Calcein as a Fluorescent Probe for Ferric Iron
APPLICATION TO IRON NUTRITION IN PLANT CELLS*

(Received for publication, October 20, 1998, and in revised form, January 29, 1999)

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The recent use of calcein (CA) as a fluorescent probe for cellular iron has been shown to reflect the nutritional status of iron in mammalian cells (Breuer, W., Epsztejn, S., and Cabantchik, Z. I. (1995) J. Biol. Chem. 270, 24209–24215). CA was claimed to be a chemosensor for iron(II), to measure the labile iron pool and the concentration of cellular free iron(II). We first study here the thermodynamic and kinetic properties of iron binding by CA. Chelation of a first iron(III) involves one aminodiacetic acid and a phenol. The overall stability constant \( \log \beta_{11} \) of Fe(III)CAH is 33.9. The free metal ion concentration is \( pFe^{III} = 20.3 \). A (Fe(III)2 CA complex can be formed. A reversible iron(III) exchange from Fe(III)CAH to citrate and nitrilotriacetic acid is evidenced when these ligands are present in large excess. The kinetics of iron(III) exchange by CA is compatible with metabolic studies. The low reduction potential of Fe(III)CAH shows that the ferric form is highly stabilized. CA fluorescence is quenched by 85% after Fe(III) chelation but by only 20% using Fe(II). Real time iron nutrition by Arabidopsis thaliana cells has been measured by fluorimetry, and the iron buffer Fe(III)CAH + CA was used as source of iron. As a siderophore, Fe(III)CAH promotes cell growth and regreening of iron-deficient cells more rapidly than Fe(III)EDTA. We conclude that CA is a good growth and regreening of iron-deficient cells more rapidly than Fe(II). We discuss the interest of quantifying iron buffers in biochemical studies of iron, in vitro as well as in cells.

Physiologists have described many examples of cells that present symptoms of iron deficiency, although containing large amounts of iron (1). In order to explain the capricious biological efficiency of cellular iron, an ill defined key operator of iron metabolism, the so-called “labile” iron has been invoked (2). The LIP is the available cellular iron to which has been assigned at least four functions: (a) cellular iron transport, (b) expression of iron regulatory genes (transferrin receptor, ferritin), (c) control of the activity of iron containing proteins, (d) catalysis of the Fenton reactions (3).

How much iron is available? How tightly is it bound and to which biological ligands? Cabantchik et al. (4) have recently used a fluorescent probe that may be an opportune tool to measure the LIP. They have shown that calcein (CA; see structure formula in Fig. 3) is dynamically sensitive to metabolic changes of iron pools, under various nutritional conditions. Its use is therefore a methodological breakthrough. Nevertheless, they assumed that physiodynamic data concerning iron (III and II) complexation with calcein were the same as those of EDTA, because calcein and EDTA both carry dianiminetetraacetic chelating arms (4). By the use of acetoxymethyl esters of CA, they could discriminate endocellular signals. Since calcein chelates cellular iron which is shared by all the complexes of the same compartment, the level of fluorescence of free calcein reflects the variations of the available iron of the compartment (4–9). Calcein was claimed to reflect the ferrous iron pool (10).

Such an approach is reminiscent of the model based on the notion of metal buffers and \( pFe \) calculations, by which ligands and complexes tend to keep metal ions in homeostatic availability (11–17); biochemical iron exchanges can be regarded as the result of competitions for iron between cellular ligands, according to their various affinities. The biosynthesis of iron ligands during growth of cells creates a driving force for the nutritional uptake of the metal. Then, after iron uptake, the competition of iron complexes with free ligands leaves only trace amounts of iron ions, in equilibrium with all the iron potential ligands. Within each cellular compartment, in which diffusion occurs freely, a single overall concentration of unchelated residual iron results from these multiple equilibria; it can be expressed as \( pFe \). The term \( pFe \) is the best quantitative expression of the overall concentrations of unchelated iron in solution, in specific pH and ionic strength conditions. CA is a fluorescent chemosensor that could reflect these equilibria.

First attempts to measure \( pFe^{III} \) in plant cells have been made by competition experiments between radiolabeled standard iron buffers and cellular ligands (18), but these measurements were done after disruption of cellular compartments and therefore had lost most of their physiological significance. Nondestructive tools are needed for \( pFe^{III} \) and \( pFe^{II} \) determinations.

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The abbreviations used are: LIP, labile iron pool; CA, calcein; SCE, standard calomel electrode; CAH, monoprotinated calcein; NTA, nitrilotriacetic acid.

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\[ pFe = -\log[Fe] = \log \beta_{11} + \log ([L]_T + [L] + [FeL]_1 + [Fe] + [FeL]_2 + [FeL]_3), \]

where \( \beta_{11} \) is the overall stability constant for the Fe + L \( \rightarrow \) FeL equilibrium, \( [L]_T = [L] + [FeL]_1 + [Fe] + [FeL]_2 + [FeL]_3 \), and \( \alpha_{FeL} \) and \( \alpha_{Fe} \) are the Ringbom coefficients (22). Chaberek and Martell (11) and others adopted this logarithmic notation as convenient for low values and reminiscent of the familiar pH scale. In the presence of free ligand, a metal concentration is buffered. Its concentration (i.e. \( pFe \)) is least affected by disturbances, which tend to add or to remove metal ions from the medium. Raymond and co-workers (15) proposed conventional condition to compare the efficiency of iron chelators.

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The aim of this work is to complement the cytological approach which uses calcein as a chemosensor, by establishing the thermodynamic and kinetic data concerning iron(III) and II complexation with calcein. We then discuss the use of CA as a fluorescent probe for studying the pools of ferrous and ferric iron. A new application using CA for measurements of the kinetics of iron(III) nutrition of plant cells is developed, and a physiological study of CA as a nutritional siderophore is presented.

MATERIALS AND METHODS

**Potentiometric and UV-visible Spectrophotometric Experiments**—All the measurements were made at 25 °C. The ionic strength was fixed at 0.1 M with sodium perchlorate. Potentiometric titration employed a DMS 716 Titrino (Metrohm) equipped with glass and calomel electrodes and connected to an IBM Aptiva microcomputer. The electrodes were calibrated to read pH according to the classical method. A calcein solution and a 1:1 Fe(III):calcein solution of ~0.001 M were titrated with standardized 0.05 M sodium hydroxide. The titration data were refined by the nonlinear least squares refinement program SUPERQUAD (19) to determine the equilibrium constants. UV-visible spectra were recorded on a Lambda 2 Perkin-Elmer spectrophotometer. Light path length is 1 cm. The acquisition was made with the UV Winlab Perkin-Elmer software. Temperature was maintained at 25 °C with the variable temperature unit. For calcein-iron spectrophotometric titrations, 0.1 M iron(III) perchlorate in 0.1 M HClO₄ solution was added to a 10⁻⁴ M calcein solution (0.05 M Tris-Cl, pH 7.2, 0.05 M NaClO₄); pH was controlled before and after each measurement. Iron concentration was obtained spectrophotometrically by using a molar extinction coefficient \( \varepsilon = 4160 \, \text{M}^{-1} \cdot \text{cm}^{-1} \) at 240 nm (20); CA concentration was obtained using the molar extinction coefficient provided by Molecular Probes. The total volume was 500 µl, and its variations due to iron additions from stock solutions were less than 2%. Equilibria after competitions were measured using the same equipment.

**Stopped Flow Experiments**—A Biologic SFM3 stopped flow module (Claix, France), controlled by a Tandem computer using Biokine software, is used for stopped flow kinetic experiments. Its temperature control unit (M3 Lauda) is set at 25 °C.

**EPR Experiments**—EPR studies have been conducted using a ESP 300E Bruker apparatus with a variable temperature unit. Spectra were treated by using either the software of the apparatus or the winEPR software (Bruker). Each sample (200 µl) contained 10% glycerol, 0.1 M Tris-Cl buffer, pH 7.2, 10⁻³ M calcein, and increasing amounts of iron(III) perchlorate. Temperature was 100 K and field was scanned from 1000 to 5000 gauss.

**Electrochemical Studies**—The electrochemical investigations by cyclic voltammetry (CV) were performed in 0.05 M Tris-Cl, pH 7.2, 0.05 M NaClO₄ aqueous solution containing millimolar concentration of the iron(III)–calcein complex. CV curves were recorded using an EGG 273 Potentiostat coupled with a Kipp & Zonen x-y recorder. Electrochemical experiments were performed in a three-compartment cell, at room temperature, under an argon atmosphere. Potential are referred to an aqueous SCE reference electrode. The working electrode was a vitrous carbon disc (5 mm diameter) polished with 1-µm diamond paste.

**Calcein + Fe(II) Preparations**—The 1:1 Fe(II)+CA solution for UV-visible spectrophotometry and fluorimetric experiments has been prepared under argon in a glove box, starting from the solids (CA, Fe(II) as (NH₄)₂(SO₄)Fe·6H₂O) and a degassed 0.1 M Tris-HCl, pH 7.2, solution. The Fe(II) + CA solution was diluted to the appropriate concentration and placed into an hermetically closed quartz cell. Cells were taken out to the fluorimeter or the spectrophotometer, and their spectra were recorded. This prevents further Fe(II) oxidation, but not ferric contaminants from the powders.

**Cell Culture and Growth Measurements**—Plant cells were grown in 24-well sterile cell culture plates (Nunc); each independent axenic culture contains 1.5 ml of culture medium. This medium (MS) was a modified Murashige and Skoog (21): for 1000 ml of medium: 4.3 g of macro- and micro-elements powder (provided by Duchefa, catalog number M0221, Haarlem, Netherlands), 10 ml of H₃PO₄ (20 g/liter), 0.5 ml; kinetin (0.1 g/liter), 0.5 ml; (2,4-dichlorophenoxy)acetic acid (0.2 g/liter); 1 ml × 1000 vitamin solution (Duchefa M 0409), and 30 g of saccharose. Iron-free MS medium (MS-Fe) has the same content, made from reagent grade products, omitting Fe(II)+EDTA. Calcein is commercially available (Molecular Probes, Eugene, OR). Wells were inoculated at an absorbance of 0.003 (450 nm) with early stationary phase Arabidopsis thaliana (var. Columbia) cell suspensions. For axeny, plates where sealed with "Magic Scotch" tape. 24-well plates were agitated at 220 rpm at 25 °C in a New Brunswick "innova” 4230 refrigerated incubator shaker. Light was supplied 18 h a day by two growlux Sylvania 15-watt fluorescent tubes in the "photosynthes" accessory.

The measurements of cell density were made daily. It was often hampered by the occurrence of condensation droplets on the cover of the 12-well plates. To minimize light scattering during densitometry, the digitized images of the cultures were recorded from beneath through a
The Bio-Rad video camera system Geldoc 1000 was used, with the "molecular analyst" software for quantification. To record images of the plates, the hood of the camera was rotated to a horizontal position. A mirror was introduced through the open door of the hood, at 45° across the horizontal light path, held by a special stand, which also maintained the 12-well culture plate horizontally on a glass plate and held the Geldoc light source, upside down, 10 cm above the cultures. Full frame images were captured in constant light conditions, i.e. white light being adjusted so that just a few saturated pixels appeared in red in the empty optical field. In these conditions, a reproducible relationship was found between the absorbance of wells in densitometric scanning profiles of recorded images and the amount of cells in the wells. Images of culture plates without cells were used as baseline reference. They showed an uneven light background. This fact was compensated by the subtraction of an adapted baseline. Measurements and baseline profiles were drawn using the rectangle tool of the image analysis software. Base lines were drawn by joining the crest highlight points in each image between the wells and then subtracted. This method was found more accurate and reproducible than the use of 24 grids or the elliptical objects tools proposed by the software. When possible and necessary, condensation was fully eliminated by replacement of the cover in axenic conditions.

Fluorimetric Measurements of Iron Uptake in Culture Wells—Cells remained in their culture plates during fluorimetric iron uptake measurements. Respiration of the cells was maintained by aeration of the iron uptake medium in the return tubing from the fluorimeter cuvette. The drained cells received 1.5 ml of IUM and were connected to the 7-ml fluorimeter circuit described above. Excitation was made at 488 nm (slit: 1.5 nm) and emission recorded at 511 nm (slit: 2 nm). After recording the initial fluorescence level in the absence of cells for 200 s, the culture well and cuvette circuit were connected, and changes of fluorescence were recorded kinetically. The same spectrofluorimeter was used for in vitro iron exchange studies.

Prior to fluorimetric measurements, cells were drained by pressing a plastic pipette on the bottom of the wells and by aspiration of the culture medium through this leaky filtering contact. After three washes and drainings with iron-free culture medium (MS-Fe), the well was ready to be connected to a 7-ml circuit, filled with the iron uptake medium IUM (IUM = iron-free culture medium (MS-Fe) supplemented with Tris-maleate NaOH buffer (0.02 M), pH 5.8, 4 × 10⁻⁷ M CA, and 2 × 10⁻⁷ M ammonium iron(II) sulfate; the iron-CA complex was synthesized previously at low pH and in a 100 times more concentrated solution. It fully oxidizes upon dilution at pH 5.8. IUM is stored in 50-ml fractions at -20 °C, protected from light, and used immediately after thawing. An aspiration filter made with a 20-ml pipette tip filled with glass wool is connected to a peristaltic pump. The medium is pumped out at a rate of 0.42 ml/min into the cuvette of the fluorimeter. The rate of the return pumping was twice as fast, therefore air was swallowed by the tubing, allowing both the level control in the cuvette and an intimate aeration of the medium. The filter was agitated to prevent cell stacking and to improve aeration. The 400-μl cuvette of the fluorimeter (Jobin-Yvon Spec Fluoromax) is connected to the circuit with two Teflon tubes.

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![Fig. 3. Formulae and EPR spectra of Fe^{III}CAH (1), Fe^{III}calcein blue (2) and Fe^{II}EDTA (4). Curve 3 is the difference (1 - 2); 2' and 4' are expanded images of the major signal (1 mM concentration, in 10% glycerol, 0.1 M Tris-Cl buffer, pH 7.2; T = 100 K).](image-url)
RESULTS

Iron Binding Properties of CA

Thermodynamic Studies

$p_K_a$ Determination of CA and Fe$^{III}$CAH—The thermodynamic analysis of the Fe(III)-calcein complexation equilibria required the determination of the ligand deprotonation constants and an equilibrium study of the ferric complexes as a function of the pH. The deprotonation constants of the ligand have been determined by potentiometric (Fig. 1) and UV-visible spectrophotometric titrations. Analysis of the titration curves yield the $p_K_a$ values: 3.09 (3), 3.67 (3), 5.29 (3), 9.57 (2) (from potentiometry) and 12.3 (1) (from spectrophotometry). Numbers in parentheses represent the S.D. in the last significant digit. The $p_K_a$ values of three out of four of the carboxylic acids of the aminodiacetic moieties are too low to be determined from potentiometric measurement. The potentiometric titration curve of a 1:1 iron(III):calcein solution (Fig. 1) exhibited a buffer region at $a = 3–4$ ($a$ is the number of moles of added base per moles of ligand) over the pH range 5–7. Another buffer region is found at $a = 4.5–5$ over the pH range 8–9, indicating that the predominant species at pH $< 8$ is the monoprotonated complex, Fe$^{III}$CAH. Its $p_K_a$ has been determined to be 8.90 (5) from the analysis of the titration curve. The precipitation of the complex below pH 5 precluded the use of the titration data in refinement program. The complex stability constant was thus determined from spectrophotometric photometry. The UV-visible spectrum of the Fe$^{III}$CAH complex at pH 7.2 exhibits a band at 500 nm with a shoulder at 600 nm (Fig. 2).

In Vitro Equilibria and Iron Exchange Competitions—Competitions were carried out in order to determine the relative efficiency of calcein and EDTA, NTA, and citrate anions with regard to iron chelation and were observed spectrophotometrically. The Fe$^{III}$CAH solution was obtained by adding equimolar amount of Fe(III) perchlorate to CA at low pH, followed by a 0.05 M Tris-Cl, pH 7.2, 0.05 M NaClO$_4$ buffer. Equimolar amounts of Fe$^{III}$CAH complex and tested ligands were left in the dark for 4 days at 25 °C. The reverse exchange was done using the tested iron complex in competition with calcein. UV-visible light spectra were recorded when exchanges in both directions had led to similar results. The spectra in Fig. 2A show (i) a complete transfer of iron from Fe$^{III}$CAH to EDTA; (ii) an exchange with citrate, too low for quantification; (iii) a significant exchange with NTA allowing the quantification of iron(III) distribution between the two competing ligands and the calculation of thermodynamic constants. In addition, similar experiments were made in the presence of 2, 5, 10, and 20 molar eq of NTA with respect to Fe$^{III}$CAH. The metal displaced was measured to be 33% in the presence of 20 NTA eq. The competition equilibrium can be expressed by the following equations:

$$\text{FeCAH} + \text{NTA} \rightleftharpoons \text{FeNTA} + \text{CA} + \text{H}^+$$  \hspace{1cm} (Eq. 1)

with $K = (\text{[FeNTA]}_{\text{tot}}\text{[CA]}_{\text{tot}}\text{[H}^+])/(\text{[FeCAH]}_{\text{tot}}\text{[NTA]}_{\text{tot}}\text{[CA]}_{\text{tot}})$ and $K = (\beta_{110}\text{[FeNTA]}_{\text{p11}}\text{[CA]}_{\text{p11}})/(\beta_{110}\text{[FeCAH]}_{\text{p11}}\text{[CA]}_{\text{p11}})$. where FeNTA, CA, and NTA refer to all forms of Fe$^{III}$ complexed with NTA, free CA, and free NTA, at equilibrium and pH 7.2, respectively. Here $\alpha$’s are the usual Renbom coefficients (22) calculated from the deprotonation constants of the ligands CA and NTA (23) or from the complexation constants of Fe$^{III}$NTA (23). The average formation constant log $B_{110}$ of Fe$^{III}$CA determined to be 33.9(1). The typical $p_K_a$ value of calcein was calculated to be 20.3 (pH = 7.4, [Fe]$_{tot} = 1 \mu M$, [ligand]$_{p11} = 10 \mu M$). It is thus clear that Fe$^{III}$CAH is almost a thousandfold weaker complex than Fe$^{III}$EDTA (p$K_a$ Fe$^{III}$EDTA = 23.5).

UV-visible and EPR Studies

The UV-visible spectrum Fe$^{III}$CAH exhibits a phenol to iron charge transfer band at 600 nm ($\epsilon = 900$ M$^{-1}$cm$^{-1}$), which is not present in the Fe$^{III}$EDTA spectrum (Fig. 2). In order to gain information about the mode of coordination of the calcein with Fe(III), the spectrum was compared with that of the Fe$^{III}$calcein blue. Calcein blue is a fluorescent ligand having a single aminodiacetic complexing arm (see Fig. 3) instead of the two complexing arms of the diaminetetraacetic in EDTA and calcein. The UV-visible spectrum of Fe$^{III}$calcein blue (Fig. 2B) exhibits a phenolate to iron charge transfer band at 490 nm. Fe$^{III}$CAH thus provides a coordination to iron significantly different from EDTA unlike suggested previously (4).

Differences in coordination were also shown by EPR spectra of Fe$^{III}$complexes of CA, EDTA, and calcein blue at pH 7.2; EPR spectra of calcein and calcein blue are similar, as shown in Fig. 3 (the differential spectrum is very close to the base line). They are both centered at $g = 4.293$, and they are dissymetric. The spectrum of Fe$^{III}$EDTA is symmetrical and centered at $g = 4.272$.

These results suggest a coordination with one aminodiacetic arm and the phenolate group of the calcein, while the second aminodiacetic arm remains uncoordinated. Since its amine $pK_a$ has been determined to be 9.6 in the free ligand, it is still protonated at pH 7.2. Consequently, calcein is found to act as a tetradentate ligand like calcein blue.

Then, the question is raised of the possible binding of a second iron cation by the second aminodiacetic arm. During the spectrophotometric titration of CA by iron(III), a sharp and intense peak at 550 nm increased up to the addition of 2 eq (Fig. 4).

EPR titration experiments were performed under the same experimental conditions. Fig. 5 shows that the EPR signal intensity reaches a maximum at one iron equivalent per ligand and then decreases until it vanishes when 2 eq of iron have been added. It can be assumed that two successive bindings of iron cation occur, involving the two aminodiacetic arms. The dierferic complex is EPR silent revealing antiferromagnetic coupling of the two iron ions.

Further physiological experiments will be limited to conditions where only one iron(III) cation is complexed.

Electrochemical Studies

CV was performed in a 5 mM aqueous solution of the 1:1 Fe$^{II}$ and Fe$^{III}$calcein complex in the presence of 0.1 M Tris-Cl, pH
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FIG. 5. Evolution of the intensity of EPR signals of FeIII-calcine along iron additions (1 mM concentration, in 10% glycerol, 0.1 M Tris-Cl buffer, pH 7.2; T = 100 K).

FIG. 6. Cyclic voltammetric curve of iron CA (5 mM) in Tris-Cl, pH 7.2, 0.1 M NaClO4 aqueous solution; scan rate 0.1 V s⁻¹; working electrode: vitrous carbon disc electrode (5 mm diameter); E versus SCE.

FIG. 7. Quenching of fluorescence after addition to CA of FeIII or FeII in equimolar amount. Concentrations are 2.5 × 10⁻¹⁷ M, pH 7.2. a = CA, b = CA + FeII, and c = CA + FeIII. b is prepared using a glovebox in argon atmosphere.

Kinetic Iron Exchange Studies (in Vitro)

Citrato is known to be very abundant in vivo (1–8 mM citrate compared with 2–100 μM iron in root sap (24)) and serves as the main intercellular iron transporter in plants. The affinity of citrate for iron(III) is characterized by a log β₁₁₁₉₁₉₉₉₉₉ value of 11.8. Thus, this value seems low as compared with that of calcein which is measured here, log β₁₁₁₁₉₁₉₉₉₉₉ = 33.9; one would expect calcein to take up iron from citrate. Ideally, a fluorescent chemosensor should not withdraw too much iron from iron transport molecules, which are the precursor of most biological iron ligands. NTA (log β₁₁₁₁₉₁₉₉₉₉₉ = 15.9) has an intermediate affinity, maybe close to that of many cellular iron ligands. Experiments are necessary to test if citrate has the potential to share iron with calcein (and NTA) in an irreversible equilibrium, which evolves quickly enough to reflect metabolic competition for iron.

A stopped flow experiment of iron(III) complexation by calcein and calcein blue has shown that the reaction is completed in 1 s (Fig. 8). CA fluorescence is therefore expected to be a fast reporting tool of the iron(III) availability.

A stopped flow experiment of iron(III) complexation by citrate and NTA has shown that the reaction is completed in 1 s (Fig. 8). CA fluorescence is therefore expected to be a fast reporting tool of the iron(III) availability.

Kinetics of iron exchange between calcein and NTA or citrate have been followed by measuring the variations of the fluorescence intensity of the solution during incubation with a large excess of NTA or citrate (10 or 6320 molar eq, respectively) in a buffer 40 mM Hepes at pH 7.2, 150 mM NaCl, at 25 °C in the presence of 4 × 10⁻¹⁷ M FeIIICAH. These conditions were chosen because citrate is much more abundant in vivo as compared with CA, when introduced as a probe. Fig. 9 shows that fluorescence intensity at 511 nm increases, indicating iron release from calcein. The kinetics of iron release from FeIII-CAH to NTA or to citrate is easily measurable in the time scale of minutes. Calcein is shown able to reflect both iron loading or release by citrate, in the range of concentrations expected to be found in vivo. We conclude that despite its high affinity, calcein at low...
analysis of Fe III complexation by CA or calcein blue ([Fe III] pH 7.2 in the presence of 40 mM Hepes, pH 7.2, and 150 mM NaCl. [H+] = 0.03 m, [calcein] = 2·10⁻⁵ m, [calcein blue] = 2.5·10⁻⁴ m) temperature is 25 °C, and absorbance is monitored at 522 nm for calcein and 530 nm for calcein blue; curves are normalized.

FIG. 9. Kinetics of the fluorimetric changes of Fe IIICAH 4·10⁻⁷ m after the addition of either NTA 2·10⁻⁵ m or citrate 2.5·10⁻⁵ m at pH 7.2 in the presence of 40 mM Hepes, pH 7.2, and 150 mM NaCl.

Concentrations seem able to reflect changes in pFe III induced by ligands like citrate and NTA, which cover a wide range of affinity for iron, presumably that of metabolic iron ligands.

In Vivo Kinetics of Iron Nutrition by Plant Cell Suspensions

Because of the high sensitivity of fluorescent probes, real time measurements of iron(III) uptake by plant cells have been carried out. Radioactive techniques are generally used for milligram amounts of plant material for nutritional studies. They require destructive assays on aliquoted samples. Fluorescent assays instead are neither destructive nor harmful and allow real time observations without sampling. However, fluorimeter cuvettes are not adapted for plant cell cultures and for discriminating signals coming from the cells from those coming from the medium. A system in which plant cell growth can be maintained was preferred, which allowed a separate measurement of the fluorescence of the medium. Contrary to its acetoxymethyl ester derivatives, unesterified CA is not able to cross the membranes and remains extracellular. The apoplast, the compartment mainly composed of cell walls, is extracellular and is known to have an important iron storage function in iron nutrition for plants (25–27). The device used here has the advantage of measuring both intracellular and apoplastic iron uptake. A [Fe]/[CA] = 1/2 mixture was used because it presents the strongest iron buffering power, and it can reflect both iron release and uptake. Culture plates were connected to the fluorimeter cuvette by a system allowing respiration with full oxygenation of the medium. In some experiments, the ionophore A 23187 (Sigma) was added to check if the cell membrane could be a limiting factor in the uptake process. Fig. 10 shows that upon contact with the cells, the fluorescence of the nutrient solution raises regularly for more than 30 min, indicating iron release from Fe IIICAH. The rate of iron uptake by cells is thus directly readable from the increase of fluorescence. The addition of the ionophore accelerates the rate of the uptake. The sensitivity of the method is high, and the rate of iron uptake is so high (up to 0.93 nmol·h⁻¹/g of fresh cells) that it cannot be considered as a steady state rate of iron nutrition. Therefore, CA is potentially a good siderophore.

Growth and Greening of Plant Cells Using Fe IIICAH as Iron Source

In order to test the physiological significance of the iron uptake observed using fluorescence monitoring, we have followed the growth of cell suspensions fed with Fe IIICAH as the only source of iron. The inocula derived either from cell suspensions prestarved with iron (presenting limited symptoms of chlorosis) or from controls provided with sufficient iron.

Results are shown in Fig. 11. Using iron-prestarved cells or normally fed cells, Fe IIICAH was found to be a very good iron source. In 7 days, growth and regreening after chlorosis was much faster than when control Fe IIIEDTA was used. However, faster growth or greening were hardly noticeable when, in other experiments (not shown), nonprestarved inocula were used. A special experiment was set up to test the physiological function of the iron apparently taken up at high rate by cells during the initial contact with Fe IIICAH; we wanted to check that neither precipitation nor other nonphysiological artifact interfered with iron uptake. Inocula from iron-prestarved and iron sufficient cultures were pretreated for 1000 s with 50 μm iron as Fe IIICAH or as Fe IIIEDTA. A series was then washed and cultivated without iron, another was maintained in the presence of 50 μm iron as Fe IIICAH or Fe IIIEDTA. Growth and chlorotic symptoms (Fig. 12 and Table I) were followed along the cultures.

The growth of iron-sufficient inocula was not affected after a 8 days culture without iron (wells 13–15) but cells be-
came chlorotic. Nevertheless, a pretreatment of 1000 s with Fe\textsuperscript{III}CAH was sufficient to prevent a further appearance of chlorosis (wells 19–21), while after the same treatment using Fe\textsuperscript{III}EDTA, the cells became chlorotic (wells 22–24). The fast initial uptake had an efficient nutritional function.

Using prestarved cells, pretreatment of 1000 s with Fe\textsuperscript{III}CAH limited chlorosis (wells 7–9 compared with 1–3), but did not restore normal growth. Pretreatment of 1000 s with Fe\textsuperscript{III}EDTA had no positive effect (wells 10–12). In controls, the continuous presence of 50 \mu M Fe\textsuperscript{III}EDTA restored growth and greening (wells 16–18). The same amount of Fe\textsuperscript{III}CAH killed the inoculum (wells 4–6) during the lag time period, at high dilution of cells.

We conclude that Fe\textsuperscript{III}CAH is a more readily available source of iron for plant cells than Fe\textsuperscript{III}EDTA, since it favors a faster growth and regreening (Fig. 11). The iron taken up from Fe\textsuperscript{III}CAH at high initial rates, during the 1000 s treatments (or during fluorescence uptake measurements), can be physiologically mobilized later, and is therefore efficiently stored in cells or in the apoplasm. Fe\textsuperscript{III}EDTA does not allow such fast iron storage loading.

Nevertheless, toxic effects have been evidenced at 5\times10^{-5} M Fe\textsuperscript{III}CAH, only on iron prestarved cells, in a dilute weakened inoculum, where the amount of iron per cell is higher than in normal growth or during iron uptake experiments. This toxicity may be correlative of an easy uptake of iron and may be due to Fenton reactions after iron reduction. Some photolability of Fe\textsuperscript{III}CAH have been observed in vitro (not shown). It may be due to photoreduction followed by Fenton reactions as observed with Fe\textsuperscript{III}citrate and Fe\textsuperscript{III}EDTA (28).

**DISCUSSION**

From a chemical point of view, calcein is an interesting iron ligand whose fluorescence is quenched upon binding to iron. As a consequence, it has been studied as a sensor for iron and suggested to be a useful tool for monitoring cytosolic iron and assessing the dynamics of intracellular iron in living cells; interesting observations were evidenced (4 to 10). Nevertheless, no firm characterization of its thermodynamic, dynamic, and structural properties had been carried out previously and this was the first aim of this work.

We show for the first time that calcein is a specific Fe\textsuperscript{III} ligand and binding of Fe\textsuperscript{II} is scarcely sensed by fluorescence extinction. The affinity for Fe\textsuperscript{II} is shown from the large \(b_{111}\) value and the large pFe\textsuperscript{III} value. This is not surprising since calcein is providing in addition to carboxylates, a phenolate for binding iron (29) as shown from the characteristic phenoxo-to-iron charge transfer band (\(e = 900 \text{ M}^{-1} \text{cm}^{-1}\) at 600 nm) in its visible spectrum. This band is also present in the spectrum of the calcein blue-iron complex (\(e = 1200 \text{ M}^{-1} \text{cm}^{-1}\) at 490 nm).

This combination of carboxylates and phenolate is known to greatly stabilize the Fe\textsuperscript{III} form. This is reflected in the high stability constant and the very low redox potential, much comparable with those for siderophores. It is clear that calcein is significantly different from EDTA as a ligand: different coordination (thus different visible and EPR spectra), lower redox potential of the Fe\textsuperscript{III}/Fe\textsuperscript{II} couple. An unexpected property of calcein is its ability to bind 2 Fe\textsuperscript{III} atoms; the absence of EPR
signal and the very intense absorption band at 550 nm of the 2:1 iron:calcein complex are remarkable physicochemical characteristics.

From a biochemical point of view, another interesting property of calcein is its fast exchangeability as an iron ligand. This is reflected in the fast kinetics of iron complexation during incubation of Fe(III) and calcein and in the fast exchange with other iron(III) ligands, such as NTA or citrate the iron transporter in plants, over a wide range of affinities. Thus the calcein/iron-calcein system is an iron buffer sensitive to competition with other ligands. This makes it a kinetically competent, simple, sensitive, and nondestructive fluorescent probe for monitoring Fe(III) in cells.

Our finding that calcein is a poor fluorescent probe for Fe(II) is in marked contrast with conclusions from several papers (4, 10). The redox conditions required to form a Fe(II)-calcein are not physiologically relevant and thus any binding, if any, of Fe(II) will result in oxidation and formation of the Fe(III)-calcein complex, disrupting the balance between Fe(III) and Fe(II). Our data thus do not support the assumption that calcein fluorescence has the potential to reflect the variations in the ferrous iron cytosolic pool. Experiments using 59Fe and organic solvent extractions of iron complexes were presented in Ref. 4 as corroborating the hypothesis of a ferrous probe. They are not convincing since tissue grinding is necessary, therefore cellular compartmentation is destroyed, oxygen is introduced, redox potential and pH are averaged, and metabolic iron fluxes are interrupted. No consensus appears in the literature on estimations of cellular Fe(III) concentrations: from 10^{-5} m or 10^{-8} m (10) to 10^{-17} m as estimated through the affinity for nicotinamine, the Fe(II) transporter in plants, which is suspected to be the natural Fe(II) sensor (30).

From a physiological point of view, we demonstrate two new applications of the Fe(III)-CAH complex. We show (i) that it provides the first real time tool for monitoring iron(III) uptake by intact plant cells. The contribution of the apoplasmin, which is physiologically important for iron storage (25–27), is not bypassed by the proposed method. Furthermore, (ii) a physiological study of iron-calcein as a nutritional siderophore demonstrates that CA is better than EDTA for restoring normal growth after iron starvation and chlorosis; its toxicity only appears in unusual high concentrations. The nutritional properties of Fe(III)-CAH reflect its chemical affinity for iron: an intermediate between those of citrate and EDTA. Calcein is a fast source and the iron taken up for 1000 s from calcein is further functional for growth and prevention of chlorosis and therefore stored in a physiologically efficient compartment, presumably ferritins and the apoplasmin. CA is therefore expected to bind iron in the range of affinity of the ferric cellular iron pool.

Iron buffering conditions are very often disregarded. In many in vitro experiments, iron is introduced either as a salt or a more stable 1/1 iron complex. In these conditions, iron is not buffered and a small exchange of iron or ligand results in drastic changes in free iron concentrations. An appropriate excess of ligand should be introduced to buffer the iron donor system (as it is done for calcium standards (31)). pFe calculations are generally possible in vitro. They are directly predictive of a hierarchy in actual binding efficiencies. The quantification of metal buffering could help biologists to describe and compare their experimental conditions. Like pH is generally controlled in the experiments describing acidosis-buffer reactions, buffered iron reagents (i.e. in known pFe limits), are desirable in experiments on iron metabolism.

In animal cells, the limit between iron deficiency and sufficiency is sensed naturally by iron regulatory proteins (IRP 1 and IRP 2), which are ligands for Fe(II). Fe(II) loading of IRPs act as a molecular switch for iron uptake or storage. According to Epsztejn et al. (10), the definition of the LIP is the sum of free and CA bound iron. This definition invokes Fe(III) and is not based upon the iron buffering power of the cells. We propose a physiological definition of the LIP: the LIP is the amount of buffered iron that can be released by the cell before the IRPs loose their iron.

New fluorescent ligands whose affinity for iron(II) is close to that of the IRPs must be characterized for the quantitative estimation of cellular iron(II). Some fluorescent probes become available for iron(III) (32, 33). A fluorescent form of desferrioxamine B has been synthesized. It was not found able to measure directly iron nutrition by plants, except when used in symbiosis by the microflora of the roots (34). A ferrichrome analog has been used to measure iron uptake by bacteria (35). A search for ferrous fluoroprobes is needed.

Acknowledgements—We thank Dr. A. Martre for technical assistance and Dr. A. Deronzier for his interest on this work.

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