NADH:Fe(III)-chelate reductase of maize roots is an active cytochrome b_5 reductase

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Abstract Microsomal NADH:Fe(III)-chelate reductase (NFR) of maize roots has been purified as a monomeric flavoprotein of 32 kDa with non-covalently bound FAD. In the presence of NADH, NFR efficiently reduced the physiological iron-chelate Fe(III)-citrate ($K_{cat}/K_{m(Fe(III)-citrate)} = 6.0 \times 10^6$ M^{-1} s^{-1}) with a sequential reaction mechanism. Purified NFR was totally inhibited by the sulfhydryl reagent PHMB at 10^{-9} M, and it could use cyt b_5 as alternative electron acceptor with a maximal reduction rate as high as with Fe(III)-citrate. We conclude that in maize roots the reduction of Fe(III)-citrate is chiefly performed by a cytochrome b_5 reductase, mostly associated with intracellular membranes and in part with the plasma membrane.

Key words: Iron reduction; NADH:Fe(III)-chelate reductase; Fe(III)-citrate; Cytochrome b_5 reductase; *Zea mays*

1. Introduction

Iron is an essential element of plants, being incorporated into heme proteins, iron-sulfur proteins and other iron-containing enzymes. Iron is often abundant in natural soils, although it is mostly present as insoluble Fe(III)-oxides and is consequently unavailable for root adsorption. Free ionic iron is extremely rare under such conditions and soluble iron is chiefly represented by Fe(III)-chelates [1].

Many fungi and microorganisms and most plants, including dicots and non-graminaceous monocots, can take up iron from the soil as ferrous ion (strategy I, as defined by Römheld [2]). An NADH-dependent trans-plasma membrane Fe(III)-chelate reductase is generally believed to be responsible for the reduction of Fe(III)-chelates in the rhizosphere [3], although the purification of such plasma membrane (PM) spanning oxidoreductase has never been achieved. Following chelate splitting, free Fe(II) is then adsorbed by a permease with high affinity for Fe(II) [4]. In these plants the capacity of reducing external iron is strongly stimulated by iron starvation, together with further biochemical and anatomical adaptations such as increased proton extrusion and differentiation of rhizodermal transfer cells [5].

Following a different ecophysiological strategy, grasses meet their iron need by importing Fe(III) as a soluble chelate with particular amino acids known as phytosiderophores, through a specific transport system of the plasma membrane [6]. Phytosiderophores are highly efficient Fe(III) chelators and they are secreted by the plant itself to increase the concentration of available iron forms in the soil. Graminaceous plants respond to iron starvation by an upregulation of phytosiderophore production and secretion, and by increasing the uptake of Fe(III)-phytosiderophore complexes (strategy II [2]). However, in vivo reduction of external Fe(III)-chelates was also observed in strategy II plants [7], and a putative Fe(II)-transporter was recently suggested to be present in rice [4].

Recent experimental efforts to purify NADH:Fe(III)-chelate reductases (NFR) in plants resulted in the identification of 28–35 kDa polypeptides in both monocots and dicots [8]. In particular, a tomato NFR purified from crude microsomes as a 32 kDa protein was suggested to be induced by iron starvation in tomato root PM [9]. An NFR with the same molecular mass and the same substrate and inhibitor specificities was also purified from maize roots, but this was basically insensitive to iron deprivation [10].

The very fact that maize and tomato NFRs are 32 kDa proteins, specific for NADH, and strongly inhibited by low levels of *p*-hydroxymercuribenzoic acid (PHMB) [9,10], suggested a possible relationship to cytochrome b_5 reductase (b_5R) which, in turn, belongs to the ferredoxin:NADP reductase family of flavoproteins [11]. In animal cells b_5R, together with its substrate cyt b_5, participate in fatty acid desaturation [12], cholesterol biosynthesis [13], and drug metabolism [14]. Moreover, the reduced state of hemoglobin in erythrocytes is chiefly controlled by the action of the b_5R-cyt b_5 complex [15], and reduction of ferric chelates, such as Fe(III)-EDTA, by animal b_5R has also been reported [16].

Here it is shown that maize root NFR, suggested to be the major Fe(III)-citrate reductase in this tissue, is indeed an active NADH:cyt b_5 reductase and that further structural and kinetic properties are shared by the two proteins.

2. Materials and methods

2.1. Purification of membrane fractions from maize roots

Maize seedlings (Kw4432×LH213, KWS Italia) were grown for two weeks at 21°C with a 12/12-h light/dark cycle, at a constant PPFD of 250 μmol m^{-2} s^{-1}. Following harvesting of whole roots, the 9000×*g* precipitate and the microsomal fraction (9000–110000×*g* precipitate) were obtained as described [10]. Purified PM and intracellular membranes (ICM) were obtained by two-phase partitioning of the microsomal fraction [17]. The phase system consisted of 6.5% (w/w) polyethylene glycol 3350, 6.5% (w/w) dextran T-500, 5 mM potassium phosphate, 5 mM KCl, 250 mM sucrose, pH 7.8. Upper (PM) and lower phases (ICM) were purified by a three-step partition procedure, diluted 10-fold in 5 mM potassium phosphate, 3 mM KCl, 330 mM sucrose, pH 7.8, centrifuged at 110000×*g*, and resuspended in 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 250 mM sucrose. Purity of PM preparations was checked by marker enzymes [10].

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Abbreviations: NFR, NADH:Fe(III)-chelate reductase; b_5R, cytochrome b_5 reductase; ICM, intracellular membranes; PHMB, *p*-hydroxymercuribenzoic acid; PM, plasma membrane

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2.2. NFR and cyt b_5 purification

Microsomal NFR was purified by the procedure of Bagnaresi and Pupillo [10] with some modifications reported here. Microsomes were solubilized on ice with 2% Triton X-100 (w/v) for 1 h under stirring. Following a 10 000×*g* (60 min) centrifugation, the resulting supernatant was loaded on a Blue Sepharose CL-6B column (FPLC, Pharmacia, Uppsala, Sweden) equilibrated with 15 mM Tris-MES, pH 6.8, 10% glycerol (v/v), 0.1 mM DTT, 1 μM FAD, 1 μM FMN and 0.1% Triton X-100 (w/v) and eluted with a linear salt gradient (0–1.0 M KCl). Active fractions were pooled, concentrated and applied on a Superdex 200 16/60 column (FPLC, Pharmacia) equilibrated with 20 mM TEA-HCl, pH 7.8, 150 mM KCl, 2 mM EDTA, 10% glycerol (v/v), 1 μM FAD, 1 μM FMN and 0.1 mM DTT. The NFR activity peak, corresponding to 28 kDa on gel filtration [10], was concentrated and buffer was exchanged to 20 mM piperazine, pH 9.9. This preparation was loaded on a Mono Q HR 5/5 column (Smart System, Pharmacia) equilibrated with the same buffer and eluted in a pure form with a linear KCl gradient (0–0.35 M KCl, 30 ml).

Cyt b_5 was purified following the procedure of Beck von Bodman et al. [18] from *Escherichia coli* TB-1 cells transformed with a recombinant pUC13 plasmid harboring the gene for the soluble core of rat hepatic cyt b_5 [18]. This *E. coli* strain was a gift of Prof. S.G. Sligar (University of Illinois, Urbana, IL).

2.3. Enzyme activity assays

NFR activity was routinely assayed as NADH:Fe(III)-citrate reductase as in Bagnaresi and Pupillo [10]. Steady-state kinetic data were treated by non-linear regression analysis [19]. Inhibition by PHMB was recorded following 5 min preincubation at room temperature in 50 mM MOPS, pH 7.0, plus PHMB as indicated.

The NADH:cyt b_5 reductase activity of membrane fractions was assayed in 10 mM Tris-HCl, pH 7.0, 0.05% (w/v) Triton X-100, 0.2 mM NADH, 50 μM cyt *c*. Reduction of cyt *c* was followed at 550 nm ($\varepsilon = 20\ mM^{-1}$) before and after the addition of 8 μM cyt b_5. The activity of purified NFR (0.2 nM) with cyt b_5 (2 μM) was qualitatively monitored in 10 mM Tris-HCl, pH 7.0, by following the spectral change of cyt b_5 in the 400–600 nm region upon addition of 0.2 mM NADH. Maximal NFR activity with cyt b_5 was measured by following at 550 nm the reduction of 50 μM cyt *c* carried out by NFR (1.5 nM) in the presence of 0.2 mM NADH and 0–12 μM cyt b_5. NADH:cyt *c* reductase activity by NFR itself (about 3% of the activity observed in the presence of saturating cyt b_5) was subtracted.

All measures were repeated on at least two independent preparations. Data are presented as mean ± S.D.

2.4. Determination of the flavin cofactor and other analytical techniques

The NFR flavin cofactor was identified by fluorometric analysis following heat denaturation of NFR holoprotein [20]. Observed fluorometric spectra at neutral and acidic pH were compared with those of authentic FAD and FMN [19]. Protein measurements, SDS-PAGE and chromatofocusing were performed as described elsewhere [21].

3. Results

The NADH:Fe(III)-citrate reductase activity of maize roots is mostly associated with membrane fractions: on average, 30% of total activity was recovered in 9000×*g* pellets (specific activity: 44 ± 17 nmol min^{-1} mg^{-1}) and 35% in microsomes (9000–110 000×*g* pellets; 135 ± 30 nmol min^{-1} mg^{-1}). Membrane-bound NFR activity was characterized by strong sensitivity to the cysteine reagent PHMB (95% inhibition at 5 μM), whereas NFR activity in the soluble fraction (110 000×*g* supernatants; 52 ± 10 nmol min^{-1} mg^{-1}) was only partly affected under the same assay conditions (56% inhibition).

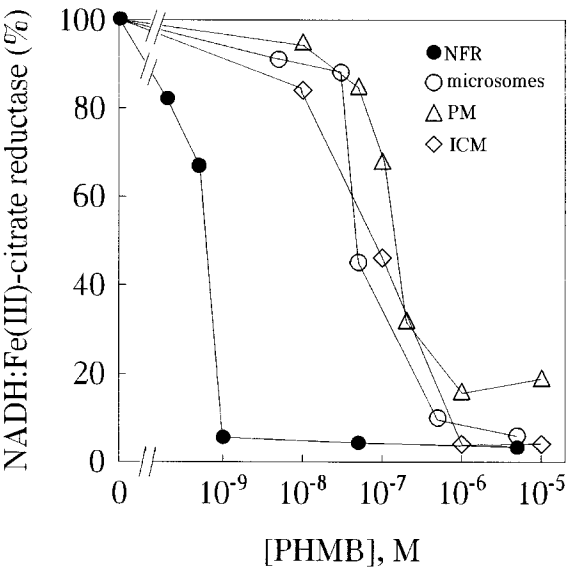

Fig. 1. PHMB inhibition of NADH:Fe(III)-citrate reductase activity of microsomes, PM, ICM and purified microsomal NFR. All assays (1.0 ml) were performed with the same amount of NADH:Fe(III)-citrate activity (1.75 nmol min^{-1}) incubated in MOPS 50 mM, pH 7.0, for 10 min at room tenperature with PHMB as indicated. Total protein concentration in the assays was 15 μg (microsomes), 30 μg (PM), 8 μg (ICM), and 8 ng (pure NFR). The reaction was initiated by adding 0.2 mM NADH, 0.6 mM Fe(III)-citrate and 0.7 mM bathophenanthroline disulfonic acid (standard conditions).

Microsomes were fractionated into highly purified PM and ICM [10,17]. NFR activity was found associated with both vesicle populations, with higher specific activity in ICM than in PM (Table 1). The sensitivity of NFR activity to PHMB was very similar when either PM or ICM or microsomes were tested (Fig. 1). Half inhibition was observed at PHMB levels around 10^{-7} M. Both PM- and ICM-associated redox activities could reduce a heterologous cyt b_5 form, which was purified from *E. coli* cells overexpressing rat liver cyt b_5 [18]. In fact, exogenous cyt b_5 stimulated the NADH:cyt *c* reductase activity of both PM and ICM (Table 1), indicative of the NADH-dependent enzymatic reduction of cyt b_5, followed by the non-enzymatic re-oxidation of cyt b_5 by cyt *c* [22]. In

Table 1
NADH:Fe(III)-citrate and NADH:cyt b_5 reductase activities of different membrane fractions from maize roots

Membrane fraction	NADH:Fe(III)-citrate reductase (nmol min^{-1} mg^{-1})		NADH:cyt b_5 reductase (nmol min^{-1} mg^{-1})	
	control	+PHMB	control	+PHMB
Microsomes	114 ± 19	14 ± 1	–	–
PM	74 ± 16	10 ± 5	48 ± 6	< 0
ICM	215 ± 10	9 ± 1	129 ± 10	< 0

For these assays, each membrane fraction was resuspended in the same buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 250 mM sucrose) and blanks with no enzyme were subtracted. Assays with 25 μg protein ml^{-1} NADH:Fe(III)-citrate reductase were run under standard conditions with the addition of 0.05% (w/v) Triton X-100. NADH:cyt b_5 reductase activity is assayed in 10 mM Tris-HCl, pH 7.0, 0.05% (w/v) Triton X-100, 0.2 mM NADH, by following the reduction of 50 μM cyt *c*, stimulated by 8 μM cyt b_5. Activity is expressed as the difference between cyt *c* reduction rates before and after cyt b_5 addition. Inhibition by 5 μM PHMB.

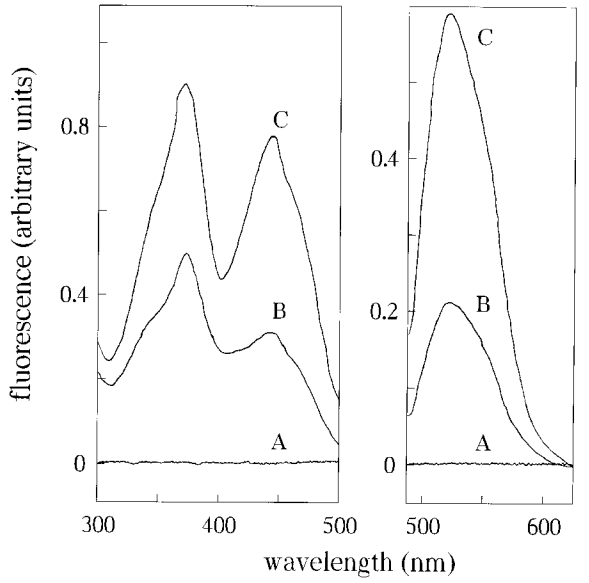

Fig. 2. Fluorometric spectra of purified NFR. Left panel: excitation spectra recorded with an emission wavelength of 525 nm. Right panel: emission spectra following excitation at 468 nm. No fluorescence signal was produced by 0.3 nM purified NFR holoprotein (trace A). The NFR sample (0.3 nM) was heat denatured, ultrafiltrated (Amicon PM10), adjusted to pH 8.0 and analyzed (trace B). Finally, the pH was lowered to 1.4 and resulting spectra are indicated as C.

these membranes, the NADH:cyt b_5 reductase activity was also totally inhibited by PHMB (Table 1). These results are consistent with a single type of oxidoreductase, bound to different types of membranes, responsible for the NADH-dependent and PHMB-sensitive reduction of both Fe(III)-citrate and cyt b_5.

Maize root microsomes have been used as starting material for purification of NFR. Pure NFR, representing the sole PHMB-sensitive NADH:Fe(III)-citrate reductase of this material, consisted of one single polypeptide of 32 kDa (SDS-PAGE) with a pI of 8.5 (chromatofocusing). In good agreement with a monomeric structure of the enzyme, native molecular weight on Superdex 200 was 28 kDa [10].

The possible flavoprotein nature of NFR was investigated by fluorometry. When analyzed in the native state, microsomal NFR gave no fluorescence signal in the expected wavelength range of flavoproteins (Fig. 2, trace A). However, typical flavin fluorescence spectra were recorded following heat denaturation of NFR holoprotein, indicating strong quenching of the flavin fluorescence by the non-covalently bound apoprotein. Excitation spectra of NFR prosthetic group peaked at 373 and 447 nm, and emission spectra showed a maximum at 525 nm (Fig. 2, trace B), closely recalling those of authentic flavins [20]. To discriminate between FAD and FMN, the same measurements were performed at acidic pH, according to the method of Siegel [20]. As a result, the fluorescence intensity of the isolated prosthetic group was strongly increased (Fig. 2, trace C), clearly demonstrating that FAD, and not FMN, was the flavin molecule under study [19]. Since no fluorescence was detectable before NFR denaturation, it can be excluded that the fluorescence signals shown in Fig. 1 are due to exogenous flavins added to buffer solutions during the first steps of NFR purification.

Basic kinetic parameters of purified NFR were obtained by steady-state kinetic analysis in the presence of Fe(III)-citrate as electron acceptor. By varying the concentrations of both NADH and Fe(III)-citrate, linear double reciprocal plots were observed, except for the inhibition detected at very high substrate concentrations. As shown in Fig. 3, plots converged close to the abscissae, strongly suggesting a sequential reaction mechanism [23]. As determined by non-linear regression analysis, K_m values for NADH and Fe(III)-citrate were 96 ± 30 and 36 ± 12 μM respectively, and limiting V_{max} was 403 ± 46 μmol Fe(III)-citrate min^{-1} mg^{-1}.

Purified NFR was very sensitive to PHMB inhibition. Half inactivation of purified NFR was attained at 5×10^{-10} M PHMB, corresponding to a PHMB to NFR molar ratio around 3 under assay conditions (Fig. 1). It is conceivable that the lower sensitivity of membrane-bound NFR to PHMB (Fig. 1) might be due to partial protection given by endogenous reductants in membrane preparations and/or to PHMB sequestration by foreign sulfhydryl groups.

The activity of purified NFR with cyt b_5 as electron acceptor was tested. In the presence of NADH, NFR catalyzed the complete reduction of cyt b_5, as shown by the shift of the Soret band from 410 nm (oxidized) to 422 nm (reduced),

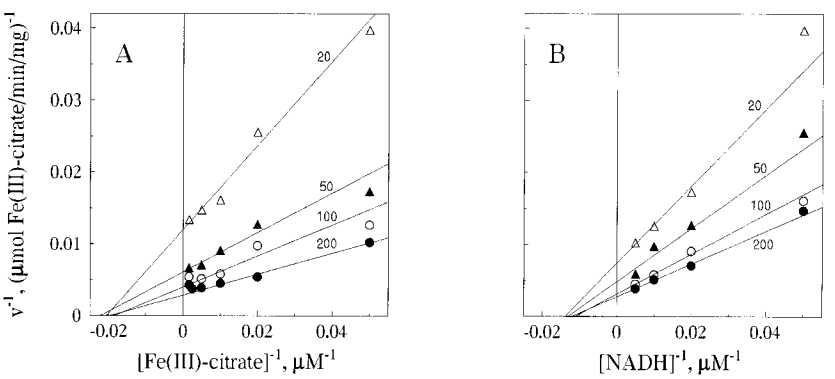

Fig. 3. Double reciprocal plots of NFR activity assayed as NADH:Fe(III)-citrate reductase. Initial velocity measurements were conducted at 25°C in 50 mM MOPS, pH 7.0, in the presence of 0.7 mM bathophenanthroline disulfonic acid, 20 ng of NFR and varying concentrations of Fe(III)-citrate and NADH in a total volume of 1.0 ml. A: Dependence of NFR activity on Fe(III)-citrate concentration (20–600 μM) at four different NADH levels as stated by numerals (μM). The same data are shown in B as a function of varying NADH concentration (20–200 μM) at four fixed Fe(III)-citrate levels (μM). Linear interpolations were drawn on the basis of the kinetic parameters obtained by non-linear regression analysis performed on original (s/v) data.

and by the appearance of α- and β-bands at 555 and 527 nm respectively (Fig. 4). Since cyt b_5 was slowly auto-oxidizable, the steady-state rate of electron transfer between NFR and cyt b_5 was estimated in the presence of cyt c as an oxidant of cyt b_5 [24]. The activity of NFR at varying cyt b_5 concentrations was then determined by following the reduction of cyt c at 550 nm (Fig. 5). NFR saturation curve was hyperbolic with an apparent K_m for cyt b_5 of 2.2 ± 1.1 μM. The maximum interpolated rate of cytochrome reduction was 545 ± 137 μmol min^{-1} mg^{-1}, i.e. not significantly different from the V_{max} with Fe(III)-citrate as electron acceptor (Fig. 3).

4. Discussion

As shown by the present results, microsomal maize root NFR consists of a 32 kDa flavoprotein with non-covalently bound FAD as prosthetic group. The enzyme is specific for NADH as electron donor and can efficiency reduce Fe(III)-citrate, a common ferric chelate of plants [25]. The reaction mechanism of NFR is sequential and it is strongly inhibited by PHMB. NFR can also reduce cyt b_5 with hyperbolic concentration dependence, and maximal activity with cyt b_5 was comparable to the V_{max} obtained in the presence of Fe(III)-citrate. Therefore NFR is, in a kinetic sense, an active cytochrome b_5 reductase.

Several biochemical features of NFR are also in common with those of animal b_5R. These include molecular mass [22], FAD content, sequential reaction mechanism, specificity for NADH, activity with Fe(III)-chelates, and inhibition by PHMB [26]. Far less is known about b_5Rs of plants [24,27], but the few data available, including those referring to a PHMB-sensitive juglone reductase of 31 kDa purified from onion roots plasma membrane [28], are again fully consistent with a strict relationship to NFR.

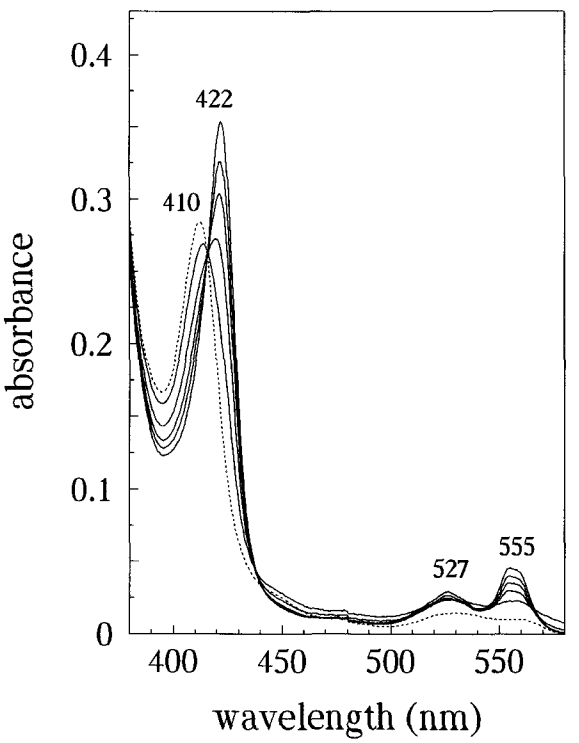

Fig. 4. Cyt b_5 reduction catalyzed by NFR in the presence of NADH. Assay medium was 10 mM Tris-HCl, pH 7.0, purified cyt b_5 concentration was 2 μM and purified NFR was 0.2 nM. Oxidized cyt b_5 is characterized by a Soret band at 410 nm (dotted line). Spectra were recorded every minute following the addition of 0.2 mM NADH (solid lines). Full reduction of cyt b_5 (peaks at 422, 527 and 555 nm) was observed after 10 min under the present conditions. No spectral change was observed upon dithionite addition at the end of the experiment.

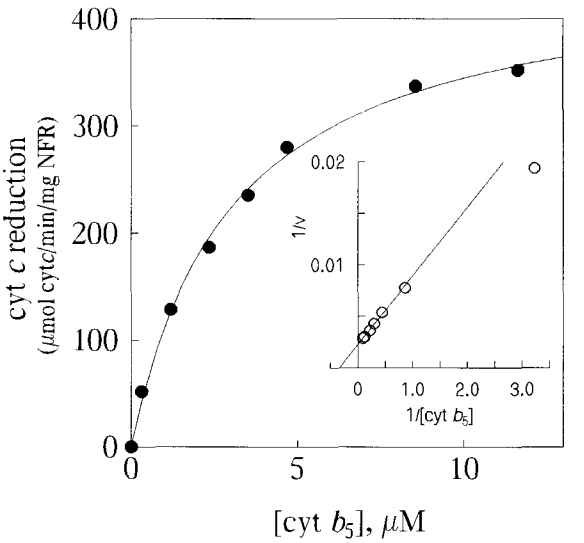

Fig. 5. Dependence of the cyt c reductase activity of NFR on the concentration of cyt b_5. Activity measured at 25°C in 10 mM Tris-HCl, pH 7.0 in the presence of 50 μM cyt c. NFR concentration was 1.5 nM and NADH was 0.2 mM. NADH:cyt c reductase activity of NFR in the absence of cyt b_5, corresponding to 14 μmol min^{-1} mg^{-1} under the present conditions, was subtracted.

In mammals, both structure and intracellular distribution of b_5Rs have been studied in detail. These b_5Rs exist in closely related isoforms with different localizations including the endoplasmic reticulum, the outer mitochondrial membrane [29] and the PM [30]. Mature b_5Rs consist of a hydrophilic catalytic domain at the C-terminus and a short N-terminal hydrophobic domain responsible for membrane anchoring [29]. Myristoylation of the N-terminal glycine has a role in the post-translational targeting of b_5R isoforms [31]. At least in erythrocytes, a soluble isoform, lacking the hydrophobic anchor, is also known [15,29].

In maize roots, the NADH-dependent reduction of ferric chelates is mostly catalyzed by ICM vesicles, the activity in PM vesicles being lower but clearly detectable. Similar distribution was found in iron-sufficient tomato roots [9]. Here it is shown that the NADH:cyt b_5 reductase activity is also not exclusive to ICM, but associated with the PM to some extent. Although NFR has been purified from crude microsomal preparations, its kinetic and inhibition properties are fully consistent with related NFR isoforms being responsible for the NADH:Fe(III)-citrate and the NADH:cyt b_5 reductase activity of both PM and ICM. Therefore also the intracellular localization of NFR seems to follow that of animal b_5R.

A ferric chelate reductase of similar size as NFR (34–36 kDa) was partially purified from the PM of iron-starved tomato roots [32], and a PM protein with some similarities to maize NFR was recently found to be stimulated in tomato plants subjected to iron shortage [9]. Following purification from crude microsomes, tomato NFR proved to share with maize NFR both molecular weight and substrates and inhib-

ition properties, suggesting an unexpected relationship between ferric chelate reductases of strategy I and strategy II plants [9,10].

By analogy with animal b_5R structure, it can be inferred that NFR is not a trans-membrane reductase, as also indicated by the fact that detergent is not required to keep the purified protein in solution. We suggest that the physiological role of NFR could be the symplastic reduction of iron chelates. In plants, the incorporation of iron into macromolecules is driven by ferrochelatases which require Fe(II) as the substrate [33]. Ferrous iron, either free or chelated to low molecular weight organic compounds, is readily oxidizable. Therefore, an iron reduction step cooperating with ferrochelatase activity seems to be necessary in both strategy I and strategy II plants, and NFR may well be involved in this reduction.

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