4 (25 mM), ferredoxin–NADP oxidoreductase (0.1 units/ml), NADPH (2 mM) and EDTA (100 μM). or DTPA (100 μM). Reaction mixtures were made anaerobic, when required, by degassing and flushing with argon. Reaction was started by adding ferredoxin (degassed separately) using a syringe.

DTPA, diethylenetriaminepenta-acetic acid.

DISCUSSION

Our results indicate some of the problems that may have arisen in previous studies of the ability of ferredoxin to stimulate generation of ·OH. Firstly, the protein does not degrade on prolonged incubation with H_2O_2 (Fig. 1). The circular dichroism (CD) spectrum of ferredoxins is extremely sensitive to even minor alterations in the iron-sulphur cluster (R. Cammack, personal communication), yet no significant CD changes were observed. Ferredoxins are often regarded as labile proteins, but their ability to resist H_2O_2 may be significant in relation to their location in the chloroplast, a major site of oxidative stress in illuminated leaves [1].

Incubation of oxidized (ferric) ferredoxins with H_2O_2 generates a deoxyribose-degrading species that is not ·OH; it could not be scavenged by any of the ·OH scavengers tested. Its formation was not inhibited by the iron-chelating agents desferrioxamine and apotransferrin, and so could not involve contamination of the ferredoxin preparation with iron salts. The properties of this oxidizing species remain to be characterized, but it must be a fairly strong oxidant in order to degrade deoxyribose [20]. It may be identical to the '·OH-like' species reported by Elstner *et al.* [10], who used methionine degradation to ethylene gas as an assay for oxidants generated from ferredoxin plus H_2O_2. It is very interesting to note that formation of this species is partially inhibited by superoxide dismutase (it is known that SOD does not interfere with the formation of TBA-reactive material from deoxyribose). Superoxide dismutase has previously been shown to inhibit formation of oxidizing species from the reaction of ferric chelates with H_2O_2 [25, 26].

When ferredoxin is incubated with ferredoxin-$NADP^+$ oxidoreductase and NADPH, H_2O_2 will be generated in the reaction mixture [8, 9].

$$Fd_{ox} + \text{enzyme} \rightarrow Fd_{red} \quad (1)$$

$$Fd_{red} + O_2 \rightarrow Fd_{ox} + O_2^- \quad (2)$$

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (3)$$

The reduced form of oxidoreductase itself reacts only very slowly with O_2 under our reaction conditions, so O_2^- production by the enzyme can be neglected [27, 28]. Incubation of ferredoxin with the oxidoreductase and NADPH did not cause any significant increase in the rate of deoxyribose degradation over that seen in the presence of oxidized ferredoxin and H_2O_2 alone. This suggests that reaction of reduced ferredoxin with H_2O_2 to form ·OH does not occur, or at least occurs much more slowly than reaction of reduced ferredoxin with O_2 (eqn 2). It should be noted that reduced ferredoxin is generated in illuminated chloroplasts, in which O_2 concentrations are higher than ambient because of photosynthetic O_2 production. Thus, even if reaction of reduced ferredoxin with H_2O_2 occurs at a slow rate, it is unlikely to be physiologically significant.

Several authors who have studied reaction of ferredoxin with H_2O_2 have added chelating agents, such as DTPA [12, 13] to their reaction mixtures. EDTA in-

creases the reactivity of iron ions with both O_2^- and H_2O_2, by favourable changes in the redox potential of the iron and by maintaining iron ions in solution (discussed in [29]). Deoxyribose degradation in the presence of ferredoxin, oxidoreductase, NADPH and EDTA (or DTPA) requires O_2 (Table 4) and H_2O_2 (inhibition by catalase, Table 4). It appears to be mediated by ·OH radicals, in that it is inhibited by a range of molecules known to react with this species, but not by urea, which does not significantly scavenge ·OH. The simplest explanation of our data is that addition of EDTA or DTPA to the reaction mixtures somehow solubilizes traces of iron, contaminating the ferredoxin preparation or other components of our reaction mixture, in a redox-active form. However, if ferredoxin was omitted from reaction mixtures containing EDTA or DTPA and replaced by ferric chloride, deoxyribose degradation was much decreased (Table 4). It may be that ferredoxin-$NADP^+$ oxidoreductase can directly reduce iron chelates; it is also possible that reduced ferredoxin can do the same [13]. Our data can thus be explained by the following reaction mechanism, in which it is assumed that iron contaminating the reaction mixture is present as Fe^{3+} (which chelates to EDTA or DTPA) and in which reaction (5) is faster than reaction (6) under our conditions.

$$Fd_{ox} + \text{enzyme} \rightarrow Fd_{red} \quad (4)$$

$$Fd_{red} + Fe^{3+} - EDTA \rightarrow Fe^{2+} - EDTA + Fd_{ox} \quad (5)$$

$$Fe^{3+} - EDTA + \text{enzyme} \rightarrow Fe^{2+} - EDTA \quad (6)$$

$$Fd_{red} + O_2 \rightarrow Fd_{ox} + O_2^- \quad (7)$$

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (8)$$

$$Fe^{2+} - EDTA + H_2O_2 \rightarrow Fe^{3+} - EDTA + \cdot OH + OH^- \quad (9)$$

$$\cdot OH + \text{deoxyribose} \rightarrow \text{TBA-reactive fragments} \quad (10)$$

Similar equations can be written for the Fe^{3+} – DTPA complexes. The ability of O_2^- to reduce Fe^{3+} – EDTA [30, 31] does not appear to be important in this system, since superoxide dismutase had little inhibitory effect. In addition, Fe^{3+} – DTPA is reduced much more slowly by O_2^- [32], although Fe^{2+} – DTPA reacts with H_2O_2 to form OH in a reaction analogous to that shown in eqn (9) [33].

To summarize, addition of chelating agents to reaction mixtures to 'suppress iron-dependent reactions' can give misleading results, as has been stressed previously [34]. Our data indicate that reduced ferredoxin does not react with H_2O_2 to form ·OH detectable by the deoxyribose assay, in agreement with the conclusions of Morehouse and Mason [13]. Although a slow reaction (such as that implied by the results of Hosein and Palmer [11]) cannot be ruled out by our experiments, it would not be likely to be significant under physiological conditions, when O_2 concentrations are high. Our studies show that a species reactive enough to degrade deoxyribose, but not identical to ·OH (and thus presumably not detectable in the spin-trapping experiments) is formed by reaction of oxidized ferredoxin with H_2O_2. Further characterization of this species is required in order to assess the likelihood of its damaging the chloroplast *in vivo*. One function of superoxide dismutase in chloroplasts may be to diminish formation of this species (Table 1).

EXPERIMENTAL

Assays of deoxyribose degradation were performed essentially as described by Puppo *et al.* [5]; full details are given in Table legends. Desferal ® (desferrioxamine B methanesulphonate) was a gift from CIBA-Geigy, Horsham, Sussex. Porcine apotransferrin was a gift from Speywood Laboratories, Wrexham, Wales. Superoxide dismutase (bovine copper-zinc enzyme), catalase, ferredoxin-NADP oxidoreductase (spinach) and ferredoxin (spinach type F5875; *Porphyra umbilicalis* type F5257) were from Sigma. Units of superoxide dismutase were as defined by McCord and Fridovich [35], units of catalase and ferredoxin reductase as defined in the Sigma catalogue. The purity of ferredoxins was checked by determining their absorption spectra (absorbance ratio A_{420}/A_{275} for both proteins was 0.34–0.38). Circular dichroism was performed on a Jasco J-40 CS CD Spectrometer, by courtesy of Dr Alex Drake (Optical Spectroscopy Unit, University of London Intercollegiate Research Services). All solns were prepared in water purified by passage through a Milli R04 and Milli Q apparatus.

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