Selective determination of mitochondrial chelatable iron in viable cells with a new fluorescent sensor

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Mitochondrial chelatable ('redox-active') iron is considered to contribute to several human diseases, but has not yet been characterized in viable cells. In order to determine this iron pool, we synthesized a new fluorescent indicator, rhodamine B-[(1,10-phenanthroline-5-yl)aminocarbonyl]benzyl ester (RPA). In a cell-free system, RPA fluorescence was strongly and stoichiometrically quenched by Fe²⁺. RPA selectively accumulated in the mitochondria of cultured rat hepatocytes. The intramitochondrial RPA fluorescence was quenched when iron was added to the cells in a membrane-permeant form. It increased when the mitochondrial chelatable iron available to the probe was experimentally decreased by the membrane-permeant transition metal chelators pyridoxal isonicotinoyl hydrazone and 1,10-phenanthroline. The concentration of mitochondrial chelatable

iron in cultured rat hepatocytes, quantified from the increase in RPA fluorescence after addition of pyridoxal isonicotinoyl hydrazone, was found to be $12.2 \pm 4.9 \mu\text{M}$. Inhibition of haem synthesis with succinylacetone did not alter the signal obtained in hepatocytes, but a rapid increase in the concentration of mitochondrial chelatable iron was observed in human erythroleukaemia K562 cells. In conclusion, RPA enables the selective determination of the highly physiologically and pathophysiologically interesting mitochondrial pool of chelatable iron in intact cells and to record the time course of alterations of this pool.

Key words: chelators, fluorescence, indicators, mitochondria.

INTRODUCTION

Iron is essential for many biological processes, but is also detrimental as it fosters the generation of highly destructive oxygen species. Iron-dependent oxidative processes in cells (e.g. lipid peroxidation, oxidative alterations of proteins and DNA damage) are usually believed to be the result of increases in the release of superoxide anion radicals (O₂^{•-}) and/or H₂O₂. In recent years, however, evidence has accumulated indicating that alterations in the intracellular pool of 'redox-active' iron (called 'chelatable iron' for experimental reasons) can contribute substantially to a variety of injurious processes (e.g. ischemia-reperfusion injury, ethanol toxicity and oxidative injury after nutrient deprivation [1–5]) or can even constitute the primary injurious event [6]. On the other hand, chelatable iron ions are increasingly found to be decisively involved in several intracellular signalling pathways, i.e. in physiological processes such as O₂ sensing [7].

Over the last five decades, attention was almost exclusively focused on a cytosolic pool of chelatable, redox-active iron, which is generally regarded to constitute the cellular chelatable iron pool [8–10]. However, a 'transit' pool of chelatable iron must also be assumed to exist within the mitochondria; during haem synthesis, iron is incorporated into protoporphyrin IX within the mitochondrial matrix. In addition, there is now increasing evidence for a role of mitochondrial chelatable iron in the pathogenesis of various human diseases. Examples include Friedreich ataxia [11,12] and neurodegenerative diseases, such as Parkinson's and Alzheimer's diseases [13–16].

Although of high physiological and pathophysiological interest, mitochondrial chelatable iron is still very poorly defined.

Several methods have been established to determine mitochondrial chelatable iron, but all of them are prone to false readings and none is applicable to viable cells [17–19]. Recently, we explored the spatial distribution of chelatable iron in single intact cells for the first time using a method based on laser scanning microscopy and detected a substantial pool of chelatable iron within mitochondria of cultured rat hepatocytes ($\geq 4.8 \mu\text{M}$) and rat liver endothelial cells ($\geq 9.2 \mu\text{M}$) [20]. However, these measurements shared the drawbacks that the fluorescent transition-metal indicator Phen Green SK (PG SK) (1) did not show sufficient intramitochondrial accumulation, and (2) had a rather uniform intracellular distribution [20]. Therefore we attempted to develop a selective mitochondrial iron indicator which overcomes these problems. Deprotonated rhodamine B (rhodamine B base) was chosen for mitochondrial targeting and as a fluorophore, and was coupled with 4-(bromomethyl)-N-(1,10-phenanthroline-5-yl)benzamide for iron chelation. In the present study, we report the synthesis, characterization and application of rhodamine B-[(1,10-phenanthroline-5-yl)aminocarbonyl]benzyl ester (RPA), the first indicator allowing selective determination of mitochondrial chelatable iron in viable cells.

EXPERIMENTAL

Animals

Male Wistar rats (200–350 g) were obtained from the Zentrales Tierlaboratorium (Universitätsklinikum, Essen, Germany). Animals were kept under standard conditions with free access to food and water. All animals received humane care in compliance with the institutional guidelines.

Abbreviations used: DTPA, diethylenetriaminepenta-acetic acid; FAS, ferrous ammonium sulphate/citric acid trisodium salt dihydrate; FCS, fetal-calf serum; HBSS, Hanks balanced salt solution; α -MEM, α -minimum essential medium; PG SK, Phen Green SK; PIH, pyridoxal isonicotinoyl hydrazone; RPA, rhodamine B-[(1,10-phenanthroline-5-yl)aminocarbonyl]benzyl ester; SA, succinylacetone; TMRM, tetramethylrhodamine methylester.

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Materials

1,10-Phenanthroline monohydrate, 4-bromomethyl-benzoic acid and rhodamine B base were purchased from Acros-Organics (Geel, Belgium), and hydrazine monohydrate and phosphorus tribromide were from Fluka (Buchs, Switzerland). TLC was carried out on pre-coated neutral aluminium oxide 60 F₂₅₄ TLC plates from Merck (Darmstadt, Germany). Neutral aluminium oxide for column chromatography, obtained from Fluka (Buchs, Switzerland), was deactivated with 7% (w/w) water. Leibovitz L-15 medium and α -minimum essential medium (α -MEM) were obtained from Gibco-BRL (Eggenstein, Germany); collagenase, collagen (Type R), dexamethasone, BSA and gentamicin were from Serva (Heidelberg, Germany); and DMSO, Triton X-100 and citric acid trisodium salt (dihydrate) were from Merck (Darmstadt, Germany). SDS was obtained from Bio-Rad (Richmond, CA, U.S.A.); fetal-calf serum (FCS), ferrous ammonium sulphate, ferric chloride, 8-hydroxyquinoline, poly-L-lysine and propidium iodide came from Sigma (Deisenhofen, Germany); and Chelex 100 (iminodiacetic acid chelating resin), succinylacetone (SA), 1,10-phenanthroline and 4,7-phenanthroline were from Sigma-Aldrich (Steinheim, Germany). The fluorescent dyes tetramethylrhodamine methyl ester (TMRM) and rhodamine 123 were purchased from Molecular Probes Europe BV (Leiden, The Netherlands). Pyridoxal isonicotinoyl hydrazone (PIH), a generous gift from Professor P. Ponka (Lady Davis Institute for Medical Research, Montreal, Canada), was dissolved in 0.1 M NaOH, diluted with Hanks balanced salt solution (HBSS; 137 mM NaCl, 5.4 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 0.4 mM KH₂PO₄, 0.4 mM MgSO₄, 0.3 mM Na₂HPO₄ and 25 mM Hepes) and the pH adjusted to 7.35 at 37 °C. Falcon cell culture flasks and 6-well cell culture plates were obtained from Becton Dickinson (Heidelberg, Germany), and glass coverslips were from Assistent (Sondheim/Röhn, Germany).

Analysis of synthesized compounds

The synthesized compounds (see below) were analysed using the following techniques: ¹H- and ¹³C-NMR spectroscopy (Avance DRX 500; Bruker, Rheinstetten, Germany); electron impact ionization and fast atom bombardment MS (VG Pro Spec 3000; Fisons Instruments Manchester, U.K.); high-resolution MS (electrospray ionization; Bio TOF II; Bruker, Boston, MA, U.S.A.); IR spectroscopy (Series FTS 135 FT-IR; Bio-Rad, Cambridge, MA, USA); UV/visual spectroscopy (Spectralys; J&M, Aalen, Germany); and fluorescence spectroscopy (FL3095 spectrofluorometer; J&M). Melting points (uncorrected) were determined using a Büchi 510 (Flawil, Switzerland).

Synthesis of 4-(bromomethyl)-*N*-(1,10-phenanthroline-5-yl)benzamide and RPA

The following compounds were prepared as previously described: 5-nitro-1,10-phenanthroline [21], 1,10-phenanthroline-5-amine [22] and 4-bromomethyl-benzoyl bromide [23].

4-(Bromomethyl)-*N*-(1,10-phenanthroline-5-yl)benzamide

A solution of 4-bromomethyl-benzoyl bromide (2.39 g, 8.60 mmol) in 50 ml of dry acetonitrile (dried over calcium hydride and distilled before use) was slowly added to 100 ml of acetonitrile containing 1,10-phenanthroline-5-amine (1.30 g, 6.66 mmol). After having stirred the suspension overnight at 22 °C, the product was filtered off and washed with dry acetonitrile, followed by cold 5.0% sodium bicarbonate and finally

with water, and dried in a desiccator under vacuum overnight. 4-(Bromomethyl)-*N*-(1,10-phenanthroline-5-yl)benzamide was obtained as a light yellow powder (yield, 2.32 g, 5.91 mmol, 88.0%). Decomposition 230 °C. ¹H-NMR (500 MHz): δ [(D₆) DMSO] 4.82 (s, 2 H), 7.67 (d, 2 H, *J* 8.2), 8.13–8.16 (m, 3 H), 8.20 (dd, 1 H, *J* 8.2, *J* 5.1), 8.49 (s, 1 H), 8.98 (dd, 1 H, *J* 8.6, *J* 1.1), 9.09 (dd, 1 H, *J* 8.2, *J* 1.0), 9.24 (dd, 1 H, *J* 4.9, *J* 1.3), 9.31 (dd, 1 H, *J* 4.6, *J* 1.1), 10.99 (s, 1 H). ¹³C-NMR (125 MHz): δ [(D₆) DMSO] 33.39, 121.59, 125.06, 125.48, 126.36, 128.52, 129.21, 129.39, 133.48, 133.62, 136.67, 139.17, 141.96, 142.22, 146.46, 148.92, 166.31. IR (KBr): $\tilde{\nu}$ (cm⁻¹) = 3432 (NH), 3088 (CH_{ph}), 2986 (CH_{aliph}), 1678 (CO_{amide}), 1594 (CO_{amide}). MS (electron impact ionization): *m/z* (%) = 392 (2) [*M*⁺], 313 (30) [*M*⁺ – Br], 195 (37) [*M*⁺ – C₈H₆BrO], 119 (100) [*M*⁺ – C₁₂H₈N₃Br].

RPA

A solution of rhodamine B base (1 g, 2.26 mmol) and 4-(bromomethyl)-*N*-(1,10-phenanthroline-5-yl)benzamide (0.89 g, 2.26 mmol) in 15 ml of dry dimethylformamide (distilled before use) was stirred for 64 h at 100 °C. Dimethylformamide was removed by rotary evaporation with a bath temperature < 50 °C. The residue was dissolved in 20 ml of dichloromethane, undissolved material was filtered off and the solvent was evaporated. The crude product was purified by column chromatography on deactivated aluminium oxide, and eluted with dichloromethane/n-hexane/ethanol (10:4:1–10:2:1, by vol.). The deeply coloured fractions were analysed by TLC with dichloromethane/n-hexane/ethanol (8:1:1, by vol) as the solvent. The combined product fractions (*R_f* = 0.4) were evaporated to give RPA as a dark-purple solid (yield, 370 mg, 0.44 mmol, 20%). M.p., 182 °C. ¹H-NMR (500 MHz): δ [CDCl₃] 1.20 (t, 12 H, *J* 7.1), 3.41–3.62 (m, 8 H), 4.88 (s, 2 H), 6.72 (dd, 2 H, *J* 9.6, *J* 2.4), 6.82 (d, 2 H, *J* 8.3), 6.89 (d, 2 H, *J* 2.4), 6.93 (d, 2 H, *J* 9.6), 7.18–7.20 (m, 1 H), 7.48–7.53 (m, 2 H), 7.67–7.78 (m, 2 H), 8.11 (s, 1 H), 8.15 (dd, 1 H, *J* 8.0, *J* 1.2), 8.28–8.30 (m, 1 H), 8.39 (d, 2 H, *J* 8.2), 8.61 (dd, 1 H, *J* 8.3, *J* 1.7), 9.02–9.05 (m, 2 H), 11.81 (s, 1 H). ¹³C-NMR (125 MHz): δ (CDCl₃) 12.57, 46.03, 67.13, 96.94, 113.24, 113.86, 122.30, 122.76, 123.03, 126.35, 127.78, 128.36, 129.36, 129.81, 130.02, 130.26, 130.81, 131.60, 132.75, 133.04, 133.05, 133.49, 133.91, 135.88, 136.93, 145.02, 146.32, 149.31, 149.59, 155.28, 157.34, 157.50, 165.00, 166.78. IR (KBr): $\tilde{\nu}$ (cm⁻¹) = 3437 (NH), 2976 (CH_{aliph}), 1715 (CO_{ester}), 1646 (CO_{amide}), 1589 (CO_{amide}), 1180 (CO_{ester}). MS (fast atom bombardment): *m/z* (%) = 754 (88) [*M*⁺ – Br], 443 (21) [*M*⁺ – C₂₀H₁₄BrN₃O]. High resolution MS (electrospray ionization, positive ion mode, methanol): calculated value for C₄₈H₄₄N₅O₄ was 754.33933 and the observed value was 754.33755. UV [performed in a 'simple buffered solution'; 2 mM freshly prepared ascorbate, 0.15% (w/v) SDS and 10 mM Tris/HCl (pH 8.2 at 22 °C)]: λ_{max} (lg ϵ) = 564 nm (4.95), 510–535 nm (broad shoulder).

Fluorescence measurements in a cell-free system

Fluorescence spectra of RPA (20 μ M) were scanned in the absence and presence of Fe²⁺ {added from a stock solution of ferrous ammonium sulphate/citric acid trisodium salt dihydrate (FAS) [24]} in a 'simple buffered solution' (see above). Spectra were not corrected for light intensity or detector sensitivity.

A laser scanning microscope (LSM 510; Zeiss, Oberkochen, Germany) equipped with a helium/neon laser was used to determine the effects of: (1) Fe²⁺ and Fe³⁺; (2) Na⁺ (NaCl), K⁺ (KCl), Mg²⁺ (MgCl₂), Ca²⁺ (CaCl₂), Mn²⁺ (MnCl₂), Ni²⁺ (NiSO₄), Cu⁺ (CuCl), Cu²⁺ (CuCl₂), Co²⁺ (CoCl₂) and Zn²⁺ (ZnCl₂); (3)

ionic strength (0 or 150 mM KCl); (4) pH (pH 7.0–9.5); and (5) viscosity of the medium (altered by addition of 1.75 M sucrose) [25] on RPA fluorescence at 37 °C in two different media: (1) a 'simple buffered solution' (see above); and (2) a medium designed to simulate the composition of the mitochondrial matrix ('mitochondrial medium' containing 30 mM KCl, 6 mM MgCl₂, 4 mM P_i, 1.1 mM CaCl₂ and 1 mM NaCl [26], 6.85 mM glucose, 0.23 mM citrate and 6 mM ATP [17], 6 mM NADPH, 4 mM NADH and 10 mM glutathione (freshly prepared) [27], and 1 mM ascorbate [28] (freshly prepared) and 10 mM Tris/HCl buffer (pH 8.2 at 37 °C)). Part of the concentrations given were calculated from the mitochondrial content of the respective compound assuming that approx. 7.2×10^9 rat liver mitochondria contain 1 mg of protein and the volume of a single mitochondrion is $0.71 \mu\text{m}^3$ [26]. All components of the media ('mitochondrial medium' and 'simple buffered solution') were dissolved in double-distilled water. The final media (without SDS) were treated with Chelex [29] in order to minimize their heavy metal contamination. Following the treatment with Chelex, 0.15 % (w/v) of the anionic detergent SDS was added as a solvent for RPA.

A portion (1 ml) of the medium was transferred into thermostated (37 °C) Eppendorf cups and RPA (5–100 μM ; stock solutions of 1 mM or 5 mM in DMSO) was added and mixed with the medium. Subsequently, the ions were added from their 100-fold stock solutions. After incubation for 2 min, a portion (100 μl) of the medium was placed on Chelex-treated glass coverslips and the fluorescence was determined using the laser scanning microscope as described below for the cellular measurements.

Cell isolation and culture

Hepatocytes were isolated from male Wistar rats as described previously [30]. The cells were seeded on to collagen-coated 6.2 cm² glass coverslips in 6-well cell culture plates or on to collagen-coated 12.5 cm² culture flasks, and cultured in L-15 medium supplemented with 5 % (v/v) FCS, 2 mM L-glutamine, 8.3 mM glucose, 0.1 % BSA, 14.3 mM NaHCO₃, 50 mg/l gentamicin and 1 μM dexamethasone, as described previously [31]. Human erythroleukaemia K562 cells (accession no. ACC 10) were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany) and cultured as described previously [31] in α -MEM medium supplemented with 7 % (v/v) FCS and 2 mM L-glutamine.

Application of RPA in the cellular system

Experiments with hepatocytes were started 20–24 h after isolation of the cells. The glass coverslips were transferred to a modified Pentz chamber and cells were washed twice with HBSS (pH 7.4) at 37 °C. The cells were incubated with RPA (0.01–10 μM ; 10 μM or 1 mM stock solutions in DMSO) for 10 or 20 min at 37 °C in HBSS and then washed three times with HBSS. K562 cells (10×10^6 cells/ml) were washed once with HBSS by centrifugation (170 g for 1 min at 37 °C) and attached to poly-L-lysine-coated (5 $\mu\text{g}/\text{cm}^2$) glass coverslips 10 min before loading the cells with RPA. Adherent K562 cells were incubated with 0.5 μM RPA for 10 min in HBSS at 37 °C and then washed with HBSS. As RPA adsorbed strongly to the surface of the chambers (most likely because of its positive charge), the glass coverslips with the adherent cells were transferred to a second (indicator-free) Pentz chamber after loading of the cells. Cells were then incubated for another 15 min (hepatocytes) or 30 min (K562 cells) in HBSS. This incubation period strongly improved the selectivity of the mitochondrial loading with RPA; intracellular indicator, which

was still located within the cytosol and the nucleus at the end of loading, almost completely accumulated within the mitochondrial matrix space during this additional incubation period. Hepatocytes were then covered with HBSS or L-15 medium, and K562 cells were covered with α -MEM (37 °C). In some experiments, cultured hepatocytes and K562 cells in suspension were incubated with SA (1 mM) in L-15 medium or α -MEM respectively, for 1, 3 or 24 h before fluorescence measurements were started.

Intramitochondrial RPA fluorescence was determined by using quantitative laser scanning microscopy (see above). The objective lens was a 63 $\times$ (numerical aperture 1.40) Plan-Apochromat. The red fluorescence of RPA excited at 543 nm was collected through a 560 nm long-pass filter. The scanning parameters for quantitative fluorescence measurements were as follows: the pinhole was set at 130 μm , producing confocal optical slices of less than 1.0 μm in thickness. Confocal images (scanning time 3.9 s; zoom-factor 0.7–2.5) were collected at 10 min intervals with the power of the helium/neon laser set at 1 %. Qualitative fluorescence measurements were performed with different parameters (longer scanning times, a higher pixel density and laser power and use of image averaging). Mitochondrial RPA fluorescence of large numbers of organelles was determined for every field of vision after background subtraction; fluorescence of single mitochondria was determined by confining the regions of interest manually to individual mitochondria (some cases of dark images required electronic brightening of the images for proper positioning of these regions). Image processing and evaluation were performed using the 'physiology evaluation' software of the LSM 510 imaging system.

The intramitochondrial level of chelatable iron was manipulated 5–10 min after the beginning of the measurements by addition to the supernatant of the highly membrane-permeant FeCl₃/8-hydroxyquinoline complex (5–15 μM) [31] or membrane-permeant iron chelators PIH (2 mM) or 1,10-phenanthroline (2 mM). In some controls, RPA (0.2 μM in HBSS at 37 °C) was supplemented with the hydrophilic, almost membrane-impermeant, Fe³⁺ chelator diethylenetriaminepenta-acetic acid (DTPA; 200 μM) [31], or RPA (0.2 μM , diluted from a 100 μM stock solution in DMSO) was pre-complexed with Fe²⁺ (1 mM; added as FAS to the stock solution [24]) prior to its addition to the cells. In other experiments, SA (1 mM) was added to the supernatant (L-15 medium or α -MEM respectively).

Ex situ calibration of intramitochondrial RPA concentrations

The intramitochondrial RPA concentration was determined by comparing the mitochondrial fluorescence (in arbitrary units) after 'dequenching' with PIH (2 mM) with the fluorescence of RPA standards (5–80 μM) dissolved in the 'mitochondrial medium' (see above). In order to obtain a calibration curve, portions (100 μl) of the medium at 37 °C containing known concentrations of RPA were placed on Chelex-treated glass coverslips (of the same type as used to collect cellular micro-fluorographs). The fluorescence measurements were performed in a focal plane 10 μm above the surface of the coverslips, safely within the medium. This focal plane and the laser scanning parameters used for the fluorescence measurements in a cell-free system were the same as used for the quantitative cellular measurements.

Ex situ calibration of Fe²⁺-induced quenching of RPA fluorescence

A portion (1 ml) of the 'mitochondrial medium' was transferred into thermostated (37 °C) Eppendorf cups and RPA (20–80 μM)

Scheme 1 Reaction scheme for the synthesis of RPA

was added and mixed with the medium. In order to obtain a calibration curve, known concentrations of FAS were added from a freshly prepared stock solution (1 mM) that additionally contained 20 mM ascorbate [24]. After incubation for 2 min (i.e. after the formation of the RPA/ Fe^{2+} complex), portions of the medium were placed on Chelex-treated glass coverslips and the fluorescence was determined as described for the cellular measurements using the laser scanning microscope.

Identification of mitochondria and recording of the mitochondrial membrane potential

Mitochondria were identified and their functional integrity confirmed by membrane-potential-dependent staining with TMRM. Hepatocytes were incubated with TMRM (0.1–1 μM) for 10 or 20 min in HBSS at 37 °C and intracellular TMRM fluorescence was detected as described above for the experiments with RPA (i.e. under control conditions). In some experiments, including all experiments with SA, hepatocytes and K562 cells were incubated simultaneously with RPA (0.2 or 1 μM in hepatocytes; 0.5 μM in K562 cells) and rhodamine 123 (0.2 or 1 μM in hepatocytes; 0.5 or 1 μM in K562 cells). In experiments with double-stained mitochondria, red fluorescence of RPA ($\lambda_{\text{excitation}} = 543 \text{ nm}$; $\lambda_{\text{emission}} \geq 585 \text{ nm}$) and green fluorescence of rhodamine 123 ($\lambda_{\text{excitation}} = 488 \text{ nm}$; $\lambda_{\text{emission}} = 505\text{--}530 \text{ nm}$) were optically isolated in successive scans.

Cell viability

The uptake of the vital dye, propidium iodide (5 $\mu\text{g}/\text{ml}$), was routinely determined at the end of the experimental procedures in order to detect loss of cell viability. The red fluorescence of propidium iodide excited at 543 nm using the helium/neon laser was collected through a 560 nm long-pass filter. Injurious effects of RPA (0.2 μM) were further excluded by determination of released lactate dehydrogenase activity using a standard assay.

Statistics

All experiments with hepatocytes were repeated at least three times with cells from different animals; experiments with K562 cells were performed in duplicate and repeated three times; and

experiments in a cell-free system were repeated at least twice. Cellular microfluorographs and traces shown in the Figures are representative of all the corresponding experiments performed. Results are expressed as means $\pm$ S.D. or S.E.M. For the assessment of the effect of SA, data were compared by means of Student's *t* test for paired samples.

RESULTS

Synthesis and characterization of RPA

RPA was prepared by linking the iron-chelating component 1,10-phenanthroline-5-amine to the fluorescent probe rhodamine B base as described in the Experimental section. Briefly, 1,10-phenanthroline-5-amine was synthesized by nitration of 1,10-phenanthroline [21] followed by reduction of the nitro-derivative with hydrazine [22]. The bifunctional linker 4-bromomethylbenzoyl bromide was attached to 1,10-phenanthroline-5-amine via a stable amide bond. The coupling reaction of the amide with equimolar amounts of rhodamine B base in dimethylformamide at 100 °C for 64 h yielded RPA (Scheme 1).

The excitation maximum of RPA (20 μM , in a 'simple buffered solution') was at 564 nm and the emission maximum at 602 nm (Figure 1, upper panel). The addition of Fe^{2+} strongly decreased the fluorescence intensity of RPA (Figure 1, lower panel). Neither quenching by Fe^{2+} nor addition of PIH (2 mM) or 1,10-phenanthroline (2 mM) led to a shift in the excitation or emission spectra. Using quantitative laser scanning microscopy, the various RPA concentrations (5–80 μM) plotted against their fluorescence intensities gave a linear function (Figure 2, upper panel). When RPA (20–45 μM) was incubated with Fe^{2+} (1–18 μM), a constant amount of iron was found to quench a constant amount of RPA (i.e. 1 μM Fe^{2+} quenched $3.2 \pm 0.3 \mu\text{M}$ RPA), irrespective of the total RPA concentration, provided that excess RPA was present (Figure 2, lower panel). At a molar ratio of 3:1 for RPA and Fe^{2+} , RPA fluorescence was almost completely quenched and did not respond to increases in Fe^{2+} concentration. These results suggest a stoichiometry of 3:1 for the RPA/ Fe^{2+} complex. This constant stoichiometry of 3:1 for the RPA/ Fe^{2+} complex is consistent with data for the binding of phenanthroline to Fe^{2+} [32] and with our previous results [24] for the fluorescent 1,10-phenanthroline derivative PG SK.

Figure 1 Spectral properties of RPA in the absence and presence of Fe^{2+}

Upper panel: absorbance (.....) and emission (—) spectra for RPA ($20 \mu\text{M}$; $\lambda_{\text{excitation}} = 564 \text{ nm}$ for emission spectrum) were recorded in a 'simple buffered solution' [2 mM freshly prepared ascorbate, 0.15% (w/v) SDS and 10 mM Tris/HCl (pH 8.2 at 22°C)]. Lower panel: emission spectra of RPA in the presence of 0–6.6 μM Fe^{2+} . Fe^{2+} was added as FAS from a freshly prepared stock solution (1 mM) that additionally contained 20 mM ascorbate. Fluorescence intensity is shown in arbitrary units (a.u.).

The quenching effect of Fe^{2+} was the same in both the 'simple buffered solution' and a medium designed to simulate the composition of the mitochondrial matrix (results not shown). This indicates that the iron chelators in the 'mitochondrial medium' (e.g. citrate, phosphate and ATP), i.e. those that might chelate Fe^{2+} within the mitochondrial matrix, do not compete with the iron-binding site of RPA. In the 'mitochondrial medium', RPA fluorescence and its quenching by Fe^{2+} were not affected by changes in pH (pH 7.0–9.5), and were only slightly affected by changes in viscosity due to addition of sucrose (1.75 M). Likewise, the ionic strength, altered by addition of KCl (150 mM) to the 'simple buffered solution' as well as changes in osmolarity ('simple buffered solution' compared with 'mitochondrial medium') did not influence RPA fluorescence and its quenching by Fe^{2+} (results not shown). Besides Fe^{2+} , RPA fluorescence was markedly quenched by Cu^{2+} , but was only slightly affected by Fe^{3+} , Co^{2+} and Ni^{2+} ; the other metal ions tested (Mn^{2+} , Cu^+ and Zn^{2+}) had little effect on RPA fluorescence (Table 1).

Subcellular distribution of RPA

When cultured rat hepatocytes were incubated with 0.01–10 μM RPA for 10–20 min at 37°C , the intracellular indicator fluorescence was almost exclusively detectable within the mitochondria, as indicated by its co-localization with the mito-

Figure 2 Calibration curve for the determination of the intramitochondrial concentration of RPA and Fe^{2+} -induced quenching of the indicator fluorescence

Upper panel: in a cell-free system, RPA (5–80 μM) standards were dissolved in a medium (pH 8.2, 37°C) designed to simulate the composition of the mitochondrial matrix. Portions of this medium containing known concentrations of RPA were placed on Chelex-treated glass coverslips and the fluorescence [arbitrary units (a.u.)] was recorded using a laser scanning microscope ($\lambda_{\text{excitation}} = 543 \text{ nm}$; $\lambda_{\text{emission}} \geq 560 \text{ nm}$). The scanning parameters used were the same as those for the quantitative intramitochondrial measurements described in the text. Lower panel: the effect of Fe^{2+} on RPA (20–45 μM) fluorescence was studied in a 'simple buffered solution' (pH 8.2; see Figure 1). Fe^{2+} , added as increasing concentrations of FAS from a freshly prepared stock solution (1 mM) that additionally contained 20 mM ascorbate, was rapidly mixed with the medium and the fluorescence [in arbitrary units (a.u.)] of portions of the medium containing known concentrations of RPA and Fe^{2+} was determined 2 min later, i.e. after complete formation of the RPA/ Fe^{2+} complex. Zero fluorescence is equal to fluorescence of medium without dye. Values shown represent means $\pm$ S.D. of three to four experiments.

chondrial marker rhodamine 123 (Figure 3). The strong accumulation of RPA within these organelles was dependent on the concentration of dye used for loading (results not shown); findings consistent with TMRM [33], a related cationic rhodamine derivative without an iron-chelating ligand. Loading of the cells with 0.2 μM RPA for 20 min in HBSS at 37°C followed by incubation for a further 15 min in dye-free HBSS led to an intramitochondrial RPA concentration of $64.3 \pm 18.6 \mu\text{M}$ [mean $\pm$ S.E.M.; $n = 4$ experiments from four different animals; measurements performed after addition of PIH and fluorescence intensities compared with RPA standards (Figure 2, upper panel) using the same scanning parameters]. Under these conditions, which were applied for all of the following quantitative measurements, mitochondrial RPA fluorescence after loading (i.e. baseline fluorescence) substantially exceeded the very weak autofluorescence of these organelles. The final RPA concentration of $64.3 \pm 18.6 \mu\text{M}$ was comparable with that achieved after loading the cells for 20 min with 0.2 μM TMRM (results not shown). Using this RPA concentration, no detrimental effects on cell

Table 1 Responsiveness of RPA to various metal ions in a cell-free system

RPA (20 μ M) was dissolved in a medium (pH 8.2, 37 °C) designed to simulate the composition of the mitochondrial matrix (see the Experimental section). The ions were added as their corresponding salts at the final concentrations indicated and rapidly mixed with the medium. A portion (100 μ l) of the medium was placed on chelex-treated glass coverslips 2 min later and the fluorescence was determined using the same scanning parameters as for the quantitative intramitochondrial fluorescence measurements (see Figures 4A, 4B and 5). The relative emission intensities shown are expressed as a percentage of the initial fluorescence intensity (set at 100%). Fluorescence of medium without dye was taken as zero. Values shown are means $\pm$ S.D. from three experiments

Ion	Added ion concentration ...	RPA fluorescence (% of initial fluorescence)	
		2 μ M	20 μ M
Mn ²⁺		96.6 $\pm$ 3.9	97.4 $\pm$ 3.5
Fe ²⁺		63.5 $\pm$ 2.9	4.5 $\pm$ 1.2
Fe ³⁺ *		93.5 $\pm$ 4.3	87.9 $\pm$ 3.4
Co ²⁺		87.0 $\pm$ 3.9	72.2 $\pm$ 3.0
Ni ²⁺		96.4 $\pm$ 2.6	77.5 $\pm$ 1.5
Cu ⁺		96.4 $\pm$ 1.3	91.0 $\pm$ 2.5
Cu ²⁺ *		76.3 $\pm$ 3.7	22.7 $\pm$ 2.6
Zn ²⁺		95.9 $\pm$ 1.1	92.6 $\pm$ 0.5

* Measurements were performed in the absence of the reducing equivalents ascorbate and glutathione.

viability (as detected by released lactate dehydrogenase activity or by propidium iodide uptake) or the mitochondrial function (as assessed by recording the mitochondrial membrane potential) were apparent.

Responsiveness of intramitochondrial RPA to chelatable iron

The addition of the transition metal chelator PIH (2 mM) strongly increased the mitochondrial RPA fluorescence within 120–220 min, indicating a redistribution of physiological mitochondrial chelatable iron from the indicator to the chelator PIH (Figures 4A, 4B and 5). In controls, PIH (2 mM) had no effect on the mitochondrial TMRM fluorescence (results not shown). RPA fluorescence was also increased after addition of the Fe²⁺ chelator 1,10-phenanthroline (2 mM), but was not affected by the non-chelating 1,10-phenanthroline analogue 4,7-phenanthroline (2 mM; results not shown). The maximum RPA fluorescence intensity following addition of 1,10-phenanthroline was clearly lower than the one reached after addition of PIH, thus indicating that ‘dequenching’ was incomplete when 1,10-phenanthroline was used (results not shown). Therefore, PIH (2 mM) was preferred for all of the quantitative measurements performed with RPA. After complete dequenching, RPA fluorescence solely depends on its mitochondrial concentration,

Figure 3 Subcellular distribution of RPA in cultured rat hepatocytes

Cells were cultured on glass coverslips and loaded with both RPA (1 μ M) and the mitochondrial marker rhodamine 123 (1 μ M) for 20 min in HBSS at 37 °C. Hepatocytes were then incubated for a further 15 min in indicator-free HBSS. The intracellular fluorescence of (A) RPA ($\lambda_{\text{excitation}} = 543$ nm; $\lambda_{\text{emission}} \geq 585$ nm) and (B) rhodamine 123 ($\lambda_{\text{excitation}} = 488$ nm; $\lambda_{\text{emission}} = 505$ –530 nm) was imaged 120 min after addition of the transition metal chelator PIH (2 mM) using a laser scanning microscope equipped with a helium/neon laser and an argon laser. The subcellular distribution of the RPA (A) and rhodamine 123 (B), which is known to stain (intact) mitochondria, are the same, as can be seen from an overlay (C) of (A) and (B). Bar indicates 5 μ m.

Figure 4 Effects of alterations in the intramitochondrial chelatable iron on the fluorescence of mitochondrial RPA in cultured rat hepatocytes

Cells were cultured on glass coverslips and loaded with the fluorescent dye RPA either at $0.2 \mu\text{M}$ for 20 min (**A, B**) or $1 \mu\text{M}$ for 10 min (**C, D**) in HBSS at 37°C . Cells were subsequently covered with HBSS (**A, B**) or L-15 cell culture medium (**C, D**) (without FCS, 37°C). The intramitochondrial fluorescence of RPA was imaged using laser scanning microscopy ($\lambda_{\text{excitation}} = 543 \text{ nm}$; $\lambda_{\text{emission}} \geq 560 \text{ nm}$). After establishing the baseline fluorescence for 5 min, images were collected before (**A**) and after complete equilibration (160 min later) following addition of PIH (2 mM) (**B**), or before (**C**) and 2 min after (**D**) the addition of the highly membrane-permeant Fe^{3+} /8-hydroxyquinoline complex ($10 \mu\text{M}$). Bar indicates $5 \mu\text{m}$.

whereas mitochondrial RPA fluorescence after loading (i.e. baseline fluorescence) depends on both the mitochondrial RPA concentration and the concentration of chelatable iron within these organelles. Therefore the difference in RPA fluorescence before and after complete dequenching is a measure of the mitochondrial chelatable iron (see below).

The intramitochondrial RPA fluorescence was not only re-established by the lipophilic transition metal chelators PIH and 1,10-phenanthroline, but was also quenched when the mitochondrial chelatable iron pool was experimentally increased. The addition of the highly lipophilic Fe^{3+} (1)/8-hydroxyquinoline(2) complex ($5\text{--}15 \mu\text{M}$), the ferric ions of which undergo rapid intracellular reduction to the ferrous state (I. Lehen, F. Petrat, H. de Groot and U. Rauen, unpublished work), strongly decreased the indicator fluorescence by approx. 70–90% within 1–3 min [Figures 4C and 4D; for the baseline fluorescence (Figure 4C), note that loading was higher than in Figure 4A]. In

controls, the mitochondrial rhodamine 123 fluorescence of cells loaded with both RPA ($1 \mu\text{M}$) and rhodamine 123 ($1 \mu\text{M}$) for 20 min as well as the mitochondrial fluorescence of cells loaded with TMRM ($0.2 \mu\text{M}$ for 20 min) remained completely unaffected for > 15 min when the iron complex was added to the supernatant (results not shown). Furthermore, the mitochondrial RPA fluorescence could be completely restored under these conditions when PIH (2 mM) was added finally (results not shown). Thus the decrease in mitochondrial RPA fluorescence resulted from quenching by iron and not from damage to the organelles (i.e. decrease in membrane potential) by this redox-active iron complex.

As reported previously for the cellular uptake of the phenanthroline derivative PG SK [20], RPA precomplexed with Fe^{2+} (1:10 molar ratio) was barely taken up by the mitochondria (results not shown), suggesting that the RPA/ Fe^{2+} complex is only weakly membrane-permeant, due most likely to its high molecular

Figure 5 Time course of 'dequenching' mitochondrial RPA fluorescence of cultured rat hepatocytes upon addition of the iron chelator PIH

Cells were cultured on glass coverslips and loaded with the fluorescent dye RPA ($0.2 \mu\text{M}$) for 20 min in HBSS at 37°C . The intramitochondrial fluorescence of RPA was imaged using laser scanning microscopy ($\lambda_{\text{excitation}} = 543 \text{ nm}$; $\lambda_{\text{emission}} \geq 560 \text{ nm}$). After establishing the baseline fluorescence (10 min), the iron chelator PIH (2 mM) was added to the supernatant and confocal images were collected at 10 min intervals. The concentration of mitochondrial chelatable iron was determined from the increase in mitochondrial RPA fluorescence (i.e. Δ fluorescence) after addition of PIH, as described in the text. The trace shown is the average of 33 cells and is representative of four experiments using hepatocytes from different animals.

mass (three RPA molecules and one Fe^{2+}) and the low lipophilicity of iron complexes formed by bidentate chelators [34–36]. Therefore the risk that RPA might transfer chelatable iron from the cytosol into the mitochondria during dye-loading should be a minor one. However, when RPA ($0.2 \mu\text{M}$) was preincubated with the Fe^{3+} chelator DTPA ($200 \mu\text{M}$), the increase in mitochondrial RPA fluorescence upon addition of PIH (2 mM) was approx. 20 % lower than the one provided by the indicator under standard conditions, indicating that traces of contaminant iron from the buffer might be loaded into mitochondria by the indicator. Therefore in experiments intended for determination of the physiological pool of mitochondrial chelatable iron (see below), cell loading with RPA ($0.2 \mu\text{M}$) should be performed (and was performed here) in the presence of DTPA ($200 \mu\text{M}$).

Intramitochondrial concentration of chelatable iron

The difference in mitochondrial RPA fluorescence before and after complete 'dequenching' with PIH (2 mM) is directly proportional to the intramitochondrial concentration of chelatable iron, as a constant amount of iron was found to quench a constant amount of RPA (Figure 2, lower panel). The concentration of the mitochondrial chelatable iron (in μM) was thus calculated from the increase in mitochondrial RPA fluorescence after addition of PIH (2 mM; Figure 5) using the *ex situ* calibration shown in Figure 2 (upper panel); the increase in RPA fluorescence (Δ fluorescence in arbitrary units) was corrected for leakage and photobleaching of the dye and converted into the corresponding RPA concentration. The latter was then divided by three, as $1 \mu\text{M}$ Fe^{2+} quenches $3 \mu\text{M}$ RPA (Figure 2, lower panel). Cells in which the nuclei were stained with propidium iodide at the end of the measurements were excluded from the evaluation. Using this calibration procedure, the concentration of intramitochondrial chelatable iron was determined to be $12.2 \pm 4.9 \mu\text{M}$ (mean $\pm$ S.E.M.; $n = 4$ experiments from four different animals). The values for the mitochondria of different cells were distributed over a wide range

Figure 6 Frequency distribution of the concentration of mitochondrial chelatable iron in single hepatocytes

Rat hepatocytes were cultured on glass coverslips and loaded with the fluorescent dye RPA ($0.2 \mu\text{M}$) for 20 min in HBSS at 37°C . The concentration of chelatable iron of individual mitochondria was determined from the increase in fluorescence after the addition of PIH (2 mM), as described in the text. Mitochondria ($n = 50$) from three hepatocytes were classified according to their concentration of chelatable iron.

(0.3 – $19.2 \mu\text{M}$). Interestingly, the values for the mitochondria of an individual cell also differed greatly (Figure 6), although to a somewhat smaller extent, suggesting differences in iron homeostasis between individual mitochondria within the same cell.

Mitochondrial chelatable iron after inhibition of haem synthesis

In order to demonstrate the suitability of the probe in assessing alterations of the mitochondrial chelatable iron pool during physiological manipulation of the cells, we determined the effect of the inhibition of haem synthesis on the mitochondrial chelatable iron pool in hepatocytes (a cell type with a relatively low rate of haem synthesis compared with erythroid cells) and the human erythroleukaemia cell line K562 (a cell type that is known to have a high rate of haem synthesis [37]). When haem synthesis in cultured rat hepatocytes was inhibited with the well-known δ -aminolaevulinic acid dehydratase inhibitor SA (1 mM), no changes in the intramitochondrial concentration of chelatable iron were detectable with RPA even after 24 h of inhibition (results not shown); incubation with SA (1 mM) for 24 h began to give rise to some cytotoxicity. In contrast with cultured rat hepatocytes, SA treatment of K562 cells led to a surprisingly rapid decrease in mitochondrial RPA fluorescence intensity (RPA

Figure 7 Effect of SA on the mitochondrial fluorescence of RPA in human erythroleukaemia K562 cells

K562 cells were used for continuous (upper panel) and discontinuous (lower panel) measurements. Upper panel: cells were attached to poly-L-lysine-coated ($5 \mu\text{g}/\text{cm}^2$) glass coverslips, loaded with RPA ($0.5 \mu\text{M}$) and rhodamine 123 ($0.5 \mu\text{M}$) for 10 min in HBSS at 37°C , incubated for further 30 min in indicator-free HBSS and then covered with α -MEM at 37°C . The intracellular fluorescence of RPA ($\lambda_{\text{excitation}} = 543 \text{ nm}$; $\lambda_{\text{emission}} \geq 585 \text{ nm}$) and rhodamine 123 ($\lambda_{\text{excitation}} = 488 \text{ nm}$; $\lambda_{\text{emission}} = 505\text{--}530 \text{ nm}$) was recorded using laser scanning microscopy and is given in arbitrary units (a.u.). After establishing the baseline fluorescence, α -MEM (control) or SA (1 mM ; + SA, 1 mM ; from a 10-fold stock solution in α -MEM, adjusted to pH 7.35) was added (indicated by the arrow) to the supernatant. Confocal images were collected at the intervals shown. The traces shown are the mean of approx. 100 cells (field of vision after background subtraction) and are representative of four experiments. Lower panel: in longer discontinuous measurements, K562 cells in α -MEM were incubated for 3.5 h with SA (1 mM) before cells were loaded with RPA and rhodamine 123 and the fluorescence was measured as described above. Results represent the means $\pm$ S.D. of approx. 900 cells in nine fields of vision from three independent experiments. * $P \geq 0.05$, significantly different from control.

also selectively stained mitochondria in this cell type), whereas the mitochondrial membrane potential, as recorded with rhodamine 123, was not affected (Figure 7). Thus the decrease in RPA fluorescence was not a result of cytotoxic side-effects, but reflects a rapid increase in the mitochondrial chelatable iron pool in this cell type.

DISCUSSION

Several methods have been established to determine the highly physiologically and pathophysiologically important pool of mitochondrial chelatable iron, but all of these methods are very prone

to false readings, as they are exclusively applicable to (mechanically) isolated organelles and therefore have the risk of artificial increases in the chelatable iron during pretreatment of the organelles [20]. For example, isolated, osmotically preswollen rat liver mitochondria were incubated for 3 h with the hydrophilic transition metal chelator bathophenanthroline sulphonate and released iron was detected using a colorimetric method [18], or the desferrioxamine-available iron pool of isolated mitochondria in suspension was measured by ESR spectroscopy [17]. Methods based on fluorescence microscopy do not share these drawbacks as they are minimally invasive and therefore applicable to living cells and tissues. The indicator RPA used in the present study is the first to enable selective detection of chelatable iron in single intact mitochondria of viable cells: intramitochondrial RPA responds well to both increases and decreases in available iron. RPA strongly and selectively accumulates within the matrix space of the mitochondrial compartment and is therefore advantageous over the indicator PG SK [20]. Due to its mitochondrial accumulation, RPA sufficiently (> 3 -fold) exceeds the mitochondrial concentration of chelatable iron, ensuring complete formation of the indicator(3)/ Fe^{2+} (1) complex [24]. Furthermore, the use of a long-wavelength indicator, such as RPA, minimizes the risk of cell damage during excitation and reduces interference with mitochondrial autofluorescence. RPA is non-toxic and its (high) quantum yield and response to Fe^{2+} are insensitive to alterations in factors of biological relevance, such as changes in pH, ionic strength, viscosity and osmolality as well as to high concentrations of K^+ , Na^+ , Ca^{2+} and Mg^{2+} . Besides Fe^{2+} , RPA fluorescence was only markedly affected by Cu^{2+} and slightly responded to Fe^{3+} , Co^{2+} and Ni^{2+} in a cell-free system. The contribution of these ions in the cellular system is likely to be negligible because the intramitochondrial concentrations of these metals are very low. The selectivity of the present method for detecting mitochondrial chelatable iron is greatly improved by the selectivity of the chelators used for fluorescence 'dequenching' (see below). Furthermore, mitochondrial RPA fluorescence was found to be strongly dequenched by the highly specific iron chelator deferoxamine (10 mM), thus suggesting a major contribution by iron (F. Petrat, H. de Groot and U. Rauen, unpublished work).

The exact determination of the mitochondrial chelatable iron requires complete redistribution of the iron associated with RPA to a second chelator, which therefore must have a high affinity for iron and has to be added in excess. 1,10-Phenanthroline (2 mM), which has previously been demonstrated to complex mitochondrial chelatable iron associated with PG SK [20], provided lower increases in RPA fluorescence compared with PIH, possibly due to some toxic effects of the high-chelator concentrations during the (long) incubation periods required. However, when PIH (2 mM) was used for 'dequenching', the risk of incomplete redistribution, and consequently an underestimation of the mitochondrial chelatable iron pool, is most unlikely as PIH (2 mM) is non-toxic and was found to completely restore RPA fluorescence when the latter had been almost completely quenched previously with $\text{FeCl}_3/8$ -hydroxyquinoline. This high iron-chelating activity of PIH within the mitochondrial compartment is in line with the findings of Huang and Ponka [38].

Mobilization of iron from haem or FeS-clusters by RPA itself, leading to an overestimation of the mitochondrial chelatable iron pool, is unlikely because almost no toxic effects were apparent when cells were loaded with RPA ($0.2 \mu\text{M}$ for 20 min) and then incubated for 24 h in dye-free Krebs–Henseleit buffer (F. Petrat, H. de Groot and U. Rauen, unpublished work). Furthermore, the incubation period with RPA was fairly short

(20 min), the final intramitochondrial indicator concentration was relatively low ($64.3 \pm 18.6 \mu\text{M}$) and prolonged incubation after loading with the indicator did not increase the detectable concentration of chelatable iron (F. Petrat, H. de Groot and U. Rauen, unpublished work).

The mitochondrial RPA concentration strongly depends on the membrane potential of these organelles (F. Petrat, H. de Groot and U. Rauen, unpublished work), as also recognized for other cationic dyes, such as TMRM and rhodamine 123. For this reason, a decrease in mitochondrial membrane potential results in indicator leakage from the organelles and, finally, in decreased RPA fluorescence intensity, which might be taken for (iron-induced) quenching of RPA fluorescence. Therefore controls of the mitochondrial membrane potential have to be included, e.g. by simultaneously recording rhodamine 123 and RPA fluorescence intensity. This is especially important when measurements with RPA are performed under pathological conditions where the mitochondrial membrane potential might be affected by mitochondrial injury (see below).

The concentration of the mitochondrial chelatable iron obtained in the present study ($12.2 \pm 4.9 \mu\text{M}$) is clearly lower than the iron concentrations given in previous studies with isolated mitochondria: $\approx 330 \pm 60 \mu\text{M}$ ($1.7 \pm 0.3 \text{ nmol/mg}$ of mitochondrial protein) [19] and $\approx 50\text{--}60 \mu\text{M}$ ($0.29 \pm 0.04 \text{ nmol/mg}$ of mitochondrial protein) [17]; values recalculated assuming that approx. 7.2×10^9 rat liver mitochondria contain 1 mg of protein and the volume of a single mitochondrion is $0.71 \mu\text{m}^3$ [26]. These higher values arise most likely from artificial iron mobilization during organelle isolation (see above). The value for mitochondrial chelatable iron obtained with RPA is consistent with the one determined previously ($\geq 4.8 \mu\text{M}$) using PG SK [20]. The concentration of the mitochondrial chelatable iron as determined with PG SK has to be regarded as a lower limit because: (1) the mitochondrial indicator concentration provided by loading the cells with PG SK was too low to detect all of the chelatable iron within these organelles [20]; and (2) the 'dequenching' of mitochondrial PG SK fluorescence by 1,10-phenanthroline (2 mM) was incomplete. In the present study, we were able to record both decreases and increases in mitochondrial chelatable iron, as the sensitivity of the method can be adapted experimentally to the individual requirements. Even at high mitochondrial iron concentrations, complete formation of the RPA(3)/Fe²⁺(1) complex can be ensured by loading the organelles with higher indicator concentrations (Figures 4C and 4D).

Under physiological conditions, the transport of iron into the mitochondrial matrix space or from there back into the cellular cytosol is an actively regulated process [11,39]. Therefore mitochondria have to be considered as separate metabolic compartments with regard to iron homeostasis. For the synthesis of iron-containing molecules (e.g. haem) within the mitochondrial matrix, a 'transit' pool of chelatable iron is required within this compartment. Consequently, some iron chelators, including PIH, are known to be inhibitors of haem synthesis, the rate of which is suggested to be correlated with the concentration of 'available' iron [37,40]. In the present study, the concentration of chelatable iron from different mitochondria of the same hepatocyte was found to vary greatly (Figure 6). These differences in the mitochondrial iron concentration might suggest that iron metabolism is not synchronized between the mitochondria of the same cell.

In line with the suggestion that the mitochondrial chelatable iron pool represents a pool of iron 'in transit', inhibition of haem synthesis with SA, an inhibitor of δ -aminolaevulinic acid dehydratase, strongly increased the concentration of mitochondrial chelatable iron in human erythroleukaemia K562 cells.

This is consistent with previous studies (for review see [37]), where iron appeared to be specifically targeted towards mitochondria in haemoglobin-synthesizing cells, even when haem synthesis was markedly suppressed. In contrast with K562 cells, in hepatocellular mitochondria, i.e. in mitochondria of cells which are known to have far lower rates of haem synthesis than erythroid cells [37], the chelatable iron pool was not affected by SA, possibly because the physiological flux of iron into these mitochondria is lower, in line with the lower requirement for iron [37]. With regard to the rapidity of the increase in mitochondrial chelatable iron in K562 cells upon addition of SA, the increased concentration of mitochondrial chelatable iron may at least partly represent a redistribution of iron from extramitochondrial (storage) sites to mitochondria rather than an increased cellular uptake of iron.

Besides being a major site for the incorporation of iron into biologically important molecules, mitochondria are known to be the main site of intracellular generation of the reactive oxygen species O₂⁻ and H₂O₂, the latter of which can be converted into the highly reactive hydroxyl radical ([•]OH) or related species when chelatable, redox-active iron is present. All the more astonishing is the fact that the concentration of mitochondrial chelatable iron was found to be very high, even higher than determined previously for the cytosolic pool of chelatable iron ($5.8 \pm 2.6 \mu\text{M}$ in rat hepatocytes) [20]. Besides being a potential site of high [•]OH/iron-oxygen-species formation, mitochondria are also very vulnerable to attack from these species. They contain large membraneous areas which are essential for function, but can easily be damaged by lipid peroxidation and, in contrast with nuclear DNA, damage to the mitochondrial DNA are hardly reversible as regenerating systems are insufficient [41]. Accordingly, disturbances in mitochondrial iron homeostasis are increasingly discussed to be an important event in the pathogenesis of various human diseases and conditions, such as gentamicin-induced nephrotoxicity [42,43], Friedreich ataxia [11,12], neurodegenerative diseases (such as Parkinson's and Alzheimer's diseases [13–16]) and aging [44].

In spite of its importance, the nature and regulation of the mitochondrial chelatable iron pool is only partially understood. Under pathological conditions, where comprehension of these mechanisms should form the basis for preventing iron-mediated oxidative damage within the mitochondrial compartment, knowledge is almost completely lacking. Even for the clinically used Fe³⁺ chelator desferrioxamine the effect on the mitochondrial chelatable iron in viable cells is unknown at present [11]. Our method permits the ability to quantify mitochondrial chelatable iron, to detect alterations in this pool under pathological conditions and to study the effect of iron chelators on mitochondrial chelatable iron, and should therefore be useful in assessing the contribution of this pool to injurious processes. Furthermore, the method should allow the regulation of mitochondrial iron uptake and release by specific iron transporters in the inner mitochondrial membrane to be investigated.

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