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HYDROXYLAMINE OXIDOREDUCTASE OF *NITROSOMONAS*

PRODUCTION OF NITRIC OXIDE FROM HYDROXYLAMINE

ALAN B. HOOPER and K.R. TERRY

Department of Genetics and Cell Biology, University of Minnesota, St. Paul, MN 55108 (U.S.A.)

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Summary

As shown previously, highly purified hydroxylamine oxidoreductase (hydroxylamine:oxygen oxidoreductase, EC 1.7.3.4) from *Nitrosomonas* catalyzes the aerobic oxidation of NH_2OH to a mixture of NO_2^- and NO_3^- in the presence of phenazine methosulfate. The present work shows that N_2O and NO are also products of the oxidation of NH_2OH. A nitrite reductase, present in less purified samples of the hydroxylamine oxidoreductase is shown to catalyze the reduction of NO_2^- to a mixture of NO and N_2O with leucopyocyanine as electron donor. The possible reduction, by purified hydroxylamine oxidoreductase, of NO_2^- or NO_3^- to form NO (with NH_2OH-reduced enzyme or phenazine methosulfate as potential electron donors) was eliminated; ^{15}NO was shown not to be produced in the presence of $^{15}NO_2^-$ and $^{15}NO_3^-$. NO thus appears to be a product of NH_2OH oxidation by purified hydroxylamine oxidoreductase and is thus a possible intermediate in the production of nitrite.

Mn^{2+} in the concentration range 10^{-6}—10^{-5} M progessively and completely inhibits the formation of nitrite and nitrate by purified hydroxylamine oxidoreductase while concomitantly stimulating the rate of dehydrogenation of hydroxylamine three-fold. The presence of Mn(II) results in decreased formation of NO and increased formation of N_2O.

The present results are consistent with a mechanism of NH_2OH oxidation in which the oxidation of an intermediate compound of the oxidation state of (HNO) occurs by dehydrogenation rather than direct addition of O. The dehydrogenation step is inhibited in the presence of Mn(II).

Abbreviations: PMS, phenazine methosulfate; DCIP, 2,6-dichlorophenolindophenol.

Introduction

Nitrosomonas europaea catalyzes the oxidation of NH_3 to HNO_2 with NH_2OH as an enzyme-bound intermediate. The enzyme hydroxylamine oxidoreductase (hydroxylamine:oxygen oxidoreductase, EC 1.7.3.4) catalyzes the removals of two electrons from NH_2OH to form a compound of the oxidation state of nitroxyl, [HNO], with the concomitant reduction of enzyme-bound *c*-type hemes [1]. The oxidation of enzyme-bound nitroxyl to HNO_2 does not occur under anaerobic conditions and is thus assumed to involve O_2. The mechanism of oxidation of [HNO] is unclear. It may occur by the direct addition of an atom of O ([HNO] + 1/2 $O_2 \rightarrow HNO_2$) or by the initial removal of an electron to form NO followed by the net addition of OH to form nitrite ([HNO] $\rightarrow$ H; NO + OH $\rightarrow HNO_2$). Support for the second pathway has come from the work of Anderson [2—4]. He has shown that incubation of crude extracts of *Nitrosomonas* with substrate quantities of NH_2OH and methylene blue under very low oxygen concentrations results in the production of nitrite-, NO- and N_2O-N in the ratio of 5 : 34 : 72 [2]. Extracts of *Nitrosomonas* contain an enzyme which catalyzes the reduction of HNO_2 to a mixture of N_2O and NO with NH_2OH or leucopyocyanine as electron donor [5]. Thus, NO produced from NH_2OH aerobically by crude extracts in the presence of an artificial electron acceptor may have originated from the reduction of HNO_2 (NH_2OH + oxidized dye + 1/2 $O_2 \rightarrow HNO_2$ + reduced dye $\rightarrow$ NO + oxidized dye). Ritchie and Nicholas [6], in fact, demonstrated that a partially purified enzyme preparation in the presence of phenazine methosulfate (PMS) catalyzed the formation of an equimolar mixture of ^{15}NO and ^{14}NO from $^{15}NH_2OH$ and $H^{14}NO_2$ demonstrating that at least half of the NO had originated from HNO_2. Thus, it is not clear whether NO is a direct product of the oxidation of NH_2OH by hydroxylamine oxidoreductase.

Utilizing a sample of purified hydroxylamine oxidoreductase containing low nitrite reductase activity, we show that NO is a product of the oxidation of NH_2OH and is not produced by reduction of HNO_2. In addition, Mn(II) is shown to inhibit the oxidation of HNO to NO. This provides an important means of experimentally separating the steps of oxidation of hydroxylamine: $NH_2OH \rightarrow$ [HNO] (inactivated by H_2O_2, [7]); [HNO] $\rightarrow$ NO (blocked by Mn); NO $\rightarrow HNO_2$ (blocked anaerobically).

Methods

N. europaea (Schmidt strain) was grown and extracts were prepared from 8 to 30-g batches of cells by freezing and thawing as previously described [8]. To prepare partially purified hydroxylamine oxidoreductase, solid ammonium sulfate was added to the crude 20 000 × *g* supernatant fraction and the protein precipitating between 70% and 80% saturation was resuspended in 0.05 M phosphate solution, pH 7.5, and retained. The ammonium sulfate precipitation was repeated twice. Partially purified nitrite reductase was prepared as described previously [5], except that an additional step employing chromatography on DEAE-cellulose resulted in a preparation enriched in nitrite and containing low hydroxylamine oxidoreductase activity.

Nitrite was estimated by diazotization [9]. Nitrate was assayed as nitrite [10], following reduction by one of three methods: reduction on a copper-cadmium column, reduction by zinc, or reduction by nitrate reductase. Hydroxylamine was assayed by reaction with 8-hydroxyquinoline [11].

Pyocyanine-nitrite reductase activity was assayed as described previously [5]. A rubber-stoppered Thunberg cuvette containing 2 ml of citrate/phosphate solution, pH 6.0, $5 \cdot 10^{-4}$ M pycocyanine was prepared under an atmosphere of nitrogen. The reaction was started by the successive injection of 8 mM NADH, 10 mM nitrite and enzyme. After 30 min the reaction was stopped by dilution with a known volume oxygen-free water which was used to displace the gas phase into an evacuated Thunberg tube. Following the removal of nitrogen dioxide by immersion of the vessel in a solid CO_2/butanol bath, the gas was sampled by syringe into a Fisher Hamilton model 25 gas molecular seive 13X with partitioner. The amount of each gas was estimated chromatographically utilizing columns of Porapak 'S' and samples of pure NO or N_2O as standards. The nitrate content was determined in the diluted reaction mixture.

Routine measurement of hydroxylamine oxidoreductase activity was carried out at 25°C in an open vessel. The standard reaction mixture contained 0.2 μg (PMS reaction) or 5 μg (DCIP reaction) protein/ml of hydroxylamine oxidoreductase, 100 μM hydroxylamine and 5 μM PMS or 100 μM DCIP in 0.05 M tris(hydroxymethyl)aminomethane sulfate, pH 8.0, total volume 3—14 ml. When used, $MnSO_4$ was present at $3 \cdot 10^{-5}$ M (PMS) or $5 \cdot 10^{-4}$ M (DCIP).

For analysis of gaseous products, the hydroxylamine oxidoreductase reaction was carried out in Thunberg tubes under an atmosphere of He. The gas and solution phases were of equal volumes (4 or 8 ml) and the final total amount of oxygen (350 μmol/ml gas) was approximately twice the total amount of NH_2OH substrate. The NH_2OH in the standard reaction mixture was 95% ^{15}N enriched (Stohler Isotopes, Rutherford, NH). When run to completion, a 2 ml aliquot of the gaseous phase was withdrawn by gas-tight syringe and injected into an LKB-9000 gas chromatographic mass spectrometer (Porapak Q, 70°C). To quantify gases, the peak heights of masses 31 (^{15}NO), 30 ($^{15}N_2$, ^{14}NO), 44 ($^{14}N_2O$), 45 ($^{15/14}N_2O$) and 46 ($^{15}N_2O$) were determined. To estimate $^{15}N_2$, the mass 30 peak was corrected for the contribution of ^{14}NO and the natural abundance of $^{15}N_2$. An $^{15}NO_2$ peak was not observed. If present in the gas mixture NO_2 may have been converted to NO in the presence of H_2O on the Porapak Q column ($2\ NO_2 + H_2O \rightarrow HNO_2 + HNO_3$; $3\ HNO_2 \rightarrow HNO_3 + 2\ NO + H_2O$). To estimate the relative amounts of N_2, NO and N_2O on a molar basis, all N not accounted for by HNO_2 and HNO_3 was assumed to be N_2, N_2O or NO. A correction was made for the differential solubility of each gas in solution. All values were corrected for small changes occurring in the absence of enzyme.

Results

Production of pyocyanine-nitrite reductase activity

The addition of 20 μmol of nitrite to an anaerobic reaction mixture containing purified nitrite reductase and leucopyocyanine resulted in the progressive reoxidation of pyocyanine as shown earlier [5]. When the reaction was stopped during the linear part of the reaction when approximately 7.5

TABLE I

PRODUCTION OF N-OXIDES DURING THE OXIDATION OF $^{15}NH_2OH$ CATALYZED BY *NITROSOMONAS* HYDROXYLAMINE OXIDOREDUCTASE IN THE PRESENCE OF PMS OR DCIP

Values (nmol/4 ml reaction mixture) were determined as described in Methods. When present, the concentration of $MnCl_2$ was 0.1 mM.

Experiment	Utilized NH_2OH	Produced			
		HNO_2	HNO_3	$^{15}N_2O$	^{15}NO
1. PMS	720	370	260	36	11
2. PMS + $MnCl_2$	760	60	37	330	3.2
3. DCIP	180	93	66	8.9	7.7
4. DCIP + $MnCl_2$	190	15	9.3	82	0.78

μmol of nitrite had been utilized, 86% of the nitrite-N was recovered as N_2O and NO in a ratio of 1.6 : 1 (values the average of five experiments). With NH_2OH as electron donor, NH_2OH and HNO_2 are utilized in equal amounts and the products are N_2O and NO in the N ratio of 29 : 1 [5]. Thus, with leucopyocyanine rather than NH_2OH as electron donor, NO is a much more prevalent product of the reaction. NADH, NADPH or hydroquinone did not serve as electron donors for enzymic reduction of nitrite. At a concentration of 6 mM, nitrate, sulfate, thiosulfate or formate were not enzymically reduced by leucopyocyanine.

Product of hydroxylamine oxidation as catalyzed by hydroxylamine oxidoreductase

Oxidation of NH_2OH by hydroxylamine oxidoreductase results in the production of a mixture of HNO_2/HNO_3 [10] and a nitrogenous gas which is not usually measured. To identify the nitrogenous gas formed, the oxidation of hydroxylamine was carried out in the presence of purified hydroxylamine oxidoreductase, catalytic quantities of PMS or substrate amounts of DCIP and a slight excess of oxygen. The products were determined by a combination of gas chromatography and mass spectrometry (Table I). The use of $^{15}NH_2OH$ allowed the identification of $^{15}N_2$ as a possible product in the presence of a background of $^{14}N_2$. The rate of oxidation of NH_2OH with DCIP was 1/20 of the rate with PMS. With either PMS or DCIP as electron acceptors HNO_2 and HNO_3 accounted for 87% of the NH_2OH-N utilized (Expts. 1 and 3 of Table I). The remaining N included N_2O and NO in a ratio of 6.5 : 1. The small amount of $^{15}N_2$ produced was not significantly greater than the amount produced in a reaction mixture lacking enzyme.

Although nitrite reductase activity was not detected in the preparations of hydroxylamine oxidoreductase utilized for these experiments, it was necessary to clearly rule out the possibility that NO was produced by reduction of HNO_2 or HNO_3 by reduced PMS or DCIP, a reaction analogous to the reduction of nitrite by pyocyanine. With this in mind, $^{15}NH_2OH$ (200 μM) was oxidized completely to $H^{15}NO_2$ and $H^{15}NO_3$ by the enzyme in the presence of PMS or DCIP in the standard hydroxylamine oxidoreductase reaction mixture open to the atmosphere so that the products $^{15}N_2O$ and ^{15}NO were lost. Fresh enzyme

TABLE II

PRODUCTION OF N_2O AND NO FROM $^{14}NH_2OH$ IN THE PRESENCE OF $H^{15}NO_2$ AND $H^{15}NO_3$

In a standard hydroxylamine oxidoreductase reaction mixture containing 5 μM PMS or 200 μM DCIP, 200 μM of $^{15}NH_2OH$ were oxidized to completion. The solution was open to the atmosphere and gas exchange facilitated by continuous rotation of the nearly horizontal test tubes. The solution was transferred to a Thunberg tube, fresh hydroxylamine oxidoreductase, 200 μM $^{14}NH_2OH$ and 1 μM fresh PMS added. The second reaction was then carried out and products analyzed as in Table I. It was not necessary to add fresh DCIP as the enzyme has DCIP oxidase activity which regenerated the dye. When present, Mn^{2+} was added to the second reaction only. Values are expressed as nmol of product/8 ml of reaction mixture.

Experiment	Utilized NH_2OH	Produced						
		HNO_2	HNO_3	^{14}NO	^{15}NO	$^{14}N_2O$	$^{15}N_2O$	$^{15}/^{14}N_2O$
1. PMS	1000	320	340	160	0	100	0	0
2. PMS + Mn	970	0	0	8.8	0	460	0	0
3. DCIP	1354	330	200	280	12	270	2	1.4
4. DCIP + Mn	1180	41	24	280	0	360	1	22

and $^{14}NH_2OH$ (200 μM) were then added to the reaction carried out under the conditions described in Table I. As shown in Table II, Expts. 1 and 3, less than 1% of the resulting NO could be accounted for as ^{15}NO. We conclude that NO is produced by direct oxidation of NH_2OH rather than reduction of HNO_2 or HNO_3.

Effect of Mn(II) on hydroxylamine oxidoreductase

Fig. 1 shows that addition of 10^{-5} M $MnSO_4$ to the hydroxylamine oxidoreductase reaction resulted in the complete inhibition of nitrite synthesis and a concomitant three-fold stimulation of the rate of oxidation of NH_2OH. The concentration of NH_2OH oxidized or HNO_2 produced (in the control reaction) was ten-fold greater than the concentration of Mn(II) indicating that Mn(II)

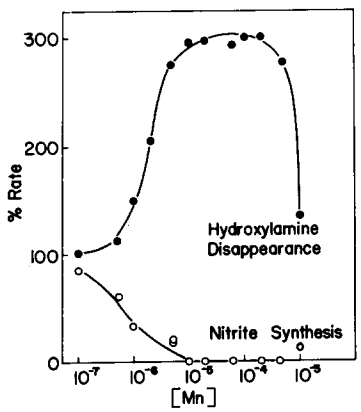

Fig. 1. Effect of Mn^{2+} on hydroxylamine oxidoreductase. The reaction was carried out in a reaction mixture containing 0.2 μg enzyme/ml, 100 μM hydroxylamine, 5 μM PMS and $MnSO_4$ at the concentrations noted.

functioned non-stoichiometrically. At concentrations of Mn(II) of 1 mM or greater the stimulation of NH_2OH dehydrogenase as well as inhibition of nitrite synthesis was diminished. The effect of Mn(II) was completely reversed by dialysis of the enzyme.

Inhibition by 10^{-4} M Mn(II) of production of nitrite from NH_2OH was observed in the presence of a catalytic quantity of PMS ($+O_2$) or a substrate concentration (10^{-4} M) of DCIP (Table I), cytochrome *c* or ferricyanide as electron acceptors. The production of nitrate from NH_2OH by hydroxylamine oxidoreductase [8] was inhibited by Mn(II) in a manner closely parallel to inhibition of nitrite synthesis (Table I).

Hydroxylamine oxidoreductase also utilizes NH_2NH_2 as electron donor (see references). Mn(II) also stimulated the rate of enzymic dehydrogenation of 100 μM NH_2NH_2 as determined in the presence of 5 μM PMS by the rate of utilization of O_2. With NH_2OH or NH_2NH_2 as electron donors, the rate of reduction of 100 μM DCIP, alone or in the presence of 5 μM PMS, was 10% or 50% of the rate of NH_2OH oxidation in the standard reaction mixture and stimulation by Mn(II) was 50% or less. The presence of 10^{-5} M Mn did not change the oxidized, dithionite-reduced or NH_2OH-reduced absorption spectrum of the enzyme-bound *c*-type hemes or heme *P*-460, a moiety which has been implicated in the hydroxylamine dehydrogenase reaction [7].

The Mn(II) effect was affected somewhat by pH and anions. The stimulation of NH_2OH utilization by 1 μM Mn(II) was approximately 40% greater at pH 6 and 9 as compared with the stimulation at pH values of 7 and 8. In contrast, the percent inhibition of HNO_2 synthesis by 1 μM Mn(II) was the same at pH values of 6, 7, 8 and 9. At a particular pH the Mn(II) effects were essentially the same in 0.05 M phosphate, citrate or glycine solution. In 0.05 M pyrophosphate solution the stimulatory effect of Mn(II) on hydroxylamine dehydrogenase was enhanced by 50% while the inhibitory effect on nitrite synthesis was diminished.

The effect was not completely specific for Mn. At a concentration of 10^{-4} M, $Ce(NH_4)_2(NO_3)_6$ stimulated the rate of oxidation of NH_2OH 2.6-fold and inhibited production of HNO_2 by 40%. In the presence of 10^{-4} M $Co(NO_3)_2$ the rate of non-enzymic oxidation of NH_2OH was too great to determine a stimulatory effect though the rate of nitrite synthesis was inhibited by 70%. In contrast, 10^{-4} M $CuCl_2$, CuCl, $MgCl_2$, $CaCl_2$, Zn acetate or $FeCl_2$ were without effect on either part of the reaction. $MnSO_4$ or $MnCl_2$ had the same effect.

Product of the oxidation of NH_2OH in the presence of manganese

In the presence of Mn(II) and with PMS or DCIP as electron acceptors, only 13% of the oxidized NH_2OH-N was recovered as HNO_2 and HNO_3 (Table I, Expts. 2 and 4). In the presence of Mn(II) the enzyme was apparently unable to convert a compound of the oxidation state of N_2O, presumably [HNO], to NO as indicated by the fact that production of NO was diminished whereas the production of N_2O was greatly stimulated. The data of Table II (Expts. 2 and 4) show that, in the presence of Mn(II), N_2O or NO originated from the oxidation of NH_2OH rather than the reduction of HNO_2 or HNO_3 by PMS, DCIP or Mn which may have been reduced during the enzyme reaction; less than 1% of

the NO or N_2O produced from $^{14}NH_2OH$ in the presence of $H^{15}NO_2$ and $H^{15}NH_3$ was accounted for as ^{15}No, $^{15}N_2O$ or $^{14/15}N_2O$.

Discussion

NO as a product of oxidation of NH_2OH

The aerobic formation of NO from NH_2OH catalyzed by extracts of *Nitrosomonas* in the presence of a suitable electron acceptor [2—4,6] suggests that NO is a possible intermediate in the oxidation of NH_2OH to HNO_2. The presence, in extracts of *Nitrosomonas*, of an enzyme which catalyzed the reduction of nitrite with NH_2OH or reduced dye as electron donor [5] raised the possibility that the NO production previously demonstrated occurred by the reduction of enzymically produced nitrite with NH_2OH or reduced dye as electron donor ($NH_2OH \rightarrow HNO_2 \rightarrow NO$). The present work demonstrates that, in fact, with leucopyocyanine as electron donor, NO is a major product of the enzymic reduction of nitrite as catalyzed by nitrite reductase from *Nitrosomonas*. The ratio of N_2O-N/NO-N [1—6] produced here by the reduction of HNO_2 by leucopyocyanine is in the same range as the corresponding ratio (0.94) resulting from the oxidation of NH_2OH under low O_2 and in the presence of methylene blue [2]. The demonstration that HNO_3 is a product of NH_2OH oxidation [10] also raised the possibility that NO is a product of nitrate reduction.

In the present work we have employed a purified sample of hydroxylamine oxidoreductase containing very low nitrite reductase activity and shown that ^{14}NO but not ^{15}NO is produced during the hydroxylamine oxidoreductase-catalyzed oxidation of $^{14}NH_2OH$ in the presence of [^{15}N]nitrate and [^{15}N]-nitrite. Although we cannot exclude the possible reduction of enzyme-bound [^{14}N]nitrite or [^{14}N]nitrate which had been produced from $^{14}NH_2OH$ and did not equilibrate with [^{15}N]nitrite or [^{15}N]nitrate in solution, we conclude that NO is probably a product of the oxidation of NH_2OH by hydroxylamine oxidoreductase.

As pointed out by Ritchie and Nicholas [6], NO cannot yet be considered an obligatory intermediate (presumed to be enzyme bound) until the demonstration of enzyme-catalyzed conversion of NO to nitrite. Because of the reactivity of NO, the significance of the anaerobic uptake of NO demonstrated by Anderson [2] is difficult to assess. To illustrate, in preliminary experiments from this laboratory, the complete anaerobic enzymic oxidation of NH_2OH with mammalian cytochrome *c* as electron acceptor resulted in the production of stoichiometric amounts of a derivative of cytochrome *c* (possibly ferrous-NO) which, in the presence of oxygen, non-enzymically formed HNO_2.

The demonstration of NO as a product indicates that oxidation of NH_2OH can occur by the successive removal of three electrons. If NO is an intermediate in the oxidation of NH_2OH, then the subsequent production of nitrite must involve reaction with a reduced form of oxygen. The requirement for O_2 for nitrite production suggests that nitrite is not produced by the reaction of OH^- with nitrosyl cation ($NO \rightarrow NO^+ + H^\cdot$; $NO^+ + OH^- \rightarrow HONO$). Rather, the data is consistent with an internal mixed-function hydroxylation of NO: $(HNO) \rightarrow (H^\cdot) + NO$; $(H^\cdot) + O_2 \rightarrow O_2H^*$; $O_2H^* + NO \rightarrow HONO + (O^\cdot)$. All intermediates are assumed to be enzyme bound. The reduced and reactive form of oxygen

(possibly superoxide, hydroxyl radical or peroxyl radical denoted here as O_2H^*) may be the same as the reactive form of oxygen which is generated during the oxidation of NH_2OH and oxidized either an N-oxide to nitrate or diethyldithiocarbamate to the disulfide [10]. It has been proposed that in vivo the same reactive oxygen acts to oxygenate both NH_3 and NO [1].

Effect of manganese on hydroxylamine oxidoreductase

The present work shows that the presence of Mn(II) results in inhibition of the rate of production of NO and HNO_2 from NH_2OH catalyzed by hydroxylamine oxidoreductase. The simultaneous alteration of NO and nitrite production is consistent with the role for NO as intermediate in nitrite production. Mn(II) apparently blocks the putative dehydrogenation of HNO resulting in the accumulation of N_2O. The use of Mn now allows studies of the initial steps of NH_2OH dehydrogenation in the absence of the subsequent oxygen-addition steps and has the additional advantage of preventing the production of small amounts of free NO. Because of its great reactivity with hemes, NO may cause artifacts in spectral or kinetic studies with hydroxylamine oxidoreductase.

It is simplest to assume that Mn(II) exerted a simultaneous effect on hydroxylamine oxidation and inhibition of nitrite synthesis by interaction at a single site rather than two distinct sites. This is supported by the closely parallel increase in rate of dehydrogenase and decrease in rate of nitrite synthesis as the Mn(II) concentration was increased. The same portion of the enzyme is assumed to carry out the dehydrogenation of either NH_2OH or NH_2NH_2 [7] although NH_2NH_2 or its dehydrogenation product does not react with the O-addition part of nitrite synthetase. The fact that NH_2NH_2 dehydrogenase was stimulated by Mn(II) indicates that the effect on dehydrogenase activity was direct and not a secondary result of the altered metabolism of N-oxides originating from NH_2OH.

The effects of Mn observed here are not taken as evidence for a role for Mn in the action of the enzyme because: (a) the effect is not completely specific to Mn but is obtained with Co^{2+} and Ce^{2+}, and (b) hydroxylamine oxidoreductase contains multiple hemes but does not contain Mn as determined by atomic absorption analysis [12] or the EPR spectrum [13].

By analogy with other reports in the literature, Mn(II) may act by (a) changing the structure or state of aggregation of the enzyme or (b) by undergoing oxidation and subsequent reduction (by electrons originating in the dehydrogenation of an N-compound). The former possibility is supported by preliminary observations that incubation with Mn(II) caused a decrease in the buoyant density of hydroxylamine oxidoreductase. Precedent for a redox role for Mn is found with the effect of Mn on peroxidase [14], where the oxidase mode of substrate oxidation is stimulated or with illuminated chloroplasts [15] where Mn pyrophosphate inhibits dye reduction by reducing the photogenerated electron donor, O_2^-, to H_2O_2. We note that the low dissociation constant for phosphate derivatives of Mn suggest that the Mn effect on hydroxylamine oxidoreductase may not be mediated by free Mn.

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