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High-Level Expression of Rabbit 15-Lipoxygenase Induces Collapse of the Mitochondrial pH Gradient in Cell Culture†

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ABSTRACT: A critical step in the development of mammalian erythroblasts into mature red blood cells is the extrusion of the nucleus, followed by intracellular degradation of the remaining organelles. It has been hypothesized that the breakdown of cellular organelles in rabbit reticulocytes is initiated by 15-lipoxygenase. In vitro, the purified rabbit reticulocyte 15-lipoxygenase binds and permeabilizes organellar membranes, thereby releasing the lumenal contents of the organelle. Here, we demonstrate that ectopic expression of 15-lipoxygenase leads to the collapse of the mitochondrial pH gradient in nonerythroid cells, using a novel reporter of mitochondrial pH, mito-pHluorin. No change in mitochondrial pH was observed with a mutant of 15-lipoxygenase that lacks enzymatic activity. These data demonstrate that 15-lipoxygenase is capable of disrupting the pH gradient maintained by mitochondria in living cells without additional factors specific for red blood cell development.

Lipoxygenases form a family of lipid peroxidizing enzymes that catalyze the dioxygenation of polyunsaturated fatty acids containing the 1-*cis*-4-*cis*-pentadiene moiety to form conjugated hydroperoxides. Different lipoxygenase (LOX)[1] isozymes can react with arachidonic acid at carbon 5, 8, 12, or 15 and are therefore designated 5-, 8-, 12-, or 15-lipoxygenases (5-, 8-, 12-, or 15-LOX) (*1*). Lipoxygenase metabolites, including hydroperoxy and hydroxy fatty acids, have been implicated in intracellular signaling affecting cell proliferation, differentiation, and apoptosis (*2*−*6*). Arachidonic acid 5-lipoxygenation is the rate-determining step in the biosynthesis of leukotrienes and lipoxins, which constitute important mediators of inflammatory processes (*7*, *8*).

Structurally, lipoxygenases are folded into two domains, the small N-terminal β-barrel domain and the large C-terminal catalytic domain that contains the substrate binding pocket and the non-heme iron complex (*9*, *10*). Similar to that of the plant soybean lipoxygenase-1, the rabbit enzyme coordinates the metal atom by an octahedral ligand sphere, which is completed by five amino acid residues (His361, His366, His541, His545, and the C-terminal residue Ile663) and a water molecule (*9*−*11*). Deletion of Ile663 of murine 12-LOX renders the protein enzymatically inactive, but it is still soluble and very likely to be correctly folded (*12*).

Among the LOX isoforms described so far, the rabbit 15-LOX is unique because of its capability of oxygenating esterified fatty acids, biomembranes, and lipoproteins without the prior action of an ester lipid cleaving enzyme, phospholipase (*13*, *14*). It has been reported that stressed reticulocytes can achieve up to 4 mg/mL 15-LOX, in vivo, prior to the degradation of organelles in the developing red blood cell (*8*). At sufficiently high concentrations 15-LOX binds and integrates into the membrane lipid bilayers, disrupting membrane integrity and releasing lumenal contents (*15*−*18*). The process of organelle degradation has been suggested to be of physiological importance in at least two mammalian cell types: (i) reticulocytes, which constitute an intermediate in red blood cell development (*8*, *19*, *20*), and (ii) central fiber lens cells of the eye, which are involved in lens formation (*21*, *22*). In both cases, 15-LOX is expressed in cells where organelle degradation is in progress but not in the immature precursors nor in the completely differentiated cells.

Using the 15-LOX purified from rabbit reticulocytes, we have shown that in vitro the enzyme is capable of integrating into organellar membrane preparations of the rough ER, the smooth ER, the Golgi, peroxisomes, and mitochondria (*15*). Under our experimental conditions, the plasma membrane appears to be spared, but the reasons for this remain unclear. When integrating into membrane lipids, 15-LOX, which

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[1] Abbreviations: LOX, lipoxygenase; ER, endoplasmic reticulum; ETYA, 5,8,11,14-eicosatetraynoic acid; GFP, green fluorescent protein; EKRM, ER-derived microsomes, washed with EDTA and 0.5 M potassium acetate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; UTR, untranslated region; PDI, protein disulfide isomerase; HEK, human embryonic kidney.

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normally is a soluble, cytosolic protein, acquires properties of an integral membrane protein and produces pore-like structures of irregular size, as visualized by electron microscopy. Coincident with integration of the enzyme, the membranes are rendered permeable, and the lumenal contents are released. Interestingly, incubation of 15-LOX with multilamellar liposomes prepared from purified lipids also led to permeabilization of the vesicles. The only requirements for membrane permeabilization were the catalytic activity of the enzyme and the presence of polyenoic fatty acids (arachidonic acid) in the lipid bilayer.

The biological relevance of 15-LOX for red blood cell maturation was demonstrated in primary cultures of rabbit reticulocytes. Using 15-LOX inhibitors, the breakdown of mitochondria, which is accompanied by loss of cellular respiration, was retarded (*23*). In these experiments, freshly isolated rabbit reticulocytes were incubated with eicosatetraynoic acid (ETYA), a suicide inhibitor of 15-LOX (*24*). After 48 h in culture, control cells lacking ETYA had progressed to late reticulocytes or beyond, and virtually no organelles remained in the cytosol. In contrast, cells cultured in the presence of ETYA preserved recognizable mitochondria, as indicated by electron microscopy and measurements of cellular respiration. These studies suffer from the caveat that no inhibitor is 100% specific. Therefore, we sought to confirm these results by introducing 15-LOX into a heterologous cell type and assessing damage to mitochondrial membranes.

We report here that 15-LOX, overexpressed in nonerythroid cells in culture, is able to disrupt the pH gradient maintained by the mitochondrial inner membrane without the need for additional factors necessary for the maturation and development of the red blood cell.

EXPERIMENTAL PROCEDURES

Plasmids. The ratiometric GFP was the kind gift of Dr. J. E. Rothman (Memorial Sloan Kettering Cancer Center, New York). Mito-pHluorin was created using PCR to place the mitochondrial targeting sequence from cytochrome *c* oxidase subunit IV (MLSLRQSIRFFK) and a linker sequence (RSGI) at the N-terminus of the pHluorin protein (*25*). PCR on the pHluorin template used, as forward primer, 5′-ATTAAGCTTATAATGCTTTCACTACGTCAATCTATAAGATTTTTCAAGAGATCTGGAAAGGAAGTAAAGGAGAAGAACTTTTCA-3′ and, as reverse primer, 5′-ATTGATAGATATCTTTTTATTTGTATAGTTCATCCATGCCATGTG-3′. The PCR product was cloned between the *Hin*dIII and *Eco*RV sites in pcDNA3 (Invitrogen). Rabbit 15-LOX was cloned from a library of rabbit reticulocyte cDNA (a gift of Dr. J. Chen, Harvard) by screening with a 210 nt LOX-specific PCR product. The full-length cDNA was subcloned between the *Hin*dIII and *Xba*I sites of pcDNA3 to produce p15-LOX. Site-directed mutagenesis was used to generate the construct, pΔ663, encoding the enzymatically inactive mutant of 15-LOX missing the last amino acid Ile663. This amino acid is involved in coordinating a non-heme iron molecule responsible for enzymatic activity (*9*, *10*). PCR was performed using p15-LOX as template, T7 oligonucleotide as forward primer, and 5′-GCATTGTGGAGAACAGTGTGGCCTGATCTAGAG-3′ as reverse primer. This effectively deleted the C-terminal codon of 15-LOX, Ile663. All constructs were sequenced.

Antibodies. The 15-LOX C-terminal antibody was previously described (*15*). Antibodies to an internal peptide of 15-LOX (CLRPTRNK) were raised in rabbits and affinity purified using the same peptide.

Cell Transfection. Human embryonic kidney 293 (HEK 293) cells were seeded in 10 cm^2 tissue culture dishes and, when 80% confluent, transfected with 25 μg of DNA using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol. Forty-eight hours posttransfection, cells were placed on ice, washed three times with ice-cold PBS, and scraped into the appropriate buffer (see below). All subsequent procedures were performed at 4 °C. Protein estimates were performed on cell extracts using the amido black assay (*26*). CV-1 green monkey kidney cells were seeded onto glass coverslips and transfected with either 5 μg of mito-pHluorin DNA alone or cotransfected with 5 μg of mito-pHluorin and 15 μg of p15-LOX or pΔ663 DNA using calcium phosphate precipitation.

SDS−PAGE and Western Analysis. Cells were lysed in 100 μL of buffer L (50 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride) plus 0.01% Triton X-100. After a 30 min incubation, debris was pelleted, and supernatants were analyzed by 12% SDS−PAGE followed with Coomassie Brilliant Blue R staining or immunoblotting with ECL detection (Amersham). 15-LOX purified from rabbit reticulocyte lysate as previously described (*15*) was used as a positive control.

Enzymatic Assay. Transfected cells were scraped in 300 μL of 50 mM HEPES, pH 7.4, 50 mM KOAc, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM phenylmethanesulfonyl fluoride and dounce homogenized 30 times with a type B pestle. Lysates underwent three successive centrifugations, all at 4 °C: 2000*g* for 15 min, 16000*g* for 15 min, and then 100000 rpm for 15 min in a Beckman Optima TLX ultracentrifuge, TLA 100.1 rotor. The resulting cytosolic extracts were normalized for wild-type or mutant 15-LOX expression by immunoblotting using antibody raised against an internal peptide sequence of 15-LOX (Internal Ab). In a total assay volume of 400 μL, extracts were incubated with 100 μM arachidonic acid (Sigma) in the presence of 10 mM Tris, pH 7.5, at room temperature. Absorbance readings at 234 nm were taken every 10 s over 7 min to measure product formation. The negative control was cytosolic extract from nontransfected cells.

Binding and Release Assays. ER microsomes washed with EDTA and high salt (EKRMs) were prepared as previously described (*27*), as were the binding and release assays (*15*). Briefly, EKRMs were incubated with purified 15-LOX or cytosolic extracts containing wild-type 15-LOX or mutant 15-LOX and then reisolated by pelleting through a 0.5 M sucrose cushion. Where indicated, EKRMs were preincubated with ETYA, a specific inhibitor of 15-LOX, at 26 °C for 20 min prior to addition of purified 15-LOX or cell extracts.

Fluorescence Imaging. For cytochrome *c* staining, cells were fixed with 3% paraformaldehyde in PBS for 20 min at room temperature, washed extensively with 50 mM NH_4Cl in PBS, and then permeabilized with 0.2% Triton X-100 and 50 mM NH_4Cl in PBS. Cells were blocked with 1% BSA and 50 mM NH_4Cl in PBS (blocking buffer) for 15 min at room temperature. After incubation with a 1:100 dilution of the cytochrome *c* antibody (Pharmingen) for 1 h, cells were

washed 3 × 15 min in blocking buffer and incubated with a Cy-2 conjugated goat anti-mouse secondary antibody for 1 h and again washed 3 × 15 min with blocking buffer. Coverslips were mounted with Fluoromount G (Electron Microscopy Sciences). Cells were viewed with a Zeiss Axiovert 200M microscope using a 40× objective. Images were acquired with MetaMorph 5.0 (Universal Imaging) software.

Ratiometric and Live Cell Imaging. After 48 h, washed cells were viewed in imaging buffer (30 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM glucose) at 37 °C. Imaging was performed as described previously (*28*) using a Zeiss Axiovert 135TV microscope with a 40×, 1.3 NA Plan-Neofluar objective and a 1.6× Optovar insert. Image acquisition and processing were performed using MetaFluor 3.0 (Universal Imaging). Excitations were given at 410 and 470 nm, delivered as 12 nm bands by a Polychrome II monochromator. Light was passed through a dichroic mirror and band-pass filter combination to capture the green light emitted from mito-pHluorins while rejecting the 410 and 470 nm excitation wavelengths (respectively 500DCXR and HQ535/550; Chroma Technology). The images were captured by a low light level camera (Pentamax 512EFT frame transfer camera with fiber-coupled Gen IV image intensifier; Princeton Instruments cooled 12-bit EEV 37 charge-coupled device chip). For the pH titration, cells were preincubated for 5 min at 37 °C in 30 mM HEPES (pH 5.0−9.0), 125 mM KCl, 20 mM NaCl, 0.5 mM $CaCl_2$, 25 mM glucose, and 10 μM nigericin. Alternatively, cells were incubated with 10 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). The statistical analysis for the ratiometric determinations was performed using an unpaired, two-tailed Student's *t*-test of equal variance.

RESULTS

Overexpression of Recombinant Rabbit Wild-Type and Mutant 15-LOX in Mammalian Cells. Reticulocytes from anemic rabbits contain about 4 mg of 15-LOX per milliliter of packed cells (*8*). One concern was the feasibility of achieving such high expression levels with transfected constructs. To this end, the full-length 15-LOX cDNA was cloned into the mammalian expression vector pcDNA3, which utilizes the CMV promoter to drive high levels of expression, producing the construct p15-LOX. It has been reported before that sequences in the 3′ UTR of the 15-LOX mRNA inhibit translation in reticulocytes due to binding of specific regulatory proteins, hnRNPs E1 and K (*29*). Therefore, a second construct was produced, p15-LOXΔUTR, which lacked the 3′ UTR. Surprisingly, in all the cell lines tested, we did not find major differences in expression levels of 15-LOX (data not shown). For all of the following experiments, the full-length construct was used.

A catalytically inactive variant of 15-LOX was constructed to serve as a negative control for the following experiments. The crystal structure of 15-LOX has revealed that the carboxylate moiety of the C-terminal isoleucine is involved in coordinating the non-heme iron at the enzyme's active site (*9*−*11*). In addition, it has been shown, for murine 12-lipoxygenase, that deletion of the C-terminal isoleucine abolishes enzymatic activity (*12*). Therefore, we generated a mutant 15-LOX construct, pΔ663 as described in Experimental Procedures, which encodes a protein lacking the C-terminal isoleucine. Human embryonic kidney (HEK) 293 cells transfected with p15-LOX or the pΔ663 mutant were analyzed by 12% SDS−PAGE and either stained with Coomassie Brilliant Blue R for total protein (Figure 1A) or blotted and probed with antibody against 15-LOX (Figure 1B). Both wild-type and mutant constructs resulted in a major protein band of ca. 75 kDa (Figure 1A, lanes 3 and 4), which was not seen with vector alone (Figure 1A, lane 2). Western blots probed with antibody raised against an epitope from the interior of the protein (Internal Ab, Figure 1B) allow a more quantitative estimation of 15-LOX expression. The results of this and similar experiments consistently showed that the amount of recombinant protein was ca. 5% of the total protein content in the cell lysate (compare lanes 3 and 4 of Figure 1B with lane 1, representing purified 15-LOX). A second antibody, raised against the C-terminal 15 amino acids of 15-LOX, appeared to have a decreased affinity for the C-terminal deletion mutant, since the last amino acid probably contributes to the antibody epitope (Figure 1B, C-term Ab).

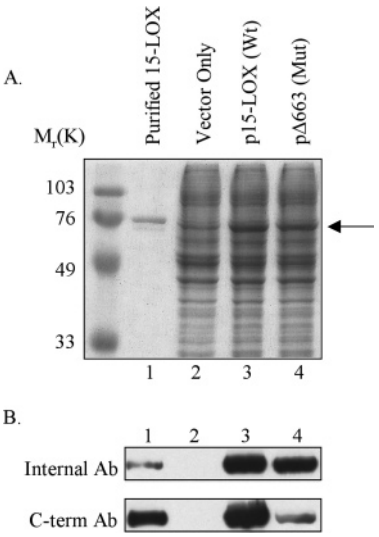

FIGURE 1: HEK 293 cells express recombinant 15-LOX. Total cell lysates analyzed by SDS−PAGE (see Experimental Procedures) were either stained with Coomassie Brilliant Blue R (A) or immunoblotted (B) with antibodies to an internal peptide (Internal Ab) or the C-terminal peptide (C-term Ab). For panel A, samples loaded were 1 μg of purified 15-LOX from rabbit reticulocyte lysate (lane 1) and 20 μg of lysates from cells transfected with vector alone (lane 2), p15-LOX (lane 3), or pΔ663 (lane 4). The arrow indicates the position of the 15-LOX protein. For panel B, 50 ng of purified 15-LOX and 4 μL of cell lysate were loaded for Western blot analysis. An unidentified protein of similar migration to 15-LOX is visible by Coomassie in extracts prepared from cells transfected from vector alone, but no 15-LOX protein is expressed in these cells, as shown in the immunoblots.

Wild-Type Recombinant 15-LOX Protein, but Not the C-Terminal Deletion Mutant, Exhibits Enzymatic Activity and Permeabilizes Membranes in Vitro. Cell extracts from transfected HEK 293 cells were used to assay for 15-LOX enzymatic activity. This was measured by incubating extracts of transfected cells with 100 μM arachidonic acid as soluble substrate and following the time-dependent formation of conjugated dienes (increase in absorbance at 235 nm). The purified 15-LOX (1 μg) was used as a positive control. Immunoblot analysis of the extracts was used to ensure that an equivalent amount of 15-LOX was used in each assay.

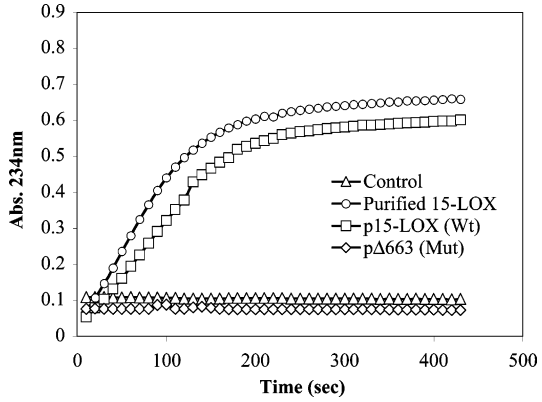

FIGURE 2: Recombinant wild-type but not mutant 15-LOX exhibits enzymatic activity. Enzymatic assays were performed as described in Experimental Procedures using purified rabbit 15-LOX (Purified 15-LOX) (open circle) or lysates of HEK 293 cells after transfection with vector DNA (control) (open triangle), p15-LOX (Wt) (open square), or pΔ663 (Mut) (open diamond). Product formation over time was monitored by measuring absorbance at 234 nm.

As shown in Figure 2, the wild-type recombinant enzyme displayed an activity comparable to that of the purified protein. In contrast, the C-terminal deletion mutant had no detectable enzymatic activity.

Previous data from our laboratory demonstrated that a minimal concentration of 100 μg/mL purified 15-LOX could permeabilize microsomes, in vitro (*15*). To estimate the concentration of recombinant p15-LOX expressed in our transiently transfected cells, we compared HEK 293 cell extracts containing p15-LOX with a titration of purified 15-LOX, as analyzed by Coomassie Brilliant Blue R staining. As seen in Figure 3A, increasing amounts of transfected p15-LOX DNA corresponded to an increase of p15-LOX expression in the transfected cells. In an effort not to overestimate the amount of p15-LOX expressed in our system as judged by Coomassie staining, we compared titrated purified 15-LOX juxtaposed with cell lysate containing p15-LOX by Western blot analysis using the C-Term antibody (Figure 3B). We calculated that approximately 25 ng of p15-LOX was expressed per 10000 HEK 293 cells assuming a 100% transfection efficiency. On the basis of an approximate HEK 293 cell volume of 4 pL, we conservatively estimated the cytosolic p15-LOX concentration to be 0.6 mg/mL. Our observed transfection efficiency was ~50% using the C-Term antibody, based on immunofluorescence studies, which was in good agreement with HEK 293 cells transfected with green fluorescent protein (GFP). This observation suggests that the actual concentration of p15-LOX per cell is roughly 2-fold higher than our above estimation.

Enzymatic activity is necessary but may not be sufficient for p15-LOX to bind and permeabilize membranes, in vitro. To test whether the recombinant p15-LOX and its C-terminal deletion mutant are able to permeabilize membranes, extracts of transfected cells were incubated with high-salt-stripped ER membranes (EKRMs). Subsequent centrifugation over a sucrose cushion separated soluble (supernatant) and membrane-associated (pellet) components. The pellets were further analyzed by SDS−PAGE and immunoblotting for the presence of 15-LOX as shown in Figure 4. Blotting with an antibody against the 15-LOX (Figure 4, 15-LOX blot) revealed that both the purified native (Figure 4, lane 5) and the recombinant (Figure 4, lane 7) wild-type 15-LOX were

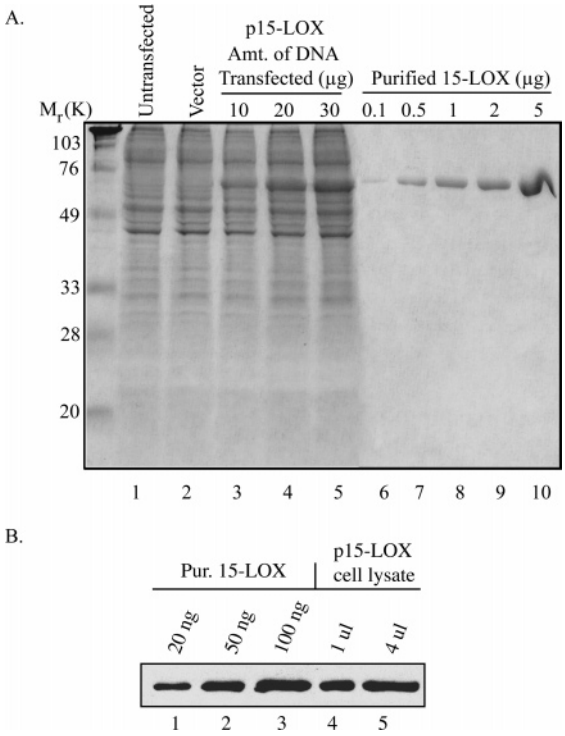

FIGURE 3: Semiquantitative estimation of recombinant 15-LOX concentration in HEK 293 cells. (A) Increasing amounts of purified 15-LOX were compared to lysates from HEK 293 cells transfected with different amounts of p15-LOX DNA. Proteins were separated by 12% SDS−PAGE and visualized by Coomassie Brilliant Blue R staining. For the lysates, 100 μg of protein was loaded per lane while the amount of purified 15-LOX was loaded as indicated. (B) Western blot analysis of titrated purified 15-LOX and HEK 293 cell lysate using the C-term antibody (see Figure 1B).

recovered in the membrane pellet. In contrast, only traces of the deletion mutant were found in the membrane pellet (Figure 4, lane 9). ETYA, an irreversible inactivator of 15-LOX, substantially decreased the amount of the enzyme that was membrane associated (compare lanes 6 and 8 of Figure 4 with lanes 5 and 7) although 100% inhibition was not achieved. Negative controls established that virtually no 15-LOX was recovered in the pellet in the absence of added membranes (Figure 4, lanes 2−4), indicating that the enzyme had not precipitated and merely cosedimented with the membranes in the pellet fraction.

The ability of 15-LOX to render membranes permeable to proteins was assayed by probing the blots for the presence of PDI, a lumenal protein present in preparations of EKRMs. When EKRMs are incubated with ETYA alone, PDI is recovered in the membrane pellet (Figure 4, lane 1). In contrast, no PDI was found in the pellet fraction after incubation of EKRMs with either purified (Figure 4, lane 5) or recombinant (Figure 4, lane 7) wild-type 15-LOX, indicating efficient membrane permeabilization. In both cases, PDI release was partially blocked if the assay included the LOX inhibitor, ETYA (Figure 4, lanes 6 and 8). The C-terminal deletion mutant did not mediate PDI release (Figure 4, lane 9).

Wild-Type 15-LOX but Not the C-Terminal Deletion Mutant Disrupts the Mitochondrial Inner Membrane in Intact Mammalian Cells. Maintenance of a pH gradient across the inner mitochondrial membrane is essential for the generation of ATP during oxidative phosphorylation. In fact, the

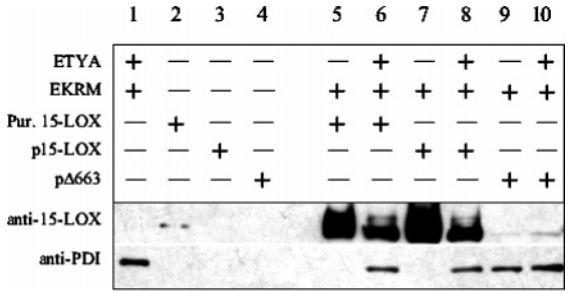

FIGURE 4: Recombinant wild-type 15-LOX specifically binds and permeabilizes microsomes (EKRMs) in vitro. Incubations were performed (see Experimental Procedures) with lysates of HEK 293 cells after transfection with p15-LOX or pΔ663 or with purified rabbit 15-LOX as indicated. EKRMs recovered by pelleting through a sucrose cushion were analyzed by immunoblotting for 15-LOX or PDI.

cytosolic proton concentration in healthy cells is higher than that of the mitochondrial matrix. However, disruption of mitochondrial membranes by 15-LOX is predicted to collapse the pH gradient, lowering the matrix pH to approach that of the cytosol. To monitor the matrix pH in living cells, we used ratiometric pHluorin, an engineered GFP variant for which the ratio of green fluorescence intensities emitted as a result of excitation at 410 and 470 nm (the excitation ratio, $R_{410/470}$) reflects the ambient pH in the vicinity of the molecule (*28*). For our experiments, it was necessary to target the pHluorin to the mitochondrial matrix. To do this, the pHluorin cDNA was fused at the 5′ end to an oligonucleotide encoding a 12 amino acid long mitochondrial targeting signal derived from the N-terminus of cytochrome *c* oxidase subunit IV (*25*, *30*). The resulting construct encodes a protein we designated mito-pHluorin (Figure 5A). Live fluorescence imaging of CV-1 cells expressing mito-pHluorin exhibited a filamentous, wormlike expression pattern typical of mitochondrial staining in mammalian cells (Figure 5B). Staining of fixed and permeabilized CV-1 cells for cytochrome C, a protein normally associated with the mitochondrial intermembrane space, revealed a similar pattern (Figure 5C).

To calibrate mito-pHluorin $R_{410/470}$ values under our experimental conditions, CV-1 fibroblasts expressing mito-pHluorin were incubated in buffers ranging from pH 5.0 to pH 9.0, in the presence of 10 μM nigericin. Nigericin, a K^+/H^+ antiporter, allows equilibration of pH across membranes in the presence of extracellular K^+. The standard curve obtained from these measurements (Figure 5D) is in agreement with the calibration curve obtained previously using pHluorin in solution (*28*). In addition, as shown in Figure 5E, cells were incubated with CCCP, a protonophore, to equilibrate mitochondrial and external pH and incubated in buffers at pH 7.0 or pH 7.4. Measurements taken at 5 and 10 min after addition of the protonophore indicate that pH equilibrium had been established.

CV-1 cells expressing mito-pHluorin under physiological conditions and in the absence of ionophore gave a measurement of 7.7 ± 0.19 (mean ± SD) for the pH of the mitochondrial matrix (see Table 1). This is in good agreement with results previously obtained using fluorescent dyes (*31*, *32*) and nonratiometric GFP (*25*). CV-1 cells were cotransfected with the mito-pHluorin construct and either p15-LOX or pΔ663. To verify expression of the LOX constructs in the transfected cells, immunofluorescence studies using an

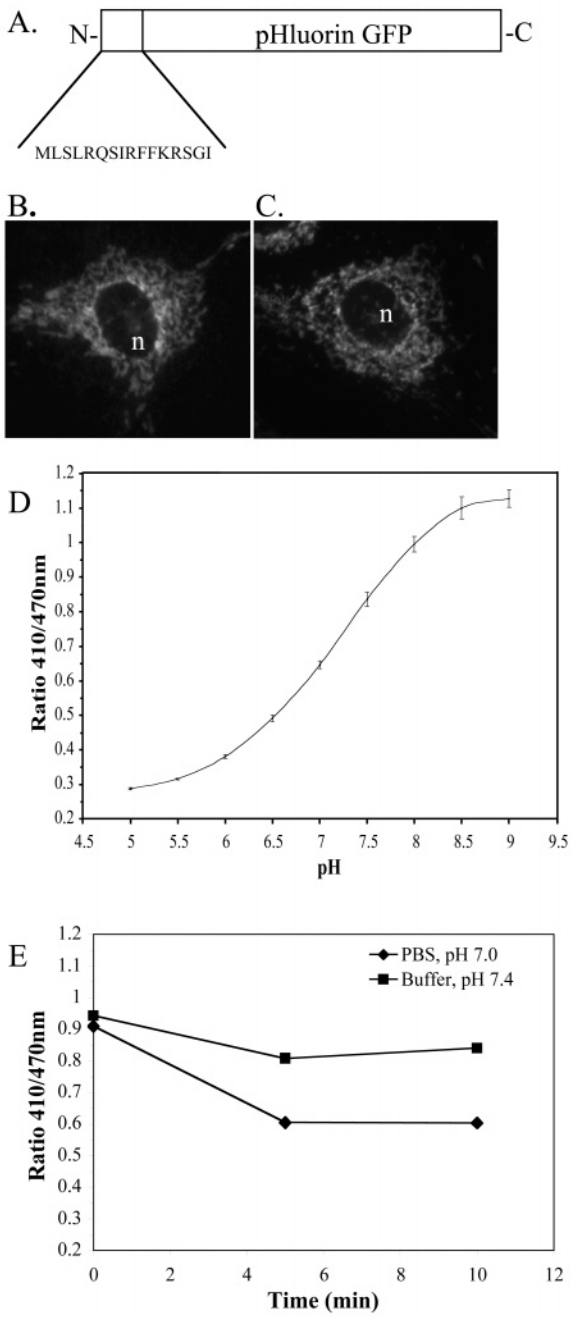

FIGURE 5: Ratiometric mito-pHluorin reflects the pH of the mitochondrial matrix. (A) The cDNA for mito-pHluorin consists of a 12 amino acid mitochondrial localization signal fused via an Arg-Ser-Gly-Ile linker sequence to the N-terminus of pHluorin. Live cell fluorescence image of the CV-1 fibroblast showing expression and localization of mito-pHluorin (B) and cytochrome *c* (C) in a fixed fibroblast; n indicates the cell nucleus. (D) Calibration curve of the 410/470 nm ratio for CV-1 cells expressing mito-pHluorin and incubated with the ionophore, nigericin. Cells were imaged in buffers adjusted to pH values between 5.0 and 9.0. (E) Measurement of the 410/470 nm ratio for mito-pHluorin expressing CV-1 cells incubated with the protonophore, CCCP, and incubated at pH 7.0 or 7.4.

affinity-purified 15-LOX antibody were carried out (data not shown). When cells were transfected with wild-type 15-LOX, the matrix pH was found to be 7.3 ± 0.3, essentially the pH of the cytosol. These data suggest disruption of the mitochondrial membranes. In contrast, the pH in the mitochondrial matrix of cells transfected with the inactive C-terminal truncation mutant, pΔ663, remained alkaline at a pH of 7.8

Table 1: Effects of Recombinant 15-LOX Expression on the Mitochondrial Matrix pH of CV-1 Fibroblasts[a]

transfected constructs	$R_{410/470}$ (mean ± SD)	matrix pH (mean ± SD)
mito-pHluorin ($n = 23$)	0.92 ± 0.07	7.7 ± 0.19
p15-LOX + mito-pHluorin ($n = 25$)	0.75 ± 0.11 ($p < 3 \times 10^{-7}$)	7.3 ± 0.3
pΔ663 + mito-pHluorin ($n = 24$)	0.93 ± 0.06	7.8 ± 0.15

[a] Fibroblasts were transiently transfected with either the ratiometric mito-pHluorin construct alone or cotransfected with either wild-type p15-LOX or mutant pΔ663. Fluorescence determinations were performed 2 days posttransfection on individually transfected cells. $R_{410/470}$ is the ratio between green fluorescence emission intensities when mito-pHluorin was excited at 410 and 470 nm. In each cell, the matrix pH was determined by a pH calibration curve as shown in Figure 5D. In cells cotransfected with p15-LOX, the $R_{410/470}$ value was lower than in cells transfected with the mito-pHluorin construct alone, reflecting a significant change in the matrix pH. This difference was statistically significant ($p < 3 \times 10^{-7}$) as determined by Student's *t*-test. In contrast, there was no significant difference between cells cotransfected with pΔ663 and cells transfected with mito-pHluorin alone. SD = standard deviation; n = number of cells used for ratiometric determination.

± 0.15. This indicates that the 15-LOX-induced dissipation of the mitochondrial pH gradient across the inner mitochondrial membrane requires the functional enzyme and is not an unspecific effect of protein overexpression.

DISCUSSION

More than 20 years ago, 15-LOX was purified from rabbit reticulocytes and identified as an "inactivator" of cellular respiration during reticulocyte maturation (*8*). Electron microscopy of rat liver mitochondria treated in vitro with a crude enzyme preparation indicated severe structural alterations (*1*), which provided a structural explanation for these functional changes. Since then, biochemical and ultrastructural studies have provided additional evidence that 15-LOX is able to bind and permeabilize organellar membranes, while sparing the plasma membrane, in vitro (*15*, *16*). More recently, electron microscopy studies on rat liver peroxisomes have shown the involvement of 15-LOX in the disruption of peroxisomal membranes (*16*). Similarly, an in vivo study demonstrated the delay of mitochondrial degradation in the presence of the 15-LOX inhibitor, ETYA, in rabbit reticulocytes (*23*). On the basis of these data, it was proposed that the action of 15-LOX in rabbit reticulocytes is vital to the loss of organelles that occurs during maturation of red blood cells. Here, we report for the first time that high-level expression of 15-LOX alone in a nonerythroid cell is able to induce a collapse of the mitochondrial pH gradient maintained by the inner membrane in the absence of other components required for red blood cell development. We had previously shown that purified 15-LOX permeabilization of liposomes required both enzymatic activity of the enzyme and the presence of 15-LOX substrate fatty acids in the membrane preparation. More recently, sequence determinants have been identified which are required for membrane binding (*18*).

To test whether similar effects can be observed in vivo, we adopted an experimental strategy which relied on the recently developed technology to measure pH using a GFP-based indicator, called ratiometric pHluorin, capable of measuring pH by displaying changes in the ratio of its excitation maxima at 410 and 470 nm ($R_{410/470}$) in live cells (*28*). Addition of a mitochondrial targeting signal produced "mito-pHluorin", a reagent for measuring the pH of the mitochondrial matrix. Although other components of the reticulocyte, such as endogenous enzyme inhibitors, may impact the efficiency and rate of organelle degradation, these experiments establish that ectopic expression of 15-LOX is sufficient to disrupt mitochondrial membranes as evidenced by the equilibration of the matrix pH to that of the cytosolic pH.

Earlier, we had demonstrated that a critical concentration of approximately 100 μg/mL purified 15-LOX was necessary for EKRM permeabilization in vitro (*15*). For these studies, we overexpressed 15-LOX in human and monkey cell lines. On the basis of Coomassie staining of total cell lysates in Figure 1, we observed a high level of 15-LOX expression which approximated 5% of the total cellular protein. The level of 15-LOX expression achieved was independent of the presence of the 15-LOX 3′ UTR in the construct. It has been shown that, in erythroblasts, regulatory proteins that bind to the 3′ UTR inhibit translation of 15-LOX mRNA. It is possible that these regulatory proteins are not expressed in the cell lines that we used. Alternatively, the transfected constructs may have produced high levels of mRNA that were in excess of the regulatory proteins.

Extracts from transfected cells were found to have 15-LOX enzymatic activity (Figure 2). The 15-LOX present in the extracts was also capable of binding and permeabilizing microsomal membranes in vitro (Figure 4). This was not the case for the soluble, yet enzymatically inactive variant of 15-LOX (pΔ663) that lacked the C-terminal isoleucine required to coordinate the active site iron. The disruption of mitochondrial membranes in vivo was assessed by measuring the pH of the mitochondrial matrix. Collapse of the normal pH gradient across the inner mitochondrial membrane was evidence of damage to the membrane. Taking all of the data together, we conclude that 15-LOX can damage mitochondrial membranes in vivo and functions independently of proteins that are specific for the developing red blood cell.

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