Cloning and characterization of a maize cytochrome-*b*₅ reductase with Fe³⁺-chelate reduction capability

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We previously purified an NADH-dependent Fe³⁺-chelate reductase (NFR) from maize roots with biochemical features of a cytochrome-*b*₅ reductase (*b*₅R) [Sparla, Bagnaresi, Scagliarini and Trost (1997) FEBS Lett. **414**, 571–575]. We have now cloned a maize root cDNA that, on the basis of sequence information, calculated parameters and functional assay, codes for NFR. Maize NFR has 66 % and 65 % similarity to mammal and yeast *b*₅R respectively. It has a deduced molecular mass of 31.17 kDa and a pI of 8.53. An uncharged region is observed at its N-terminus but no myristoylation consensus site is present. Taken together, these results, coupled with previous biochemical evidence, prove that NFR belongs to the *b*₅R class and document *b*₅R from a plant at the molecular level for the first time. We have also identified a putative *Arabidopsis thaliana* NFR gene. Its organization (nine exons) closely resembles mammalian *b*₅Rs. Several NFR isoforms are expected to exist in maize. They are

probably not produced by alternative translational mechanisms as occur in mammals, because of specific constraints observed in the maize NFR cDNA sequence. In contrast with yeast and mammals, tissue-specific and various subcellular localizations of maize *b*₅R isoforms could result from differential expression of the various members of a multigene family. The first molecular characterization of a plant *b*₅R indicates an overall remarkable evolutionary conservation for these versatile reductase systems. In addition, the well-characterized Fe³⁺-chelate reduction capabilities of NFR, in addition to known Fe³⁺-haemoglobin reduction roles for mammal *b*₅R isoforms, suggest further and more generalized roles for the *b*₅R class in endocellular iron reduction.

Key words: ferric reductase, iron, plant.

INTRODUCTION

Mammal NADH-dependent cytochrome-*b*₅ reductases (*b*₅Rs) are well-known flavoproteins involved as electron donors in a variety of physiological processes. In most cases, *b*₅Rs reduce cytochrome *b*₅, which in turn delivers electrons for a variety of functions, including fatty acid desaturation [1] and elongation [2], cholesterol biosynthesis [3], drug metabolism [4] and methaemoglobin iron reduction [5]. However, no clear function has been yet assigned to well-known *b*₅R isoforms, suggesting additional roles for these proteins. Despite the fact that one gene is reported to code for mammal *b*₅Rs, various isoforms have been observed with differential tissue and subcellular localizations.

Mammal *b*₅Rs are found in the endoplasmic reticulum, the outer mitochondrial membrane, the plasma membrane [6] and the cytosol. Sophisticated mechanisms give rise to these various subcellular localizations [7–10]. Among these mechanisms, two start codons present in the erythroid transcript give rise, through alternative translation initiation, to a putative membrane-bound isoform and to the major soluble reductase. This latter, also known as methaemoglobin reductase, is the best functionally characterized *b*₅R and is responsible for keeping haemoglobin iron in the reduced state, a prerequisite condition for O₂ binding.

The wealth of information concerning mammal *b*₅R has no

counterpart in other organisms, with the sole exception of yeast, in which two *b*₅Rs, CBR and MCR1, of unclear function have been described. CBR was identified as a novobiocin-binding protein of unknown cellular localization [11]. MCR1 exhibits complex sorting mechanisms: a leaky stop-transfer process targets this protein both to the outer mitochondrial membrane and to the intermembrane space [12,13].

Although the characterization of yeast *b*₅Rs provides some hints concerning *b*₅R features outside mammals, no sequencing or cloning has until now been reported for a *b*₅R belonging to the plant kingdom.

Our previous studies of Fe³⁺-chelate reduction in plants led to the purification of a root reductase showing the biochemical features of *b*₅Rs and a remarkable selectivity for Fe³⁺-citrate as electron acceptor over other Fe³⁺-chelates [14–17]. Fe³⁺-reductases have a key role in iron assimilation, and iron uptake mechanisms have been extensively studied with yeast as a model [18,19]. However, the routes followed by iron once inside the cell are at best poorly defined, although it is well established that reduction steps of the metal are strictly necessary in all organisms.

Here we demonstrate by molecular cloning and sequencing that maize NADH-dependent Fe³⁺-chelate reductase (NFR) belongs to the *b*₅R group. This result provides the first molecular clues for this important enzyme class in the plant kingdom,

Abbreviations used: *b*₅R, cytochrome-*b*₅ reductase; EST, expressed sequence tag; FCR, NADH:ferricyanide reductase; NFCTR, NADH:Fe³⁺-citrate reductase; NFR, NADH-dependent Fe³⁺-chelate reductase; psNFCTR, *p*-hydroxymercuribenzoic acid-sensitive NADH:Fe³⁺-citrate reductase.

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giving a tool for probing the evolutionary conservation of various intriguing properties of b_5R systems. In addition, a potential involvement of b_5R s in endocellular Fe^{3+} -chelate reduction is discussed.

EXPERIMENTAL

Plant cultures

Maize plants (*Zea mays*, var AMO 406; Maisadour, Mont de Marsan, France) were grown hydroponically under iron-free conditions ($-Fe$) or iron-sufficient conditions ($100 \mu M Fe^{3+}$ -EDTA; $+Fe$) as described previously [20].

$+Fe$ and $-Fe$ plants 10 days old were harvested 3–4 h after the beginning of the photoperiod. Roots were cut 2 cm under and leaves 2 cm above the collar. Samples were frozen in liquid nitrogen and stored at $-80^\circ C$ for further experiments.

cDNA library construction and screening

Total RNA was extracted from 10 g of frozen 10-day-old maize roots as reported by Whestoff et al. [21]. Poly(A)⁺ RNA was then purified by using oligo(dT)-cellulose (Pharmacia, type 7) by the method of Sambrook et al. [22]. A λ -Zap II cDNA library was constructed starting with $5 \mu g$ of poly(A)⁺ RNA from 10-day-old ($-Fe$) maize roots with a ZAP-cDNA Gigapack II Gold cloning kit (Stratagene). An 800 bp *SalI*–*NcoI* fragment obtained from rice EST D23879 (MAFF DNA bank, Tsukuba, Japan) was radiolabelled by random priming and used for library screening under conditions of high stringency (final filter washes at $65^\circ C$ in $0.5 \times SSC/0.1\%$ SDS). The pBluescript SK(–) phagemid was excised from positive phages by co-infection of SOLR strain with helper ExAssist phage.

NFR amino acid sequencing

Microsomal maize root NFR was purified by the method of Sparla et al. [17]. After SDS/PAGE, the 32 kDa band was excised and digested with trypsin. Peptides were purified by reverse-phase HPLC and sequenced with an automated sequencer (Beckman LF 3000 protein sequencer).

DNA sequencing

cDNA nucleotide sequence was determined by the dideoxynucleotide chain termination method [23]. Appropriate oligonucleotides were used as primers to walk along the sequence. Both DNA strands were independently and entirely sequenced. Sequence computer analysis was performed with DNA strider software and by connecting to the BLAST Network Service at NCBI (<http://www.ncbi.nlm.nih.gov>). Protein sequence alignments and phylogenetic trees were generated by ClustalW (<http://www2.ebi.ac.uk/clustalw/>) and the dendrogram was drawn by the Treeview program [24].

Southern analysis

Genomic DNA from maize seedlings was ethanol-precipitated from the supernatant of a LiCl fractionation of total nucleic acids [25]. DNA samples ($10 \mu g$) were digested by various enzymes overnight at $37^\circ C$, and subjected to electrophoresis on a $0.04 M$ Tris/acetate/ $0.002 M$ EDTA (TAE)/ 0.7% agarose gel. After depurination by soaking the gel in $0.25 M$ HCl, DNA was transferred to $0.45 \mu m$ pore-size Biotrans⁺ nylon membranes (ICN) in accordance with the supplier's recommendations. Hybridization was performed under standard conditions [26] with random primed (Ready To Go; Pharmacia) ^{32}P -labelled

DNA fragments (probe P1, 988 bp *EcoRI*–*EcoRI*; probe P2, 265 bp *SacI*–*DraI*; see Figure 2A). After high-stringency washes ($0.2 \times SSC/0.1\%$ SDS at $65^\circ C$), autoradiography was performed for 6 days at $-70^\circ C$ to Royal X-Omat film (Kodak) with an intensifying screen.

RNA preparation and Northern blot analysis

Total RNA, from frozen maize roots or leaves, was extracted as described previously [25], with some modifications [27]. Total RNA ($10 \mu g$) for each sample was subjected to electrophoresis through a formaldehyde/ 1.2% (w/v) agarose gel [27] and blotted on a nylon membrane (Hybond N+; Amersham). Blotted RNA was hybridized with probes labelled with $[\alpha\text{-}^{32}P]dCTP$ with a random priming labelling kit (Ready To Go DNA labelling kit; Pharmacia Biotech).

Yeast functional assay

Maize NFR cDNA was subcloned as a *NotI*–*XhoI* fragment in the yeast vector pYES2 (Invitrogen) for expression under the control of the *GAL1* promoter. Yeast strain W303a (MATa leu2-3,112 his 3-11,15 ade2-1 ura3-1 trp2-2 can1-100) was transformed by the lithium acetate procedure [28] and cells were pregrown for 24 h at $30^\circ C$ in a synthetic defined medium [SD; 0.17% YNB-AA/AS/ 0.5% $(NH_4)_2SO_4$] with required auxotrophic components and 2% (w/v) glucose. SD media ($100 ml$), containing either 2% (w/v) glucose or 2% (w/v) galactose were then inoculated with $1 ml$ of preculture. After 20 h of growth, cells were harvested by centrifugation. Crude extracts were prepared by disrupting cells with glass beads as described [28], but dithiothreitol was omitted from buffers to avoid interference with reductase assays. NADH:ferricyanide reductase (FCR), NADH: Fe^{3+} -citrate reductase (NFCTR) and *p*-hydroxymercuribenzoic acid-sensitive NFCTR (psNFCTR) were assayed as described previously [16,17]. The protein concentration of crude extracts was determined by Bradford's method [29].

RESULTS

NFR cloning

Microsequencing of the purified microsomal protein was undertaken. Because no N-terminal sequence could be obtained, internal peptides were generated by trypsin digestion and purified by reverse-phase HPLC. The sequences of two of these peptides, 14 and 18 residues long (underlined in Figure 1A), were determined. Expressed sequence tag (EST) database searches revealed near-complete identity of those two peptides within predicted polypeptide sequences of rice ESTs D23879 and C73512 respectively (DDBJ accession numbers). The first peptide showed a perfect match over its entire length, whereas the second presented only one mismatch (see Figure 5A). The greatest similarity of predicted proteins from the rice ESTs was to mammal [30–32] and yeast [11,12] b_5R s, strengthening our previous proposal of identity or close relationship between maize NFR and b_5R [15–17]. The 400 bp sequenced region of rice EST D23879 did not contain a start codon and encoded a peptide located within the most N-terminal half of b_5R , whereas the deduced protein sequence from EST C73512 (452 bp) corresponded to the most C-terminal part of b_5R , encompassing a putative stop codon. To clarify the relationship between the two ESTs, 195 bp of the 3' end of rice EST D23879 were sequenced and found to be 95% identical with the 3' UTR of rice EST C73512 (results not shown). The two EST entries were therefore attributable to identical or closely related mRNA species; we decided to use an 800 bp *SalI*–*NcoI* DNA fragment encompassing

Figure 1 Sequence analysis of maize NFR cDNA

(A) Nucleotide sequence and deduced amino acid sequence of NFR cDNA. Arrows indicate the 5' ends of two additional NFR cDNA clones (see the text for details). The regions of the polypeptide for which the amino acid sequence was obtained by direct sequencing of the protein are underlined. The FAD-binding site (residues 96–102) and the NADH-binding site (residues 155–166 and 250–256) are in bold type. The N-terminal stretch of 21 uncharged residues is shown in italics. A conserved cysteine residue involved in sensitivity to thiol reagents is boxed. (B) Hydropathy plot of the deduced NFR product. The plot was made by the method of Kyte and Doolittle with a window of 11 residues.

the whole putative coding sequence region of rice EST D23879 as a probe for subsequent screening experiments.

A cDNA library prepared from roots of 10-day-old iron-deficient maize was screened with the above-mentioned probe under conditions of high stringency. Three positive plaques were revealed out of 60 000 phages plated. Restriction analysis of the three DNA inserts revealed slightly different sizes, ranging from 1.15 to 1.1 kb, and a very similar restriction map; the only difference consisted of the lack of an *Nco*I site in the extreme 5' end of the shortest DNA insert. DNA sequencing of their 5' regions revealed that the three clones were identical, differing only in the length of their 5' extremities. DNA inserts 1 and 2 contained a putative start codon, whereas the third DNA insert was interrupted at the G of this putative ATG, thereby accounting for the absence of the *Nco*I site. The longest DNA insert was fully sequenced and contained an open reading frame (see below) encompassing the two peptide sequences determined from the purified maize root NFR (see Figure 1A; arrows indicate the 5' ends of the shorter cDNA species).

Features of maize NFR

NFR cDNA is 1147 nt long and presents an open reading frame encoding a 279-residue protein with a calculated molecular mass

Figure 2 Northern analysis of maize NFR mRNA

(A) Restriction map of NFR cDNA. The putative coding sequence is shown as an open box. A full-length probe (P1; *Eco*RI fragment) or a 3' probe (P2; *Sac*I–*Dra*I fragment) as indicated was used in hybridization experiments. Abbreviations: E, *Eco*RI; EV, *Eco*RV; S, *Sac*I; D, *Dra*I. (B) RNA gel-blot hybridizations of maize transcripts from 10-day-old plants. Total RNA from roots and shoots was hybridized with probe P1 (left panel) or probe P2 (right panel). The labels +Fe and –Fe refer to iron-sufficient and iron-deficient plant growth conditions respectively.

of 31.17 kDa and a pI of 8.53. Both these values are in excellent agreement with previous determinations with purified microsomal NFR, which migrates as a 32 kDa polypeptide on SDS/PAGE [14] and has a pI of 8.5 as determined by chromatofocusing experiments [17].

As depicted in Figure 1(A), the NFR coding sequence is flanked by a 40 bp 5' UTR and by a 269 bp 3' UTR ending by a poly(A) tail. Despite the absence of an in-frame stop codon upstream of the putative start codon, NFR cDNA is very likely to encode the full protein, for reasons given in the Discussion section. For hybridization experiments, an *Eco*RI (probe P1, corresponding to the full coding sequence) or a *Sac*I–*Dra*I (probe P2, derived from the 3' UTR) DNA fragment was used. In Northern blots from 10-day-old maize root and shoots, both probes revealed one transcript of approx. 1.3 kb (Figure 2). In a manner consistent with previous observations relative to maize NFR activity in response to iron deficiency [14], no substantial increase in NFR transcript abundance was observed as a consequence of iron starvation. The accumulation of NFR transcript in iron-deficient shoots as visible in Figure 2 is accounted for mostly by differences in RNA loading (results not shown). By using the same probes as for Northern analysis, a Southern blot analysis of maize genomic DNA digested with *Eco*RI, *Eco*RV (which cut within probe P2) and *Hind*III revealed a relatively complex pattern (Figure 3), suggesting that maize NFR(s) are encoded by a small gene family. This is in sharp

contrast with the unique hybridized DNA fragment revealed in mammal and yeast genomic DNA Southern blots probed for *b₅R* [11,12,30].

Expression and functional identification in yeast

As determined previously, purified NFR can use a variety of different electron acceptors, including ferricyanide and Fe^{3+} -citrate. Ferricyanide can be reduced by a variety of distinct reductases, whereas Fe^{3+} -citrate reduction is far more specific to NFR. Indeed, psNFCTR was reported to be the most selective assay for NFR [14,16]. In addition, purified NFR was shown to present an FCR-to-psNFCTR ratio of 2.5:1, clearly distinguishable from other ferricyanide reductases bearing an FCR-to-psNFCTR ratio of 10:1 to 20:1 [14,16]. To confirm the functional identity of the cloned maize cDNA, a DNA fragment comprising the whole NFR coding sequence was cloned in the pYES2 yeast expression vector, under the control of the *GAL1* galactose-inducible promoter (Figure 4). Transformed yeast cells were grown in the presence of glucose (repressing conditions) or galactose (inducing conditions). A 4-fold stimulation of psNFCTR, the most specific assay for NFR activity, was detectable in crude extracts from induced over-repressed cells. Furthermore the ectopically expressed reductase showed a low ratio of FCR to psNFCTR activities (approx. 2.5:1), which is consistent with previous biochemical studies. The psNFCTR activity observed in repressed cells can be attributed to endogenous yeast NFR-like proteins, such as CBR and MCR1.

NFR comparison with known and inferred *b₅R* sequences

Maize NFR exhibits significant and comparable similarity to known members of mammal and yeast *b₅R*, showing 37.9% identity (66% similarity) to bovine *b₅R*, and 37.5% identity (65.2% similarity) to yeast CBR. High similarity is also observed with the C-terminal domain of several plant nitrate reductases (35% identity with maize nitrate reductase), which is consistent with previously observed remarkable similarities between nitrate reductase C-terminal domain and human *b₅R* [33]. Several internal gaps in NFR account for the slightly lower molecular mass of NFR than that of other *b₅R*s. In both yeast and plant *b₅R*s a hinge region is shorter than in mammal *b₅R*s, whereas a specific, additional stretch in the C-terminal region is found only in yeast *b₅R*s (Figure 5A). CBR, the yeast *b₅R* showing the greatest similarity to NFR, is of unknown subcellular localization; however, NFR also exhibits a lower degree of similarity to a second yeast *b₅R*, MCR1, for which a mitochondrial localization has been established [12,13]. However, a similar subcellular localization for maize NFR seems unlikely owing to the presence of several acidic residues and the paucity of Arg and Ser residues in the N-terminus [34].

Searching the *Arabidopsis thaliana* genomic database revealed, in a recent entry from chromosome V (MVA3 clone, GenBank accession number AB006706; Mb 49 to 46), several putative exons closely resembling the NFR product. Computer-aided and text-based exon assembly led us to predict a 281-residue product showing remarkable similarity with NFR (75.1% identity and 90% similarity, see both the alignments and the dendrogram in Figure 5). This gene seems to be transcribed, as indicated by the existence of at least one *Arabidopsis* EST (R83968) showing more than 95% identity at the nucleotide level with the *Arabidopsis* assembled gene product. Interestingly, this *A. thaliana* *b₅R* is organized in nine exons, as reported for rat and human genes [31,35].

Figure 3 Southern blots of maize DNA (10 µg per lane)

Genomic DNA cut with *Eco*RI (E), *Eco*RV (EV) or *Hind*III (H) was hybridized at high stringency with the full-length probe (P1, left panel) or 3' probe (P2, right panel; see also Figure 2A).

Figure 4 NFR functional detection in yeast

The NFR coding sequence was inserted into the galactose-inducible yeast expression vector pYES2. Transformed yeast cells were grown in the presence of glucose (repressing conditions; open bars) or galactose (inducing conditions; filled bars). Crude extracts were assayed for reductase activities known to be catalysed by NFR and other proteins [14–16]: in increasing order of selectivity for NFR, they were FCR, NFCTR and psNFCTR. FCR and NFCTR activities are reported at 1/100 and 1/10 scales respectively. Measurements were conducted in quadruplicate.

DISCUSSION

Structural comparison between NFR and known *b₅R*s

Maize NFR cDNA contains a 279-residue open reading frame whose deduced parameters are in excellent agreement with previously defined NFR biochemical features. A poly(A) tail is present at the 3' end but there is no stop codon in frame with the putative product in the relatively short (40 bp) 5' leader. Indeed, NFR cDNA might be incomplete in its 5' part, as suggested by Northern experiments showing a 1.3 kb transcript (Figure 2A), whereas the cDNA size is 1.14 kb. Nevertheless the indicated ATG is likely to be the authentic start codon as indicated by the following observations: (1) despite a striking sequence conservation with the open reading frame in a recent rice EST entry (OS-3 in Figure 5A; C25971), the 5' regions upstream of the

A

Figure 5 Evolutionary relationship between maize NFR and other reductases

(A) Multiple alignments of maize NFR. Known or putative *b*₅Rs were aligned; the consensus to maize NFR (ZM-NFR) is shown. AT-chrV is a putative product assembled from an *A. thaliana* gene (MVA3 clone, GenBank accession number AB006706; 49 to 46 Mb). OS-EST1, OS-EST2 and OS-EST3 are respectively rice EST D23879, C73512 and C25971 (DDBJ accession numbers). BOVIN-b5R [37], HUMAN-b5R [35] and RAT-b5R *b*₅R [31] are known mammalian *b*₅Rs, whereas SC-B5R (CBR [11]) and SC-MB5R (MCR1 [12]) are yeast *b*₅Rs. SC-FRE1 is the C-terminal 294 residues of yeast surface ferric reductase FRE1 [49]. (B) Phylogenetic tree of known and putative *b*₅Rs. Yeast *b*₅Rs MCR1 and CBR are respectively indicated as yeast mb5R and yeast b5R. The tree file was generated with CLUSTALW and drawn by the Treeview program [24].

proposed start codon diverge abruptly; (2) the putative *A. thaliana* NFR has an in-frame stop codon 47 nt upstream of the proposed start codon; (3) an N-terminal extension would generate a protein with a molecular mass in disagreement with our previous NFR molecular mass estimation by SDS/PAGE. Incomplete or short 5' UTRs are common for *b*₅R cDNA species, as maize NFR cDNA presents the longest 5' region so far detected [30,31].

As expected, NFR protein presents NAD(P)H-binding and FAD-binding domains, which is consistent with its catalytic properties and the previous fluorimetric determination of a FAD prosthetic group [17]. These two motifs are localized respectively within the C-terminal and N-terminal domains of *b*₅R (see Figure 1A). When aligned to mammal *b*₅R, NFR shows a major gap (residues 152–167 in human *b*₅R; Figure 5A). In mammals this sequence encompasses a two-stranded anti-parallel β -sheet hinge region that influences the relative positioning of the NADH and FAD domains [36]. This suggests distinctive structural

properties for NFR. In addition, this region has been implicated through lysine residues in efficient charge pairing with cytochrome *b*₅ haem propionyl carboxylate groups, resulting in fast electron delivery to the cytochrome [37]. Such a modification might be critical for electron acceptor specificities. Indeed, in contrast with mammal *b*₅R, maize NFR is able to reduce cytochrome *c* without the intervention of cytochrome *b*₅ [14,17].

The NFR N-terminus, after a short stretch containing polar residues, presents an uncharged region of 21 residues, as is also evident from the hydrophobicity plot (Figure 1B). Such membrane anchors are a distinctive feature of membrane-associated *b*₅R members and have been shown to be both necessary and sufficient for membrane association in mammals [9]; this is consistent with the microsomal source of purified NFR protein [17]. In contrast with mammal *b*₅Rs, however, no myristoylation consensus is present in plant NFR, as in yeast *b*₅Rs.

Current investigation on *b*₅R is focused mainly on the refined cell-sorting mechanisms applying to this class of proteins. In

mammals, one single b_5R gene leads to a variety of isoforms owing to alternative promoter utilization and alternative splicing. These isoforms differ in their tissue-specific and subcellular localizations [8,9]. The generation of soluble forms relies on the presence of a second AUG start codon located in the second exon, immediately downstream of the membrane anchor domain and upstream of the FAD and NADH domains. In contrast, the second in-frame AUG is found in the middle of the NFR transcript in maize and *A. thaliana*. Its utilization as start codon would lead to an NFR form lacking the FAD domain. It is therefore unlikely that plant b_5R mRNA could give rise to a soluble isoform by means of alternative translation initiation as observed in rats.

The fact that NFR cDNA could code for only one form of b_5R is somewhat unexpected, because NFR-like reductases seem to be associated with a wide range of subcellular territories in both maize and tomato roots [14] and shoots, by analogy with the various localizations of mammal b_5R isoforms. Furthermore we previously purified soluble b_5R isoforms starting from soluble extracts from plant roots rather than microsomal protein extracts, strongly suggesting the existence of differentially targeted b_5R isoforms [14–16]. Such discrepancies could be explained by the existence of more than one b_5R gene in maize. Plant b_5R isoform production could be therefore achieved by the differential transcription of various b_5R genes. In contrast, in 10-day-old roots and shoots only one major mRNA species was detected, suggesting either a different developmental expression pattern for other putative transcripts or several transcripts of nearly identical size expressed at the developmental stage analysed here.

Are b_5R s involved in Fe^{3+} -chelate reduction *in vivo* ?

In erythrocytes, soluble b_5R keeps haem iron in the reduced state, a prerequisite for oxygen binding. Reductants such as reduced glutathione and ascorbate have only a marginal role in this process. As a consequence, the alteration of soluble b_5R from erythrocytes is responsible for a form of recessive congenital methaemoglobinemia [5].

In plants, cytochrome b_5 reduction rates seem to be well in excess of those that are strictly necessary for electron delivery to desaturases [38]. In addition, an increasing number of recently identified desaturases seem to bring their own cytochrome b_5 as an N-terminal or C-terminal extension [39], raising the question of alternative roles for free cytochrome b_5 . These observations would be consistent with additional b_5R roles, either with the intervention of cytochrome b_5 , as with methaemoglobin, or by the direct reduction of other acceptors [6].

Maize NFR reduces Fe^{3+} *in vitro*, with a preference for Fe^{3+} -citrate over non-physiological Fe^{3+} -chelators (such as Fe^{3+} -EDTA or ferricyanide) [14,17]. In dicotyledonous plants an obligatory iron-reduction step is a prerequisite for uptake of the metal [40–42]. Although not itself capable of a transmembrane electron flow, NFR could interact for this purpose with a membrane partner, such as a quinone [6] or a cytochrome. Alternatively, if a permease/oxidase complex such as is found in yeast is operative in dicotyledons, NFR could account for immediate iron reduction, after its entry into the cell in the oxidized form.

Ferric iron is generated spontaneously under aerobic conditions at physiological pH; the oxidized form of the metal is known to be involved in various steps of iron trafficking [18,19,41]. However, iron cannot escape a final reduction step because there is an absolute requirement for its ferrous form for insertion in prosthetic groups [43,44]. Some of these reduction requirements could be exerted by non-enzymic reductants but in

some cases the involvement of a thiol-reagent-inhibited NADH-dependent reductase has been proposed. Indeed, iron mobilization from endocytic vesicles involves a reduction in which an NADH-dependent, *p*-hydroxymercuribenzoic acid-inhibited reductase has been reported to take part, and suggested to be a b_5R [45]. Additionally, a thiol-sensitive reductase has been reported to account for reductive iron uptake in membrane vesicles of cultured brush border cells [46]. Although the molecular identification of these reductases remains to be achieved, b_5R s do fulfil the necessary requirement *in vitro* for at least part of such catalytic activities. In fact, in addition to well-documented acceptor specificities of NFR, purified mammal b_5R can reduce a wide range of Fe^{3+} -chelates *in vitro* [47,48].

In conclusion, several observations argue in favour of additional roles for plant and animal b_5R s in various Fe^{3+} -chelate reduction steps. An intracellular enzymic reductant such as b_5R might present several advantages over the uncontrolled action of other reductants. It can be speculated that iron reduction relying on b_5R could be regulated in a tissue-specific manner, as well as being effected by appropriate and differently sorted endocellular isoforms. This would permit the fine tuning of iron reduction at discrete endocellular targets, controlling the iron redox state and thus the well-known toxic properties of this metal.

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REFERENCES

- Strittmatter, P., Spatz, L., Corcoran, D., Rogers, M. J., Setlow, B. and Redine, R. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4565–4569
- Keyes, S. R. and Cinti, D. L. (1980) *J. Biol. Chem.* **255**, 11357–11364
- Reddy, V. R., Kupfer, D. and Capsi, E. (1977) *J. Biol. Chem.* **252**, 2797–2801
- Hildebrandt, A. and Estabrook, R. W. (1971) *Arch. Biochem. Biophys.* **143**, 66–79
- Mota Vieira, L., Kaplan, J. C., Kahn, A. and Leroux, A. (1995) *Blood* **85**, 2254–2262
- Villalba, J. M., Navarro, F., Cordoba, F., Serrano, A., Arroyo, A., Crane, F. and Navas, P. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 4887–4891
- Pietrini, G., Aggujaro, D., Carrera, P., Malyszko, J., Vitale, A. and Borgese, N. (1992) *J. Cell Biol.* **117**, 975–986
- Borgese, N., D'Arrigo, A., De Silvestris, M. and Pietrini, G. (1993) *FEBS Lett.* **325**, 70–75
- Borgese, N., Aggujaro, D., Carrera, P., Pietrini, G. and Bassetti, M. (1996) *J. Cell Biol.* **135**, 1501–1513
- Mota Vieira, L., Kaplan, J. C., Kahn, A. and Leroux, A. (1994) *Eur. J. Biochem.* **220**, 729–737
- Csukai, M., Murray, M. and Orr, E. (1994) *Eur. J. Biochem.* **219**, 441–448
- Hahne, K., Haucke, V., Ramage, L. and Schatz, G. (1994) *Cell* **79**, 829–839
- Haucke, V., Santos Ocana, C., Honlinger, A., Tokatlidis, K., Pfanner, N. and Schatz, G. (1997) *Mol. Cell. Biol.* **17**, 4024–4032
- Bagnaresi, P. and Pupillo, P. (1995) *J. Exp. Bot.* **46**, 1497–1503
- Bagnaresi, P. and Basso, B. (1996) *J. Plant Nutr.* **19**, 1171–1177
- Bagnaresi, P., Basso, B. and Pupillo, P. (1997) *Planta* **202**, 427–434
- Sparla, F., Bagnaresi, P., Scagliarini, S. and Trost, P. (1997) *FEBS Lett.* **414**, 571–575
- Kaplan, J. and O'Halloran, T. V. (1996) *Science* **271**, 1510–1512
- Askwith, C. and Kaplan, J. (1998) *Trends Biochem. Sci.* **23**, 135–138
- Thoirion, S., Pascal, N. and Briat, J. F. (1997) *Plant Cell Environ.* **20**, 1051–1060
- Whetstall, P., Nelson, N., Bunemann, H. and Hermann, R. G. (1981) *Curr. Genet.* **4**, 109–120
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Sanger, F., Nicklen, S. and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463–5467
- Page, R. D. M. (1996) *Comp. Appl. Biosci.* **12**, 357–358
- Logeman, J., Shell, J. and Willmitzer, L. (1987) *Anal. Biochem.* **163**, 16–20
- Fobis-Loisy, I., Loidon, K., Lobreaux, S., Lebrun, M. and Briat, J. F. (1995) *Eur. J. Biochem.* **231**, 609–619
- Lobreaux, S., Massenet, O. and Briat, J. F. (1992) *Plant Mol. Biol.* **19**, 563–575
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1995) *Current Protocols in Molecular Biology*, John Wiley, New York

- 29 Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- 30 Pietrini, G., Carrera, P. and Borgese, N. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7246–7250
- 31 Zenno, S., Hattori, M., Misumi, Y., Yubisui, T. and Sakaki, Y. (1990) *J. Biochem. (Tokyo)* **107**, 810–816
- 32 Ozols, J., Korza, G., Heinemann, F. S., Hediger, M. and Strittmatter, P. (1985) *J. Biol. Chem.* **260**, 11953–11961
- 33 Hyde, G. E. and Campbell, W. H. (1990) *Biochem. Biophys. Res. Commun.* **168**, 1285–1291
- 34 Sjöling, S. and Glaser, E. (1998) *Trends Plant Sci.* **3**, 136–140
- 35 Tomatsu, S., Kobayashi, Y., Fukumaki, Y., Yubisui, T., Orii, T. and Sakaki, Y. (1989) *Gene* **80**, 353–361
- 36 Nishida, H., Inaka, K., Yamanaka, M., Kobayashi, K. and Miki, K. (1995) *Biochemistry* **34**, 2763–2767
- 37 Strittmatter, P., Kittler, J. M., Coghill, J. E. and Ozols, J. (1992) *J. Biol. Chem.* **267**, 2519–2523
- 38 Smith, M. A., Cross, A. R., Jones, O. T. G., Griffiths, W. T., Stymne, S. and Stobart, K. (1990) *Biochem. J.* **272**, 23–29
- 39 Sayanova, O., Smith, M. A., Lapinskas, P., Stobart, A. K., Dobson, G., Christie, W. W., Shewry, P. R. and Napier, J. A. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 4211–4216
- 40 Yi, Y. and Guerinot, M. L. (1996) *Plant J.* **10**, 835–844
- 41 Briat, J.-F. and Lobreaux, S. (1997) *Trends Plant Sci.* **2**, 187–193
- 42 Römheld, V. (1987) *Physiol. Plant.* **7**, 231–234
- 43 Chow, K. S., Singh, D. P., Roper, J. M. and Smith, A. G. (1997) *J. Biol. Chem.* **272**, 27565–27571
- 44 Rouault, T. A. and Klausner, R. D. (1996) *Trends Biochem. Sci.* **21**, 174–177
- 45 Scheiber, B. and Goldenberg, H. (1993) *Arch. Biochem. Biophys.* **305**, 225–230
- 46 Ekmekcioglu, C., Feyertag, J. and Marktl, W. (1996) *J. Nutr.* **126**, 2209–2217
- 47 Yang, M. X. and Cederbaum, A. I. (1996) *Arch. Biochem. Biophys.* **331**, 69–78
- 48 Miura, A., Tampo, X. and Yonaha, M. (1995) *Biochem. Mol. Biol. Int.* **37**, 141–150
- 49 Dancis, A., Roman, D. G., Anderson, G. J., Hinnebusch, A. G. and Klausner, R. D. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 3869–3873

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