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Iron reductase systems on the plant plasma membrane–A review

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Abstract

Higher plant roots, leaf mesophyll tissue, protoplasts as well as green algae are able to reduce extra-cellular ferricyanide and ferric chelates. In roots of dicotyledonous and nongraminaceous, monocotyledonous plants, the rate of ferric reduction is increased by iron deficiency. This reduction is an obligatory prerequisite for iron uptake and is mediated by redox systems localized on the plasma membrane. Plasma membrane-bound iron reductase systems catalyze the transmembrane electron transport from cytosolic reduced pyridine nucleotides to extracellular iron compounds. Natural and synthetic ferric complexes can act as electron acceptors.

This paper gives an overview about the present knowledge on iron reductase systems at the plant plasma membrane with special emphasis on biochemical characteristics and localisation.

Abbreviations: FeCh – ferric chelate(s), FeCh-R – ferric chelate reductase, FeCit – Fe^{III}-Citrate, FeCN – ferricyanide, FeCN-R – ferricyanide reductase, FeEDTA – Fe^{III}-EDTA, LPC – lysophosphatiolylcholine, NR – nitrate reductase, PM – plasma membrane, TX 100 – Triton × 100

Introduction

Plant roots enzymatically reduce ferric chelates (FeCh) of both natural and synthetic origin, as well as non-physiological electron acceptors with high redox potentials, like ferricyanide (FeCN). The necessary redox systems are localized on the plasma membrane (PM). These iron reductase activities transfer electrons from cytoplasmatic reduced pyridine dinucleotides to the apoplast. Iron-efficient plant species are characterized by their ability to grow at low soil iron concentrations. In many of these species, the reductase activity measured with intact roots (in vivo rates) is inducible by iron deficiency. In the present article, we will summarize those experimental results, which are significant for the comprehension of the obligatory ferric reduction step which precedes the uptake of ferrous iron. In the past, apart from the physiological reduction of FeCh, the non-physiological reduction of FeCN also has often been associated with the iron-uptake mechanism. Therefore, this review will consider experimental results on both processes. Most of the available data rely on measurements made on tissues or intact membranes. These systems contain several different PM redox activities, a fact that makes it difficult to determine which redox system is involved in a given measured process and attached to a specific physiological role. Nevertheless, in the following we summarize the available information to identify and possibly discern different PM bound iron reductase systems.

Iron reduction by intact roots

Intact roots of all plant species investigated so far are able to reduce extracellular ferric compounds. This reducing capacity is either based on the extrusion of reducing substances or on a reduction step at the cell surface, or on the simultaneous occurrence of both processes. Iron-efficient plant species can be classified into two groups: The grass family species (*Poaceae*, "Strategy II" plants) exhibit an increased excretion of

Table 1. Characteristics of ferricyanide reduction by intact roots (for easy comparison only data on freshweight basis are depicted)

Species	Growth conditions +Fe/−Fe	Activity [nmol Fe min^{-1} (g fw)$^{-1}$]	Stimulation (-fold)	Days of iron deficiency	pH Optimum	K_m (μM) FeCN	Assay conditions	References
Strategy I								
Phaseolus	+Fe	61.6					n.m.	Bienfait, 1988
vulgaris	−Fe	125.0	2.0					
Lycopersicon	+Fe	20.0					0.5 m*M* FeCN	Bienfait, 1987
esculentum FER	−Fe	68.3	3.4					
Lycopersicon	+Fe	16.0					0.5 m*M* FeCN	Bienfait, 1987
escul. fer T3820	−Fe	18.3	1.1					
Gossypium	+Fe	13.3					0.8 m*M* FeCN;	Chosack et al., 1991
hirsutum	−Fe	50.0	3.8	7			pH 6.4	
Geum urbanum	−Fe	348.3		21		290	n.m.	Schmidt and Janiesch, 1991
Strategy II								
Hordeum	+Fe	63.3					n.m.	Bienfait, 1988
vulgare	−Fe	63.3	1.0					
Zea mays	+Fe	64.1			7.0–8.3		0.8 m*M* FeCN	Qui et al., 1985
Zea mays	+Fe	78.8					0.8 m*M* FeCN; pH 6.1	Rubinstein and Stern, 1986

n.m. = not mentioned.

Table 2. Characteristics of Fe-EDTA reduction by intact roots (for easy comparison only data on freshweight basis are depicted)

Species	Growth conditions	activity [nmol Fe min^{-1} (g fw)$^{-1}$]	Stimulation (-fold)	Days of iron deficiency	pH Optimum	K_m (μM) Fe-EDTA	Assay conditions	References
Strategy I								
Arabidopsis thaliana	+Fe	14.50					0.2 m*M* Fe-EDTA	Moog et al., 1994
	−Fe	54.50	3.8	5–7	5.5	45	0.3 m*M* BPDS; pH 5.5	
Arachis hypogeae	+Fe	0.83					0.1 m*M* Fe-EDTA	Römheld and Marschner, 1983
	−Fe	71.00	85.2	13–14	5.0		0.3 m*M* BPDS; pH 6.0	
Arachis hypogeae	+Fe	n.d.					0.1 m*M* Fe-EDTA	Chaney, 1989
	−Fe	31.66		6		55	0.4 m*M* PDTS; pH 6.0	
Malus domestica	+Fe	0.86					0.09 m*M* Fe-DTPA;	Ao et al., 1985
	−Fe	10.00	12	14–21	5.0		0.2 m*M* BPDS; pH 5.0	
Lycopersicon esculentum FER	+Fe	1.67					0.1 m*M* Fe-EDTA	Bienfait, 1987
	−Fe	6.67	4.0					
Lycopersicon escul. T3820 fer	+Fe	0.33					0.1 m*M* Fe-EDTA	Bienfait, 1987
	−Fe	0.50	1.5					
Lycopersicon esculentum	+Fe	14.50					0.2 m*M* Fe-EDTA;	Buckhout et al., 1989
	−Fe	106.80	7.4	8			1 m*M* PDTS; pH 6.0;	
Lycopersicon esculentum	+Fe	5.90					0.5 m*M* Fe-EDTA;	Brüggemann et al., 1990
	−Fe	20.20	3.4	9		230	0.3 m*M* BPDS; pH 6.0	
Solanum tuberosum	+Fe	0.67					0.1 m*M* Fe-EDTA;	Bienfait, 1987
	−Fe	15.00	22.4				0.4 m*M* BPDS; pH 5.3	
Phaseolus vulgaris	+Fe	3.30					0.3 m*M* Fe-EDTA;	Sijmons and Bienfait, 1983
	−Fe	66.66	20.2	7–10			1.2 m*M* BPDS; pH 5.5	
Phaseolus vulgaris	+Fe	8.30					n.m.	Bienfait, 1988
	−Fe	83.30	10.0			120		
Phaseolus vulgaris	+Fe	1.40					0.1 m*M* Fe-EDTA;	Schmidt and Janiesch, 1991
	−Fe	16.30	12.0	7–10			0.3 m*M* BPDS; pH 6.0	
Plantago lanceolata	+Fe	0.75					0.5 m*M* Fe-EDTA;	Schmidt et al., 1990
	−Fe	1.25	1.7	42			0.6 m*M* BPDS; pH 7.0	
Cucumis sativus	+Fe	7.92					0.1 m*M* Fe-EDTA;	Alcantara et al., 1991
	−Fe	76.66	9.7	2			0.3 m*M* BPDS, pH 5.5	
Ficus benjamima	+Fe	n.d.					0.1 m*M* Fe-EDTA;	Rosenfield et al., 1991
	−Fe	91.66		42	5.5 – 6.7		0.3 m*M* BPDS, pH 5.5	
Geum urbanum	+Fe	1.83			5.0 – 5.5		0.3 m*M* Fe-EDTA;	Schmidt and Janiesch, 1991
	−Fe	26.66	15	21	5.0 – 5.5	87	0.9 m*M* BPDS, pH 5.5	
Glycine max	+Fe	n.d.					0.1–0.8 m*M* Fe-EDTA;	Cornett and Johnson, 1991
	−Fe	n.c.				28	0.22 m*M* BPDS, pH 5.5	
Pyrus amygdaliformis	+Fe	1.87					0.1 m*M* Fe-EDTA;	Dolcet-Sanjuan et al., 1992
	−Fe	3.35	1.8	20			0.3 m*M* BPDS, pH 6.0	
Cydonia oblongata	+Fe	0.23					0.1 m*M* Fe-EDTA;	Dolcet-Sanjuan et al., 1992
	−Fe	0.29	1.3	20			0.3 m*M* BPDS; pH 6.0	
Strategy II								
Hordeum vulgare	+Fe	1.67					n.m.	Bienfait, 1988
	−Fe	1.67	1.0					

n.c. = data on dryweight basis; n.d. = not determined; n.m. = data not mentioned.

specific ferric chelators, named phytosiderophores (i.e. mugineic acid, Tagaki et al., 1984), which allow the enhanced solubilization of Fe^{III} and the subsequent uptake of the ferric chelate complex (cf. reviews by Marschner et al., 1986; Römheld, 1987). Iron-efficient members of all other systematic groups revealed an increased ability to reduce extracellular ferric compounds at the cell surface ("Strategy I" plants). This reduction step is obligatory before chelate splitting and uptake of Fe^{2+} occur (Chaney et al., 1972). In this article, we will only consider those data, which are relevant for understanding Strategy I.

Based on data obtained with intact roots, Chaney et al. (1972) proposed that the ability to reduce extracellular iron compounds is due to an enzyme activity probably localized on the plant PM. In Tables 1 and 2 the characteristics of FeCN and Fe^{III}-EDTA (FeEDTA) reduction are summarized. All tested iron-sufficient grown plants showed an in vivo activity of FeCN and FeEDTA reduction. There were large differences between species and lines in the extent to which iron deficiency increased these reduction rates. The (non-physiological) reduction of FeCN by intact roots was investigated in less detail than the reduction of iron nutrition sources like FeEDTA or other FeCh. In general, higher activities were obtained with FeCN than with FeEDTA. Upon iron deficiency, FeCN reduction is increased up to 3.8 times, while FeEDTA reduction can be stimulated up to 20-fold. In iron-sufficient plants, FeCh reduction rates varied between 0.2 and 14.5 nmol Fe g fw^{-1} min^{-1}, while iron-deficient plants reduced up to 100 nmol Fe g fw^{-1} min^{-1}. Interestingly, as depicted in Table 2, the Strategy II grass *Hordeum vulgare* also shows an in vivo activity of FeEDTA reduction, which lies in the order of magnitude of iron-sufficient dicots. However, in agreement with the Strategy I/II model, this activity was not inducible by iron deficiency. The iron-inefficient tomato mutant T3820fer, investigated by Brown and Ambler (1974), revealed very low rates under both control and iron-deficient conditions, indicating that the FER gene affects the reductase activity and that this is the reason for the ineffective iron uptake of the mutant (Bienfait, 1988a). Compared to the large sets of data showing the potential for induction of the in vivo activity by iron-deficiency, there are only few reports available on other characteristics of the activity:

pH dependence For intact oat (Rubinstein et al., 1984) and bean (Sijmons et al., 1984a) roots it has been shown that the rate of FeCN reduction is rather insensitive to pH and does not show a distinct optimum. In contrast, FeEDTA reduction always had a distinct pH optimum around pH 5.5, i.e. the apoplastic pH.

Michaelis-Menten kinetic Only Schmidt and Janiesch (1991a) published kinetic data of in vivo FeCN reduction and reported a Michaelis-Menten kinetic with a K_m (FeCN) of 290 μM for *Geum urbanum*. FeEDTA reduction in vivo also followed Michaelis-Menten kinetics, as found by several authors. The reported Km values vary between 28 and 230 μM, depending on the species, and, possibly, on assay conditions. Since no concentration-independent component was found in the kinetic plots, the in vivo reaction is not limited by electron supply from the cytoplasm.

Time-course of induction As reported by Brown and Jolley (1988), tomatoes showed a maximal reduction capacity between the 7th and 10th day of iron deficiency. In beans, highest reduction rates were found after 6-8 days (Schmidt, 1993), and in *Ficus benjamina*, this response occurred around the 56th day of iron deficiency (Rosenfield et al., 1991). The day of the maximum activity obviously depends on the species, and this induction can be inhibited by the application of the translation inhibitor cycloheximide (Schmidt, 1993).

Inhibition or stimulation Although there are many data on inhibition and stimulation of FeCN reduction on different plant cells and tissues, it is not possible to name a general inhibitor or stimulans. The effects of any tested substance depends on the plant species, type of tissue, or assay condition (for a review see Barr and Crane, 1991). Recently, Schmidt (1993) reported the inhibition of FeEDTA reduction by the PM H^+-ATPase inhibitor orthovanadate (500 μM) in roots of both iron deficient and iron sufficient bean plants, while FeCN reduction was only inhibited in iron deficient plants. However, when the same experiments were performed with *Plantago lanceolata*, FeCN reduction was not inhibited but even increased in the presence of vanadate (Schmidt, 1994). The data on pH dependence, Michaelis-Menten kinetics, time-course of induction and inhibition / stimulation of ferric reduction by intact roots clearly point to an enzymatic process. A putative FeCh reductase (FeCh-R) could be localized either in the apoplast or on the PM. The findings of Tipton and Thowsen (1985) on crude cell wall preparations first indicated that a cell wall-bound enzyme mediates the

ferric reduction. However, in this case the source of electrons remained unclear. Sijmons et al. (1984a) demonstrated that the extracellular reduction of both FeCN and FeCh was an electrogenic process, depolarizing the negative potential of the cytoplasm with respect to the apoplast (for a review see Prins and Elzenga, 1991). This finding pointed to a trans-PM electron transport system, using a cytoplasmic electron donor for the reaction. Positive evidence was presented by the identification of FeCN reduction in purified PM from corn roots (Buckhout and Hrubec, 1986) and by comparing the FeCh-R in vivo activities with the FeCh-R activities obtained with purified PM (in vitro activities) (Brüggemann and Moog, 1989; Buckhout et al., 1989). Today there is no doubt that iron reductase systems are localized on the root PM.

Iron reductase systems in root plasma membranes

Based on the data obtained in experiments with intact roots, Bienfait (1985) extended the hypothesis of Chaney et al. (1972) and proposed a model in which two types of PM bound redox systems are discerned, the so-called "Standard" and the "Turbo" system (for comprehensive reviews see Bienfait, 1985, 1988b, 1989; Böttger et al., 1991; Chaney and Bell, 1987).

In his definition, the "Standard" system can reduce external electron acceptors with high redox potential like FeCN (+360 mV) and possibly oxygen (+820 mV), which might be the natural electron acceptor. It is constitutive in all plants and does not reduce FeCh, which exhibit lower redox potentials (ca.+100 – +250 mV). The biological role is seen in functions other than iron uptake like modulation of membrane potential or production of highly active oxygen species.

The "Turbo" system is a transmembrane FeCh-R, which is induced by iron deficiency in the epidermis of young roots of dicotyledonous and monocotyledonous, non-graminaceous plants. It reduces both FeCN and FeCh outside intact cells with electrons originating from cytoplasmic NADPH. The tenfold increase in ferric chelate reduction of intact bean roots under iron deficiency led to the expression "Turbo" system. The physiological function is definitely a mechanism to increase the Fe^{2+} concentration at the cell surface for uptake by the roots.

Since this hypothesis was proposed, advanced membrane purification techniques have been developed, which allow purified plasma membranes without contaminants to be isolated (for reviews see Larsson and Møller, 1990). Investigations on ferric reduction of purified PM from iron-deficient and iron-sufficient plants were compared with in vivo data and brought the original model into question. In their study, Brüggemann et al. (1990) could not confirm the synthesis of a biochemically distinct enzyme formed under iron stress, but proposed that the in vivo response to iron starvation relies on the enhanced synthesis or activation of a constitutively existing redox system. However, till the time of this work (July 1993), no iron chelate reductase system has been isolated from PM and purified to homogeneity so far. Therefore the correlation of kinetic and other biochemical data available now (mostly obtained on purified PM or partially purified redox systems) will be used in this article to answer the following questions: How many different iron reductase systems are localized on the PM? – Is there a difference between "Standard" reductase and "Turbo" reductase as proposed by Bienfait (1985)? – Is there possibly an ubiquitous PM bound iron redox system?

Characteristics of ferricyanide reduction

Reduction of the membrane impermeable oxidant FeCN is the most frequent PM redox reaction investigated and was found in PM of all higher plant roots investigated so far. The characteristics of root PM FeCN reductase (FeCN-R) are depicted in Table 3. Generally, reduction rates are higher with NADH than with NADPH as electron donor. Purified PM obtained by two-phase-partitioning (Larsson et al., 1987) forms vesicles which are largely right-side-out oriented, meaning that the cytoplasmatic side of the PM is facing the inside of the vesicle. Since FeCN and NAD(P)H are membrane impermeable, they can not reach reaction sites on the inside of the PM vesicle. Treatment with detergents makes PM vesicles permeable for the reaction partners and allows the determination of latent activities on the cytoplasmic side. PM bound FeCN-R from iron-sufficient roots was 50–96% latent (Table 3), indicating that at least one relevant reaction site is located at the cytoplasmic side of the PM. The specific activity on average revealed around 600 nmol Fe (mg protein)$^{-1}$ min^{-1}, K_m (FeCN) ranged from 90 to 200 μM and K_m (NADH) from 20 to 150 μM, depending on species and PM preparations.

A specific inhibitor for FeCN reduction has not been identified yet. As extensively reviewed by Buckhout and Luster (1991), FeCN reduction is largely insensitive to inhibitors of mitochondrial electron

Table 3. Characteristics of ferricyanide reduction by root plasma membranes

Species	Growth conditions +Fe/-Fe	NADH SA[a]	NADH Stimulat. (-fold)	NADH Latency[b]	NADPH SA[a]	NADPH Stimulat. (-fold)	NADPH Latency[b]	Km (μM) FeCN	Km (μM) NADH	pH Optimum	Assay conditions (NAD(P)H conc; pH; detergent)	References
Strategy I												
Lycopersicon esculentum	+Fe	697		93	162						0.16 m*M*; 7.0; 0.025% TX 100	Buckhout et al., 1989
	−Fe	1474	2.1	96	197	1.2						
Lycopersicon esculentum	−Fe	5000								6.5–8.0	0.16 m*M*; 7.0; 0.025% TX 100	Holden et al., 1991
Lycopersicon esculentum FER	+Fe	507			439						0.15 m*M*; 7.5; 1% LPC	Valenti et al., 1991
	−Fe	1292	2.6		467	1						
Lycopersicon escul. fer T3820	+Fe	411			466						0.15 m*M*; 7.5; 1% LPC	Valenti et al., 1991
	−Fe	572	1.4		483	1						
Phaseolus vulgaris	+Fe	863		52	687		58		153	6.8	0.75 m*M*; 7.0; 0.02% TX 100	Schmidt and Janiesch, 1991; Schmidt, 1992
	−Fe	1915	2.2	60	1132	1.7	68					
Gossypium hirsutum	+Fe	3852		50							0.1 m*M*; 7.0; 0.05% TX 100	Chosack et al., 1991
	−Fe	10404	2.7	68								
Geum urbanum	+Fe	650						208			0.77 m*M*; 7.0; 0.02% TX 100	Schmidt, 1992
Strategy II												
Avena sativa	+Fe	1000									0.1 m*M*; 7.5	Rubinstein et al., 1984
Zea mays	+Fe	168		87					24.3[c]		0.16 m*M*; 7.0; 0.025% TX 100	Buckhout and Hrubec, 1986
Zea mays	+Fe	284			134					7.5	0.16 m*M*; 7.0; 0.01% TX 100	Luster and Buckhout, 1988
Zea mays	+Fe	1300						92.0	93.3	7.0	0.25 m*M*; 7.0;	Bourdil et al., 1990
Zea mays	+Fe	6400		80				92.3	75.0	5.0	0.25 m*M*; 7.0; LPC: Prot. = 5	Bourdil et al., 1990
Hordeum vulgare	+Fe	480		64							0.25 m*M*; 7.3; 0.015% TX 100	Askerlund, 1989
Hordeum vulgare	+Fe	365									0.5 m*M*; 6.8; 0.02% TX 100	Brüggemann and Moog, 1989

[a] Specific activity [nmol Fe min^{-1} (mg protein)$^{-1}$].

[b] If data available, latency was calculated by the equation: [(rate with detergent)-(rate without detergent) × 100 (rate with detergent)$^{-1}$.

[c] Authors report a K of 0.5 μM for NADH in the absence and a K of 20.4 μM for NADPH in the presence of detergent.

Table 4. Biochemical characteristics of partially purified plasma membrane-bound iron reductases

PM source	Acceptor	SA[a] of partially purified protein	kDa[b]	pI values	Method[c]	References
Strategy I						
Spinacia oleracea leaves	FeCN	78000	22, 30, 37, 40, **43**, 120	below 5.6	Anion exchange chromatography (Mono Q), SDS-Page	Askerlund et al., 1991
Lycopersicon esculentum roots	FeCN		28, **34**	4.8, 5.7	preparative IEF	Holden et al., 1991
Lycopersicon esculentum roots	FeCN		35, **70**, 130		Molecular sieve chromatography (Superose 12)	Valenti et al., 1991
Hordeum vulgare roots	FeEDTA		**94**, 106, 120, 205	7.4	native IEF, SDS-Page	Brüggemann and Moog, 1989
Lycopersicon esculentum roots	FeEDTA	458	55, 64, 100		native Page SDS-Page	Brüggemann et al., 1990
Lycopersicon esculentum roots	FeEDTA	4600	31, **35**, 56, 58 62, 74, 104	5.5, 5.7	native Page, native IEF, electroelution, SDS-Page	Moog and Brüggemann, 1992a
Lycopersicon esculentum roots	FeEDTA	108000	**29**, 56, **96**	5.5, 5.7	native Page, electroelution 2-D-PAGE, Anion exchange chromatography (Mono Q)	Moog and Brüggemann, 1992b Moog et al., unpublished
Lycopersicon esculentum roots	FeCit		28, **34**	5.5, 5.8, 6.2	preparative IEF	Holden et al., 1991
Lycopersicon esculentum roots	FeCit	800	27, 34, 38, 55, 64, 66	5.5, 5.8, 6.2	IEF, electroelution, SDS-PAGE	Holden et al., 1992
Lycopersicon esculentum roots	FeCit		27, 29, **35**		Chromatofocussing, SDS-PAGE	Holden et al., 1994
Strategy II						
Zea mays roots	FeCN	35400	28, 40, **44**		Dye-ligand affinity chromatography, SDS-Page	Luster and Buckhout, 1988

[a]Specific activity [nmol min^{-1} (mg protein)$^{-1}$].

[b]Prominent bands printed in bold.

[c]Prior to any protein purification method, the enzyme was solubilized from PM by detergent.

Table 5. Characteristics of Fe^{3+}-EDTA reduction by root plasma membranes

Species	Growth conditions +Fe/-Fe	NADH SA[a]	NADH Stimul. (-fold)	NADH Latency[b]	NADPH SA[a]	NADPH Stimul. (-fold)	NADPH Latency[b]	Km (μM) Fe^{3+} Chelate	Km (μM) NADH	pH Optimum	Assay condions (NAD(P)H conc; pH; detergent)	References
Strategy I												
Lycopersicon	+Fe	293		85	18			200	230	6.8	0.5 m*M*; 6.8;	Brüggemann et al.,
esculentum	−Fe	463	1.6	88	14	0		220	225	6.8	0.02% TX 100	1990
Plantago	+Fe	69		79	6			172		6.8	0.3 m*M*; 7.0;	Schmidt et al., 1990
lanceolata	−Fe	90	1.3					178	200	6.8	0.02% TX 100	
Lycopersicon	+Fe	74									0.16 m*M*; 6.0	Holden et al., 1991
esculentum	−Fe	98	1.3								0.03% TX 100	
Gossypium	+Fe	1812									0.1 m*M*; 7.0;	Chosack et al., 1991
hirsutum	−Fe	3816	2.1								0.05% TX 100	
Phaseolus	+Fe	83		51	24		13	300	223	6.8	0.75 m*M*; 7.0;	Schmidt and Janiesch,
vulgaris	−Fe	214	2.6	52	27	1.1	15				0.02% TX 100	1991; Schmidt, 1992
Geum	+Fe	105		45					107	7.0	0.77 m*M*; 7.0;	Schmidt, 1992
urbanum											0.02% TX 100	
Plantago	+Fe	37		69				46			0.77 m*M*; 7.0;	Schmidt, 1992
maritima											0.02% TX 100	
Vigna unguiculata	+Fe	120–180						151		6.8	0.5 m*M*; 6.5;	Brüggemann et al.,
											0.02% TX 100[c]	1993
Beta vulgaris	+Fe	160			50				23		0.5 m*M*; 7.0;	Susin et al., 1993
	−Fe	380	2.4		130				21	7.0	0.02% TX 100	
Cucumis sativus	−Fe	129		88						7.0	0.5 m*M*; 7.0;	Walter et al., 1993
											0.015% TX 100	
Strategy II												
Hordeum	+Fe	128		68	42			120	125	6.8	0.5 m*M*; 6.8;	Brüggemann and Moog,
vulgare											0.02% TX 100	1989

[a]Specific Activity [nmol Fe min^{-1} (mg protein)$^{-1}$].

[b]If data available, latency was calculated by the equation: [(rate with detergent)-(rate without detergent)] × 100 (rate with detergent)$^{-1}$.

[c]Assayed in the absence of oxygen.

transport and ionophores. These authors reported a 70–90% inhibition of FeCN-R after preincubation with 50 μM *p*-chloromercuriphenyl sulfonate (PCMS) in the presence of detergent (0.01% TX 100). This indicated the presence of a sulfhydryl group on the part of the redox protein that faces the cytoplasmic side. pH optima were found from 6.8 to 7.5 or over a broad range (Holden et al., 1991) without a specific optimum. Evidence for the existence of more than one FeCN-R activity comes from the occurrence of biphasic kinetics in maize root PM (Bourdil et al., 1990). This hypothesis is supported by differential elution of FeCN-R activities from Cibachrom blue affinity columns (Luster and Buckhout, 1988) and different pI values of FeCN-R activities of 4.8 and 5.7 (Table 4) reported by Holden et al. (1991). In attempts to identify the molecular weight by using partially purified FeCN-R activity, different protein patterns were found, reflecting obviously the different purification methods of the different laboratories. However, comparison of the molecular weights of the obtained polypeptides suggests that (one) FeCN-R at least contains a subunit in the 28–35 kDa region.

The comparison of PM from iron-sufficient and iron-deficient plants revealed another difference between FeCN-R activities, depending on whether NADH or NADPH was used as electron donor. While NADH-FeCN reduction in PM from iron-deficient plants generally increased as compared to iron-sufficient controls, most authors did not detect a substantial increase in activity when NADPH-FeCN reduction was tested (Table 3). Only Schmidt and Janiesch (1991b) reported a 1.7-fold increase in NADPH-FeCN reductase activity for PM from iron-stressed bean roots. For wildtype tomato, NADH-FeCN-R activity was increased 2- to 3-fold by iron deficiency, while NADPH-FeCN-R activity remained nearly unaffected by the nutritional status of the plant (Buckhout et al., 1989; Valenti et al., 1991), again indicating two different FeCN-R activities. Valenti et al.(1991) concluded from their data that PM-bound NADPH-FeCN and duroquinone reductase activities were performed by the same enzyme and Chosack et al. (1991) demonstrated that PM-bound duroquinone reductase activity did not increase under iron starvation. Consequently, these data also provide evidence for at least two PM-bound FeCN-R activities. In contrast to the definitions of the so-called "Standard" and "Turbo" system, the NADPH-FeCN-R is not generally activated by iron stress, but the NADH-FeCN-R responds to iron deficiency with increased activity. To evaluate if NADH-FeCN-R activity is part of or identical with the increased ability of the "Turbo" system to reduce FeCh, it is necessary to analyze the data available on FeCh reduction.

Characteristics of ferric chelate reduction

Investigations on PM bound ferric chelate reductase have mostly been carried out with either FeEDTA or FeCit as electron acceptors (Tables 5 and 6). For both iron chelates, the reduction rates are in the same order of magnitude when the same plant species is examined. The pH optima range from 6.5 to 7.0, frequently determined to be 6.8, and there is a clear preference for NADH over NADPH as the electron donor. Reduction rates with NADPH on average reach 5–10% of the rates measured with NADH as electron donor. Detergent latencies ranged from 51 to 97% and did not differ when PM vesicles from iron-sufficient and iron-deficient plants were compared. With FeCit, there were always lower K_m values (30–50 μM) than with FeEDTA (120–300 μM), but this difference is rather due to chemical dissimilarities of the two chelates than to different enzymes. From the ecophysiological point of view, a high specificity for a certain FeCh would not be expected, since the natural substrate of the enzyme occurring in the soil will be a cocktail of many different FeCh.

Data on inhibition of FeCh reduction are scant. Brüggemann and Moog (1989) reported an 80% inhibition with 60 μM PCMS indicating the involvement of sulfhydryl groups in the reaction chain. Holden et al. (1991) demonstrated the inhibitory effects of six different triacine dyes, which can act as competitive inhibitors for NADH. Inhibitors of mitochondrial electron transport had no effect on FeCh reduction (Holden et al., 1992). A specific inhibitor for the enzyme has not been identified so far.

In contrast to the proposed model of the "Turbo" system, FeCh-R is constitutively present in all plants investigated so far. The activity is inducible upon iron-deficiency in "Strategy I" plants, and it is also present in "Strategy II" plants when grown under iron-sufficient conditions (Table 5). When root PM from iron-deficient plants is compared to that of iron-sufficient controls, with both iron compounds the reduction activity was 2- to 3-fold increased when NADH was present in the assay. In contrast, no increased reduction rates were found when NADPH was tested. It is concluded that NADPH-FeCN-R can also reduce FeCh to some extent, but the activity is not inducible by iron deficiency.

Table 6. Characteristics of Fe^{3+}-Citrate reduction by root plasma membranes

Species	Growth conditions +Fe/-Fe	NADH SA[a]	NADH Stimul. (-fold)	NADH Latency[b]	NADPH SA[a]	NADPH Stimul. (-fold)	NADPH Latency[b]	Km (μM) Fe^{3+}-Chelate	Km (μM) NADH	pH Optimum	Assay conditions (NAD(P)H conc; pH; detergent)	References
Lycopersicon esculentum	+Fe	121		97	3.7						0.16 m*M*; 7.0; .025% TX 100	Buckhout et al., 1989
	−Fe	234	1.9	96	4.2	1.1						
Lycopersicon esculentum FER	+Fe	57									0.15 m*M*; 7.5; 1% LPC	Valenti et al., 1991
	−Fe	179	3.1									
Lycopersicon escul. fer T3820	+Fe	157									0.15 m*M*; 7.5; 1% LPC	Valenti et al., 1991
	−Fe	199	1.3									
Lycopersicon esculentum	+Fe	331						31			0.16 m*M*; 6.0; 0.03% TX 100	Holden et al., 1991, 1992
	−Fe	626	1.9					35		6.5		
Vigna unguiculata	+Fe	120–180						50	62	6.8	0.5 m*M*; 6.5; 0.02% TXl00;	Brüggemann et al., 1993

[a]Specific Activity [nmol Fe min^{-1} (mg protein)$^{-1}$].

[b]If data available, latency was calculated by the equation: [(rate with detergent)-(rate without detergent)] × 100 (rate with detergent)$^{-1}$.

[c]Assayed in the absence of oxygen.

An apparent contradiction exists between the amount of stimulation of in vivo and in vitro FeCh-R activity. While in intact roots, iron deficiency may stimulate the activity by up to tenfold or more, in isolated PM usually much smaller increases were found. This may be the result of dilution: the induced activity is only localized at distinct root zones (ca 1 cm above the apex) and possibly bound to certain cell types, whereas PM preparations are homogenized from all cell types present in the roots.

Studies on biochemical characteristics of FeCh-R were done with the detergent- solubilized and partially purified enzyme. As summarized in Table 4, native IEF followed by enzyme activity staining revealed two or three pI values of 5.5, 5.8 (and 6.2), indicating either degradation products or different isoforms. PM bound FeCh-R has not been purified to homogeneity yet. From the comparison of the molecular weights reported for the partially purified enzyme by different purification methods (Table 4), it can be concluded that the enzyme probably at least contains subunits of 29 and 35 kDa. Holden et al. (1994) concluded from their studies with the partially purified enzyme that FeCh-R has no glycosylated components, in contrast to many other PM proteins.

Comparing the characteristics of NADH-FeCN-R and NADH-FeCh-R of roots, five concrete similarities for both activities can be stated (see Tables 3, 4, 5, 6):

- 2- to 3-fold induction by iron deficiency
- high latencies (at least one cytoplasmic binding site)
- pI around 5.6
- pH-optimum around 6.8
- identical MW in the 28/29 and 34/35 kDa region

Furthermore, in their attempt to separate and purify the different redox systems from iron-stressed tomato roots by gel filtration, Valenti et al. (1991) lost the FeCh-R activity during chromatography, but found a third NADH-FeCN-R peak, which was ten times increased as compared to non-stressed roots. Taking the five identical properties into account, this result can be interpreted as an indication that FeCN and FeCh are reduced by the same inducible enzyme, but FeCh reduction requires a component which is cut off or inactivated during the separation procedure and is not necessary for FeCN reduction. This argument leads to the identity of (one) NADH-FeCN-R with the FeCh-R and associates the FeCN activity to the "Turbo" system.

In evaluating the stated similarities, two points should be made. First, the in vitro pH-optimum of 6.8 has not been demonstrated to be the one for the electron transfer from the enzyme to the ferric compound. When the data on in vivo pH optima of the FeCh-R reaction of 5.0–5.5 (Table 1b), on the apoplastic side are taken into account, it is most probable that the pH optimum at 6.8 is the one for the NADH-dehydrogenase reaction at the cytoplasmic side. This implies that the electron transfer from the enzyme to the ferric compound is the limiting step over a broad pH range in vivo (i.e. between pH 5 and 7), whereas the NADH oxidase reaction always takes place at the same (optimal) cytoplasmic pH (i.e. 6.8–7.0). Second, the agreement between different laboratories on the molecular weight of presumed subunits of the FeCH-R does not imply that there is positive evidence for a functional association of these polypeptides to an iron reductase system.

On the other hand, there are results indicating that there exists an NADH-FeCN-R system different from the FeCh-R system. In their study, Holden et al. (1991) tested a series of triazine dyes to inhibit PM FeCh-R and FeCN-R from iron-deficient tomato roots. Triacine dyes are known to act as competitive inhibitors for NADH. The pattern of inhibition was different between FeCh-R and FeCN-R, indicating that the NADH binding site for FeCh reduction is not the same as at least one NADH binding site for FeCN reduction, thus belonging to two different enzymes. Furthermore, in their study preparative IEF resulted in a predominant peak at pH 4.8 for FeCN-R activity, not correlating with FeCh-R activity (although this may be the peak of a FeCN reducing degradation product of FeCh-R). The proposal that NADH-FeCN reduction and FeCh reduction are mediated by two distinct enzymes, the latter one also being able to reduce FeCN to some extent, is supported by results obtained by Schmidt (1993, 1994). The author demonstrated that the in vivo reduction of FeCN and FeEDTA is inhibited by the translation inhibitor cycloheximide in iron-deficient and iron-sufficient bean and *Plantago lanceolata* roots. From the pattern of turnover rates the existence of at least two different reductase systems could be concluded.

From the present state of knowledge on the level of the PM data, as reflected in the contradictory discussion above, it is not possible to decide if there is more than one reductase system inducible under iron-deficiency. Nevertheless, from the data available now, it seems about time to actualize the definition of the "Turbo" and "Standard" system into a 1994 version:

Table 7. Characteristics of iron reduction by non-root plasma membranes

PM source	NADH		NADPH		Km (μM) Fe-comp.	Km (μM) NADH	pH Optimum	Assay conditions (NAD(P)H conc; pH; detergent)	References
	SA[a]	Latency[b]	SA[a]	Latency[b]					
FeCN									
Glycine max hypocotyl	105–375		339		28.6	77	6.3–7.8	0.05–0.1 m*M*; 7.0	Barr et al., 1986
Glycine max hypocotyl	83–450	47–64	170		50[c]	50[c]		0.1 m*M*; 7.0 0–0.01% TX 100	Sandelius et al., 1986
Raphanus sativa germinating seeds	330		78					0.1 m*M*; 5.5	Hassidim et al., 1987
Brassica oleracea inflorescences	860	92						0.25 m*M* 7.3 0.025% TX 100	Askerlund et al., 1989
Spinacea oleracea leaves	1120	93						0.25 m*M*; 7.3 0.025% TX 100	Askerlund et al., 1989
Beta vulgaris leaves	1560	64			33			0.25 m*M*; 7.3 0.025% TX 100	Askerlund et al., 1988
Nicotiana tabacum leaves	349							0.15 m*M*; 8.0 0.03% TX 100	Valenti et al., 1989
Fe^{3+}-EDTA									
Glycine max hypocotyl	56		40					0.05–0.1 m*M*; 7.0;	Barr et al., 1986
Vigna unguiculata leaves	480–780			173		6.8		0.5 m*M*; 6.5; 0.02% TX 100[d];	Brüggemann et al., 1993
Fe^{3+}-Citrate									
Vigna unguiculata leaves	435				137	22	6.8	0.5 m*M*; 6.5; 0.02% TX 100;	Brüggemann et al., 1993

[a]Specific Activity [nmol Fe min^{-1} (mg protein)$^{-1}$].

[b]If data available, latency was calculated by the equation: [(rate with detergent)-(rate without detergent)] × 100 (rate with detergent)$^{-1}$.

[c]Authors report K_m values for NADPH as electron donor of K_m (FeCN) 100 μM and K_m (NADPH) 20 μM.

[d]Assayed in the absence of oxygen.

Standard system At the root cell PM, there are at least two constitutive transmembrane (ferric) reductase activities which can reduce external electron acceptors like FeCN and FeCh, using either NADH or NADPH as electron donors. The natural electron acceptors of these systems may be oxygen and FeCh. The biological role can be seen in iron uptake under iron-sufficient conditions and other functions like membrane polarization, control of cell elongation or proliferation etc.

Turbo system Upon iron deficiency, the activity of the NADH-dependent constitutive iron reductase system is increased either by enzyme activation or by induced protein synthesis. This inducible redox activity is located on the plasma membrane in distinct root zones of dicotyledonous and monocotyledonous, non-graminaceous plants. It reduces apoplastic FeCN and FeCh with electrons delivered by cytoplasmic NADH. The physiological function is the mobilization of iron for uptake by the roots.

Iron reduction by PM from non-root tissue

The capability of PM to reduce FeCN seems to be an ubiquitous feature of higher plant cells, since such activities have been reported for stem, leaf and flower tissues so far (cf. Table 7). The specific activities were similar (or somewhat higher) to those obtained with root PM, and showed similar biochemical characteristics (pH-dependence, K_m, latency). The first report was published by Barr et al. (1986), who studied redox reactions catalyzed by soybean hypocotyl PM isolated by phase partition and by free-flow electrophoresis and used mainly FeCN as electron acceptor. The authors also demonstrated for the first time that plant PM contain FeCh-R activity with NADH as electron donor, using FeCit and FeEDTA. The report by Sandelius et al. (1986) gives the only set of K_m combinations for NADPH- and NADH-dependent FeCN reduction available so far, i.e. 50 μM NADH and 20 μM NADPH, respectively. For a recent review on the general features of FeCN reduction of PM in plant cells see Barr and Crane (1991).

The electrogeneity of the trans-PM electron transport to FeCN was confirmed by Hassidim et al. (1987) by thiocyanate accumulation of ascorbate-loaded (right-side-out) PM vesicles from germinating radish seeds during FeCN reduction. Valenti et al. (1989), who were studying the duroquinone reductase activities of the PM, reported that both PM duroquinone reductase and NADH-FeCN-R activity of tobacco leaves were stimulated by the application of a bacterial protein-lipopolysaccharide complex to the plants, probably a pathogen-defense reaction. Other leaf PM preparations from spinach and sugarbeet proved to contain the FeCN-R activity (Askerlund et al., 1988a, 1991). These results led to the question of whether iron uptake by leaf cells may be mediated by a PM redox system as it is known for roots (i.e. Bienfait, 1989; Böttger et al., 1991). The need for such a redox system arises from the finding that the transport form of iron in the xylem is the 1:1 FeCit complex (Brown and Jolley, 1986). First evidence for the existence of such a redox system at the PM of mesophyll cells was presented by Brüggemann et al. (1993), who showed that an obligatory reduction step is a prerequisite for iron uptake by leaf mesophyll of *Vigna unguiculata*. The redox activity with typical enzymatic characteristics was found testing intact leaf mesophyll and purified leaf mesophyll PM. A FeCh-R activity was identified which provided the necessary enzymatic activity for the reduction. This redox activity was stimulated by photosynthesis. However, in vivo the enzymatic activity may be aided by photoreduction of FeCit by blue light, as long as this process takes place in the vicinity of the PM and uptake of the released Fe^{2+} occurs faster than reoxidation by oxygen (cf. discussion by Bienfait and Scheffers, 1992). Recently, De la Guardia and Alcántara (1993) confirmed that intact sunflower leaf mesophyll also contained a (photosynthesis-stimulated) FeCh-R activity. To summarize the data on non-root tissue, it is evident that FeCN-R and FeCh-R are integral features at least of dicotyledonous plant leaves. Today the physiological role of these enzymes for iron uptake and acquisition is an open question.

Iron reduction by algae

Studies on PM-bound redox enzymes in algae are scarce and mainly focussed on measurements with intact systems. Evidence for the existence of a trans-PM electron transport comes from electrophysiological studies in the Characean *Lampothamnium papulosum* (Thiel and Kirst, 1988). This alga reduced external FeCN with a Michaelis-Menten kinetic, exhibiting a K_m of 0.2 mM. During FeCN reduction, proton extrusion, a reversible PM depolarization and a delayed decrease of the membrane resistance could be recorded. These data were interpreted in terms of the initial phase of the transmembrane reduction being an

electrogenic process. The shift of the cells towards a depolarized state by the chemically-induced outward current would then lead to the observed decrease of the membrane resistance and finally result in a (K^+-) diffusion dominated membrane potential.

In several studies on the planctic diatom *Thalassiosira weissflogii*, a (presumably) PM-bound redox system was identified, which enables this organism to reduce external electron acceptors like Cu^{2+} complexes or FeEDTA (Anderson and Morel, 1980; Jones et al., 1987). Cu^{2+} bathocuproine disulfonate and other complexes were reduced under Michaelis-Menten kinetics with K_m around 10 μM, independently of free Cu^{2+} concentration. This indicated that the redox activity was not responsible for copper uptake, since the latter reaction is strongly dependent on free Cu^{2+} concentration and total copper concentrations in seawater are far below the observed K_m. However, testing different transition metal complexes as electron acceptors, the authors could present evidence that the E'_O of the cell surface-bound redox system ranges between +94 and -100 mV. In a subsequent study, they showed cross-reactivity of the diatom cell surface with anti-nitrate reductase (NR) antibodies and an inhibition of Cu^{2+} reduction by the same antibody, indicating that the redox process is mediated by an enzyme closely related to soluble *Chlorella* NR (Jones and Morel, 1988). In this context, it should be noted that soluble NR from higher plants also reduces FeCh (Redinbaugh and Campbell, 1983). In a similar approach, Tischner et al. (1989) showed cross-reactivity of a 60 kD protein associated with isolated PM from the Chlorophycee *Chlorella sorokiniana* with anti-spinach NR antibodies, which is the first evidence for a redox component in purified PM from algae.

In vivo FeCN reduction, which was stimulated by iron deficiency, has been reported for the green alga *Monoraphidium braunii* (Corzo et al., 1991). Earlier, Allnutt and Bonner (1987a,b) presented the first evidence for the induction of in vivo FeCh-R activity by iron deficiency in *Chlorella vulgaris* (strain 11468). They showed a close correlation of the time dependencies of iron uptake by the organism and FeCh reduction. Unfortunately, no data on the concentration dependencies of the reactions were presented. The most efficient ferric complexes in their studies were the siderophore complexes ferrioxamine B and ferric rhodotorulate, while FeCit was reduced at much lower rates. Interestingly, the authors found strong inhibitory effects of the uncoupler carbonyl cyanide m-chlorophenylhydrazone and the inhibitor of the PM proton pump, vanadate, on both ferric reduction and iron uptake. This indicates that cell wall acidification may be necessary for the redox reaction. Until now this redox activity has not been characterized further. Own studies on FeEDTA reduction by intact *Chlorella pyrenoidosa* (strain 211-8b) showed that this species also reduced FeCh at reasonable rates in vivo, but that the activity did not follow enzymatic kinetics (Fig. 1). Instead, reduction rates increased linearly with concentration, suggesting that external FeCh only reacts as an (unspecific) electron trap. Thus, in *Chlorella* reduction rates may only be limited by the capacity for electron donation at the cytoplasmic site and the alga exerts no enzymatic control over the trans-membrane electron flux.

In conclusion, there is abundant evidence for the existence of transmembrane electron transport systems in algae, which may serve in the reduction and acquisition of extracellular ferric iron. However, in the group which systematically is the closest one to higher plants, the *Chlorophyceae*, no PM-bound FeCh-R has been characterized so far.

Molecular aspects of the redox reaction

Since the ferric chelate reductase has not been isolated to homogeneity in the active form from any source so far, our knowledge about its molecular nature is very limited. However, there is experimental evidence for several molecular features of the reaction which will be summarized in this chapter.

Transmembrane electron flow and electrogeneity

In a direct attempt to assess transmembrane electron transport using ascorbate-loaded right-side-out PM vesicles from sugarbeet leaves, Askerlund and Larsson (1991) showed reduction of external FeCh and FeCN in the absence of further external reductants. When an NADH-regenerating system (NAD^+ and alcohol dehydrogenase) was trapped inside the vesicles, the system reduced external dichlorophenol indophenol sulfonate upon addition of ethanol, but not FeCN. In contrast, right-side-out PM vesicles from soybean hypocotyl, loaded with NADH by electroporation, reduced external FeCN (Böttger, 1989). Despite these somewhat contradictory in vitro results, observed with different plant materials, the following line of arguments is, in our opinion, convincing to reasonably assume transmembrane electron flow as the source of redox equiv-

alents for apoplastic FeCN-R and FeCh-R: Inside-out PM vesicles revealed only a low latency for FeCN-R upon detergent addition and, thus, contain binding sites for both the electron donor and the acceptor on the cytoplasmic side. In contrast, the high latency of the reaction in right-side-out vesicles proves that the full electron transfer reaction cannot occur at the apoplastic side and that at least one reaction partner has to reach the cytoplasmic face of the PM (Askerlund et al., 1988). Practically identical K_m (FeCh) in vivo and in vitro show, that the in vitro measurements in the presence of detergent indeed reflect the very FeCh-R activity, which in vivo definitely occurs at the apoplastic side (Brüggemann et al., 1990). The dependence of in vivo FeCh-R activity by leaf mesophyll tissue on photosynthesis shows that at least in this case the cytoplasmic redox state controls an apoplastic reduction activity (Brüggemann et al., 1993). Furthermore, the transport of electrons from cytoplasmic NADH to extracellular ferric iron is equivalent to a transmembrane current which has experimentally been shown in the fundamental electrophysiological study of Sijmons et al. (1984a) for iron-deficient bean roots. In their study, iron deficiency induced a strong increase of reversible root PM depolarization upon the addition of both FeCN and FeEDTA, followed by a slight repolarization. These and other results imply that at least the inducible FeCh-R reaction is not obligatorily coupled to proton export in an electroneutral fashion, as suggested by Böttger and Lüthen (1986) for the "Standard" system. The latter authors showed that the application of FeCN to corn roots led to proton extrusion during the reduction of the extracellular oxidant. However, experimental evidence from several laboratories rather suggests that the primary effect of electron extrusion by the reductase(s) is followed by a secondary stabilization of the charge balance. This can be achieved either by increased activity of the H^+-ATPase or by a K^+ outward current, as extensively reviewed by Prins and Elzenga (1991).

The cytoplasmic site

Electron source Our knowledge about this part of the FeCh-R is confined to the identification of NADH as the electron donor in vitro (cf. chapter: Characteristics of ferric chelate reduction). Equimolar concentrations of NADPH yielded rates of only a few percent as those obtained with saturating NADH (cf. Table 3). These results were in apparent contradiction to the findings of Sijmons et al. (1984a), who showed that iron deficiency increased the steady-state NADPH:$NADP^+$, but not the NADH:NAD^+ ratio in bean roots in vivo and reported NADH concentrations around 1 nmol g^{-1} FW or less. This may roughly correspond to 10 μM in the whole cytoplasm with an unknown, and rather lower concentration in the cytosol, if it is assumed that the cytoplasm makes up 10% of the whole cell volume. Data obtained by non-aqueous fractionation of photosynthesizing tissue even indicate that cytosolic NADH concentrations may lie around 1 μM or less (Dr. H. Heldt, pers. commun.). Sijmons et al. (1984b) reported that the NADPH pools, corresponding to 200-300 μM in the cytoplasm, decreased significantly upon extracellular FeCN reduction, while the (low) NADH pools remained nearly unaffected. In addition, the Michaelis-Menten kinetics obtained in vivo for extracellular FeCh-R indicate that the enzyme operates below saturation with respect to the dehydrogenase site. To solve this discrepancy, there are some possibilities, of which we would like to name two: It can be postulated, that a transdehydrogenase enzyme, be it a distinct enzyme or a loosely bound subunit of the FeCh-R, locally increases the NADH concentration by making use of the high NADPH:$NADP^+$ ratio. This component may be lost during PM preparation, leaving behind a purely NADH-dependent enzyme. It should also be noted that the measured pool concentrations only reflect the time- and volume-average steady-state conditions at an unknown rate of flow through the pools. Thus, an NADH-dependent reductase, apparent-

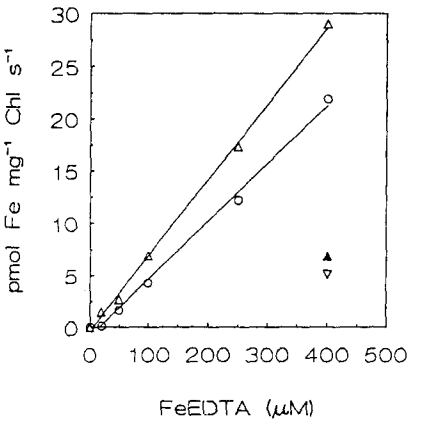

Fig. 1. FeEDTA reduction by *Chlorella pyrenoidosa* strain 211-8b. Cells were grown in Pirson and Ruppel (1962) medium in the presence of 25 μM FeEDTA (△, ▲, ▽, late exponential phase) or in iron-free medium for two weeks (o, stationary phase); (△, o) rates in the presence of 200 μM BPDS; (▲) rate in the absence of BPDS, measured by BPDS addition after pelleting the algae from the reaction solution; (▽) rate due to reductant release.

ly operating at or even below its K_m, may in fact well be working near saturation or may be regulated by the rate of electron flow from NADPH to NADH. Another explanation would be that the enzyme in vivo operates with NADPH as electron source, but may tolerate NADH as reductant. During the isolation of the PM or as a result of the detergent in the assay, the specificity of the dehydrogenase reaction is lost, turning it into an NADPH-ineffective form, while the ability to use NADH is unaffected.

pH-dependence It is very probable that the pH optima measured in vitro reflect the situation at the cytoplasmic site, since they differ significantly from those obtained with intact roots (cf. Tables 2, 5). Thus, the dehydrogenase reaction shows a pH optimum around pH 6.8, which is very similar to the cytoplasmic pH.

Binding sites for electron acceptors In an elegant study using both inside-out and right-side-out PM vesicles from sugar beet leaves, Askerlund et al. (1988a) showed that the cytoplasmic site is capable of NADH-dependent FeCN and cytochrome c (Cyt c) reduction in the absence of detergent. The presence of only one NADH/FeCN reaction site at the cytosolic side of the PM was supported by kinetic measurements, which revealed one single K_m for NADH and FeCN, respectively. However, for the reaction pair NADH / Cyt c, a much lower K_m (NADH) was obtained. It was concluded that the NADH-FeCN-R and the NADH-Cyt c-R activities are caused by different enzymes or that the differences in K_m (NADH) result from a regulation of the dehydrogenase activity by the redox state of an electron chain, in which FeCN and Cyt c reduction occur at different sites.

The integral membrane component(s)

In search for integral PM proteins that contain electron transport function, evidence came from two different laboratories, that plant PM contain at least two cytochromes with high (+100 – +150 mV) and low (-50 mV) midpoint potentials, the latter one of the b_5 type (Askerlund et al., 1989; Asard et al., 1992). The first one is ascorbate-reducible and probably identical to the blue-light-reducible Cyt. It may be involved in the blue-light perception by the PM (Askerlund et al., 1989). The low midpoint potential Cyt, which showed redox and spectral similarities to Cyt b of NR, may be responsible for the PM-dependent reduction of Cyt c in the Askerlund experiments (Askerlund et al., 1989). NR has also been reported by (low) enzymatic activity and cross-reactivity with anti-NR IgG in PM preparations from barley and corn roots (Ward et al., 1988, 1989). However, these findings could not be reproduced, when PM from spinach leaf, sugar beet leaf or tomato root were analyzed with a different anti-NR antibody (Askerlund et al., 1991; Brüggemann et al., 1990). Additionally, evidence against a Cyt nature of FeCh-R came from an experiment by Holden et al. (1994), who found no comigration of heme-staining and FeCh-R activity staining in native IEF of root PM proteins. At present, no convincing theory on the nature of an integral, electron transporting component can be formulated. It should, however, be noted that the isolation methods employed so far rule out the possibility that the transmembrane electron transport is mediated by a membrane-soluble low molecular weight component like quinones.

The apoplastic site

There is abundant evidence from the in vivo studies, that both FeCN and FeCh can be reduced at the apoplastic site of the FeCh-R. Since the FeCh-R activity is much less stable than FeCN-R and can be lost during purification, it seems reasonable to assume that, independently of an apoplastic FeCN reduction site, an additional component exists which is responsible for binding of and electron transfer to the chelate. At this point, the question of the "true" electron acceptor of the FeCh-R should be discussed. Firstly, there is experimental evidence against the (possible) hypothesis that "free" aqueous Fe^{3+} is the electron acceptor. In a comparative study, Chaney (1989) showed that ferric chelators with extremely differing stability constants, ranging from $10^{18.2}$ to $10^{31.2}$ M^{-1} revealed very similar reduction rates and K_m with intact peanut roots, i.e. regardless of changes in free Fe^{3+} concentrations by a factor of $10^{6.5}$. Of the four chelates tested, those with a net charge of -1 at assay pH 6.0 (as opposed to net charges of 0 or -2) gave the highest reduction rates. Schmidt and Janiesch (1991) showed that the apparent K_m of in vivo FeEDTA reduction by intact *Geum urbanum* roots increased from 100 to 400 μM with increasing assay pH between pH 4 and 6, that is in the pH range in which an FeEDTA solution mainly contains chelated species of slowly changing charge, and from 400 to 800 μM between pH 6 and 7, where FeEDTA releases 50% of its chelated iron (Chaney, 1988). This result confirms the rational, that FeCh (and not Fe^{3+}) is the electron acceptor.

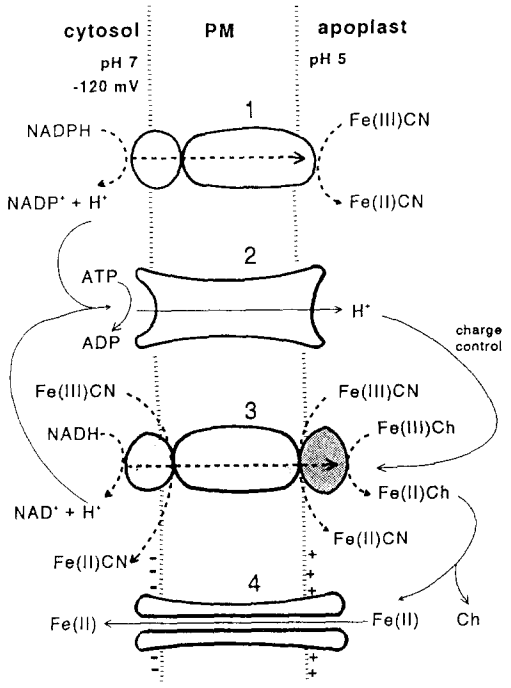

Fig. 2. Model of ferric reduction and uptake at the PM. 1) NADPH-FeCN-R; 2) H^+-ATPase; 3) NADH-FeCh-R: ▒ trans-dehydrogenase domain (subunit), ■ ferric chelate binding domain (subunit); 4) Cation channel. [ch = chelate; (- - -) electron transport and redox reactions; (—) all other transport and reactions].

Furthermore, it shows that apart from the true chelate concentration, net charges play a significant role in the determination of the reaction rates. This argument has been strengthened by Toulon et al. (1992), who showed that not only the pH, but also the buffering capacity of the reaction solution strongly influenced the reaction rates, even when the pH was maintained constant. This points to the role of the microenvironment of the apoplasmatic FeCh-R reaction site and the actual pH in the unstirred layer around the apoplast, which is controlled by the bulk pH, the buffering capacity, and the activity of the PM H^+-ATPase. The involvement of the H^+-ATPase in the FeCh-R reaction, as shown for *Chlorella* (Allnutt and Bonner, 1987b), has also been demonstrated for higher plants by Toulon et al. (1992) and Schmidt (1993). They inhibited the H^+-ATPase with vanadate and found a strong decrease of in vivo FeCh-R activity. Although a direct effect on a possible phosphorylation site of the FeCh-R cannot be ruled out *a priori*, the most likely interpretation of these results is that the acidification of the direct surrounding of the FeCh-R is a prerequisite for the reaction. Since FeEDTA carries a -1 charge between pH 3 and 6 and buffers only below pH 3, where it is decharged (Toulon et al., 1992), a single negative charge of the chelate does not hinder the reaction. The pH effect on FeEDTA reduction may thus rather be due to changes in the surface and/or enzyme charges, which are influenced by the actual proton concentration at the apoplastic side of the PM. This interpretation is supported by the finding that divalent cations, which mask negative surface charges, strongly increase in vitro reaction rates with isolated root PM (Schmidt, 1992).

As mentioned earlier, the natural transport form of ferric iron in the xylem is FeCit (Brown and Jolley, 1986), which carries one negative charge at pH 3–5 due to the abstraction of the hydroxyl proton (Warner and Weber, 1953). However, above pH 5, a polymer hydroxyl is formed and, at the same time, the complex is charged more negatively (cf. titration curves by Spiro et al., 1967; Toulon et al., 1992). The polymeric iron hydroxyl/citrate does not precipitate immediately, but is kept in solution by a citrate coat (Spiro et al., 1967), until the pH rises beyond 9. These characteristics of aqueous FeCit chemistry may have severe consequences for plants on calcareous soils, as pointed out by Bienfait and Scheffers (1992), since alkalinisation of the xylem sap by HCO_3^- will lead to (soluble) polymer formation. The polymer is no longer accessible to reduction and uptake at the leaf mesophyll PM due to its very slow diffusion rates through the cell wall. It will finally precipitate, especially when the coating citrate is broken down enzymatically or by blue light. However, in the studies on the specificity of iron uptake from FeCh and of FeCh-R by intact leaf mesophyll, a 4-fold preference for FeCit over FeEDTA has been found as judged from K_m (Brüggemann et al., 1993). It seems possible that in the case of leaf PM, where FeCh-R activity is strongly influenced in vivo by the changing cytosolic redox state through photosynthesis, the final electron donor of the FeCh-R has a midpoint potential nearer to the E'_O of FeEDTA (+130 mV) and thus higher than in the case of the roots, where it is supposed to be $\leq$ +80 mV (Sijmons and Bienfait, 1983).

The model

The characteristics summarized above led us to propose the following model of the PM-bound FeCh-R and FeCN-R with the minimum of components required to explain the experimental results (Fig. 2): Under iron-sufficient conditions (Standard system), at least two

independent transmembrane electron transport systems (Fig. 2: 1 and 3) may reduce extracellular FeCN, using specifically either NADPH (1) or NADH (3) as electron source. Of these, the NADH-dependent system is also capable of reducing FeCh at a specific binding domain or subunit. This may have different midpoint potentials in different tissues, as suggested by the different specificities for FeCh in PM isolated from roots and leaves. Under iron-deficient conditions (Turbo system) only the activity of the NADH-dependent system (3) is increased: at the cytoplasmic site, electrons are taken up from NADH possibly by a b-type cytochrome with a midpoint potential of -50 mV, which is FeCN- accessible both from the cytoplasmic and the apoplastic side, the electrons are transferred through a membrane-spanning domain to another domain (subunit), which contains a FeCh binding site and a redox component with a higher positive midpoint potential. The PM H^+-ATPase (2) assists FeCh reduction by acidifying the microenvironment of the FeCh-R and performing charge control over the PM surface, decreasing the repelling of the negatively charged FeCh. After reduction the ligand releases ferrous iron, which may then follow the membrane potential gradient through any divalent cation channel (4) into the cytosol.

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