OXYGEN SPECIES IN PARAQUAT TOXICITY: THE CRYPTO-OH RADICAL

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1. Introduction

The study of the chemistry and biochemistry of oxygen has received much attention over the past decade and was greatly stimulated by the discovery of the superoxide dismutase (SOD) activity of erythrocuprein [1]. Since then, superoxide ($O_2^{\cdot-}$) has often been cited as the species responsible for the toxicity of oxygen [2–5]. However, the low reactivity of $O_2^{\cdot-}$ [6–9] and its inability to form the free hydroxyl radical ($OH^{\cdot}$) via the uncatalysed Haber–Weiss reaction [10–12], suggest that it is not the species responsible for initiating damage in biological systems. The free OH radical and singlet oxygen have both been invoked as the damage-inducing agents [13–16], although their extreme reactivity infers that their reactions must occur close to their sites of generation [9,17]. Thus, the likelihood of *free* OH radicals being formed in vivo is regarded as being very small [9].

Paraquat, also known as methyl viologen (MV, 1,1′-dimethyl-4,4′-bipyridylium dichloride) has long been known to require oxygen in order to exert its toxic effect in both plant [18] and animal systems [19]. The damage arising from paraquat treatment is specific and is initially manifested by the breakdown of the tonoplast and plasmalemma membranes in plants [20] and by pulmonary lesions in animals [21]. In these systems, the paraquat cation is univalently reduced to the radical which rapidly undergoes autoxidation in the presence of molecular oxygen to form $O_2^{\cdot-}$ and the paraquat ion [22]. Studies into the protection of biological systems against paraquat damage which involved the removal of paraquat-enhanced superoxide levels have met with varying degrees of success [23–25], although none was completely able to inhibit the action of paraquat.

The aim of this study was to investigate further the oxygen species involved in the reactions of paraquat. The formation of ethylene from methionine in the presence of pyridoxal phosphate is dependent on pulse-radiolytically generated free OH radicals [26], or a Fenton-type oxidant [27]. In these studies, $O_2^{\cdot-}$ and H_2O_2 alone were found to be insufficient to result in ethylene production. Thus, the coupling of this reaction to another which produces reduced paraquat constitutes a model system to further investigate the reactions of active oxygen species.

2. Materials and methods

Ferredoxin–$NADP^+$-oxidoreductase (EC 1.18.1.2) was prepared from spinach [28] and superoxide dismutase (EC 1.15.1.1) was isolated from dried green peas [29]. Catalase (EC 1.11.1.6), glucose-6-phosphate and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) were obtained from Boehringer, Mannheim.

The basic reaction consisted of the dehydrogenase system producing NADPH which was then used to reduce paraquat by the ferredoxin–$NADP^+$-oxidoreductase. All experiments were conducted in Fernbach flasks with a centre well and were sealed with serum rubber stoppers. Ethylene formation from methionine in the presence of pyridoxal phosphate was determined by gas chromatography as in [30]. After the addition of the reaction components, 100 μl 2×10^{-4} M paraquat solution was placed in the centre wells of the flasks, which were then sealed. Anaerobic conditions were obtained by evacuating the flasks and returning them to atmospheric pressure using O_2-free nitrogen, which was then flushed through the vessels for 10 min. Strict anaerobic conditions were maintained by introducing 100 μl 50 mM sodium dithionite solution to the centre wells using a syringe, which resulted in the appearance of a deep blue colour due to the paraquat radical and served as an oxygen trap. Partial anaerobic

conditions were obtained by initially subjecting flasks to the above routine (omitting the addition of sodium dithionite) and then admitting 100 μl air (~1 μmol O_2) into the flask with a syringe. The flasks were incubated for 30 min at 22°C. Other conditions are described in section 3.

3. Results

Initial experiments performed in the absence of an oxygen trap in 'anaerobic' vessels often resulted in rather variable results. This was due to the ability of oxygen to diffuse through the serum rubber stoppers during the course of the experiment. The presence of the paraquat/sodium dithionite oxygen trap completely eliminated this problem.

Table 1 shows that under strict anaerobic or normal aerobic conditions (~100 μmol O_2/flask), paraquat-mediated ethylene formation from methionine and pyridoxal phosphate was minimal. However, in the presence of a low level of oxygen (~1 μmol O_2/flask-'partial' anaerobiosis), ethylene production was markedly stimulated. The effect of altering oxygen tension during an experiment is shown in fig.1. This was achieved by adding sufficient air to the reduced paraquat solution in the centre well of an anaerobic flask until the blue colour just disappeared. A further 100 μl (~1 μmol O_2) was then added to the flask

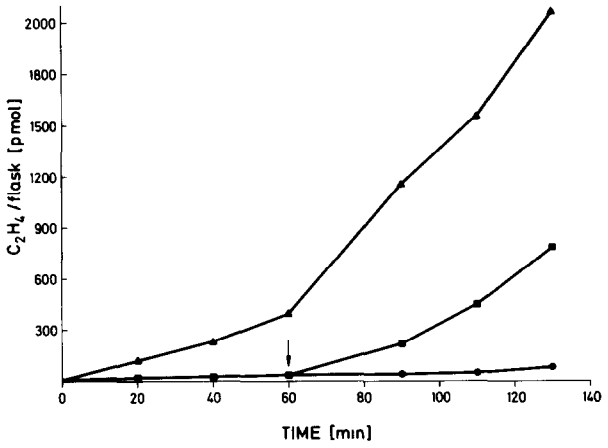


Fig.1. Effect of varying oxygen tension on ethylene formation. Reaction conditions were as described for table 1. The arrow (↓) indicates the change from strict anaerobiosis (●) to partial anaerobiosis (■). Ethylene formation in flasks maintained in partial anaerobiosis for the entire experiment is also shown (▲).

atmosphere. This clearly shows the dependence of this reaction on a low level of oxygen. Ethylene formation was strongly inhibited by catalase, whereas SOD had no effect, indicating that the oxidising species was derived from H_2O_2 rather than from $O_2^{\cdot-}$. Further evidence for a H_2O_2-derived oxidant was obtained from experiments where this oxygen species was included in the reaction mixture (table 2). Under strict anaero-

Table 1
Effect of oxygen on ethylene formation from methionine

Conditions	Ethylene formed (pmol/h)		
	Control	+SOD (50 U)	+Catalase (100 U)
Anaerobiosis	3	2	4
Partial anaerobiosis (1 μmol O_2/flask)	420	414	16
Aerobiosis (100 μmol O_2/flask)	7	8	5

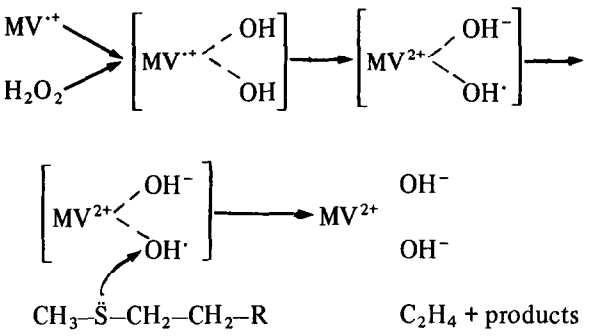
Reaction conditions – The reaction mixture contained in 2 ml: 10 μmol glucose-6-phosphate; 50 μg glucose-6-phosphate dehydrogenase; 1 μmol NADP; ferredoxin-NADP$^+$-oxidoreductase containing 0.1 mg protein; 0.2 μmol paraquat; 20 μmol methionine; 0.2 μmol pyridoxal phosphate; 100 μmol phosphate buffer (pH 7.8). The reaction was conducted in ~10 ml for 30 min at 22°C in Fernbach flasks

Table 2
Effect of Fe^{3+} and H_2O_2 on paraquat-mediated ethylene formation from methionine

Additions	Ethylene formed (pmol/h)	
	Aerobiosis (100 μmol O_2/flask)	Anaerobiosis
None	0	0
MV	0	0
H_2O_2 (1 μmol)	0	0
Fe^{3+} (0.2 μmol)	0	0
MV + H_2O_2	3	1024
MV + H_2O_2 + SOD	4	1105
MV + H_2O_2 + catalase	2	14
MV + Fe^{3+}	328	4
MV + Fe^{3+} + SOD	330	5
MV + Fe^{3+} + catalase	8	2

Reaction conditions as described in table 1

bic conditions, added H_2O_2 was able to overcome the previous inhibition of ethylene formation, but no effect was observed under normal oxygen concentrations, or in the absence of paraquat. By contrast, Fe^{3+} was able to stimulate ethylene formation only under aerobic conditions. As before, catalase was always inhibitory while SOD was without effect.



4. Discussion

These results demonstrate that under certain oxygen tensions, ethylene formation from methionine and pyridoxal phosphate can be mediated by paraquat, as shown with other redox compounds [31]. Earlier investigations with isolated chloroplast lamellae showed that although paraquat was reduced and subsequently reoxidised to form $O_2^{\cdot-}$ and H_2O_2, only small amounts of ethylene were formed [27]. This study indicates that this was probably due to an excess oxygen concentration.

The inhibition of ethylene formation by catalase and the lack of an effect with SOD indicates that the oxidant of methionine was derived from reduced paraquat and H_2O_2. However, the species responsible was not the free OH radical due to the above reasons and to the lack of inhibition found with traditional scavengers of the free OH radical, e.g., mannitol, ethanol and benzoate (not shown). Therefore, we propose the formation of an active oxygen species which mimics the free OH radical, but is more discriminating in its reactions. This will subsequently be referred to as a 'crypto-OH radical'. The reaction between reduced paraquat and molecular oxygen is very rapid ($k = 7.7 \times 10^8\ M^{-1}\ s^{-1}$) [22] compared to that with H_2O_2 ($k = 2.3 \times 10^3\ M^{-1}\ s^{-1}$) [32] and therefore crypto-OH radical formation would only be expected to occur under oxygen limiting conditions (i.e., high H_2O_2/O_2 ratio). A possible mechanism for the formation of the crypto-OH radical and subsequent ethylene production from methionine is described in the following reactions:

$$MV^{2+} + e^- \longrightarrow MV^{\cdot+}$$

$$MV^{\cdot+} + O_2 \longrightarrow MV^{2+} + O_2^{\cdot-}$$

$$2\,O_2^{\cdot-} + 2\,H^+ \longrightarrow H_2O_2 + O_2$$

The inhibition of ethylene formation in these reactions by catalase and high oxygen levels is due to the removal of the initial complex-forming species. $O_2^{\cdot-}$ has no direct role in ethylene generation via this sequence, which thus explains the lack of a SOD effect.

The ability of Fe^{3+} in mediating ethylene formation under aerobic conditions was not due to an 'iron-catalysed Haber–Weiss' reaction [14], since no inhibition was observed with SOD. It appears more likely that Fe^{3+} was directly reduced by the paraquat radical to Fe^{2+}, which then formed a Fenton-type oxidant with H_2O_2 in agreement with [27].

The implication from these results is that biological systems capable of reducing paraquat may form crypto-OH radicals under oxygen-limiting conditions. This is unlikely to occur in illuminated chloroplasts due to the high oxygen concentration present which is derived from photosynthesis. Also, studies into the mechanism of resistance of paraquat-tolerant plants have shown that these biotypes possessed increased levels of either SOD alone [33] or of both SOD and catalase [34], which infers that $O_2^{\cdot-}$ plays a role in the in vivo activity of the herbicide. Thus, crypto-OH radical formation probably accounts for only part of the herbicidal activity of paraquat. However, in [35] paraquat in alveolar macrophages was reduced at the expense of NADPH and glucose. It is thus conceivable that in certain areas of the lung which have decreased oxygen levels, crypto-OH radicals may be produced which could account for the observed toxic action of paraquat. The enhanced toxicity of paraquat towards animals under oxygen concentrations greater than normal may be derived from increased H_2O_2 levels. A higher concentration of this species could induce greater toxicity either by increasing Fenton-type oxidant production or by diffusing over a greater area and thus providing an enlarged capacity for crypto-OH radical formation.

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