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SIDEROPHORE REDUCTION CATALYZED BY HIGHER PLANT NADH:NITRATE REDUCTASE

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Squash cotyledon NADH:nitrate reductase catalyzes the reduction of the siderophore ferrioxamine B. The enzyme also reduced ferric ion in a buffer system containing the chelators oxalate and maleate. Ferrioxamine B reduction was maximal at pH 4; ferric ion reduction was maximal at pH 8. The present study indicates that iron assimilation by higher plants may occur with microbial siderophores serving as ferric ion sources and nitrate reductase functioning as the siderophore reductase.

Siderophores are microbial chelators which avidly bind ferric ion (Fe^{3+}) with formation constants that range from 10^{29}-10^{52} (1,2). Aerobic microbes employ siderophores to acquire the iron necessary for growth in ecosystems where Fe^{3+} concentrations are so low that it is otherwise unattainable (1,2). Siderophores are thus of importance in agricultural and medical milieus as both are normally deficient with respect to soluble Fe^{3+}.

Studies with plant growth promoting rhizobacteria (PGPR) have shown that these microbes actively compete for Fe^{3+} in the environment (3-5). A crucial question raised by these studies is the mechanism by which plants acquire sufficient iron for growth. Studies with the PGPR show that while plant pathogens are denied iron, the plants themselves grow well and are not chlorotic (4-6). Additionally, evidence indicates that iron assimilation via siderophore mediated uptake occurs in oats and sorghum (7).

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Higher plant NADH:nitrate reductase (EC 1.6.6.1) is of large molecular size (mol. wt. 230,000) contains heme-iron, FAD, and molybdenum (8). In addition to converting nitrate to nitrite using reduced pyridine nucleotides as electron donors, a dehydrogenase activity of the enzyme is known, and thus the enzyme catalyzes the reduction of ferricyanide, mammalian cytochrome c, and ferric citrate (9,10). These capacities of nitrate reductase and its presence in both the leaves and roots of higher plants prompted us to examine whether nitrate reductase could function as a siderophore reductase.

Materials and Methods

Enzyme source: Nitrate reductase was purified from squash cotyledons (Cucurbita maxima L. cv. Buttercup) by blue-sepharose affinity chromatography as described (11). Such preparations are enriched in excess of 200-fold over the crude extract, yielding nitrate reductase which is about 10% pure.

Enzyme assays: NADH:nitrate reductase was assayed as previously described (12). NADH:ferrioxamine B reductase and NADH:ferric ion reductase activites were assayed by a modification of the method of Dailey and Lascelles (12). The one ml assay mixture contained 25 mM buffer at the appropriate pH, 1 mM $FeCl_3$ or 0.375 mM ferrioxamine B, 0.1 mM NADH, and 0.4 mM ferrozine. The formation of Fe^{2+}-ferrozine complex was monitored at 562 nm (E_{mM} = 28). Units of activity are defined as μmoles product formed/min.

Gel electrophoresis: Polyacrylamide gel electrophoresis was performed using 5% acrylamide(13). Gels were stained for nitrate reductase activity and $FeCl_3$ and ferrioxamine B reductase activities as previously described substituting the appropriate electron acceptor (10).

Protein determinations: Protein was determined after Lowry et al. (14) using bovine serum albumin as the standard.

Materials: All chemicals were reagent grade or better. Squash seeds were obtained from W. Atlee Burpee Co. Immunoglobulins raised in rabbits, monospecific against nitrate reductase, were a kind gift from Dr. Wilbur H. Campbell, SUNY College of Environmental Science and Forestry, Syracuse, N.Y.. Deferrioxamine B (Desferal) was a gift from the CIBA Pharmaceutical Company, Division of CIBA-GEIGY Corporation, Summit, N.J. In order to assure an excess of deferrioxamine B, stock solutions of deferrioxamine B and $FeCl_3$ $6H_2O$ in 10 mM HCl were made such that the final concentration of ferrioxamine B was 3.75 mM and that of deferrioxamine B was 0.25 mM at a pH of 3.5.

Results

Squash cotyledon nitrate reductase preparations readily reduced ferrioxamine B and Fe^{3+} (Figure 1). Given the extremely

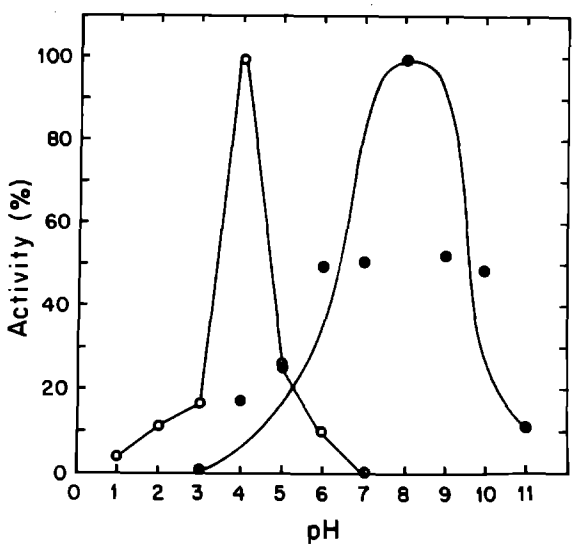

Figure 1. Relationship of pH and reduction of ferrioxamine B and Fe^{3+} by squash cotyledon nitrate reductase preparations. One hundred percent activity equals 0.12 units/mg protein and 1.3 units/mg protein for ferrioxamine B and $FeCl_3$ reductase activities, respectively.
Fe^{3+} ● (buffer system - oxalate-maleate-glycylglycine-carbonate)
ferrioxamine B ○ (buffer system - oxalate-maleate). The former buffer system interfered with the ferrioxamine B reductase assay, as did buffers prepared with Tris.

low solubility of Fe^{3+} at neutral and alkaline pH (1), it is likely that the Fe^{3+} activity noted is due to the chelating effect of the buffer employed (oxalate-maleate-glycylglycine-carbonate). The siderophore deferioxamine B, however, is an avid Fe^{3+} chelator, [log K_f = 30.5; (15)] and the activity noted thus represents removal of Fe^{3+} via reduction to Fe^{2+} by the nitrate reductase preparation. The optimal pH of ferrioxamine B and Fe^{3+} reduction differed (Figure 1) as the former activity was maximal at pH 4 while the latter was greatest at pH 8.

Squash cotyledon nitrate reductase preparations were compared with respect to their activities in the reductions of nitrate, ferrioxamine B and Fe^{3+} (Table 1). The greatest rate of reduction was with nitrate followed by Fe^{3+} and finally ferrioxamine B. Changes in nitrate reductase preparation concentrations up to

Table I. Comparative activities of nitrate, ferrioxamine B, and Fe^{3+} reduction by squash cotyledon nitrate reductase preparations [a]

Electron Acceptor	Product Formed μmoles/min mg Protein
Nitrate	1.5
Ferrioxamine B	0.12
$FeCl_3$	1.0

[a]All assays were performed as described. Ferrioxamine B was assayed at pH 4, Fe^{3+} assayed at pH 8.0, while nitrate reduction was assayed at pH 7.5.

four-fold resulted in corresponding changes in the rates observed for all three activities (data not shown).

As the nitrate reductase-enriched preparation functioned as a siderophore reductase, experiments to identify nitrate reductase as the siderophore reductase were performed. A band which stained positively for nitrate reductase activity using both the methyl viologen negative stain and the nitrite appearance stain comigrated with the band of Fe^{3+}-reductase activity in polyacrylamide gels (data not shown). However, similar results were not observed when ferrioximine B was used as electron acceptor, possibly due to Tris in the gel buffer.

To verify that nitrate reductase was the catalyst responsible for ferrioxamine B reductase, the inhibition of these enzyme activities by antibodies against nitrate reductase raised in rabbits was observed (Table II). Both the NADH:Fe^{3+} and ferrioxamine B reductase activity were inhibited by monospecific antibodies against nitrate reductase. However, an approximately 10 fold greater concentration of antibodies was required to obtain comparable inhibition. Similar results were reported for ferric citrate reductase activities (10). When these antibodies were incubated with the preparation, malate dehydrogenase, a known contaminant, displayed no inhibition of activity.

Table II. Inhibition of enzymatic activities by antibodies against squash cotyledon nitrate reductase

Activity	Antibody Concentration mg/ml	% Inhibition of Activity
NADH:nitrate reductase	0.5	83%
NADH:ferrioxamine B reductase	0.5	42%
NADH:ferrioxamine B reductase	5.0	88%
NADH:Fe^{3+} reductase	5.0	69%
NADH:malate dehydrogenase	0.5	0%
NADH:malate dehydrogenase	5.0	0%

Uninhibited nitrate reductase activity equals 1.5 moles/min mg protein.

Antibodies against nitrate reductase were incubated with squash cotyledon nitrate reductase preparations for 10 min at 22°C prior to being assayed as described.

Steady-state kinetics at fixed NADH concentrations were performed (Figure 2). The apparent Michaelis constant for ferrioximine B was 0.17 mM, which compares favorably to the K_m for nitrate of approximately 0.05 mM, but is much greater than the re-

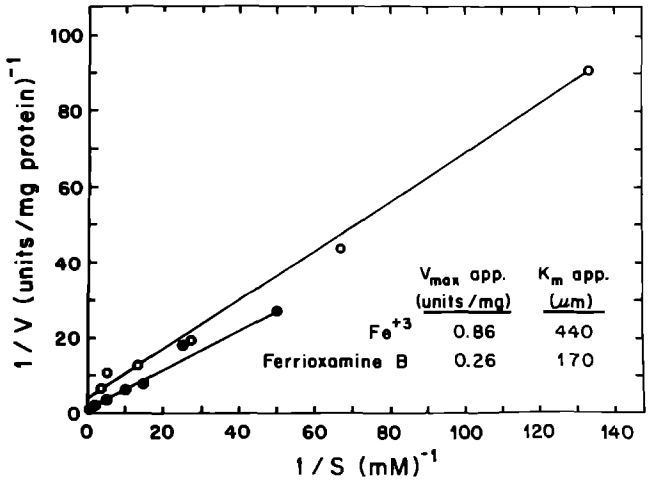

Figure 2. Determination of apparent Michaelis constants for the reduction of ferrioxamine B and Fe^{3+} by squash cotyledon nitrate reductase preparations.
Fe^{3+} ● (pH 8)
ferrioxamine B ○ (pH 4)

ported K_m for ferric-citrate reduction (0.02 mM)(10). Fe^{3+} reductase had a much higher apparent K_m (0.44 mM).

Discussion

This study demonstrates that squash cotyledon nitrate reductase is a catalyst capable of siderophore (ferrioxamine B) reduction. Flavoproteins can catalyze the reduction of inorganic iron (16) and the presence of oxalate and maleate, known metal iron chelators, in the buffer employed may be responsible for the Fe^{3+} activity observed. We assess no physiological role to the reduction of Fe^{3+} by squash cotyledon nitrate reductase.

As the majority of nitrate assimilation occurs in plant leaves, the function of nitrate reductase in plant roots has been regarded as somewhat enigmatic (17). A role for the enzyme in roots is suggested as it could assist in the assimilation of iron in an environment where active competition occurs (3,7). As plants acquire iron by siderophore-mediated uptake (7), nitrate reductase may assist in the removal of Fe^{3+} ion from siderophores via a reductive mechanism. Reductive assimilation of Fe^{3+} ion from siderophores has been shown in microorganisms (18,19,20).

The PGPR limit the availability of pathogens to acquire iron presumably by employing a more avid iron-sequestering (siderophore) system (4,5). The plant symbiont is not, however, denied iron and does not become chlorotic (4,5,6,7). Together with studies that note siderophore-mediated iron acquisition by plants (7), the current study suggests that at least one siderophore (ferrioxamine B) may be a source of iron for plant assimilation. The mechanism of siderophore-iron transport by plants and the breadth of siderophore reduction capabilities of higher plant NADH:nitrate reductase are questions currently under investigation in our laboratories.

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