

Fe³⁺-Chelate Reductase Activity of Plasma Membranes Isolated from Tomato (*Lycopersicon esculentum* Mill.) Roots¹

Comparison of Enzymes from Fe-Deficient and Fe-Sufficient Roots

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ABSTRACT

Reduction of Fe³⁺ to Fe²⁺ is a prerequisite for Fe uptake by tomato roots. Ferric chelate reductase activity in plasma membranes (PM) isolated from roots of both iron-sufficient (+Fe) and iron-deficient (–Fe) tomatoes (*Lycopersicon esculentum* Mill.) was measured as NADH-dependent ferric citrate reductase and exhibited simple Michaelis-Menten kinetics for the substrates, NADH and Fe³⁺(citrate³⁻)₂. NADH and Fe³⁺(citrate³⁻)₂ K_m values for reductase in PM from +Fe and –Fe tomato roots were similar, whereas V_{max} values were two- to threefold higher for reductase from –Fe tomatoes. The pH optimum for Fe-chelate reductase was 6.5. Fe-chelate reductases from –Fe and +Fe tomato roots were equally sensitive to several triazine dyes. Reductase was solubilized with n-octyl β-D-glucopyranoside and electrophoresed in nondenaturing isoelectric focusing gels. Three bands, with isoelectric points of 5.5 to 6.2, were resolved by enzyme activity staining of electrofocused PM proteins isolated from +Fe and –Fe tomato roots. Activity staining was particularly enhanced in the isoelectric point 5.5 and 6.2 bands solubilized from –Fe PM. We conclude that PM from roots of +Fe and –Fe plants contain Fe-chelate reductases with similar characteristics. The response to iron deficiency stress likely involves increased expression of constitutive Fe-chelate reductase isoforms in expanding epidermal root PM.

The mechanism of Fe uptake by roots of dicots and non-graminaceous monocots involves an obligatory reduction of Fe³⁺ to Fe²⁺ before uptake into root cells (14). Evidence has accumulated that this reduction is the result of enzymatic

activity on the PM² of root cells (7, 22). An “Fe-efficient” plant such as tomato (*Lycopersicon esculentum* Mill. cv Rutgers) proliferates lateral roots covered with root hairs when grown under conditions of Fe deficiency. Cytochemical evidence demonstrated that these newly formed roots are particularly enriched in Fe-chelate reductase activity (2, 8). Roots of –Fe tomatoes reduced Fe³⁺-chelates at a 4 to 7 times higher rate than did roots of +Fe plants (9, 10). Recently, NADH-dependent reduction of Fe³⁺-chelates by PM isolated from the roots of tomato (9, 10) and *Plantago lanceolata* (24) was reported. Consistent with the whole root studies, PM isolated from –Fe tomato plants demonstrated higher Fe-chelate reductase activities than PM isolated from +Fe plants (9, 10).

It has been demonstrated that plant PM contain pyridine nucleotide-dependent redox activity (see refs. 12 and 15 and references therein). Ferricyanide has been commonly used as an artificial electron acceptor to measure PM redox activity. The identity of the natural acceptor(s) has been the subject of much debate (12, 15). Bienfait (4, 5) proposed that this standard reductase (measured as NAD(P)H-ferricyanide reductase) is constitutive, does not respond to the iron status of the plant, and is unlikely to reduce ferric chelates. Bienfait (4, 5) further proposed that a high capacity reductase (“turbo” reductase), capable of both ferric chelate and ferricyanide reduction, is induced under conditions of Fe deficiency. In support of this hypothesis are reports of NADH-ferricyanide reductases in many plants including those (grasses) that do not use Fe-chelate reduction as an Fe-capture system (16). Also, –Fe soybean roots reduced Fe³⁺-chelates at a 10-fold higher rate than +Fe roots, whereas ferricyanide reduction increased by a factor of only 2 (5). Bienfait (5) acknowledged

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² Abbreviations: PM, plasma membrane(s); –Fe, iron deficient; +Fe, iron sufficient; BPDS, 4,7-di(4-phenylsulfonate)-1,10 phenanthroline; octylglucoside, n-octyl-β-D-glucopyranoside; PDTs, 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (or ferrozine); IEF, isoelectric focusing; EDDHA, ethylenediamine-di(o-hydroxyphenylacetate); HEEDTA, n-hydroxyethylethylenediamine triacetate; pI, isoelectric point.

that there is no evidence to rule out the transformation of a "standard" to a "turbo" reductase under conditions of Fe deficiency stress.

Our approach to addressing the question of whether the response of roots to Fe deficiency stress involves the induction of novel reductase synthesis was to characterize and compare NADH-dependent Fe-chelate reductase activity in PM isolated from $-Fe$ and $+Fe$ tomato roots. We wished to test the hypothesis that a reductase induced by Fe deficiency stress would likely have different biochemical properties than a constitutive reductase. We also have compared Fe-chelate reductase activities with NADH-dependent ferricyanide reductase activity, because tomato root PM exhibit ferricyanide reductase activity that may or may not be involved in Fe-chelate reduction (10).

MATERIALS AND METHODS

Membrane Isolation

Tomato (*Lycopersicon esculentum* Mill.) plants were grown in Fe-sufficient nutrient solutions for 4 weeks ($+Fe$ plants) or for 3 weeks followed by transfer to $-Fe$ nutrient solution for 1 week ($-Fe$ plants) as described in Buckhout *et al.* (10). $-Fe$ plants exhibited characteristic interveinal chlorosis and development of root hair-covered lateral roots. PM vesicles were isolated from tomato roots according to the method of Buckhout *et al.* (10) using aqueous two-phase partitioning of a 13,000 to 50,000g microsomal membrane pellet. The homogenization medium contained 0.5 M sucrose, 15 mM Hepes-KOH (pH 7.5), 1 mM EDTA, 1 mM EGTA, 0.6% PVP, 1 mM PMSF, and 5 mM DTT. To increase the yield of membranes, some of the preparations included a 10-min mortar and pestle homogenization on the brei that remained after filtration of the Polytron³-homogenized roots. This step did not change the specific activity of Fe-chelate reductase. The composition of the phases was as reported previously (10), except that the phases were increased to 40 from 36 g, two rather than three phases were used, and 1.25 mM DTT was added to the first phase to reduce oxidative browning. The final upper phases were diluted 10-fold with a wash buffer of 0.25 M sucrose and 15 mM Hepes-KOH (pH 7.5), and membranes were pelleted at 53,000g for 2 h. Membranes were stored at $-80^{\circ}C$ in wash buffer including the proteinase inhibitors PMSF (1 mM), leupeptin (1 $\mu g/mL$), pepstatin (1 $\mu g/mL$), and chymostatin (10 $\mu g/mL$).

Enzyme Assays

The standard assay for NADH-dependent Fe^{3+} -chelate reductase (Fe-chelate reductase) in 1.0 ml included 15 mM Mes-KOH buffer, (pH 6.0), 250 μM Fe^{3+} -citrate (20:1 citrate-KOH: $FeCl_3$, pH 6.0), 0.03% Triton X-100, 125 μM BPDS, and 10 mg PM protein, and the reaction was started with 160 μM NADH (prepared as a 16 mM stock in 15 mM Hepes-KOH, pH 7.5) (10). In some experiments, where indicated, other Fe^{3+} -chelates were substituted for Fe^{3+} -citrate or ratios

of citrate: Fe^{3+} were altered. Change in absorbance of BPDS resulting from chelation of Fe^{2+} was monitored at $30^{\circ}C$ and 535 nm ($\epsilon^{mM} = 22 \text{ cm}^{-1}$) for 1 to 3 min. Absorbance changes in the absence of NADH or enzyme were negligible. Alteration of the sequence of addition, such that Fe^{3+} -citrate was added last, produced lower rates. NADH-ferricyanide reductase was assayed as described by Buckhout and Hrubec (11). Cyt *c* oxidase was assayed on each batch of isolated PM to check for mitochondrial contamination by following the oxidation of Cyt *c* (0.45 mg/mL in 50 mM Hepes, pH 7.0, reduced with dithionite) at 550 nm ($\epsilon^{mM} = 21 \text{ cm}^{-1}$). The rate of Cyt *c* oxidation was corrected for KCN-insensitive oxidation (1 mM KCN in 25 mM Hepes, pH 7.0). PM contained $<0.5\%$ of total Cyt *c* oxidase activity from the microsomal pellet.

Protein was assayed by the method of Markwell *et al.* (17) following TCA precipitation (3) of the protein and reprecipitation with ethanol ($-20^{\circ}C$, 1 h). This procedure resulted in lower protein estimates and correspondingly higher Fe-chelate reductase-specific activities than previously reported (10).

BPDS was acquired from Aldrich Chemical Co. (Milwaukee, WI) and Cibacron blue (Procion blue H-B) from Fluka Chemie AG (Buchs, Switzerland). Other Procion triazine dyes were provided by ICI Americas, Inc. (Wilmington, DE). Triton X-100 (Surfact-Amps X-100, 10% solution) was acquired from Pierce Chemical Co. (Rockford, IL). All other chemicals were reagent grade.

Calculation of Substrate Chelate Species

The computer program Geochem-PC (21) was used to calculate the concentrations of five species of Fe chelated to citrate under various conditions in the assays of Fe-chelate reductase. These conditions included pH (6.0), ionic strength (0.1 M), and various Fe^{3+} and citrate concentrations. The five species⁴ were $Fe^{3+}(cit^{3-})$, $Fe^{3+}H(cit^{3-})$, $Fe^{3+}(cit-H^{4-})$, $Fe^{3+}(cit^{3-})_2$, $Fe^{3+}_2(cit-H^{4-})_2$. At an ionic strength of 0.1 M, the \log_{10} of the chelate formation constants for the five Fe-chelates are 11.2, 12.5, 8.4, 18.2, and 21.3, respectively.

IEF

Fe-chelate reductase was solubilized from PM by the addition of octylglucoside at a ratio of 22:1 (w/w) of detergent to PM protein. Incubation on ice for 30 min was followed by centrifugation at 110,000g for 60 min. Analytical IEF of octylglucoside-solubilized enzyme was performed in 5% non-denaturing polyacrylamide gels ($7 \times 10 \times 0.15$ cm) containing 50 mM octylglucoside, 10% glycerol, and 2% ampholytes with pH ranging from 4 to 7. Gels were electrophoresed for 1.5 h at 200 V followed by 1.5 h at 400 V. The temperature was maintained at $4^{\circ}C$. The pH gradient along the length of the gel was measured with a flat-bottomed electrode. Gels were stained for NADH-dependent Fe-chelate reductase activity using a reaction mixture similar to the spectrophotometric assay except that the Fe^{2+} chelator, PDTS, at a concentration of 250 μM was substituted for BPDS, Fe^{3+} -citrate was used at 100 μM , and the buffer (Mes) concentration was increased to

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⁴ cit-H⁴⁻ denotes citrate with the three carboxyl protons and the hydroxyl proton displaced.

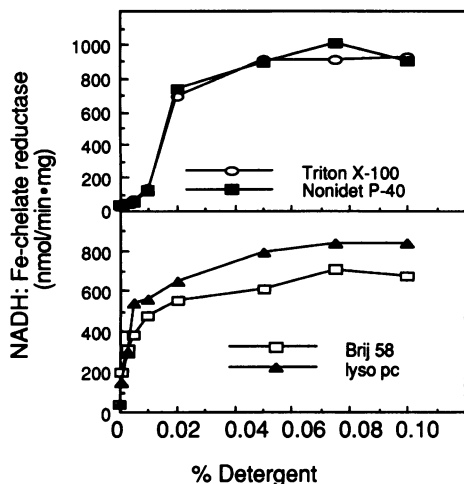


Figure 1. Detergent titration of Fe-chelate reductase activity in PM vesicles isolated from $-Fe$ tomatoes. All assays in this experiment were done on the same batch of membranes and points are the average of two to three assays. Results of experiments on PM isolated from $+Fe$ tomatoes gave identical patterns, except that the maximum activity was lower (data not shown).

150 mM. PDTS and octylglucoside were purchased from Sigma Chemical Co. (St. Louis, MO) and ampholytes from Bio-Rad (Richmond, CA).

Preparative aqueous IEF was also performed on octylglucoside-solubilized PM (Rotofor preparative IEF cell, Bio-Rad). The aqueous phase (60 mL) consisted of 1% ampholytes (pH 4–7), 30% glycerol, 28 μ M octylglucoside, 0.5 to 1 mg of octylglucoside-solubilized PM protein, 1 mM PMSF, 10 μ g/mL each of leupeptin and pepstatin, 5 μ M DTT, and 1 μ M flavin adenine dinucleotide. Electrophoresis was started under conditions of constant wattage (12 W). The wattage was then periodically decreased during the 3.5-h electrophoresis period to keep the voltage <2000 V. After completion of the electrophoresis, 20 fractions were collected. The pH of each fraction was measured, and enzyme assays were conducted immediately. The buffer concentration in the Fe-chelate reductase and ferricyanide reductase assays was increased to 100 mM to overcome the effects of the ampholytes in the electrophoresis medium.

RESULTS

Detergent Latency of PM Reductases

The Fe-chelate reductase is likely a transmembrane enzyme or enzyme complex, which uses cytoplasmic reductant (*e.g.* NADH) to reduce apoplasmic Fe^{3+} (9, 10). Thus, Fe-chelate reductase activity was latent in isolated PM vesicles in the absence of a detergent to facilitate substrate permeability to the vesicle interior (9, 10). Recent reports demonstrated that the activity of some enzymes, such as the plant PM ATPase, was affected by the detergent type (20, 23). To test the effect of detergent on Fe-chelate reductase activity, Fe-chelate reductase activity was titrated with four detergents (Fig. 1) that have diverse effects on the PM ATPase (20). Both lysophosphatidyl choline and Brij 58 increased reductase activity at

lower concentrations than Triton X-100 and Nonidet P-40, as was observed with the ATPase (20, 23). With all four detergents, the activity reached a stable maximum value. The difference in maximum Fe-chelate reductase activity between detergents never exceeded 25% (Fig. 1). Fe-chelate reductase from $-Fe$ tomatoes was used in the experiment shown in Figure 1. Identical results were obtained with $+Fe$ tomatoes (data not shown) except that the stable maximum activities were lower than those reported in Figure 1. A similar analysis of the effect of Triton X-100 and lysophosphatidyl choline conducted with ferricyanide reductase produced similar results (data not shown). Thus, Fe-chelate reductase and ferricyanide reductase did not appear to exhibit sensitivity to detergents or to be excessively activated by detergents, as was seen with the PM ATPase (20).

NADH Kinetics of Fe^{3+} Reduction by Tomato Root PM

As shown by previous studies with tomato PM, NADH was the preferred electron donor for Fe-chelate reductase (9, 10). Kinetic parameters for the substrate NADH were compared for PM Fe-chelate reductase isolated from both $-Fe$ and $+Fe$ tomato roots. Fe-chelate reductase exhibited simple Michaelis-Menten kinetics with respect to NADH (Fig. 2). The NADH K_m values were similar for enzyme from $+Fe$ and $-Fe$ PM (Table I). The V_{max} values reflected a two- to threefold increase in activity in PM from $-Fe$ treatments as compared to $+Fe$. This enrichment in specific activity was consistent with that previously reported (9, 10) but less than that measured in *in vivo* assays (7, 9, 10). Microscopic observation of the degree of homogenization of the root material indicated that a significant percentage of root hairs remained intact throughout the tissue homogenization phase of our PM isolation protocol. These root hairs were subsequently filtered out or ended up in the pellet after the initial low speed centrifugation. Because the Fe-chelate reductase activity in $-Fe$ roots is particularly concentrated in epidermis of newly

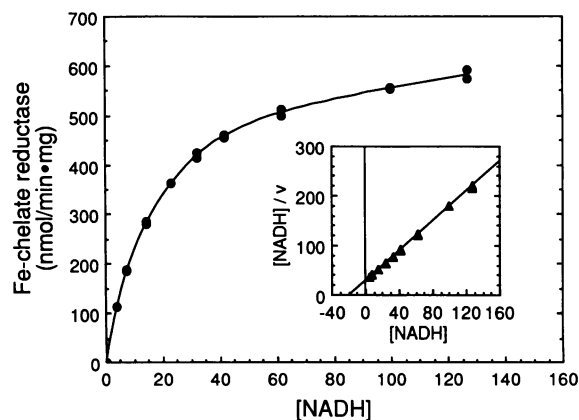


Figure 2. Kinetics of Fe-chelate reductase with respect to the substrate NADH. The concentration of the NADH (μ M) in the reaction mixture was verified by measuring the absorbance at 340 nm ($\epsilon^{mM} = 6.22$ cm^{-1}). Inset is the Hanes-Woolf transformation of the data. K_m and V_{max} values for this experiment on PM from $-Fe$ tomato roots were 19.6 μ M and 669 $nmol$ ($mg \cdot min$) $^{-1}$, respectively.

Table I. Kinetic Parameters of the Plasma Membrane Fe-Chelate Reductase with Respect to the Substrates, NADH and Ferric Dicitrate

The K_m and V_{max} values for NADH and Fe-citrate were determined from four to six separate experiments, such as shown in Figure 2, and Hanes-Woolf transformations were used for calculations. The concentration of the Fe-citrate species, $Fe^{3+}(cit^{3-})_2$, was used as the substrate concentration rather than the absolute amount of Fe^{3+} -citrate added to the assay. The concentrations of $Fe^{3+}(cit^{3-})_2$ were determined using the program Geochem-PC (21). Numbers represent means \pm SE; numbers of separate determinations are in parentheses.

	K_m	V_{max}
	μM	$nmol (min \cdot mg)^{-1}$
NADH		
-Fe	$22.6 \pm 2.3 (n = 6)$	$710 \pm 16 (n = 3)$
+Fe	$18.3 \pm 2.7 (n = 5)$	$240 \pm 12 (n = 4)$
$Fe^{3+}(cit^{3-})_2$		
-Fe	$35.2 \pm 8.3 (n = 6)$	$626 \pm 73 (n = 6)$
+Fe	$31.4 \pm 5.8 (n = 4)$	$331 \pm 37 (n = 4)$

formed lateral roots and accompanying root hairs (2, 8), PM isolated by such incomplete homogenization are not likely to be quantitatively representative of whole roots.

Chelator-Dependent Fe-Chelate Reductase Activity and Kinetics

Fe-chelate reductase activity was dependent on the nature of the Fe^{3+} chelator (Table II). The highest activity was seen with the organic acids oxalate and citrate. When Fe^{3+} was bound to the synthetic chelators EDTA, HEEDTA, and EDDHA, the activity was considerably less. The order of activity does not correlate with the stability constants for Fe^{3+} with the chelators (18, 19), published midpoint potentials from standard tables for Fe^{3+} -chelate couples (see ref. 5 and references therein), or the activity of free Fe^{3+} in the assay solutions (Table II).

We chose to use a potential physiological chelator, citrate, rather than synthetic chelators (e.g. EDTA) as Fe^{3+} chelator in our subsequent analysis. The activity of the reductase with citrate as the chelator varied as a function of the citrate: Fe^{3+}

Table II. Fe-Chelate Reductase Activity is Dependent on the Ferric Chelator

Assays were conducted as described in "Materials and Methods" with the exception that Fe^{3+} -chelates were prepared by the addition of $FeCl_3$ to stock solutions of different chelators which had been adjusted to pH 6.0 with KOH. Numbers in parentheses represent SEM of two replications from two independent membrane preparations.

Chelator	Chelator: Fe^{3+}	Activity of Free Fe^{3+}	Fe-Chelate Reductase	
			-Fe PM	+Fe PM
		$-\log (mol/L)$	$nmol (min \cdot mg)^{-1}$	
Oxalate	10:1	17.7	850 (90)	738 (81)
Citrate	20:1	17.7	530 (30)	250 (28)
EDTA	1.5:1	21.1	98 (6)	74 (4)
EDDHA	1.5:1	21.2	39 (8)	39 (0)
HEEDTA	1.5:1	18.4	25 (3)	23 (4)

Table III. Probable Substrate for Fe-Chelate Reductase is the $Fe(cit)_2$ Species of Fe^{3+} Chelated to Citrate

Assays were conducted as described in "Materials and Methods" with the exception that $FeCl_3$ was dissolved in different concentrations of citric acid-KOH (pH 6.0) to give stocks of 10 mM Fe at the ratios of citrate:Fe indicated. Final Fe concentration was 250 μM Fe. Each value for Fe-chelate reductase activity is the average of three separate assays and are from one experiment that was representative of three. Concentration of the major species of Fe citrate was determined using Geochem-PC as described in "Materials and Methods." The concentrations of two other species of Fe-citrate, $Fe(Cit)^{3-}$ and $FeH(Cit)^{2-}$, were not included in the table because they made up <0.1% of the total Fe under these conditions.

Fe-chelate Reductase	Citrate:Fe	Species of Fe-Citrate			Activity of Free Fe^{3+}
		$Fe(cit)_2$	$Fe(cit-H)$	$Fe_2(cit-H)_2$	
$nmol (min \cdot mg)^{-1}$		μM			$-\log (mol/L)$
473	5:1	143	34	37	16.4
586	10:1	204	20	13	17.0
781	20:1	233	11	4	17.7
772	25:1	237	8	2	17.9
816	30:1	240	7	2	18.0
780	35:1	241	6	1	18.1
742	40:1	243	5	1	18.3
610	50:1	245	4	<1	18.5
439	75:1	247	3	<1	18.9

ratio, with maximum activity occurring at ratios of 20:1 to 40:1 (Table III). These results suggested that the different species of Fe^{3+} -citrate present in a test solution could affect the apparent rate of reduction. To evaluate the chemical species of Fe^{3+} -citrate present, the computer program Geochem-PC (21) was used. Based on formation constants for $Fe^{3+}(cit^{3-})$, $Fe^{3+}H(cit^{3-})$, $Fe^{3+}(cit-H^4)$, $Fe^{3+}(cit^{3-})_2$, and $Fe^{3+}_2(cit-H^4)_2$, three chelate species predominate at the Fe^{3+} and citrate concentrations used in these experiments: $Fe^{3+}(cit-H^4)$, $Fe^{3+}(cit^{3-})_2$, and $Fe^{3+}_2(cit-H^4)_2$. The concentrations of these species in assays containing 250 mM Fe^{3+} are shown also in Table III. The concentration of uncomplexed citrate ion in the solution is the determining factor that drives the formation of the species. With increasing citrate concentration, the formation of ferric dicitrate ($Fe^{3+}[cit^{3-}]_2$) is favored. The ratio of citrate: Fe^{3+} is not directly related to speciation; thus, the effect of the citrate: Fe^{3+} ratio would be different at different total Fe^{3+} concentrations.

Fe-chelate reductase activity correlates positively with increasing concentrations of the predominant species and probable substrate, $Fe^{3+}(cit^{3-})_2$ (citrate:Fe ratios of 5:1 to 35:1; Table III). At very high ratios (50:1 and greater), the rate of reductase activity is lower than at intermediate ratios (Table III). This cannot be a result of the chemical speciation of the Fe^{3+} -citrate present because $Fe^{3+}(cit^{3-})_2$ constitutes nearly all the Fe^{3+} in the assay solutions.

$Fe^{3+}(cit^{3-})_2$ K_m values were determined using Fe^{3+} -citrate at citrate:Fe ratios that give high activity. The concentration of $Fe^{3+}(cit^{3-})_2$ was calculated by Geochem-PC for each assay condition, and this value was used as the substrate concentration. As with NADH, simple Michaelis-Menten kinetics were seen. The affinity of Fe-chelate reductase for the substrate $Fe^{3+}(cit^{3-})_2$ was similar for reductases from +Fe and -Fe

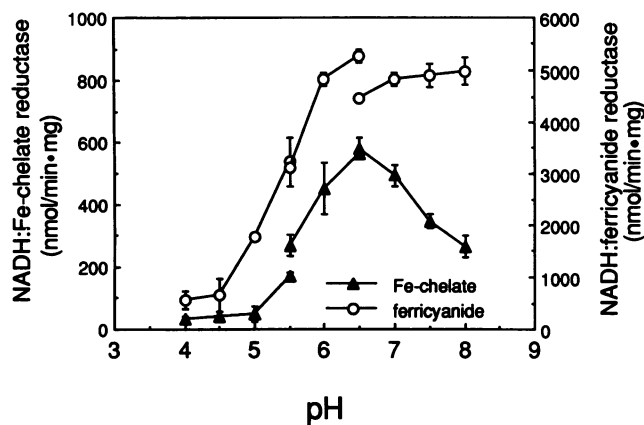


Figure 3. NADH-dependent Fe-chelate reductase shows a different pattern of activity with respect to pH than NADH-dependent ferricyanide reductase. pH was buffered using Mes (at 50 and 500 μM) in the pH range of 4.0 to 6.5 and Hepes in the pH range of 6.5 to 8.0. Each point represents the average of three assays. Data are from one experiment that was representative of three.

tomato root PM (Table I). The V_{max} for the reductase from $-\text{Fe}$ tomatoes was increased over that measured for reductase isolated from $+\text{Fe}$ tomatoes as observed with NADH as substrate (Table I).

pH Optima of PM Reductases

We investigated the pH activity profile of both the Fe-chelate reductase and NADH-ferricyanide reductase in PM from $-\text{Fe}$ roots (Fig. 3). Although both had pH optima of 6.5, the ferricyanide reductase continues to show nearly maximum activity until pH 8.0, the most alkaline pH tested. In contrast, Fe-chelate reductase activity decreased rapidly after pH 6.5. To investigate the pH optimum for the Fe-chelate reductase, it was necessary to choose buffers that have minimal binding affinity for Fe^{3+} . Mes ($\text{pK}_a = 6.1$) was used at a concentration of 50 mM in the pH range of 5.5 to 6.5, but the

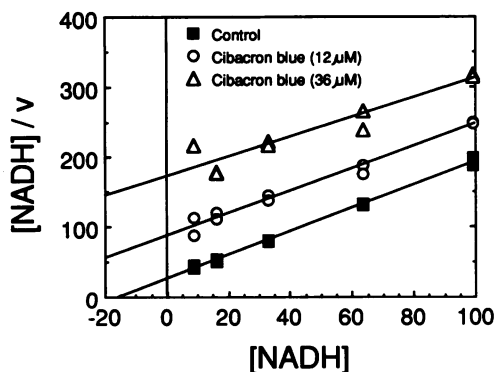


Figure 4. Cibacron blue inhibited Fe-chelate reductase activity in a manner consistent with competition for NADH. Assays were conducted as in Figure 2 in the presence or absence of Cibacron blue. Cibacron blue concentrations were 12 or 36 μM and the NADH concentration was in μM . Data are plotted as Hanes-Woolf transformations.

Table IV. PM NADH-Dependent Reductase Activity Is Inhibited by Triazine Dyes

Dye concentration (μM) required to inhibit 50% of reductase activity was determined from a graph plotting Fe-chelate reduction rate in the presence of five to six different concentrations of each dye. Dye was included in the reagent blank when the absorbance of the dye at 535 nm was significant. Data are from a single experiment representative of two.

Dye	Fe-Chelate Reductase		Ferricyanide Reductase ($-\text{Fe}$ PM)
	$+\text{Fe}$ PM	$-\text{Fe}$ PM	
Green HE-4BDA	2	1	23
Red H-8B	8	9	47
Blue MX-2G	12	9	50
Red H-3BN	10	13	110
Blue MX-R	29	23	55
Orange MX-2R	250	220	90

concentration was increased 10-fold to provide adequate buffering capacity at pH 4.0 to 5.0. Hepes ($\text{pK}_a = 7.5$) was used in the pH range of 6.5 to 8.0. Assays done with both buffers (or concentrations) at transition points (pH 5.5 and 6.5) showed that changing the buffer did not significantly affect the activity of the enzyme. For comparative purposes, the ferricyanide reductase assays were buffered in an identical manner.

Inhibition of PM Reductases by Triazine Dyes

Triazine dyes inhibit pyridine nucleotide-dependent oxidoreductases. In some cases, they are known to act as competitive inhibitors for the substrate NADH (26). We investigated whether dyes of this type inhibit Fe-chelate and ferricyanide reductase. Kinetic analyses of the effect of Cibacron blue (Procion blue H-B, reactive blue 2) on Fe-chelate reductase demonstrated behavior consistent with competitive inhibition (Fig. 4). In the presence of Cibacron blue (12 and 36 μM), an increase was seen in the K_m for NADH from 16 to 54 and 100 μM , respectively, without substantive change in the V_{max} (596–625 and 634 $\text{nmol} [\text{min} \cdot \text{mg protein}]^{-1}$, respectively). This suggests that Cibacron blue could compete with NADH for the active site of NADH oxidation.

A series of triazine dyes were tested for effectiveness in inhibiting PM Fe-chelate and ferricyanide reductase activity (Table IV). The dyes differed widely in their effectiveness on Fe-chelate reductase activity. A 100-fold higher concentration of Orange MX-2R was required to inhibit Fe-chelate reductase activity as compared to Green HE-4BDA. The others ranged between these extremes. However, individual dyes were equally effective in inhibiting Fe-chelate reductase in PM from either $+\text{Fe}$ or $-\text{Fe}$ tomato roots. In contrast, the dyes inhibited ferricyanide reductase to a much lesser degree than Fe-chelate reductase, with the exception of Orange MX-2R (Table IV). Furthermore, the pattern of inhibition by the dyes was different between the Fe-chelate and ferricyanide reductase.

Detergent Solubilization of Fe-Chelate Reductase and IEF of the Solubilized Enzyme

Octylglucoside at a detergent to protein ratio of 22:1 (w/w) effectively solubilized 95% of the Fe-chelate reductase activity, 90% of the ferricyanide reductase activity, and 50% of the PM protein. Electrophoresis of the solubilized PM protein using preparative aqueous phase IEF showed that peak of Fe-chelate reductase activity with a pI of 5.7, but activity was found over a range of fraction pH from 4.5 to 6.5 (Fig. 5). Two peaks of NADH-ferricyanide reductase activity were resolved. One was at the acid edge (pH 4.8) of the Fe-chelate reductase activity and the other peak was coincident with the Fe-chelate reductase activity. The majority of the ferricyanide reductase activity, using this technique, was separated from the Fe-chelate reductase activity.

IEF of octylglucoside-solubilized tomato root PM in non-denaturing polyacrylamide gels separated Fe-chelate reductase activity into three to four bands that could be visualized by using a NADH-Fe³⁺-chelate activity stain (Fig. 6). In preliminary experiments, in which a wide range of ampholytes were used (pH 3 to 9), no additional bands were seen. Additional enzyme forms, not resolved by this method, may exist.

The enzyme-stained bands had pI values of 5.5, 5.8, and 6.2. In some experiments, a fourth band could be faintly detected just below (0.1–0.2 pH units) the pI 5.5 band. When equal amounts of protein solubilized from –Fe or +Fe PM were loaded into adjacent lanes of IEF gels, the intensity of band staining was always higher in the –Fe PM lane (Fig. 6). In particular, pI 5.5 and 6.2 bands were obviously enhanced. The pI 5.8 band, the most intensely stained of all three bands, showed some enhancement also in the –Fe lane.

DISCUSSION

The tomato root PM Fe-chelate reductase is capable of reduction of various Fe³⁺-chelate complexes both *in vivo* and *in vitro*. The chemical nature of the Fe³⁺ substrate reduced by root cells in soil is unknown. Root cells most probably encounter Fe³⁺ which is chelated to a variety of organic acids in the rhizosphere. However, under acidic conditions, some Fe³⁺ may be present as a free ion. For Fe-chelate reductase in

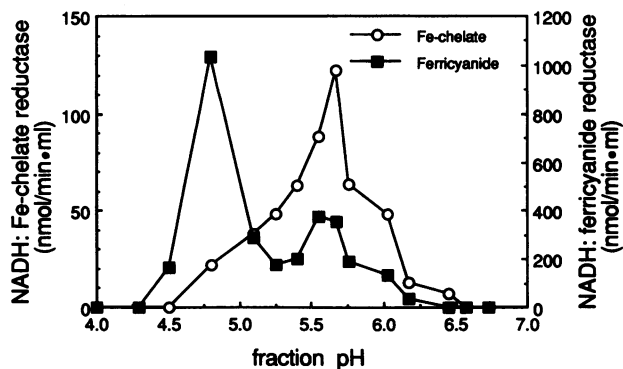


Figure 5. Analysis of Fe-chelate reductase and NADH-ferricyanide reductase activity in fractions from aqueous phase IEF plotted as a function of the pH of the fractions.

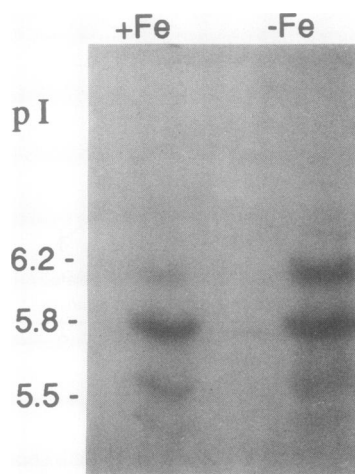


Figure 6. Native IEF gel stained for Fe-chelate reductase activity as described in "Materials and Methods." Equal amounts of protein solubilized from –Fe and +Fe PM were loaded in adjacent lanes.

tomato PM, not all Fe³⁺-chelates were reduced at an equal rate. In our experiments in which Fe³⁺-chelate speciation was carefully controlled by pH and chelate:Fe³⁺ ratios, the specific activity was higher with organic acid chelators (citrate, oxalate) of the type found in plant tissues than with the synthetic chelators (EDTA, HEEDTA, EDDHA) (Table II).

There are some data that allow comparison of Fe-chelate reductase in isolated PM with activity in whole root. In a study with intact peanut roots, EDTA and HEEDTA proved to be better substrates than citrate (22). With bean roots, the Fe-chelate reductase activity varied with Fe³⁺ chelated to oxalate > malonate > EDTA > citrate (6). With these limited data, it is not clear whether the differences in order of effectiveness of the Fe³⁺ chelators to serve as substrate were due to whole root *in vivo* assays versus *in vitro* assays done with isolated PM or due to differences between plant species.

Kinetic studies of Fe-chelate reductase activity in whole roots have been performed by Chaney (13) using peanut and Brüggemann et al. (9) using tomato. The peanut root Fe-chelate reductase showed Michaelis-Menten kinetics with apparent K_m values of 23 and 55 μM for FeEDTA and Fe-diethylenetriamine pentaacetic acid, respectively. This is close to the results for tomato PM Fe-chelate reductase (Table I) but 10-fold lower than reported by Brüggemann et al. (9) with FeEDTA as substrate. Similarly, the Fe-chelate reductase activity in PM isolated from the roots of *P. lanceolata* produced K_m values for FeEDTA and NADH that were higher than the results that we obtained with Fe³⁺-citrate (24). It is likely that differences between these studies and ours lies in the use of different Fe-chelate species.

Fe³⁺-citrate was chosen as substrate for the standard Fe-chelate reductase assay and is more likely, than synthetic chelators, to reflect the *in vivo* condition. The ability of the Fe³⁺(cit³⁻)₂ species to serve as an effective substrate for the root PM Fe-chelate reductase would be expected based on previous research. At pH levels as low as 6.0, other Fe³⁺-citrate species have very slow exchange rates, whereas Fe³⁺(cit³⁻)₂ has a very rapid Fe³⁺ exchange rate (1). Thus, one

ligand bond from one of the two citrates could be displaced and Fe^{3+} could form a ligand bond with the Fe-chelate reductase. At very high ratios of citrate to Fe^{3+} (>40), citrate could compete with the enzyme for Fe^{3+} during exchange. Furthermore, excess citrate could compete with BPDS for Fe^{2+} and catalyze the reoxidation to Fe^{3+} (25). Competition by citrate with the reductase for Fe^{3+} and citrate-catalyzed reoxidation of Fe^{2+} would explain the decrease in reductase activity at citrate: Fe^{3+} ratios higher than 40:1 (Table III). A similar pattern was reported by Bates *et al.* (1) in experiments studying the ability of transferrin to bind Fe^{3+} chelated to citrate with increasing ratios of citrate to Fe^{3+} .

The Fe-chelate reductase had a pH optimum at 6.5 when tested in isolated tomato PM. This value agrees well with similar analyses of PM Fe-chelate reductases in tomato and *Plantago* (9, 24) but was higher than the optimum determined for intact roots (6, 22). However, the optimum determined for intact roots reflects the optimum for just the Fe^{3+} reduction, whereas the pH analysis in detergent-permeabilized vesicles reflects a compromise of both substrates [NADH and $\text{Fe}^{3+}(\text{citrate}^{3-})_2$].

An early hypothesis by Bienfait (4) proposed the *de novo* synthesis of a low midpoint electrochemical potential Fe-chelate reductase on the expanding root epidermal PM in response to Fe deficiency stress. In support of this theory, a 10-fold increase in Fe^{3+} EDTA ($E_o' +130$ mV) reduction but twofold increase in FeCN ($E_o' +360$ mV) reduction was demonstrated in whole root reduction assays using iron deficiency-stressed bean plants (5). In our experiments with isolated PM, however, the reduction rate for Fe^{3+} -chelates with the lowest midpoint potentials (Fe^{3+} -oxalate, $E_o' -10$ mV; Fe^{3+} -EDTA, $E_o' +130$ mV) was not increased in $-Fe$ PM to a greater degree than the reduction rate for Fe^{3+} -chelates with higher midpoint redox potential (Fe^{3+} -citrate, $E_o' +200$ mV) (Table II). Thus, no evidence for the induction of a low midpoint potential reductase in response to Fe deficiency was found in measurements with isolated PM. It should be stressed, however, that the midpoint potentials in published standard tables may not take into account factors that influence the reduction and reoxidation of various Fe-chelate couples in the rhizosphere or in our spectrophotometric assay. Reliable values for midpoint potentials of specific chelates would have to be measured under conditions simulating either the spectrophotometric assay (*i.e.* in the presence of BPDS) or the rhizosphere, taking into account oxygen tension, chelator stability constants, and pH.

The determination of the K_m values and the identical pattern of inhibition by triazine dyes for the reductase in PM from $+Fe$ and $-Fe$ roots lends no support to the concept of the induction of a novel reductase in response to Fe deficiency. Although the V_{max} for the reductase under $-Fe$ conditions was 2 to 3 times higher than under $+Fe$ conditions (Table I), this could be explained by a higher concentration or an activation of constitutive Fe-chelate reductase in tomato root epidermal PM. Perhaps the strongest evidence against the induction of a novel reductase in response to Fe deficiency stress was obtained following the separation of the PM Fe-chelate reductases by IEF and their identification by staining for Fe-chelate reductase activity. PM from both root sources contain the same number of separable Fe-chelate reductases

with the same pIs but differ in apparent activities as indicated by the intensity of the enzyme staining. The pI 6.2 and 5.5 reductases always stained more intensely in preparations from $-Fe$ PM (Fig. 6). The pI 5.8 reductase was the most predominantly stained reductase in both $+Fe$ and $-Fe$ PM, and in most experiments there were also detectable increases in staining of the pI 5.8 reductase solubilized from $-Fe$ PM over $+Fe$ PM (Fig. 6). The relationship between the multiple forms of the Fe-chelate reductase is not yet known. Some preliminary evidence from SDS-PAGE analysis (not shown) indicated that the predominant polypeptide components of the stained regions of the IEF gels were similar.

Preparative IEF of octylglucoside-solubilized tomato root PM and enzyme analysis of the resulting fractions yielded two peaks of ferricyanide reductase activity (Fig. 5.); a predominant peak at pH 4.8 and a second smaller peak concurrent with the Fe-chelate reductase activity at pH 5.7 (consistent with the PI of the major enzyme-stained band on the IEF gel). This, along with the nonidentical pH profile and patterns of inhibition by triazine dyes, suggests that there is more than one NADH-dependent reductase complex on the PM that is capable of using ferricyanide as an artificial electron acceptor. These data are in agreement with the results of Luster and Buckhout (16) for multiple reductases in maize PM. We did not compare NADH kinetics for the Fe-chelate reductase and the ferricyanide reductase because the nature of the electron acceptor (Fe^{3+} versus ferricyanide) could alter the K_m for NADH oxidation. Therefore, any differences between K_m values might be artifactual.

The second smaller peak of ferricyanide activity, seen in preparative IEF activity profiles, is exactly concurrent with the peak of Fe-chelate reductase at pH 5.7 (Fig. 5). One interpretation of the concurrent peaks is that the Fe-chelate reductase can also use ferricyanide as a substrate. Alternatively, the two activities could be due to two different enzymes with the same pI. We are currently purifying detergent-solubilized tomato root PM Fe-chelate reductases in an attempt to resolve such questions.

In conclusion, the kinetic (K_m), triazine dye inhibition, and IEF data are more supportive of similarities than of differences between Fe-chelate reductases in PM isolated from $+Fe$ and $-Fe$ tomato roots, and there is little in the enzymology to justify the presence of novel reductases. These results are in agreement with the conclusions of Brüggemann *et al.* (9). Furthermore, our data support the hypothesis that amplification or activation of constitutive PM Fe-chelate reductase isoforms occurs in the root epidermal PM in response to Fe deficiency stress.

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