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NITRITE PRODUCTION FROM THE OXIDATION OF SALICYLHYDROXAMIC ACID BY PEROXIDASE OR Mn(II)

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Key Word Index—manganese; nitrate reductase; nitrite; oxygen consumption; peroxidase; salicylhydroxamic acid; *Selenastrum minutum*; green algae.

Abstract—Horseradish peroxidase (HRP) catalyzed the NADH-dependent and oxygen-consuming oxidation of salicylhydroxamic acid (SHAM) to generate NO_2^-. The reaction ceased when NADH was depleted, and could be re-initiated by further addition of NADH. NO_2^- production was inhibited by superoxide dismutase or catalase. Hydoxylamine could also serve as a substrate for HRP-catalyzed NO_2^- production. *Selenastrum minutum*, a unicellular green alga with high extracellular peroxidase activity, also produced small amounts of NO_2^- in the presence of SHAM and NADH. In the absence of HRP or algal cells, NO_2^- could also be produced from non-enzymatic SHAM oxidation by Mn(II), in a reaction that was not sensitive to inhibition by superoxide dismutase or catalase. These results suggest that under certain conditions it is possible to generate NO_2^- in the absence of NO_3^- or nitrate reductase activity, and that the commonly-employed peroxidase activators SHAM and Mn(II) may lead to erroneous conclusions regarding the presence of extracellular or plasma membrane-associated nitrate reductase.

INTRODUCTION

The plasma membrane (PM) of plant and algal cells is capable of catalyzing numerous redox reactions, with various electron donors and electron acceptors [1, 2]. Particularly well-known is the ferricyanide/ferric chelate reductase system(s), in which intracellular reductant is used to reduce extracellular Fe(III) to Fe(II) [3, 4]. PM redox systems are also suggested to be involved in the reduction of other extracellular cations, such as Cu(II) (e.g. [5, 6]), and in the production of active oxygen species ($O_2^{\cdot-}$, H_2O_2) [7] as a defense against pathogens (e.g. [8, 9]) or for various metabolic processes such as cell wall synthesis (e.g. [10, 11]). Production of active oxygen species is accompanied by oxygen consumption, and is likely mediated by NAD(P)H-dependent peroxidase activity and/or peroxidase-like NAD(P)H oxidase activity associated with the PM, and catalyzing an active oxygen (H_2O_2, $O_2^{\cdot-}$) dependent free radical chain reaction [7, 12]. This latter activity is sensitive to inhibition by catalase and superoxide dismutase, which scavenge active oxygen species [12, 13].

Somewhat more contentious is the suggestion that a portion of the cellular nitrate reductase (NR) activity is localized to the PM in plants and algae. While it is generally acknowledged that the majority of NR activity is cytosolic [14, 15], there is mounting evidence that a small proportion is localized to the PM [16]. PM-associated NR has been variously suggested to function in NO_3^- uptake, reduction of extracellular NO_3^- to NO_2^-, and in the reduction of various other extracellular electron acceptors [16–25].

The reductant for NR is generally considered to be NAD(P)H [14]:

$$NO_3^- + NAD(P)H + H^+ \rightarrow NO_2^- + NAD(P)^+ + H_2O \quad (1)$$

However a recent model for the functioning of PM-associated NR, resulting from experiments with plasma membranes isolated from maize roots, suggests that PM-associated peroxidase interacts with NR to provide the reducing equivalents for NO_2^- production [26]. In this model, production of the reducing equivalents needed by PM NAD(P)H-dependent NR activity is mediated by a free radical, perhaps $NAD^{\cdot}$, produced by PM peroxidase-catalyzed oxidation of NADH, according to the following reaction scheme (modified from [26]):

$$2NADH + H_2O_2 \rightarrow 2\,NAD^{\cdot} + 2H_2O \quad (2)$$

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$$NAD^{\cdot} + O_2 \rightarrow NAD^+ + O_2^{\cdot-} \quad (3)$$

$$NADH + O_2^{\cdot-} + H^+ \rightarrow NAD^{\cdot} + H_2O_2 \quad (4)$$

$$2NAD^{\cdot} + NO_3^- + 2H^+ \rightarrow 2NAD^+ + NO_2^- + H_2O \quad (5)$$

In the above scheme, reaction (2) is catalyzed by peroxidase, reaction (5) is catalyzed by PM-associated NR, and reactions (3) and (4) are non-enzymatic. PM-associated NR activity was strongly enhanced by the addition of Mn(II) and salicylhydroxamic acid (SHAM) [26], both of which are well-known activators of peroxidases (e.g. [10, 27, 28, 29]). It was suggested that the interaction between peroxidase and PM NR could modulate the rate of plant NO_3^- reduction [26].

However, it was recently demonstrated that the combination of Mn(II) and SHAM may lead to substantial rates of non-enzymatic O_2 consumption, which could be 50% inhibited by catalase or superoxide dismutase [30]. As well, given the fact that peroxidases are capable of catalyzing a wide variety of oxidation reactions, we have further investigated the potential interactions between peroxidase and the peroxidase activators SHAM and Mn(II) with respect to NO_3^- reduction. In this paper, we provide evidence that NO_2^- production is not necessarily dependent upon the presence of NO_3^- or NR, and that NO_2^- may be produced by the NADH-dependent oxidation of SHAM by peroxidase, and also by the non-enzymatic oxidation of SHAM by Mn(II). These results suggest that the generation of NO_2^- in the presence of NO_3^- is not sufficient evidence to demonstrate the presence of PM-associated NR, nor for the involvement of peroxidase activity in the reduction of NO_3^- to NO_2^-.

RESULTS AND DISCUSSION

In a cell-free system consisting of 2 U ml^{-1} HRP and 100 μM SHAM, NO_2^- production was NADH-dependent (Fig. 1). Low NADH concentrations resulted in a very rapid reaction, such that the time interval between addition of NADH and the taking of the first sample was sufficient for maximal NO_2^- production. Higher initial NADH levels (2.5 and 5.0 mM) resulted in a reaction that was initially slower, but maximal NO_2^- levels were higher (Fig. 1). Once the reaction stopped, it could be re-initiated by the addition of more NADH (Fig. 1), but not by the further addition of peroxidase or SHAM (not shown). The initial rate of NO_2^- production (first 5 min) was also affected by the amount of added HRP. Higher HRP activity resulted in higher initial rates of NO_2^- production, however lower initial HRP activity led to higher final NO_2^- concentrations (Fig. 2). The higher final NO_2^- concentration may be due to a lower rate of HRP-catalyzed NADH oxidation at lower levels of HRP activity.

Addition of 1 mM KNO_3 to the reaction mixture did not measurably increase the yield of NO_2^- (Table 1), suggesting that NO_2^- production did not occur via the reduction of NO_3^-, but rather by the peroxidase-catalyzed oxidation of SHAM. Addition of 1 mM NH_4Cl also did not increase the yield of NO_2^- (not shown), suggesting that this system was not capable of producing NO_2^- via oxidation of NH_4^+.

NO_2^- production by the peroxidase/SHAM/NADH system was accompanied by high rates of O_2 consumption (Table 1), and both the O_2 consumption and NO_2^- production could be inhibited by the presence of catalase or superoxide dismutase (Table 1). Rates of O_2 consumption were much higher than rates

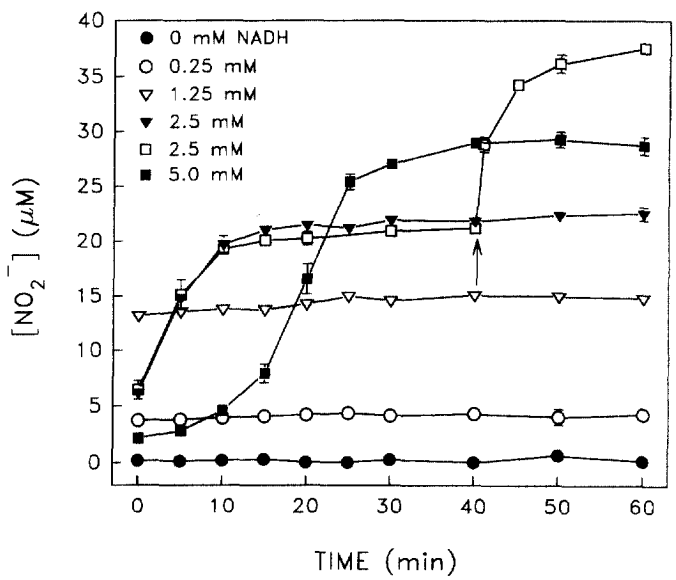

Fig. 1. Time course of NO_2^- production by the SHAM/peroxidase/NADH system. The reaction was started by addition of 2 U ml^{-1} horseradish peroxidase at time = 0. SHAM was added at a final concentration of 100 μM. Mn(II) was absent. The arrow represents the addition of 2.5 mM NADH. Bars represent SE (n = 4).

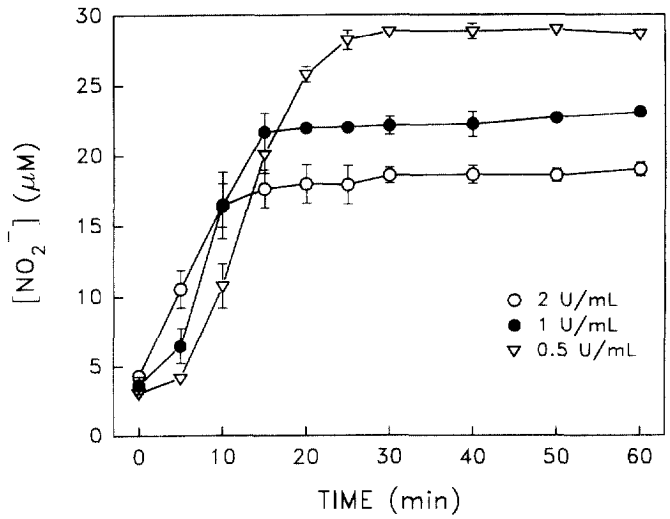

Fig. 2. Time course of NO_2^- production by the SHAM/peroxidase/NADH system, as influenced by peroxidase activity. SHAM was added at a final concentration of 100 μM, and NADH was added at 2.5 mM. Mn(II) was absent. Bars represent SE ($n = 3$).

of NO_2^- production (Table 1; [30]), and followed different time courses [30], indicating that the two processes are not stoichiometrically connected. Conversely, under anaerobic conditions the rate of NO_2^- production was greatly decreased (Table 1), indicating the O_2-dependence of NO_2^- production.

Peroxidase-catalyzed O_2-consuming reactions typically involve one or more non-enzymatic free radical-dependent steps [31]. Thus, the inhibition of NO_2^- production by catalase or superoxide dismutase suggests that active oxygen species are involved in the reaction mechanism, i.e. peroxidase may not be directly catalyzing NO_2^- production, but rather is catalyzing the production of active oxygen species (via NADH oxidation) that subsequently react non-enzymatically with SHAM. The production of nitrogen oxides by peroxidase-mediated oxidation of N-containing compounds has been demonstrated in a num-

Table 1. NO_2^- production in two different systems. (A) Peroxidase-catalyzed NO_2^- production from SHAM in the presence of NADH. SHAM and $MnCl_2$ were added at a final concentration of 5 mM. (B) Non-enzymatic NO_2^- production by the oxidation of SHAM by Mn(II). In (A), peroxidase was added at a final activity of 2 U ml^{-1}, and NADH was added at a final concentration of 5 mM. In (B), $MnCl_2$ was added at a final concentration of 5 mM. Numbers represent the mean ± SE ($n = 3$). ND, not determined; POD: peroxidase; SOD; superoxide dismutase

Experimental conditions	$[NO_2^-]$ after 30 min. (μM)	O_2 Consumption rate (μM min^{-1})
(A) SHAM/POD/NADH		
Control	41.6 ± 0.1	233.2 ± 9.3
5 mM EDTA	37.5 ± 0.4	227.1 ± 12.4
SOD (25 U ml^{-1})	1.6 ± 0.1	9.0 ± 2.0
Catalase (25 U ml^{-1})	2.8 ± 0.2	22.1 ± 4.1
5 mM KNO_3	43.6 ± 0.5	ND
Minus O_2	0.3 ± 0.1	ND
Minus SHAM	0	6.3 ± 1.6
Minus POD	0	0
(B) SHAM/$MnCl_2$		
Control	8.1 ± 0.5	10.1 ± 1.7[a]
5 mM EDTA	0.4 ± 0.2	2.5 ± 0.5[a]
SOD (1000 U ml^{-1})	7.5 ± 0.8	3.8 ± 0.6[a]
Catalase (1000 U ml^{-1})	7.7 ± 0.2	3.3 ± 0.5[a]
5 mM KNO_3	8.4 ± 1.0	ND
Minus O_2	5.8 ± 0.3	ND

[a] Similar data are presented in [30].

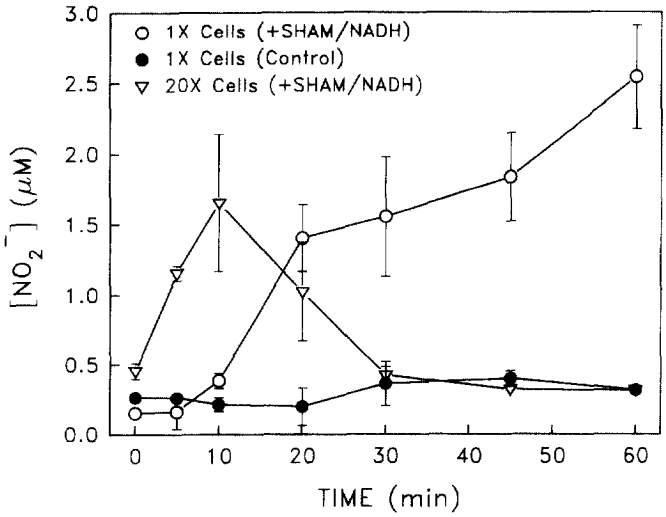

Fig. 3. Time course of NO_2^- production by cells of *Selenastrum minutum*. SHAM and NADH were added at a final concentration of 5 mM each. Mn(II) was absent. Bars represent SE ($n = 6$).

ber of systems [32–36], although to our knowledge NO_2^- production via oxidation of SHAM by peroxidase has not previously been reported.

Experiments using unicellular green algae provided mixed results. Cells of *Chlamydomonas reinhardtii* Dang. (UTEX 89), which possesses substantial extracellular peroxidase activity [37], did not produce detectable NO_2^- in the medium in the presence of 5 mM SHAM and 5 mM NADH (not shown), conditions which results in a large increase in the rate of O_2 consumption [37]. In contrast, cells of *Selenastrum minutum*, an algal species with even greater extracellular peroxidase activity [29], produced a low level of extracellular NO_2^- under those conditions (Fig. 3), which also result in a large stimulation of the O_2 consumption rate (see [29]). Part of the difficulty in demonstrating higher NO_2^- levels in the medium may lie in the fact that algae possess efficient plasma membrane NO_2^- transport systems [38], suggesting that the data in Fig. 3 underestimate the actual rate of NO_2^- production.

In the absence of peroxidase and NADH, non-enzymatic NO_2^- production could be demonstrated using a combination of SHAM and Mn(II) (Fig. 4). The reaction rate appeared to be more sensitive to the SHAM concentration than the Mn(II) concentration (Fig. 4), and could be inhibited by chelating the Mn(II) with an equivalent concentration of EDTA (Table 1). Despite the fact that this reaction consumes O_2 ([30]; Table 1), high activities of catalase or superoxide dismutase had no measurable effect on the rate of NO_2^- production (Table 1). Similarly, anaerobic conditions only slight inhibited NO_2^- production (Table 1), suggesting that active oxygen species were not required

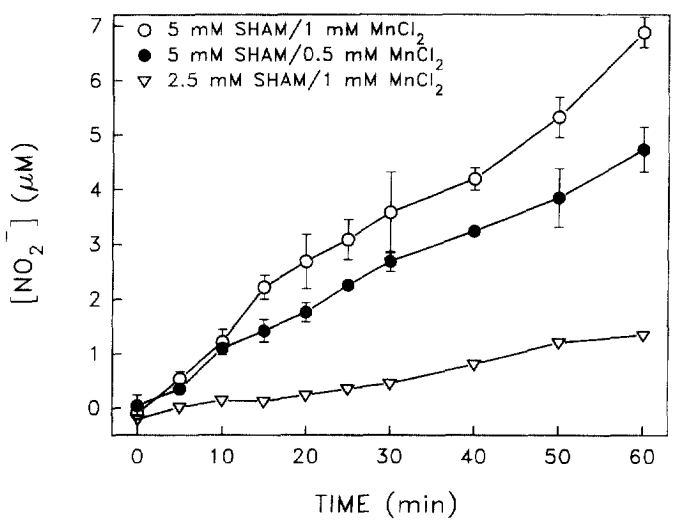

Fig. 4. Time course of NO_2^- production by the SHAM/$MnCl_2$ system. The reaction was started by the addition of $MnCl_2$ at time = 0. Horseradish peroxidase was absent. Bars represent SE ($n = 3$).

for the oxidation of SHAM by Mn(II). Similar to the SHAM/peroxidase/NADH system, addition of 1 mM KNO_3 (Table 1) or NH_4Cl (not shown) did not measurably affect the yield of NO_2^-.

Further NO_2^--producing reactions could also be demonstrated. For example, oxidation of benzohydroxamic acid by Mn(II) yielded NO_2^-, and the NADH/peroxidase system was efficient in the production of NO_2^- from hydroxylamine (not shown). Taken as a whole, the results presented in this paper suggest that PM NR is not the only potential mechanism for the production of NO_2^- by PM-associated enzymes. For example, activation of peroxidase by SHAM may lead to the peroxidase-catalyzed oxidation of SHAM, resulting in NO_2^- production. Furthermore, attempts to activate peroxidase using a combination of SHAM and Mn(II) may lead to non-enzymatic NO_2^- generation. We suggest that the potential role of peroxidase in NO_3^- reduction remains to be elucidated, and that the possibility of NO_2^- generation from the peroxidase-catalyzed oxidation of N-containing compounds may lead to over-estimation of rates of NO_3^- reduction in some circumstances.

EXPERIMENTAL

All reactions were carried out in 15 mM HEPES-KOH (pH 6.5) in a water-jacketed glass reaction vessel at 20°C in the dark. Other buffer systems (MES, Tris, phosphate; all at pH 6.5) provided similar results (not shown).

Nitrite (NO_2^-) was quantified colorimetrically (540 nm–750 nm) by diazotization with sulfanilamide and reaction with N-(1-naphthyl)ethylenediaminedihydrochloride (NNED) [39]. The two components were combined into the "colour reagent", which was mixed just prior to use. The colour reagent consisted of equal parts of 1% (w/v) sulfanilamide in 1.5 N HCl and 0.2% (w/v) NNED in distilled water. NADH interferes with colour development in this assay [40]; for experiments which included NADH, excess NADH was oxidized to NAD^+ by the addition of 20 mM pyruvate and 2 U lactate dehydrogenase. Preliminary experiments indicated that the presence of pyruvate and lactate dehydrogenase had no measurable effect on the NO_2^- standard curve.

Horseradish peroxidase (HRP; lyophilized, Sigma type VI) was added to the reaction mixtures from a 500 U ml^{-1} stock in 15 mM HEPES-KOH (pH 6.5). Catalase and superoxide dismutase (both from Sigma) were also added from stocks in 15 mM HEPES-KOH (pH 6.5). NADH was added from a 250 mM stock in 15 mM MES-KOH (pH 7.5), and SHAM was added from a 0.75 M stock in 1 N KOH. Nitrate (KNO_3) and EDTA (disodium salt) were added from stocks in distilled water. Exogenous hydrogen peroxide was not added in any of the experiments.

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 photoautrophically in semi-continuous culture, in water-jacketed, glass vessels at a temperature of 20°C and a photon fluence rate of 220 μmol quanta m^{-2} s^{-1}, as described in [29]. Growth rate of the cells was approximately 1.3 d^{-1}. For measurement of algal cell wall-peroxidase catalyzed NO_2^- production, algal cells were harvested by centrifugation (19,000 × *g* for 1 min), the supernatant was discarded, and the pellet (containing the cells) was resuspended in an equal volume of assay buffer (15 mM HEPES-KOH, pH 6.5, 300 μM $MgSO_4$, 245 μM $CaCl_2$).

For experiments utilizing horseradish peroxidase, the reaction was stopped by placing samples in a boiling water bath for 1 min. For experiments utilizing algal cells, samples were first quickly centrifuged, and the supernatant was subsequently placed in a boiling water bath. In both cases, excess NADH was destroyed, as described above, prior to adding an equal volume of colour reagent. For experiments examining the oxidation of SHAM by Mn(II), the reaction was stopped by the addition of an equal volume of colour reagent. Preliminary experiments indicated that these procedures were sufficient to halt the reactions.

For each type of experiment listed above, identification of NO_2^- was confirmed by ion chromatography, using a 4000i ion chromatograph (Dionex Corp., Sunnyvale, CA, U.S.A.) equipped with a star-ion-A300 anion column (Phenomenex Corp., Torrance, CA, U.S.A.). Flow rate was 1.5 ml min^{-1}, the eluent was 1.8 mM Na_2CO_3 and 1.7 mM $NaHCO_3$, and the regenerant for the suppressor was 50 mN H_2SO_4.

Oxygen consumption was measured in the liquid phase with an oxygen electrode (Hansatech, King's Lynn, U.K.) equipped with a water-jacketed cuvette for temperature control. Experiments monitoring oxygen consumption were conducted at 20°C and in a 1.0 ml volume. Measured rates of O_2 consumption were corrected for O_2 consumption by the electrode.

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