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# INTERACTIONS BETWEEN Cu(II), Mn(II) AND SALICYLHYDROXAMIC ACID IN DETERMINATION OF ALGAL PEROXIDASE ACTIVITY

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**Key Word Index**—*Selenastrum minutum*; algae; Cu(II); Mn(II); peroxidase; superoxide dismutase; salicylhydroxamic acid; oxygen consumption.

**Abstract**—Cells of the green alga *Selenastrum minutum* have a high capacity for $O_2$ consumption mediated by extracellular peroxidase. The rate of peroxidase-mediated $O_2$ consumption is greatly stimulated by the addition of salicylhydroxamic acid (SHAM), and the $O_2$ consumption rate is further enhanced by Mn(II) in the presence of SHAM; Mn(II) has no effect on the rate of $O_2$ consumption by cells in the absence of SHAM. Both in the presence and absence of Mn(II), SHAM-stimulated peroxidase-mediated cellular $O_2$ consumption is inhibited by Cu(II). In the absence of Mn(II), inhibition is probably via chelation of Cu(II) by SHAM. The combination of Mn(II) and SHAM results if $O_2$ consumption even in the absence of cells in a peroxidase-free system. This latter $O_2$ consumption is 50% inhibited by catalase or superoxide dismutase, and completely inhibited by low concentrations of Cu(II). In this latter case, Cu(II) is not acting by chelation of the SHAM. Contrary to some reports in the literature, in *S. minutum* there is no evidence that the inhibition of peroxidase by Cu(II) is solely due to superoxide dismutase-like properties of Cu(II). Furthermore, it is suggested that the combination of Mn(II) and SHAM should not be used to activate peroxidase activity due to the potential for high rates of non-enzymatic $O_2$ consumption. © 1997 Elsevier Science Ltd

## INTRODUCTION

Many plant and algal cells have high levels of peroxidase activity associated with the cell wall and/or the exterior face of the plasma membrane [1–4]. Peroxidases catalyse oxidation of a substrate by $H_2O_2$ via the following sequence of reactions [5]:

$$\text{Peroxidase} + H_2O_2 \rightarrow \text{Compound I} \quad (1)$$

$$\text{Compound I} + YH_2 \rightarrow \text{Compound II} + YH^{\cdot} \quad (2)$$

$$\text{Compound II} + YH_2 \rightarrow \text{Peroxidase} + YH^{\cdot} \quad (3)$$

$$YH^{\cdot} + YH^{\cdot} \rightarrow Y + YH_2 \quad \text{or} \quad HY-YH \quad (4)$$

$YH_2$ represents the substrate (electron donor) for the overall reaction. Reaction 4 is non-enzymatic.

Net $O_2$ consumption frequently accompanies peroxidase activity, and the $O_2$ consumption may contribute measurably to determinations of $O_2$ consumption rates by plant tissues, resulting in over-estimation of the rate of respiratory (mitochondrial) $O_2$ consumption [6, 7]. $O_2$ is not directly consumed by peroxidase, but rather $O_2$ consumption is a consequence of radical chain reactions associated with the production of $H_2O_2$ and $O_2^-$. For example, peroxidase-mediated NADH oxidation is suggested to occur via the following reaction scheme (modified from [5, 8]):

$$\text{NADH} + O_2 \rightarrow \text{NAD}^{\cdot} + O_2^- \quad (5)$$

$$2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2 \quad (6)$$

$$\text{Peroxidase} + H_2O_2 \rightarrow \text{Compound I} \quad (7)$$

$$\text{Compound I} + \text{NADH} \rightarrow \text{Compound II} + \text{NAD}^{\cdot} \quad (8)$$

$$\text{Compound II} + \text{NADH} \rightarrow \text{Peroxidase} + \text{NAD}^{\cdot} \quad (9)$$

$$\text{NAD}^{\cdot} + O_2 \rightarrow \text{NAD}^+ + O_2^- \quad (10)$$

$$\text{NADH} + O_2^- + H^+ \rightarrow \text{NAD}^{\cdot} + H_2O_2 \quad (11)$$

$$\text{Peroxidase} + O_2^- \rightarrow \text{Compound III} \quad (12)$$

Reactions 5, 6, 10 and 11 are non-enzymatic.

Specific inhibitors of extracellular peroxidases are lacking [7], making it difficult to quantify the contribution of peroxidase-mediated $O_2$ consumption to overall measured dark $O_2$ consumption rates. As superoxide ($O_2^-$) is an intermediate in the complex

aerobic peroxidase reaction mechanism, the superoxide scavenging (and Cu-containing) enzyme superoxide dismutase is an effective inhibitor of $O_2$ consumption by isolated peroxidase enzyme [5]. Superoxide dismutase has also sometimes been employed as a potential inhibitor of peroxidase-mediated $O_2$ consumption by intact tissues and cells, but with only partial success [3, 4, 6, 9, 10]. Recently, it has been suggested that Cu(II) could be used as a peroxidase inhibitor of purified soya bean plasma membranes [11], based on the presumed superoxide dismutation ability of this ion or its low molecular weight chelates [12–15]. Furthermore, peroxidase activity is often stimulated several-fold by Mn(II), especially in the presence of hydroxamic or phenolic compounds [e.g. 8, 16–19, 21–24]. The activating effect of Mn(II) has been frequently employed in studies of peroxidase activity.

In an effort to utilize both Cu(II) and Mn(II) as tools to further investigate extracellular peroxidase activity in green algal cells, I have been able to demonstrate that Cu(II) does indeed inhibit peroxidase, and that Mn(II) does indeed stimulate $O_2$ consumption. However their modes of action, and their interactions, were unexpected. I provide evidence that Cu(II) inhibition of salicylhydroxamic acid (SHAM) stimulated peroxidase-mediated $O_2$ consumption by cell suspensions is due to chelation of Cu(II) by SHAM, and that the combination of Mn(II) and SHAM leads to the production of active oxygen species even in the absence of cells or peroxidase. Superoxide dismutase-like properties of Cu(II) were insufficient to explain the inhibition of $O_2$ consumption.

## RESULTS AND DISCUSSION

SHAM is a well-known activator of peroxidases in plant and algal cells [1, 3, 4, 6, 9, 21, 25–29]. The green algal *Selenastrum minutum* has a particularly large capacity for SHAM-stimulated peroxidase-mediated $O_2$ consumption [4, 30]. Previous work has indicated that addition of 5 mM SHAM resulted in maximal stimulation of peroxidase-mediated $O_2$ consumption [4, 30]. Addition of 5 mM SHAM to a cell suspension culture resulted in more than a doubling of the control $O_2$ consumption rate (Fig. 1). SHAM-stimulated $O_2$ consumption was relatively insensitive to inhibition by low concentrations (0.5–2.0 mM) of $CuSO_4$; however 5 mM $CuSO_4$ almost completely inhibited the SHAM-stimulated $O_2$ consumption. Higher concentrations of $CuSO_4$ had only slight further inhibitory effects (Fig. 1). In the absence of SHAM, $CuSO_4$ concentrations up to 20 mM had no measurable effect on the $O_2$ consumption rate (Fig. 1); i.e. $CuSO_4$ specifically inhibited SHAM-stimulated $O_2$ consumption. Other Cu(II) salts, including $CuCl_2$ and Cu-acetate, were also effective at inhibiting SHAM-stimulated $O_2$ consumption at similar concentrations, but acted slightly more slowly than $CuSO_4$ (not shown).

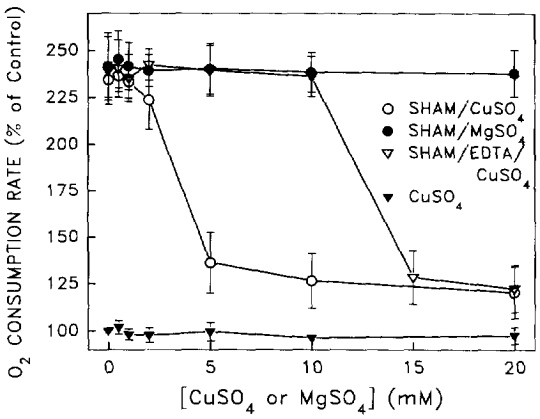


Fig. 1. Interactions between Cu(II) or Mg(II) and the rate of SHAM-stimulated oxygen consumption by algal cell suspensions. In the absence of SHAM (▼) oxygen consumption is mediated primarily by mitochondrial respiration; SHAM-stimulated oxygen consumption is mediated by peroxidase [4]. SHAM was added at a concentration of 5 mM. EDTA, when present, was added at a concentration of 10 mM, and was added to the buffer before the experiment. Control oxygen consumption rate was 358 ($\pm$21 [$n$ = 7]) nmol $O_2$ $ml^{-1}$ $h^{-1}$.

As $CuSO_4$ exhibited the most rapid inhibition of SHAM-stimulated $O_2$ consumption, various other sulphate salts were tested for their inhibitory potential. $MgSO_4$ had no detectable effect on the rate of SHAM-stimulated $O_2$ consumption by cells (Fig. 1) or on the rate of $O_2$ consumption in the absence of SHAM (not shown). Similar results were found for $ZnSO_4$ (not shown). Thus, the inhibition of SHAM-stimulated $O_2$ consumption appears to be specific for Cu(II).

While hydroxamic acids are well-known chelators of Fe(III), they are also able to chelate Cu(II) [31], and this appears to be the basis for Cu(II)-inhibition of SHAM-stimulated $O_2$ consumption by algal cells in the absence of Mn(II). While no specific data exist for the interactions between SHAM and Cu(II), in the absence of cells it is possible to measure the formation of the SHAM-Cu(II) chelate, which has an absorbance maximum at 320 nm. Titration of a 1 mM SHAM solution with increasing $CuSO_4$ results in an increase in A320 until 1 mM $CuSO_4$ is reached (Fig. 2). Similarly, titration of a 0.5 mM SHAM solution reaches a plateau at 0.5 mM $CuSO_4$ (Fig. 2). This suggests that SHAM will chelate Cu(II) in a 1:1 ratio.

This observation also provides an explanation for the results of Fig. 1. Five mM $CuSO_4$ was required to inhibit peroxidase-mediated $O_2$ consumption activated by 5 mM SHAM. This suggests that chelation of the Cu(II) prevents the activation of peroxidase by SHAM. Similarly, 2 mM $CuSO_4$ was sufficient to inhibit the lesser stimulation of $O_2$ consumption resulting from 2 mM SHAM (not shown). Furthermore, it is possible to show that if the Cu(II) is kept chelated by EDTA, it is not inhibitory of SHAM-stimulated $O_2$ consumption (Fig. 1). In the presence of 10 mM EDTA, which chelates Cu(II) on a 1:1

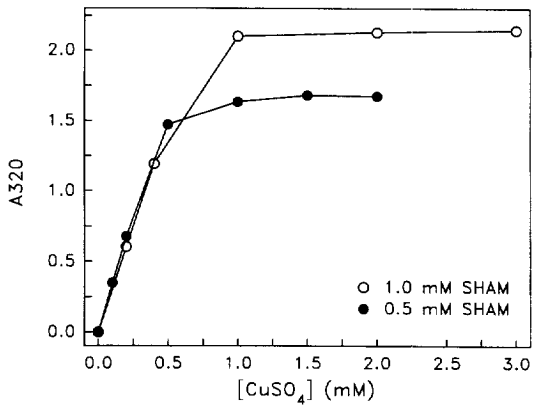


Fig. 2. Absorbance at 320 nm of SHAM/Cu(II) complexes produced in 1.0 or 0.5 mM SHAM solutions.

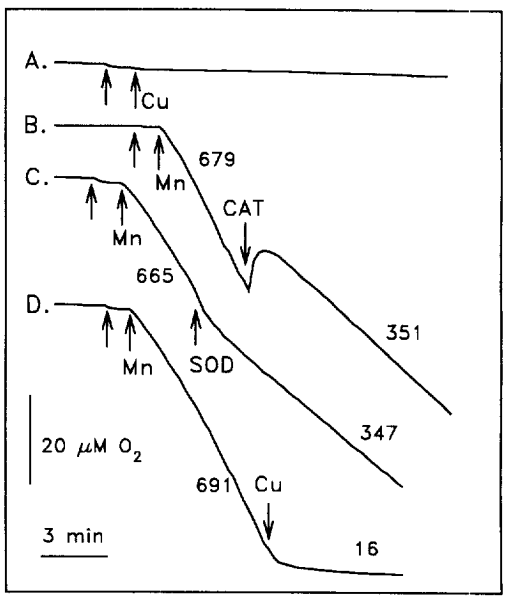


Fig. 3. Non-enzymatic oxygen consumption due to the Mn(II)-catalysed oxidation of SHAM. Experiments were performed in assay buffer, without cells. This system was entirely without peroxidase activity. The first arrow of each trace represents the addition of 5 mM SHAM. 'Cu' represents the addition of 5 mM $CuSO_4$ in (A), and 0.5 mM $CuSO_4$ in (D). 'Mn' represents the addition of 5 mM $MnCl_2$. Numbers beside the traces represent the rates of oxygen consumption in units of nmol $O_2$ $ml^{-1}$ $h^{-1}$. Catalase (CAT) and superoxide dismutase (SOD) were both added at an activity of 1000 U $ml^{-1}$. Trace A: addition of SHAM and 5 mM $CuSO_4$. Trace B: partial inhibition of Mn(II)-catalysed $O_2$ consumption by the addition of catalase. Trace C: partial inhibition of Mn(II)-catalysed $O_2$ consumption by the addition of superoxide dismutase. Trace D: almost complete inhibition of Mn(II)-catalysed $O_2$ consumption by the addition of 0.5 mM $CuSO_4$.

basis, 15 mM $CuSO_4$ was required to inhibit SHAM-stimulated $O_2$ consumption (Fig. 1). Similar results were found for other EDTA concentrations (not shown).

It has been frequently suggested that Mn(II) is a potent activator of peroxidase, especially in the presence of hydroxamic or phenolic compounds (see Introduction), although the activation mechanism has not been conclusively elucidated. Addition of 5 mM $MnCl_2$ to *S. minutum* cells, in the absence of SHAM, had no measurable effect on the $O_2$ consumption rate (Table 1). Other $MnCl_2$ concentrations (10 $\mu$M–20 mM) also had no measurable effect on the rate of $O_2$ consumption by cells (not shown). In contrast, there was a large stimulation of $O_2$ consumption when Mn(II) was added in the presence of SHAM (Table 1). However, in experiments using cell- and peroxidase-free buffer, identical to that used to measure $O_2$ consumption by cells, many of these observations could be duplicated (Fig. 3). Addition of a combination of 5 mM SHAM and 5 mM $MnCl_2$ to buffer resulted in a large stimulation of $O_2$ consumption, which could be 50% inhibited by the addition of 1000 U $ml^{-1}$ catalase [Fig. 3(B)]. 50% inhibition could also be achieved by the addition of 1000 U $ml^{-1}$ superoxide dismutase [Fig. 3(C)]. Higher activities of catalase or superoxide dismutase did not exhibit greater inhibitory effect (not shown). These results suggest that the combination of SHAM and Mn(II) results in the production of both $H_2O_2$ and $O_2^-$, both of which are intermediates in peroxidase-catalysed reactions [5].

Halliwell [8] provided evidence that the activating effect of phenolics on peroxidase-mediated NADH oxidation was to promote the conversion of inactive peroxidase Compound III into active peroxidase. He further suggested that the stimulatory effect of Mn(II) on this reaction was to promote the reaction between

Table 1. Oxygen consumption rates by cells, and the effects of SHAM, superoxide dismutase (SOD), Cu(II) and Mn(II). Cells were harvested by centrifugation and resuspended in assay buffer. SHAM was added at a final concentration of 5 mM, Mn(II) was added as 5 mM $MnCl_2$, Cu(II) was added as $CuSO_4$, and SOD was added at an activity of 1000 U $ml^{-1}$. Rates are expressed in units of nmol $O_2$ $ml^{-1}$ $h^{-1}$ ($\pm$SE [$n$])

| | Control | +SOD | 0.5 mM Cu(II) | + 5 mM Cu(II) |
|---|---|---|---|---|
| Cells | 379 ± 12(5) | 382 ± 9(3) | 368 ± 22(5) | 371 ± 9(4) |
| Cells/SHAM | 643 ± 25(6) | 638 ± 20(6) | 628 ± 10(5) | 402 ± 17(5) |
| Cells/SHAM/Mn(II) | 1086 ± 32(8) | 863 ± 12(6) | 649 ± 13(5) | 408 ± 21(7) |
| Cells/Mn(II) | 362 ± 7(4) | — | — | — |

NADH and $O_2^-$, leading to the production of a radical NADH oxidation product. NAD$^\cdot$, and $H_2O_2$:

$$Mn(II)+O_2^-+2H^+ \rightarrow Mn(II)+H_2O_2 \quad (13)$$

$$Mn(III)+NADH \rightarrow Mn(II)+NAD^\cdot \quad (14)$$

Maximum stimulation of peroxidase-mediated NADH oxidation was achieved with a combination of a phenolic and Mn(II) [8]. It is clear that Mn(II) reacts with superoxide, to produce the strong oxidant Mn(III) [32, 33] or perhaps $MnOH^{2+}$ [33].

In contrast, Yamazaki and Piette [34] suggested that the activating effect of phenolics ($XH_2$ in the reaction scheme below) on the aerobic oxidase reaction catalysed by peroxidase was due to enhancement of the production of radicals (YH$^\cdot$) of the substrate ($YH_2$):

$$XH_2+H_2O_2 \rightarrow XH^\cdot \quad (15)$$

$$YH_2+XH^\cdot \rightarrow YH^\cdot+XH_2 \quad (16)$$

$$YH^\cdot+YH^\cdot \rightarrow 1/2(YH_2+Y) \quad (17)$$

$XH_2$ is the activating phenolic, and XH$^\cdot$ is the phenolic radical. Only reaction 15 is peroxidase-catalysed; reactions 16 and 17 are non-enzymatic. Similar to the suggestion of Halliwell [8], Yamazaki and Piette [34] provided evidence that the activating effect of Mn(II) was due to the non-enzymatic oxidation of the substrate by Mn(III), leading to further $H_2O_2$ formation (modified from [34]):

$$YH_2+H_2O_2 \rightarrow YH^\cdot \quad (18)$$

$$YH^\cdot+O_2 \rightarrow Y+O_2^- \quad (19)$$

$$O_2^-+Mn(II) \rightarrow H_2O_2+Mn(III) \quad (20)$$

$$YH_2+Mn(III) \rightarrow YH^\cdot+Mn(II) \quad (21)$$

As above, $YH_2$ represents the substrate for the peroxidase reaction; only reaction 18 is peroxidase-catalysed [34].

$CuSO_4$ was even more effective than superoxide dismutase in reducing the rate of $O_2$ consumption [Fig. 3(D)]. Two points are important in this respect. First, the effect of Cu(II) in inhibiting the reaction was not due to chelation in this case, as concentrations of Cu(II) well below 5 mM were effective (500 $\mu$M in Fig. 3(D)). Secondly, if $CuSO_4$ were acting only as a superoxide dismutase, then not more than 50% inhibition of the reaction rate should be expected. The reaction catalysed by superoxide dismutase is:

$$2O_2^-+2H^+ \rightarrow H_2O_2+O_2 \quad (22)$$

The almost complete inhibition of $O_2$ consumption shown in Fig. 3(D) would suggest that $CuSO_4$ is not simply acting in the dismutation of $O_2^-$, but must have other effects. While this does not rule out the possibility that $CuSO_4$ also catalyses the dismutation of $O_2^-$, catalysis of dismutation is insufficient to explain the results [Fig. 3(D)]. Furthermore, in the absence of Mn(II), there is no detectable stimulation of $O_2$ consumption by the addition of Cu(II) to SHAM in solution [Fig. 3(A)], suggesting that Cu(II), unlike Mn(II), does not catalyse the oxidation of SHAM.

The presence of $H_2O_2$, which is the product of the superoxide dismutation reaction, may be detected by using the enzyme catalase, which catalyses the decomposition of $H_2O_2$:

$$2H_2O_2 \rightarrow H_2O+O_2 \quad (23)$$

Catalase may be used to probe for the presence of $H_2O_2$ by measuring the $O_2$ produced by $H_2O_2$ decomposition [6, 26, 29]. Both in the presence [Fig. 4(B)] and absence [Fig. 3(B)] of superoxide dismutase, addition of catalase to a cell-free system containing SHAM and Mn(II) resulted in the transient production of $O_2$, indicating the presence of $H_2O_2$. Conversely, there was very little $O_2$ produced when catalase was added to the SHAM-Mn(II) system in the presence of 500 $\mu$M Cu(II), indicating that little $H_2O_2$ was present. These results suggest that the inhibitory effects of Cu(II) on $O_2$ consumption [Fig. 3(D)] were not due to the superoxide dismutase activity of Cu(II).

It is possible to use superoxide dismutase and varying concentrations of Cu(II) to examine the contributions of different processes contributing to $O_2$ consumption by *S. minutum* cell suspensions in the presence of various combinations of effectors (Table 1). Under control conditions, neither Cu(II) nor superoxide dismutase affected the $O_2$ consumption

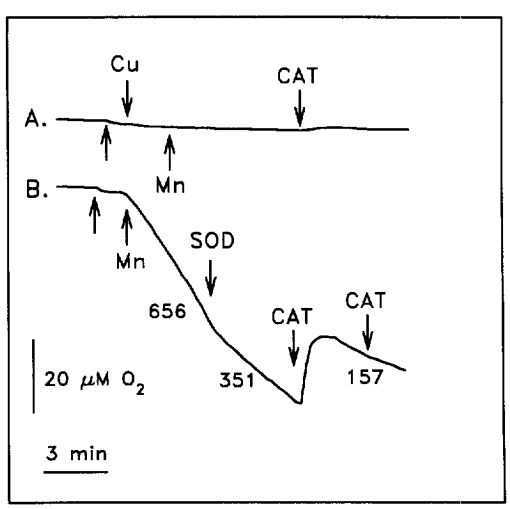


Fig. 4. Non-enzymatic oxygen consumption due to the Mn(II)-catalysed oxidation of SHAM. Experiments were performed in assay buffer, without cells. This system was entirely without peroxidase activity. The first arrow of each trace represents the addition of 5 mM SHAM. 'Cu' represents the addition of 0.5 mM $CuSO_4$. 'Mn' represents the addition of 5 mM $MnCl_2$. Numbers beside the traces represent the rates of oxygen consumption in units of nmol $O_2$ $ml^{-1}$ $h^{-1}$. Catalase (CAT) and superoxide dismutase (SOD) were both added at an activity of 1000 U $ml^{-1}$. Trace A: absence of $O_2$ production due to the addition of catalase in a system containing Cu(II). Trace B: $O_2$ production due to catalase addition to a system containing superoxide dismutase.

rate, while in the presence of 5 mM SHAM only 5 mM Cu(II), but not superoxide dismutase, decreased the $O_2$ consumption rate (Table 1). Exogenous superoxide dismutase has previously been shown to be ineffective in inhibiting cell-associated peroxidase activity in green algal cells [3, 4]. Maximal stimulation of $O_2$ consumption could be achieved by the combination of SHAM and Mn(II), and superoxide dismutase and two different concentrations of Cu(II) decreased the $O_2$ consumption rate in a predictable manner, with superoxide dismutase being effective only in the presence of Mn(II). In other words, superoxide dismutase could inhibit 50% of the $O_2$ consumption due to the reaction of Mn(II) with SHAM, but could not inhibit the peroxidase-mediated $O_2$ consumption. 500 $\mu$M Cu(II) could almost completely inhibit $O_2$ consumption due to SHAM oxidation by Mn(II), but 5 mM Cu(II) was required to inhibit $O_2$ consumption due to activation of peroxidase by SHAM (inhibition by chelation; see Fig. 1).

While there is evidence that Cu(II) [12], or low molecular weight Cu(II) complexes (e.g. [13, 14, 15]) may exhibit superoxide dismutase activity under certain specific conditions, there is also a great deal of evidence that Cu(II) may have strong pro-oxidant properties under certain conditions [35, 36]. For example, while addition of superoxide dismutase inhibits the autoxidation of dihydroxyfumarate in solution, oxidation is stimulated by addition of Cu(II) [37].

Cu(II) may generate the highly reactive hydroxyl radical ($OH^{\cdot}$) from $H_2O_2$ and a suitable reducing agent (e.g. superoxide or ascorbate), according to the following suggested reaction scheme [38, 39]:

$$Cu(II) + O_2^- \rightarrow Cu(I) + O_2 \quad (24)$$

$$Cu(I) + H_2O_2 \rightarrow Cu(II) + OH^{\cdot} + OH^- \quad (25)$$

In this case, while Cu(II) is indeed a scavenger of $O_2^-$, as in the dismutation reaction, one of the products of the reaction is highly reactive. The hydroxyl radical reacts with very high rate constants with almost every type of biological molecule, including amino acids, nucleic acids and lipids [40]. Cu(II)-mediated hydroxyl radical formation results in oxidation of DNA (e.g. [41]) and lipid peroxidation (e.g. [42]). The Cu(II) ion may also mediate oxidation of sulfhydryl groups [43] and compromise the plasma membrane permeability barrier (e.g. [44]).

In the absence of a reducing agent, addition of Cu(II) to SHAM in solution did not result in a detectable stimulation of $O_2$ consumption [Fig. 3(A)]. Cu(II) complexes (e.g. Cu-amine), in the presence of $O_2$, are well-known to be able to catalyse the oxidative coupling of phenols. In the process of phenol oxidation, Cu(II) is reduced to Cu(I), and $O_2$ is required in order to reoxidize the Cu(I) [45]. Total $O_2$ consumption is typically stoichiometric to product formation (e.g. [46]). Conversely, simple Cu(II) salts, with the exception of $Cu(NO_3)_2$ [47]. are generally catalytically inactive in phenol oxidation [45]. The data presented in Fig. 3(A) are consistent with the suggestion that $CuSO_4$, unlike Mn(II), did not catalyse SHAM oxidation.

In summary, I suggest that the combination of Mn(II) and SHAM leads to the production of active oxygen species in the absence of peroxidase, i.e. the non-enzymatic generation of active oxygen ($H_2O_2$ and/or $O_2^-$). Thus it is not possible to ascribe the Mn(II)/SHAM stimulation of $O_2$ consumption solely to peroxidase activity, even when peroxidase is present. However, active oxygen species are intermediates of the peroxidase reaction [5], leading to the possibility that at least some of the stimulation of $O_2$ consumption is peroxidase-mediated in the presence of that enzyme. Secondly, while Cu(II) can be demonstrated to inhibit SHAM-stimulated $O_2$ consumption under certain conditions, ascribing the inhibition due to the superoxide dismutase-like activity of Cu(II) is probably not warranted. I suggest that the use of Cu(II) as a peroxidase inhibitor is inappropriate. Furthermore, care must be taken in evaluating the effects of Mn(II) on apparent peroxidase activity in the presence of SHAM or other substrates potentially oxidizable in the presence of Mn(II) and $O_2$.

## EXPERIMENTAL

The freshwater unicellular green alga *Selenastrum minutum* (Näg.) Collins (UTEX 2459), originally isolated from Lake Ontario, was obtained from the culture collection of the University of Texas, Austin. Cells were grown photoautotrophically in semi-continuous culture in water-jacketted glass chemostat vessels at a temp. of 20° and continuous illumination at a photon fluence rate of 220 $\mu$mol quanta $m^{-2}$ $s^{-1}$ [4]. Cells were stirred and aerated with 1% $CO_2$ in air. Light was provided by a bank of 12 cool white, high output fluorescent lights (F48T12/CW/HO, Philips Electronics Ltd., Scarborough, Ontario, Canada). The medium was a modification of that formulated by Hughes [48], and contained 300 $\mu$M $K_2HPO_4$, 3 mM $NH_4NO_3$, and was buffered at pH 7.5 with 15 mM HEPES–KOH. $Na_2CO_3$ and $NaSiO_3$ were omitted, and minor elements were added according to Allen [49]. The iron stock contained $Na_2EDTA$ (0.4 g $l^{-1}$), citric acid (4.8 g $l^{-1}$) and $FeSO_4 \cdot 7H_2O$ (2.4 g $l^{-1}$); 2.5 ml was used per litre of medium. Cells were diluted once per day to maintain exponential growth; growth rate was approximately 1.3 $day^{-1}$.

$O_2$ consumption by cell suspensions and cell-free assay buffer was measured in the liquid phase with an oxygen electrode (Hansatech, King's Lynn, U.K.) equipped with a water-jacketted cuvette for temp. control. All experiments were conducted at 20° and in a 1.0 ml vol. Measured rates of $O_2$ consumption were corrected for $O_2$ consumption by the electrode. For experiments investigating the interactions between algal peroxidase and various effectors, 1.0 ml aliquots of cells were harvested by centrifugation (19 000 *g* for 1 min). The supernatant was discarded, and the pellet

(containing algal cells) was resuspended in 1.0 ml of assay buffer (15 mM HEPES–KOH, pH 6.5, 300 $\mu$M $MgSO_4$, 245 $\mu$M $CaCl_2$). Experiments investigating non-enzymatic $O_2$ consumption (absence of cells) due to the interactions between SHAM and Mn(II) were also performed in the above assay buffer (1.0 ml vol.); there was no peroxidase activity in the latter experiments.

SHAM was added from a 0.75 M stock in 1 N KOH. Superoxide dismutase and catalase (both from Sigma) were added from stocks in 15 mM HEPES–KOH (pH 7.0). All other effectors were added from stocks in distilled $H_2O$.

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## REFERENCES

1. Møller, I. M., Bérczi, A., van der Plas, L. H. W. and Lambers, H., *Physiologia Plantarum*, 1988, **72**, 642.
2. Bingham, I. J. and Stevenson, E. A., *Physiologia Plantarum*, 1995, **93**, 427.
3. Torkelson, J. D., Lynnes, J. A. and Weger, H. G., *Journal of Phycology*, 1995, **31**, 562.
4. Weger, H. G., Lynnes, J. A. and Torkelson, J. D., *Physiology of Plants*, 1996, **96**, 268.
5. Yamazaki, I. and Yokota, K., *Molecular and Cellular Biochemistry*, 1973, **2**, 39.
6. van der Werf, A., Raaimakers, D., Poot, P. and Lambers, H., *Planta*, 1991, **183**, 347.
7. Shugaev, A. G., Urmantseva, V. V. and Volkova, L. A., *Russian Plant Physiology—English Translation*, 1993, **40**, 381.
8. Halliwell, B., *Planta*, 1978, **140**, 81.
9. Spreen Brouwer, K., van Valen, T., Day, D. A. and Lambers, H., *Plant Physiology*, 1986, **82**, 236.
10. Bingham, I. J. and Farrar, J. F., *Physiologia Plantarum*, 1987, **70**, 491.
11. Zancani, M., Nagy, G., Vianello, A. and Macrì, F., *Phytochemistry*, 1995, **40**, 367.
12. Rabani, J., Klug, R. D. and Lilie, J., *Journal of Physical Chemistry*, 1973, **77**, 1169.
13. Brigelius, R., Spöttl, R., Bors, W., Lengfelder, E., Saran, M. and Weser, U., *FEBS Letters*, 1974, **47**, 72.
14. Weser, U. and Schubotz, L. M., *Bioinorganic Chemistry*, 1978. **9**, 505.
15. Duracková, Z., Felix, K., Feniková, L., Kepštová, I., Labuda, J. and Weser, U., *Biometals*, 1995, **8**, 183.
16. Kenten, R. H., *Biochemical Journal*, 1955, **59**, 110.
17. Maclachlan, G. A. and Waygood, E. R., *Canadian Journal of Biochemistry and Physiology*, 1956, **34**, 1233.
18. Akazawa, T. and Conn, E. E., *Journal of Biological Chemistry*, 1958, **232**, 403.
19. Klebanoff, S. J., *Biochimica et Biophysica Acta*, 1960, **44**, 501.
20. Elstner, E. F. and Heupel, A., *Planta*, 1976, **130**, 175.
21. Gross, G. G., Janse, C. and Elstner, E. F., *Planta*, 1977, **136**, 271.
22. Ishida, A., Ookubo, K. and Ono, K., *Plant and Cell Physiology*, 1987, **28**, 723.
23. Pantoja, O. and Willmer C. M., *Planta*, 1988, **174**, 44.
24. Kalyanaraman, V. S., Kumar, S. A. and Mahadevan, S., *Biochemical Journal*, 1975, **149**, 577.
25. Brooks, J. L., *Biochemical and Biophysical Research Communications*, 1983, **116**, 916.
26. Askerlund, P., Larsson, C., Widell, S. and Møller, I. M., *Physiologia Plantarum*, 1987, **71**, 9.
27. Diethelm, R., Miller, M. G., Shibles, R. and Stewart, C. R., *Plant Cell Physiology*, 1990, **31**, 179.
28. Bingham, I. J. and Farrar, J. F., *Physiologia Plantarum*, 1987, **70**, 491.
29. van der Plas, L. H. W., Gude, H. and Wagner, M. J., *Physiologia Plantarum*, 1987, **70**, 35.
30. Weger, H. G., Guy, R. D. and Turpin, D. H., *Plant Physiology*, 1990, **93**, 356.
31. Anderegg, G., L'Eplattenier, F. and Schwarzenbach, G., *Helvetica Chimica Acta*, 1963, **46**, 1400.
32. Kono, Y., Takahashi, M. and Asada, K., *Archives of Biochemistry and Biophysics*, 1976, **174**, 454.
33. Bielski, B. H. J., Arudi, R. L. and Cabelli, D. E., in *Oxygen Radicals in Chemistry and Biochemistry*, ed. W. Bors, M. Saran and D. Tait. Walter de Gruyter, Berlin, 1984, pp. 1–15.
34. Yamazaki, I. and Piette, L. H., *Biochimica et Biophysica Acta*, 1963, **77**, 47.
35. Ljutakova, S. G., Russanov, E. M. and Liochev, S. I., *Archives of Biochemistry and Biophysics*, 1984, **235**, 636.
36. Gille, G. and Sigler, K., *Folia Microbiologica*, 1995, **40**, 131.
37. Halliwell, B., *Biochemical Journal*, 1977, **163**, 441.
38. Rowley, D. A. and Halliwell, B., *Archives of Biochemistry and Biophysics*, 1983, **225**, 279.
39. Aronovitch, J., Godinger, D. and Samuni, A., in *Oxygen Radicals in Chemistry and Biochemistry*, ed. W. Bors, M. Saran and D. Tait. Walter de Gruyter, Berlin, 1984, pp. 219–223.
40. Halliwell, B. and Gutteridge, J. M. C., *Biochemical Journal*, 1984, **219**, 1.
41. Sagripanti, J. L. and Kraemer, K. H., *Journal of Biological Chemistry*, 1989, **264**, 1729.
42. Sandmann, G. and Böger, P., *Plant Physiology*, 1980, **66**, 797.
43. Salhany, J. M., Swanson, J. C., Cordes, K. A., Gaines, S. B. and Gaines, K. C., *Biochemical and Biophysical Research Communications*, 1978, **82**, 1294.

44. Ohsumi, Y., Kitamoto, K. and Anraku, Y., *Journal of Bacteriology*, 1988, **170**, 2676.
45. Gampp, H. and Zuberbühler, A. D., in *Metal Ions in Biological Systems*, Vol. 12, Properties of Copper, ed. H. Sigel. Marcel Dekker, New York, 1981, pp. 133–139.
46. Endres, G. F., Hay, A. S. and Eustance, J. W., *Journal of Organic Chemistry*, 1963, **28**, 1300.
47. Tsuruya, S., Kishikawa, Y., Tanaka, R. and Kuse, T., *Journal of Catalysis*, 1977, **49**, 254.
48. Hughes, E. D., Gorham, P. R. and Zehnder, A., *Canadian Journal of Microbiology*, 1958, **4**, 225.
49. Allen, M. M., *Journal of Phycology*, 1968, **4**, 1.