Superoxide formation in pea chloroplasts by a dioxathiadiaza-2,5-pentalene derivative, a new lipophilic Photosystem I acceptor

John R. Bowyer*, Patrick Camilleri+ and Andrew Stapleton†

Shell Research Ltd, Sittingbourne Research Centre, Sittingbourne ME9 8AG, England

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The dioxathiadiaza-2,5-pentalene derivative, HEP II, has herbicidal effects similar to those of methyl viologen. HEP II promotes superoxide formation when added to illuminated pea chloroplasts. Superoxide dismutase, but not catalase, diminished formation of the superoxide whereas cyanide and azide enhanced its formation, presumably by inhibiting the endogenous superoxide dismutase activity. DCMU, which inhibits photosynthetic electron transport, inhibited superoxide formation. Rates of superoxide formation and oxygen uptake were very similar when equal concentrations of methyl viologen or HEP II were added. At subsaturating concentrations of electron acceptor, Mg^{2+} decreased the rate of oxygen uptake with methyl viologen but not with HEP II, probably reflecting differences in their interactions with the Photosystem I electron donation site. It is likely that HEP II, by analogy with methyl viologen, is reduced by chloroplast Photosystem I and reoxidised by molecular oxygen, generating superoxide.

Photosynthesis *Heteropentalene* *Superoxide* *Photosystem I* *ESR*

1. INTRODUCTION

For many years, the bipyridinium herbicides, for example, methyl viologen (paraquat), have been used as electron acceptors in kinetic experiments on chloroplast electron transport [1], and as low-potential reduction/oxidation mediators in potentiometric studies (e.g., [2]). Reduction of the methyl viologen dication by PS I results in the formation of the monocation radical which can be reoxidised by molecular oxygen leading to formation of superoxide [3–8]. Superoxide itself is relatively unreactive, and it is generally considered [9,10] that the toxic species is the hydroxyl radical ($OH^{\cdot}$) generated by the following reactions catalysed by trace amounts of Fe(III):

$$O_2^{\cdot -} + \text{Fe(III)} \longrightarrow O_2 + \text{Fe(II)}$$

$$\text{Fe(II)} + H_2O_2 \longrightarrow \text{Fe(III)} + OH^- + OH^{\cdot}$$

H_2O_2 is generated by the dismutation of superoxide, catalysed by the enzyme SOD associated with PS I [11,12].

During a search for compound with effects on plants similar to those shown by the bipyridinium herbicides [13], but with lower mammalian toxicity, a group of compounds referred to as dioxathiadiazaheteropentalenes has been discovered

* Present address: Department of Biochemistry, Royal Holloway College, Egham Hill, Egham, Surrey TW20 0EX, England

+ To whom correspondence and reprint requests should be addressed

† Present address: Department of Biochemistry, University of Manchester, Manchester M13 9PL, England

Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; PS I, Photosystem I; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DMPO-OOH, superoxide-DMPO spin-adduct; DMPO-OH, hydroxyl-DMPO spin-adduct; SOD, superoxide dismutase; DETAPAC, diethylenetriaminepentaacetic acid; Mops, morpholinepropanesulphonic acid

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[14]. These compounds have similar electrochemical properties to those of the bipyridinium herbicides [1,14], similar herbicidal effects ([13,14] and Raven, C.A., personal communication) and similar effects on chloroplast ultrastructure ([15] and Williamson, F.A. and Camilleri, P., unpublished).

Here, we show, using a spin trap, that one of the pentalene derivatives, referred to as HEP II, generates superoxide in a DCMU-sensitive reaction when added to illuminated pea chloroplasts. The rates of superoxide formation and of associated oxygen uptake were very similar to those seen with the same concentration of methyl viologen. Mg^{2+} had no effect on the rate with HEP II present, but decreased the rate with methyl viologen. We attribute this to differences in the interaction between the two compounds and the electron-donating site on PS I.

2. MATERIALS AND METHODS

Broken chloroplasts were prepared from peas grown in a glasshouse in a loam/peat/sand compost. For measurements of oxygen uptake, 20 g freshly picked pea leaves were homogenised for 20 s in a Waring blender at full speed in 100 ml buffer (pH 6.8) at 4°C containing 0.35 M sucrose and 0.01 M KH_2PO_4. The homogenate was filtered through 4 layers of muslin and centrifuged for 7 min at 2000 × *g*. The chloroplast pellet was resuspended in 50 ml homogenising buffer and recentrifuged. The resulting pellet was homogenised for several minutes in 25 ml of 17 mM NaCl using a hand homogeniser. The resulting ruptured chloroplasts were obtained by centrifugation at 15000 × *g* for 10 min, and resuspension in 12 ml of 17 mM NaCl. Chlorophyll content was assayed as in [16]. The chloroplasts were suspended to 50 μg/ml chlorophyll in 1.5 ml buffer (0.1 M Tricine, 0.05 M methylamine, 0.002 M sodium azide, pH 8) containing varying concentrations of $MgCl_2$ ($\leqslant$10 mM). After 2 min incubation, HEP II or methyl viologen was added to 10^{-6} M, the former from a 10^{-2} M stock solution in acetone. Rates of light-induced oxygen uptake were measured using a Clark-type oxygen electrode (Hansatech) at 20°C. Actinic illumination was provided by a 150 W projector filtered by Kodak Wratten 29 red filter giving an intensity of about 400 $W \cdot m^{-2}$ at the electrode chamber.

Superoxide formation was detected by ESR spectroscopy using the spin trap DMPO, as in [15,17,18]. This method was used because it can be used to monitor simultaneously formation of superoxide and hydroxyl radicals, although care must be taken to ensure that spin-trapped hydroxyl has not arisen through decomposition of spin-trapped superoxide [19,20]. Chloroplasts for the spin-trapping experiments were prepared by homogenising 20 g pea leaves for 8 s in a Polytron at full speed in 200 ml buffer (pH 7.3) at 1°C containing 0.4 M sucrose, 0.01 M $MgCl_2$, 30 mM Hepes and 0.5% bovine serum albumin. The homogenate was filtered through 4 layers of muslin and centrifuged at 300 × *g* for 3 min. The supernatant was centrifuged at 4000 × *g* for 3.5 min and the resulting chloroplast pellet resuspended in 0.4 M sucrose, 0.5% bovine serum albumin. Chloroplasts were suspended to 75 μg/ml chlorophyll in oxygenated 50 mM Mops buffer (pH 7) to which was added 2 mM NH_4Cl (to uncouple photosynthetic electron transport from energy conservation), 1 mM DETAPAC (to chelate iron which otherwise oxidises DMPO [18]), and 0.2 M DMPO. HEP II was added to 10^{-4} M from a solution in dimethyl sulphoxide (5 μl in 1 ml buffer). Other additions are described in the figure legends. One ml of this chloroplast suspension was sucked into an ESR aqueous sample cell (Wilmad Glass) located in the ESR cavity. ESR measurements were made at room temperature using a Bruker ER-200D-9/7 X-band spectrometer with an ER $410ST_{102}$ mode rectangular cavity. Other ESR parameters are described in the figure legends. The sample was illuminated in the cavity through a grill in the cavity wall using a Volpi Intralux 250HL illuminator with a fibre optic light guide. Red light was provided using a Schott RG665 filter, at an intensity of 800 $W \cdot m^{-2}$, after aperture reduction. This subsaturating light intensity was used as the decay rate of the DMPO-OOH signal increased markedly at higher light intensities, making it difficult to obtain a complete, undistorted spectrum of the spin-adduct.

DMPO-OH was generated by UV photolysis for 90 s of 1% H_2O_2 in 1 mM Mops buffer (pH 7) and 0.1 M DMPO. The UV source was a 400 W medium-pressure mercury vapour lamp (Applied Photophysics). DMPO and DETAPAC were ob-

tained from Aldrich, SOD (bovine liver), bovine serum albumin and buffers from Sigma and catalase (bovine liver) from Boehringer. HEP II was provided by the Organic Chemistry Division, Shell Research Ltd, Sittingbourne.

The partition coefficient of HEP II was determined between 1-octanol and water by shaking the solute with these immiscible solvents and then analysing the solute concentrations in both phases using absorption spectroscopy.

3. RESULTS AND DISCUSSION

The structure of HEP II is shown in fig.1. This neutral molecule has an octanol–water partition coefficient of 20; the corresponding partition coefficient of the methyl viologen dication is expected to be much lower than this (<0).

The data in fig.2A,B show that 1 μM HEP II stimulates oxygen uptake by illuminated pea chloroplasts to a level similar to that with 1 μM methyl viologen. The oxygen uptake was inhibited by DCMU indicating that it was linked to electron transfer 'downstream' of PS II. The midpoint redox potential of HEP II (−375 mV at pH 7) [14] suggests that PS I must be the electron donor. However, while addition of $MgCl_2$ slowed the rate of oxygen uptake with methyl viologen as acceptor (see [21]), it has no effect on the rate with HEP II. The very low concentration of methyl viologen used (1 μM) means that electron transfer to methyl viologen limits the rate of electron transfer, rather than light intensity. Following a suggestion (J. Barber, personal communication), we interpret the effect of Mg^{2+} with methyl viologen as acceptor as being due to an inhibition of the interaction between the negatively charged PS I and the positively charged methyl viologen. A similar effect has been observed with ferredoxin and an electron acceptor

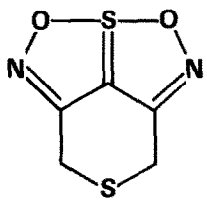

Fig.1. The structure of the dioxathiadiaza-2,5-pentalene derivative, HEP II.

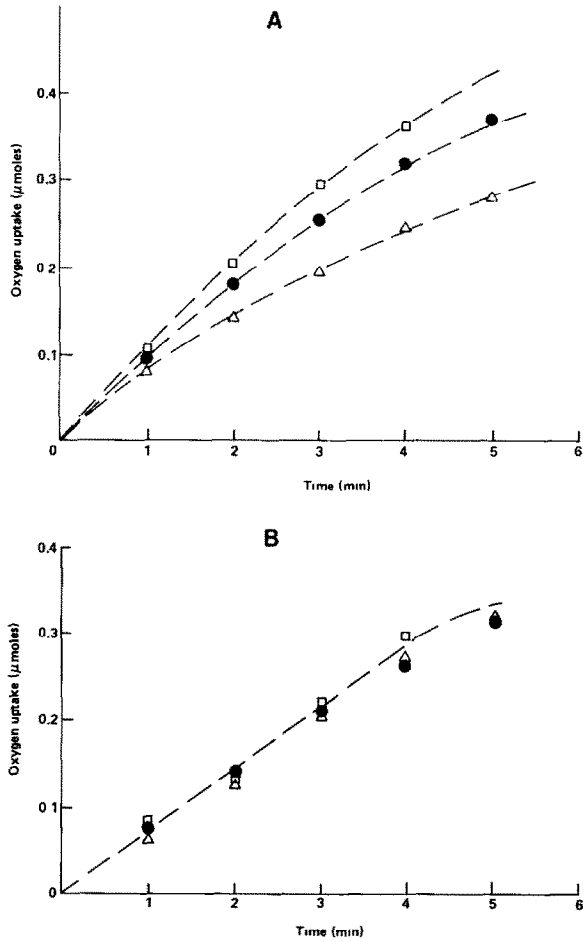

Fig.2. Effects of $MgCl_2$ on the rate of oxygen uptake by illuminated chloroplasts in the presence (A) of 1 μM methyl viologen and (B) 1 μM HEP II. $MgCl_2$ was added to 1 mM (●) or 10 mM (△), or not at all (□). Other conditions are described in section 2.

associated with cyclic electron flow in chloroplasts [22]. This interpretation is confirmed by the lack of effect of Mg^{2+} on the rate of oxygen uptake in the presence of neutral HEP II. Presumably, the ability of HEP II to act as a putative electron acceptor from PS I does not involve electrostatic interactions.

The spectrum in fig.3A was recorded 100 s after initiation of illumination of chloroplasts in the presence of DMPO with 10^{-4} M HEP II as electron acceptor. A very similar spectrum was obtained using 10^{-4} M methyl viologen as electron acceptor (not shown, see also fig.4 in [15]). The signal before illumination was negligible (fig.3D).

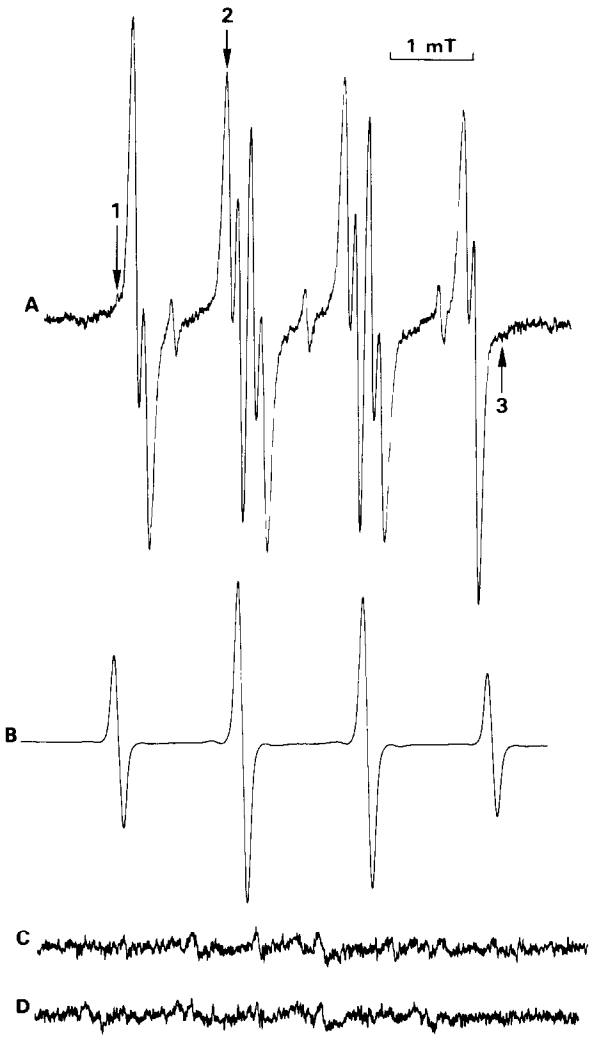

Fig.3. ESR spectra of DMPO spin-adducts. All spectra were recorded at 9.77 GHz microwave frequency, 20 mW microwave power (except B; 10 mW), 100 kHz field modulation frequency, 50 μT (0.5 G) modulation amplitude, 0.1 mT·s^{-1} scan rate and 50 ms time constant. (A) Spectrum of illuminated chloroplasts with 10^{-4} M HEP II present, as described in section 2, recorded 100 s after the onset of illumination (cf. fig.4b). Gain 2.5 × 10^5. Arrows 1 and 3 indicate the positions of signals attributable to DMPO-OH. Arrow 2 indicates the signal monitored to follow the kinetics of formation of DMPO-OOH. (B) Spectrum of DMPO-OH. Gain 8 × 10^3. (C) Spectrum of illuminated chloroplasts as in A, but with 150 units/ml SOD. Gain 3.2 × 10^5. (D) Spectrum of chloroplasts as in A, recorded before illumination. Gain 3.2 × 10^5.

The spectrum of the species generated in the absence of HEP II had the same characteristic components as those seen in its presence, but at lower amplitudes. The spectrum in fig.3A is similar to that of DMPO-OOH (fig.1 in [17]).

The spectrum in fig.3B is that of DMPO-OH. Comparison of fig.3A,B indicates that very little DMPO-OH was present 100 s after illumination of chloroplasts.

The signal at a magnetic field of 0.3464 T indicated by arrow 2 in fig.3A was used to monitor the kinetics of formation of the species tentatively attributed to DMPO-OOH. Addition of SOD inhibited formation of the signal (fig.4b–e) and of other components in the complete spectrum (fig.3C), owing to competition with DMPO for superoxide. This provides further evidence that the spectrum in fig.3A is largely due to the formation of DMPO-OOH, but some other products were also formed, which were not identified. Plots d and b in fig.5 show the rate of formation of the signal in the absence and presence of 10^{-4} M HEP II. They show that the signal reached a maximum about 100 s after the onset of illumination, and remained constant for about 100 s. The signal rise rate in the presence of 10^{-4} M HEP II was more than 6-times faster than that seen in its absence and resulted in a 6-fold greater final signal. A very similar rate was observed using 10^{-4} M methyl viologen (cf. similar rates of oxygen uptake at 10^{-6} M HEP II and methyl viologen). Addition of cyanide increased the rate of formation of DMPO-OOH, in both presence and absence of HEP II (fig.5a,c). This is probably due to an inhibition of the endogenous SOD activity [11,12]. The effect of cyanide was not due to an effect on the rate of photosynthetic electron flow (not shown). DCMU, which inhibits photosynthetic electron flow, prevented formation of DMPO-OOH (fig.5e).

Azide enhanced the formation of the DMPO-OOH signal, probably owing to inhibition of the endogenous SOD activity (fig.4a). Catalase had very little effect on the rate of formation of DMPO-OOH, when added to 1300 units/ml. H_2O_2 is produced by SOD, and an effect of catalase would only be expected if the rate of dismutation of superoxide was limited by build-up of product.

Attempts to increase the amount of DMPO-OH formed by adding H_2O_2 (1%) and/or Fe^{3+} (60 μM $FeCl_3$) in the absence of DETAPAC, failed,

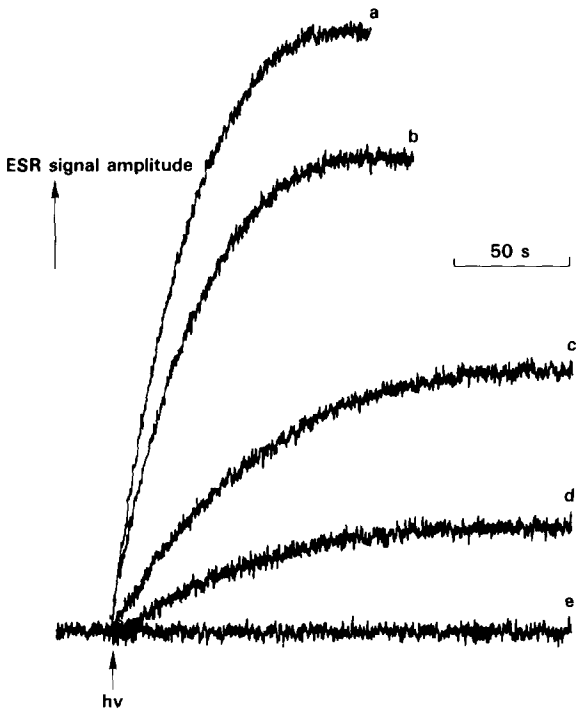

Fig.4. Effect of SOD on the rate of formation by pea chloroplasts of the signal attributed to DMPO-OOH. The traces show the amplitude of the ESR signal recorded at a fixed magnetic field as a function of time. The ESR magnetic field was set at a field corresponding to peak 2 in fig.3A (0.3464 T). ESR operating conditions were as in fig.3, except that the gain was 5×10^5, the time constant 200 ms, and the magnetic field fixed. Chloroplasts were suspended (see section 2) with the following further additions: (a) 2 mM sodium azide; (b) control; (c) 3 units SOD/ml; (d) 15 units SOD/ml; (e) 150 units SOD/ml.

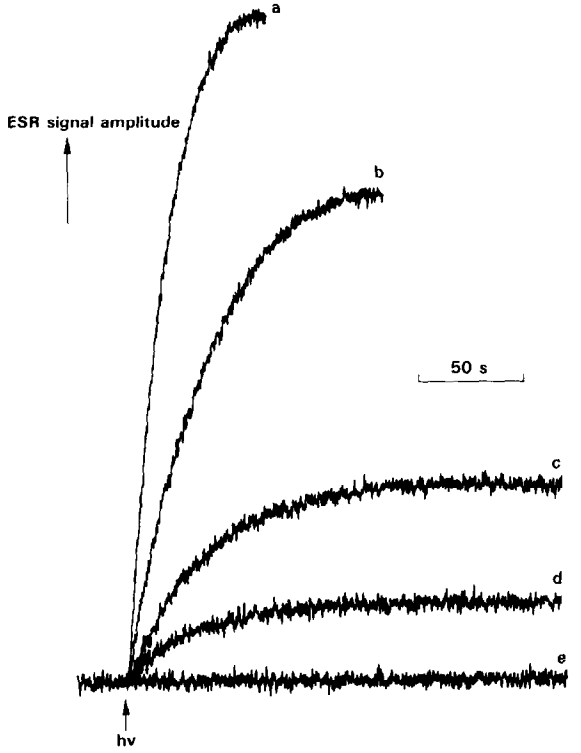

Fig.5. Effect of various treatments on the rate of formation by pea chloroplasts of the signal attributed to DMPO-OOH. The set-up was as in fig.4b. Further additions or omissions were: (a) 2 mM sodium cyanide; (b) control; (c) 2 mM sodium cyanide, no HEP II; (d) no HEP II; (e) 10 μM DCMU.

possibly because of a failure to generate sufficient Fe(II). However, experiments using DMPO are complicated by the fact that DMPO-OOH itself breaks down with a half-life of a few minutes to form DMPO-OH and a non-paramagnetic species [19,20]. Spectra recorded after the decay of the signals attributed to DMPO-OOH did include peaks corresponding to those of DMPO-OH (not shown) but they were not further characterised. Our results indicate that, like methyl viologen, HEP II is reduced by PS I, and reoxidised by molecular oxygen, with the formation of superoxide. Enhanced superoxide formation is very probably the cause of the herbicidal effects of HEP II.

The high lipophilicity and electrical neutrality of HEP II in comparison to methyl viologen, and its ability to interact with PS I, suggest that it will be a very useful reduction/oxidation mediator in potentiometric studies on chloroplasts. HEP II will also be useful in studying those effects of Mg^{2+} on electron transfer through PS I which are thought to be unrelated to interactions with the electron acceptor [21]. Furthermore, HEP II and its analogues appear to have potential applications as electron transfer catalysts in photochemical energy transducers (in preparation).

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