

Calcium promotes membrane association of reticulocyte 15-lipoxygenase

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The reticulocyte 15-lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12) is implicated in oxidative damage to reticulocyte mitochondria before their elimination by degradation during maturation to the erythrocyte. A proportion of the 15-lipoxygenase sediments with the mitochondrial-rich stromal fraction of density-gradient-fractionated rabbit reticulocytes suggesting a physical association with mitochondria before their elimination. Ca^{2+} promotes binding of reticulocyte 15-lipoxygenase to isolated rat liver and reticulocyte mitochondria

and 15-lipoxygenase-mediated lipid peroxidation of mitochondrial lipids and free linoleic acid. Association of reticulocyte 15-lipoxygenase with isolated mitochondria is not simply a consequence of Ca^{2+} -induced swelling, but implies that Ca^{2+} mediates translocation of soluble lipoxygenase to mitochondrial membranes. Therefore, Ca^{2+} may have an important physiological role in the regulation of 15-lipoxygenase-mediated targeting of reticulocyte mitochondria for degradation.

INTRODUCTION

The reticulocyte represents an advanced stage in the differentiation of the red cell, containing only residual organelles, mainly mitochondria. The maturation of the reticulocyte to the erythrocyte involves the highly selective proteolytic loss of mitochondria and a number of soluble enzymes. A 15-lipoxygenase (EC 1.13.11.12), synthesized from pre-existing mRNA during reticulocyte maturation, appears to mediate the elimination of reticulocyte mitochondria [1]. Reticulocyte lipoxygenase oxygenation of fatty acid substrates, mainly linoleic acid [2], in the mitochondrial membrane precedes degradation of mitochondrial constituents, including proteins. Disruption of the membrane structure of mitochondria may reveal cryptic signals in mitochondrial proteins for cytosolic or mitochondrial proteases. Products of the lipoxygenase reaction or free radicals may modify mitochondrial proteins, identifying them for proteolysis.

The reticulocyte 15-lipoxygenase appears to be unique among lipoxygenases in its ability to oxygenate fatty acids esterified to membrane phospholipids without prior phospholipase-mediated release of non-esterified fatty acids [3]. This implies some form of association of the reticulocyte enzyme with cell membranes. Reticulocyte 15-lipoxygenase preparations will act on rat liver mitochondria, resulting in the formation of known products of lipoxygenase action [4], and products of lipoxygenase activity have been found in the mitochondrial membranes of intact reticulocytes [5]. Interestingly, before attack on arachidonic acid, leucocyte 5-lipoxygenase translocates to membranes [6], which is dependent on the presence of an activating or docking protein (5-Lipoxygenase Activating Protein, FLAP) and Ca^{2+} [7,8]. Ca^{2+} has also been found to stimulate the activity of 15-lipoxygenase of keratinocytes [9] and polymorphonuclear neutrophils [10] against exogenous substrate. These observations led us to investigate whether reticulocyte 15-lipoxygenase associates with mitochondria during reticulocyte maturation and whether translocation and 15-lipoxygenase enzyme activity were effected by Ca^{2+} . In this paper we identify a Ca^{2+} -dependent association of reticulocyte 15-lipoxygenase with, and attack on, isolated mitochondria, which could be physiologically relevant to the enzyme's proposed role in mitochondrial breakdown.

EXPERIMENTAL

Materials

Rabbit antibody to human reticulocyte 15-lipoxygenase was generously given by E. Sigal, Cardiovascular Research Institute, University of California, San Francisco, CA, U.S.A. Rabbit antiserum to bovine succinate dehydrogenase was donated by J. G. Lindsay, Department of Biochemistry, University of Glasgow, U.K. Rabbit antiserum to a synthetic peptide derived from reticulocyte 15-lipoxygenase (GLFQKHREQEL) was raised by injection of the purified peptide conjugated to keyhole-limpet haemocyanin. Peroxidase-conjugated swine anti-rabbit immunoglobulins were from DAKO, High Wycombe, Bucks., U.K. 13(*S*)-Hydroperoxyoctadeca-9*Z*,11*E*-dienoic acid (13-HPODE) prepared by Biomol was obtained from SEMAT, St. Albans, Herts., U.K. [^{14}C]Linoleic acid, Hybond Super-C nitrocellulose and ECL chemiluminescent blotting reagents were supplied by Amersham International, Amersham, Bucks, U.K., and ICN-Biomedical silical-gel plates by Park Scientific, Northampton, U.K. Other reagents were obtained from Sigma Chemical Co., Poole, Dorset, U.K. MK-886 was generously given by A. Ford-Hutchinson, Merck Frosst Centre for Therapeutic Research, Pointe Claire-Dorval, Canada.

Density-gradient fractionation of red blood cells

Reticulocyte levels of 40% of total red blood cells were induced in New Zealand White rabbits by daily removal of 15 ml of blood/kg body wt. Blood volume was maintained by injection of the previous day's plasma diluted 1:1 with 0.9% (w/v) NaCl and iron by daily administration of a veterinary preparation of iron and vitamins. On day 8 of the bleeding schedule, blood was collected into EDTA (final concn. 3 mM), and contaminating white cells were removed by passing the whole blood through a column packed with 100% cotton-wool, pre-washed by boiling in several changes of 0.9% NaCl and primed by running through 15 ml of plasma. Eluted cells were washed twice in histidine-buffered saline (5 mM histidine/150 mM NaCl, pH 7.55), and finally suspended 1:1 (v/v) in histidine-buffered saline. Then 4 ml of red blood cells was layered on a Percoll gradient,

Abbreviation used: 13-HPODE, 13(*S*)-hydroperoxyoctadeca-9*Z*,11*E*-dienoic acid.

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pre-formed by centrifugation of 35 ml of 70% (v/v) Percoll (prepared by diluting a stock Percoll solution consisting of 1 vol. of 10 × histidine-buffered saline and 9 vol. of Percoll with histidine-buffered saline to 70% (v/v) and adjusting the osmolarity to approx. 300 mosM) at 25000 g for 15 min in an M.S.E. 8 × 50 ml rotor of an M.S.E. Prepsin 75 centrifuge at 4 °C with the brake off. Loaded pre-formed gradients were centrifuged at 800 g for 15 min at 4 °C in a swing-out rotor of a bench-top centrifuge; 2 ml fractions were then unloaded from the top of the gradient by upward displacement with Nyegaard Maxidens. Cells were collected by centrifugation and washed in 3 × 10 vol. of PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.4 mM KH₂PO₄). Cells smears were stained with Brilliant Cresyl Blue to reveal reticulum and estimate reticulocyte numbers. Cells were analysed with a Sysmex 2000 cell counter to provide estimates of cell number, volume and haemoglobin concentration. A stromal fraction of red blood cells lysed by freeze-thawing in PBS containing 1 mM EDTA was collected by centrifugation for 10000 g for 40 min at 4 °C.

Preparation of rat liver mitochondria

Rats were killed by cervical dislocation, and the liver was perfused *in situ* with ice-cold 10% (w/v) sucrose solution. After removal, the liver was chopped finely and homogenized with 8 strokes in a loose-fitting Potter–Elvehjem homogenizer. Nuclei and intact cells were removed by centrifugation at 1000 g for 10 min, and the post-nuclear supernatant was centrifuged at 7000 g for 7 min. The resulting mitochondrial pellet was resuspended in 6 ml of 250 mM sucrose/20 mM Tris/HCl (pH 7)/4 mM EGTA/3% (w/v) BSA containing 1.4 ml of Percoll, and centrifuged at 10000 g for 10 min. The resulting fluffy layer above the mitochondrial pellet was carefully removed and the pellet washed twice in 10% sucrose. All centrifugation steps were carried out at 4 °C. Mitochondrial preparations were enriched for cytochrome oxidase (EC 1.9.3.1) and succinate dehydrogenase (EC 1.3.99.1) and depleted of the lysosomal marker enzyme acid phosphatase (EC 3.1.3.2) (specific activities relative to the homogenate 6–7, 4–5 and 0.9 respectively). For some experiments, mitochondria were further purified by digitonin treatment to remove lysosomes [11]. After incubation of 0.25 ml of mitochondrial pellet at 4 °C with 1 ml of digitonin (0.3 mg/ml) in 10% sucrose/2 mM Hepes (pH 7.4)/1 mM EDTA for 5 min, the fluffy layer of lysosomes was removed from the mitochondria above them after centrifugation for 4.5 min at 19000 g and mitochondria were washed twice more in sucrose.

Mitochondrial association of lipoxygenase

Binding of reticulocyte 15-lipoxygenase to mitochondria was assessed by incubating reticulocyte cytosol with freshly prepared rat liver mitochondria. Lipoxygenase-rich cytosol was prepared by hypotonic lysis of reticulocyte-rich, white-cell-depleted, red blood cells with water and centrifugation at 100000 g for 1 h at 4 °C to remove membranes. The supernatant was stored at –70 °C before use. Incubations were conducted at 37 °C with 2 μl of pelleted mitochondria (15 μg of protein) and 30 μl of reticulocyte-rich lysate in a final volume of 100 μl of 50 mM Tris/HCl (pH 7.5)/150 mM KCl (Tris/KCl buffer). Other additions to incubations are as described in the Results section. After the prescribed incubation time, the mixture was diluted 10-fold with ice-cold Tris/KCl buffer containing 4 mM EGTA, and mitochondria were collected by centrifugation at 10000 g for

2 min, washed in 2 × 1 ml of Tris/KCl buffer and examined for bound lipoxygenase by SDS/PAGE and immunoblotting.

SDS/PAGE and Immunoblotting

Samples were dissolved in electrophoresis sample buffer and heated for 2 min at 90 °C, separated on 7.5% (w/v) acrylamide gels, and proteins were transferred to nitrocellulose at 100 mA for 1 h in a Hoeffer semi-dry blotter [12]. Immunoblotting was carried out with either a rabbit antibody to the human reticulocyte 15-lipoxygenase (1.5 μg/ml) or an affinity-purified rabbit antibody to 15-lipoxygenase-derived synthetic peptide (1.2 μg/ml). Immunoblots were developed with a peroxidase-labelled second antibody and a chemiluminescent substrate (ECL). Lack of reaction with non-immune rabbit immunoglobulins or antibody to the synthetic peptide preabsorbed with peptide indicated that the band detected on autoradiograms was specific. A single band of approx. 76 kDa was produced by both antibodies, with occasional fainter smaller bands, presumably breakdown products. Volume integration of bands on X-ray film was performed by the ImageQuant software package after scanning with a Molecular Dynamics Personal Densitometer.

Preparation of semi-purified 15-lipoxygenase

A globin-free lipoxygenase-enriched fraction was prepared from reticulocyte cytosol [3]. Briefly, the cytosol was adjusted to 55% saturation with (NH₄)₂SO₄ and the resulting precipitate was collected and washed with 55%-satd. (NH₄)₂SO₄ solution. The final pellet was dissolved in water to a concentration of about 20 mg of protein/ml and dialysed overnight against 50 mM Tris/HCl, pH 7.5 at 4 °C, and stored at –20 °C until required.

Assay of 15-lipoxygenase activity

15-Lipoxygenase activity was determined spectrophotometrically and by t.l.c. of radioactive reaction products. Incubations, conducted at 25 °C, contained 75 mM Tris/HCl buffer, pH 7.4, 0.4 mM linoleic acid and 1 μg of partially purified reticulocyte lipoxygenase in a final volume of 1 ml. Linoleic acid was dissolved in ethanol and diluted in buffer to give a final ethanol concentration of 0.02% (v/v). The A₂₃₄ of the hydroperoxy product was measured with a recording spectrophotometer. Radiochemical assays employed [1-¹⁴C]linoleic acid (1 Ci/mol, 0.4 mM) in a total volume of 100 μl. Reaction products were extracted with 2 ml of chloroform/methanol (2:1, v/v), 0.1 ml of 2 M citric acid and 0.8 ml of water. The organic phase was evaporated to dryness under nitrogen and chromatographed on silica-gel plates in diethyl ether/light petroleum (b.p. 60–80 °C)/acetic acid (99:99:2, by vol.). After autoradiography, radioactive bands corresponding to linoleic acid and 13-HPODE were identified by comparison to authentic standards, excised and quantified by liquid-scintillation counting.

Lipoxygenase activity against membrane lipids was monitored by determination of thiobarbituric acid-reactive products [3]. Incubations included mitochondrial pellet (150 μg of protein) and 90 μl of reticulocyte-rich cytosol in a final volume of 100 μl of Tris/KCl buffer. After 30 min at 37 °C incubations were stopped, and protein was precipitated by addition of an equal volume of 7% (w/v) HClO₄ containing 2 mM Fe₂(SO₄)₃. Precipitated protein was removed by centrifugation at 10000 g for 10 min, and 50 μl of 1% (w/v) 2-thiobarbituric acid in 3.5% HClO₄/1 mM Fe₂(SO₄)₃ was added to 100 μl of the supernatant. The mixture was boiled for 15 min and the A₅₃₂ measured in a microplate reader. A standard curve was produced with pure malondialdehyde.

Other enzyme assays

Succinate dehydrogenase, cytochrome oxidase, acid phosphatase and uroporphobilinogen I (EC 4.3.1.8) were determined as described previously [12,13].

RESULTS

Density-gradient fractionation of red blood cells

Some 90% of all white cells were removed by a single passage of blood through a column of cotton-wool, as observed after May-Grünwald Giemsa staining. Percoll buoyant-density-gradient fractionation of the subsequent reticulocyte-rich red-cell population (35% reticulocytes; mean cell volume 108 fl) yielded fractions containing red cells with densities ranging from 1.059 to 1.123 g/ml, and with reticulocytes, identified by Brilliant Cresyl Blue staining, appearing in the less dense fractions at the top of the gradient (Figure 1a). Red-cell density increases during maturation as a result of continued haemoglobin synthesis and cell shrinkage [14]; therefore Percoll-gradient fractionation produced fractions containing cells of increasing maturity down the gradient. Similarly, the change in mean cell volume through the gradient closely followed that of the reticulocyte count (Figure 1a). The first three or four fractions showed little change in mean cell volume or reticulocyte count. However, the density of the reticulum in the red cells, shown by staining with Brilliant Cresyl Blue, did noticeably decrease, indicating that the degradation of ribosomal material is nevertheless occurring, although cell shrinkage is not (results not shown). Reticulum is lost and cell volume decreases markedly in cells recovered from the middle fractions of the gradient, until a cell the size of an erythrocyte was formed which contained little or no ribosomes in fractions 9–12. Cytochrome *c* oxidase activity, a mitochondrial enzyme, exhibited a dramatic fall in activity in cells recovered between fractions 3 and 4 (Figure 1b), suggesting rapid loss of mitochondria in these cells. Interestingly, the number of cells containing reticulum did not show such a dramatic decline, suggesting that degradation of mitochondria may be more sudden than degradation of the protein-synthesizing machinery. The disappearance of succinate dehydrogenase protein, detected by immunoblotting, was most significant for cells from between fractions 3 and 5 of the gradient (results not shown), further suggesting a pivotal event in mitochondrial destruction in cells recovered from this region of the gradient. The activity of the cytosolic enzyme uroporphobilinogen I synthase, an enzyme which declines in activity during maturation, declined through the gradient in a similar fashion to cytochrome oxidase (Figure 1b). In contrast, the 15-lipoxygenase activity of red blood cells increased dramatically from a low level in cells from fraction 1 to a maximum in cells from fractions 4 and 5, and thereafter declined. The profile of appearance of lipoxygenase activity is compatible with 15-lipoxygenase-mediated loss of mitochondria, as judged by the decline of the cytochrome oxidase activity (Figure 1b). Overall, analysis of cells recovered from Percoll density gradients indicates that separation of red cells of different maturity has been achieved.

Association of 15-lipoxygenase with reticulocyte membranes

Immunoblot analysis of the 40000 *g*-min supernatant and pellet (stroma) fractions of cells recovered from Percoll density gradients indicated an increase in the amount of 15-lipoxygenase detectable in the 40000 *g*-min supernatant of cells from gradient fraction 3 compared with fraction 1 (Figure 2a). However, even the youngest cells that we have recovered from Percoll gradients

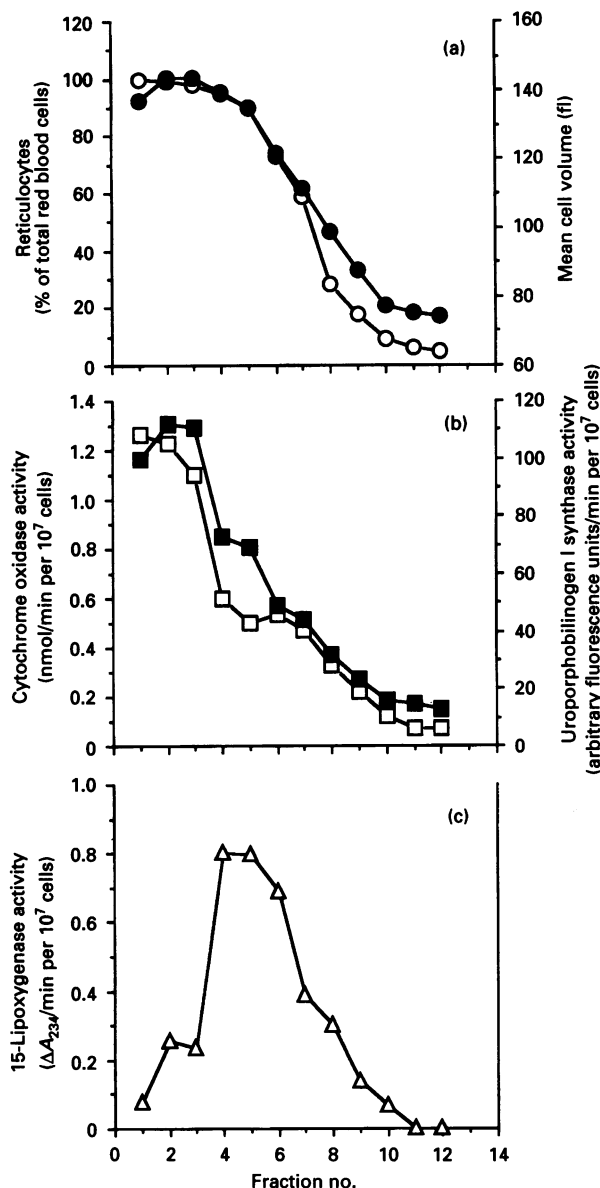


Figure 1 Percoll density-gradient fractionation of reticulocyte-rich red blood cells

White-blood-cell-free reticulocyte-rich red blood cells were fractionated on a Percoll density gradient, and 2 ml fractions were collected from the top of the gradient. Reticulocytes were detected after staining of reticulum with Brilliant Cresyl Blue (\circ ; a) and mean cell volumes were determined in a Sysmex 2000 cell counter (\bullet ; a). Cytochrome oxidase (\square) and uroporphobilinogen I synthase (\blacksquare) activities are expressed for 10^7 cells (b). 15-Lipoxygenase was assayed spectrophotometrically with the 55%-satd.-(NH_4) SO_4 precipitate of cell cytosol (\triangle ; c). The results shown are from a single density gradient, but essentially the same results were obtained with separate gradients from six separate rabbits.

do not represent a population of the very youngest reticulocytes which can be released into the circulation [15], as they already have a detectable quantity of translated lipoxygenase protein and lipoxygenase enzyme activity (Figure 1c). A high level of 15-lipoxygenase protein is maintained until relatively mature cells predominate in fraction 10, and is then rapidly lost as mature erythrocytes predominate, in a similar fashion to the loss of lipoxygenase enzyme activity (Figure 1c).

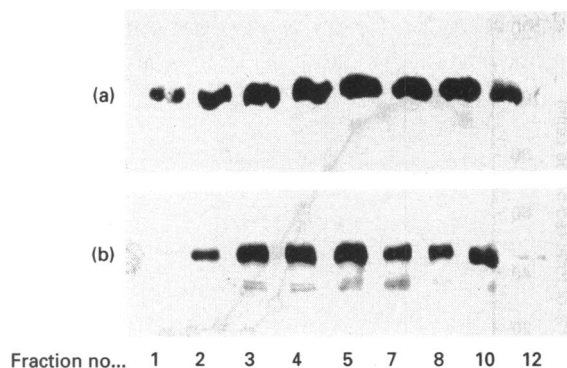


Figure 2 Immunoblot analysis of 15-lipoxygenase in Percoll-density-gradient-fractionated reticulocyte-rich rabbit red blood cells

Rabbit red blood cells recovered from a Percoll density gradient were separated into 4×10^5 μ -min supernatant (a), and mitochondria-rich pellet (b) before SDS/PAGE and probing of nitrocellulose transfers with rabbit antiserum to 15-lipoxygenase and detection of bound secondary antibody by chemiluminescence. Supernatant from equal numbers of cells from each fraction was analysed. Similarly, stroma from equal numbers of cells from each fraction was analysed, but a 5-fold greater number of cells was used. Minor bands smaller than lipoxygenase observed in this and other blots appear to be breakdown products of lipoxygenase.

Immunological detection of 15-lipoxygenase in stromal fractions (mainly plasma membrane and mitochondria) prepared from Percoll-density-gradient-separated cells indicate that a significant amount of the enzyme is membrane-associated (Figure 2b). A negligible amount of lipoxygenase is detected associated with the stromal fraction of cells from fraction 1, but a considerable amount is sedimentable in cells from fractions 3–5, and thereafter declines. Densitometric analysis of immunoblots indicate a 19-fold increase in lipoxygenase protein recovered in the stromal fraction of cells recovered from fraction 3 compared with fraction 1, whereas there is only a 4-fold increase in soluble lipoxygenase. It therefore appears that 15-lipoxygenase associates with cell membranes only when the cytosolic concentration of lipoxygenase has reached a certain critical level, or after some other limiting event. A maximum of approx. 15% of total 15-lipoxygenase protein is found associated with the stromal fraction.

Ca²⁺-promoted binding of 15-lipoxygenase to isolated mitochondria

Lipoxygenase binding to mitochondrial membranes was tested *in vitro* by incubating reticulocyte-rich red-cell cytosol, free of membranes, with mitochondria freshly prepared from rat liver. After incubations as short as 1 min with Ca²⁺ present, 15-lipoxygenase could be found with the mitochondrial pellet after centrifugation (Figure 3, lane a). EGTA decreases 15-lipoxygenase binding to 3% of that observed in the presence of 2 mM CaCl₂ (Figure 3, lane d versus lane a). Mg²⁺ or Fe²⁺ ions did not promote association of reticulocyte lipoxygenase with rat liver mitochondria (Figure 3, lanes b and c). Ca²⁺-promoted association of 15-lipoxygenase with isolated mitochondria was observed at Ca²⁺ concentrations down to 50 μ M (results not shown). Ca²⁺-promoted lipoxygenase binding to mitochondria could result from Ca²⁺-induced mitochondrial swelling or activation of phospholipases. Pre-swelling mitochondria in a hypotonic buffer did not result in increase in lipoxygenase binding above that seen with intact mitochondria in the absence of Ca²⁺ (Figure 3, lane e versus lane a), and Ca²⁺ still promoted 15-lipoxygenase binding to pre-swollen mitochondria (Figure 3,

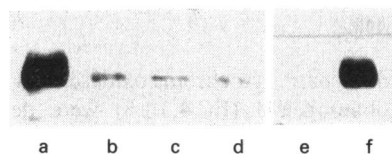


Figure 3 Effect of Ca²⁺ on rat liver mitochondria-associated sedimentability of reticulocyte 15-lipoxygenase

Freshly isolated rat liver mitochondria were incubated with reticulocyte-rich red-blood-cell cytosol, prepared from a heterogeneous population of cells, at 37 °C. Mitochondria were collected by centrifugation, subjected to SDS/PAGE, and the presence of reticulocyte 15-lipoxygenase was determined by probing nitrocellulose transfers with antibody to 15-lipoxygenase. Mitochondria were incubated simultaneously with reticulocyte cytosol and 2 mM Ca²⁺ (lane a), 2 mM Fe²⁺ (lane b), 2 mM Mg²⁺ (lane c) or 4 mM EGTA (lane d). Mitochondria pre-swollen in hypotonic buffer were incubated with reticulocyte-rich red-blood-cell cytosol lysate in the presence of EGTA (lane e) or Ca²⁺ (lane f).

Table 1 Formation of malondialdehyde from rat liver mitochondria by reticulocyte 15-lipoxygenase

Freshly prepared rat liver mitochondria were incubated at 37 °C with reticulocyte-rich cytosol at pH 7.5. Control values, obtained by incubations in the presence of the 15-lipoxygenase inhibitor salicylhydroxamate (1 mM), have been subtracted. Each value is the mean of four separate experiments.

Addition	Malondialdehyde formed (nmol/30 min per 150 μ g of mitochondrial protein)
Mitochondria	0.00 \pm 0.03
Reticulocyte cytosol	0.00 \pm 0.03
Mitochondria + cytosol + 2 mM EGTA	0.10 \pm 0.02
Mitochondria + cytosol + 2 mM Ca ²⁺	0.45 \pm 0.05

lane f versus lane e). In addition, Ruthenium Red or LaCl₃, both inhibitors of Ca²⁺ transport into mitochondria, did not prevent Ca²⁺-promoted lipoxygenase binding to rat liver mitochondria (results not shown). Furthermore, preincubation of mitochondria with Ca²⁺, followed by chelation of Ca²⁺ with EGTA before addition of reticulocyte lysate, failed to result in lipoxygenase binding (results not shown). Alternatively, Ca²⁺ may cause aggregation and sedimentability of lipoxygenase. We have found that on prolonged storage of reticulocyte lysate at -70 °C a proportion of 15-lipoxygenase aggregates, as determined by gel-exclusion chromatography, and becomes sedimentable. Self-aggregation of 15-lipoxygenase is excluded in these experiments by the observation that preincubating lysate with Ca²⁺ before addition of EGTA and mitochondria failed to result in sedimentable lipoxygenase (results not shown). Therefore Ca²⁺, lipoxygenase and mitochondria must be present simultaneously for Ca²⁺-promoted lipoxygenase binding to occur.

Incubation of lysate from reticulocytes metabolically labelled with [³⁵S]methionine indicated that, although 15% of radiolabelled 15-lipoxygenase co-sedimented with rat liver mitochondria, only 0.005% of radiolabelled haemoglobin was sedimentable, indicating that association of 15-lipoxygenase with mitochondria was not simply non-specific. Ca²⁺ also promoted 15-lipoxygenase-mediated peroxidation of mitochondrial lipids. Thiobarbituric acid-reactive products (malondialdehyde) were increased 6-fold when incubations of lysate with mitochondria included Ca²⁺ as compared with EGTA (Table 1).

Although routine rat liver mitochondrial preparations were not enriched in lysosomal acid phosphatase activity, this enzyme

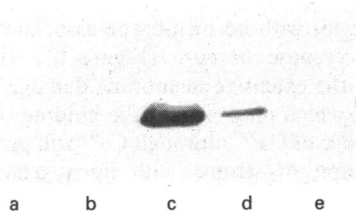


Figure 4 Ca^{2+} promotes association of 15-lipoxygenase with a mitochondrial-rich reticulocyte stroma fraction

Freeze-thawed mitochondrial-rich stroma from young reticulocytes (Fraction 1) contain no detectable lipoxygenase (lane e). After incubation with cytosol from a mixed population of reticulocytes, 15-lipoxygenase sedimented with the stroma in the presence of EGTA (lane d), but stroma-associated lipoxygenase was greatly increased in the presence of 1 mM Ca^{2+} (lane c). Little lipoxygenase was sedimentable from mixed-reticulocyte cytosol incubated without stroma in the presence (lane a) or absence (lane b) of Ca^{2+} .

was detectable, indicating contamination of mitochondria with lysosomes which could be associating with reticulocyte 15-lipoxygenase. Treatment of rat liver mitochondrial preparations with digitonin substantially decreased the acid phosphatase activity (approx. 50%), but did not decrease the amount of 15-lipoxygenase bound in the presence of Ca^{2+} (results not shown). Indeed there appears to be a small increase (approx. 15%) in bound lipoxygenase as the mitochondrial content of the preparation increased (relative specific activity of cytochrome oxidase with respect to the starting homogenate increased from 9.0 to 10.1), suggesting that most of the 15-lipoxygenase is binding to mitochondrial rather than lysosomal membranes.

Association of reticulocyte 15-lipoxygenase with reticulocyte mitochondria was tested *in vitro* by incubating reticulocyte-rich cytosol (prepared from a mixed population of reticulocytes) with mitochondrial-rich stroma prepared from the least dense reticulocytes recovered from a Percoll density gradient (Fraction 1). Reticulocytes were stored at -20°C before thawing and preparation of the stroma fraction. Lipoxygenase was undetectable in the stroma fraction (Figure 4, lane e) of these young reticulocytes. After incubation with mixed-reticulocyte cytosol and Ca^{2+} , substantial amounts of lipoxygenase sedimented with the mitochondrial-rich stroma (Figure 4, lane c). Sedimentable lipoxygenase was greatly decreased, although still detectable, in the presence of EGTA and in the absence of Ca^{2+} (Figure 4, lane d). Little lipoxygenase was sedimentable from mixed-reticulocyte cytosol in the presence or absence of Ca^{2+} but in the absence of stroma (Figure 4, lanes a and b). These results indicate that Ca^{2+} promotes association of lipoxygenase with reticulocyte mitochondria as well as rat liver mitochondria *in vitro*, although Ca^{2+} does not appear to be essential.

Ca^{2+} stimulation of lipid peroxidation by 15-lipoxygenase

A Ca^{2+} requirement of reticulocyte 15-lipoxygenase activity was assessed in a spectrophotometric assay with linoleic acid, dissolved in a small volume of ethanol and diluted in assay buffer, as the substrate. Addition of 5 mM EGTA decreased reticulocyte 15-lipoxygenase activity of semi-purified preparations to 16% of the maximal rate in the presence of Ca^{2+} . Ca^{2+} -stimulated lipoxygenase activity with a half-maximal rate at about 0.4 mM Ca^{2+} with 100% activity requiring approx. 2 mM Ca^{2+} (Figure 5). These values are similar to those published previously for 15-lipoxygenase of keratinocytes and polymorphonuclear neutrophils [9,10]. However, stimulation of reticulocyte 15-lipoxygenase was seen at concentrations of Ca^{2+} as low as 50 μM (Figure 5).

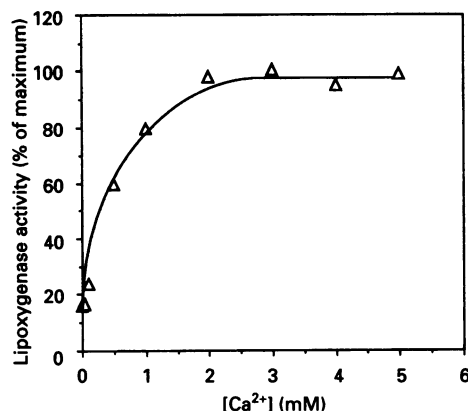


Figure 5 Ca^{2+} stimulation of reticulocyte 15-lipoxygenase activity

15-Lipoxygenase activity was measured spectrophotometrically at 234 nm with ethanol-solubilized linoleic acid as substrate and 55%-saturated- $(\text{NH}_4)_2\text{SO}_4$ precipitate of reticulocyte-rich red-blood-cell cytosol as source of enzyme. All incubations included 4 mM EGTA and, where shown, Ca^{2+} to give the indicated final concentration of free Ca^{2+} .

Table 2 Effect of calcium on reticulocyte 15-lipoxygenase activity in the absence and presence of cholate

Incubations at 25°C contained 0.4 mM linoleic acid solubilized in ethanol and 1 μg of protein of reticulocyte-rich-cytosol 55%-saturated- $(\text{NH}_4)_2\text{SO}_4$ precipitate. Lipoxygenase activity was determined spectrophotometrically at 234 nm.

Addition	15-Lipoxygenase activity (% of rate with 1 mM Ca^{2+})
1 mM Ca^{2+}	100
5 mM EGTA	40
0.2% (w/v) cholate + 1 mM Ca^{2+}	190
0.2% (w/v) cholate + 5 mM EGTA	198

Other bivalent cations (Mg^{2+} , Fe^{2+} , Mn^{2+}) failed to stimulate lipoxygenase activity (results not shown). Similar results were obtained when the activity of lipoxygenase was measured with [^{14}C]linoleic acid as substrate and determining radioactive 13-HPODE produced and linoleic acid converted (results not shown).

Solubilization of the linoleic acid substrate with sodium cholate resulted in higher enzymic rates in the absence of Ca^{2+} and abolished the Ca^{2+} stimulation of lipoxygenase activity (Table 2). Similarly, if the sodium salt of linoleic acid was used, higher rates were obtained and Ca^{2+} failed to stimulate 15-lipoxygenase activity (results not shown).

DISCUSSION

The buoyant-density fractionation of reticulocyte-rich (35%) red blood cells on Percoll gradients successfully yields a series of cell populations of progressively increasing average maturity, as demonstrated by amount of reticulum, mean cell volume and cytochrome *c* oxidase activity (Figure 1). As the reticulocyte matures it synthesizes haemoglobin, shrinks and becomes more dense. This permits exploitation of buoyant density in sub-fractionating a mixed population of red blood cells according to

age. From density-fractionated cells it was found that the loss of cytochrome oxidase activity and succinate dehydrogenase protein was concomitant with the appearance of sedimentable 15-lipoxygenase. The appearance of sedimentable lipoxygenase appears to lag behind its appearance in the cytosol, which may be due to a requirement for 'susceptible' mitochondria [16] after the action of an additional 'mitochondrial susceptibility factor' ('MSF') [17]. Indeed, we found that incubation of density-fractionated red blood cells with the Ca^{2+} ionophore A23187 in the presence of Ca^{2+} did not alter the proportion of sedimentable reticulocyte 15-lipoxygenase in cells of any age. This would suggest that, although Ca^{2+} may promote lipoxygenase association with mitochondria, it is not the rate-limiting step.

Ca^{2+} promotes reticulocyte 15-lipoxygenase association with rat liver mitochondria (Figure 3) and a freeze-thawed mitochondrial-rich stromal fraction prepared from young reticulocytes (Figure 4). Translocation of 5-lipoxygenase to cell membranes requires Ca^{2+} and the presence of a docking protein, 5-Lipoxygenase Activating Protein (FLAP) and is inhibited by the drug MK-886 [6,7]. We failed to find any inhibition of reticulocyte 15-lipoxygenase association with isolated rat liver mitochondria by MK-886 at concentrations up to 1 mM (results not shown). This would rule out FLAP or a close homologue as a docking protein for reticulocyte 15-lipoxygenase and therefore as a candidate for the mitochondrial susceptibility factor. Ca^{2+} -induced swelling of mitochondria, or a possible Ca^{2+} -induced aggregation of 15-lipoxygenase in the absence of mitochondria, does not explain the association of reticulocyte 15-lipoxygenase with rat liver mitochondria, suggesting that the effect of Ca^{2+} is specific for a direct interaction between lipoxygenase and the mitochondrial membrane. Magnani and co-workers [18] found that degradation of hexokinase bound to reticulocyte mitochondria is stimulated by Ca^{2+} and inhibited by the 15-lipoxygenase inhibitor salicylhydroxamic acid, even when reticulocytes were incubated in hypotonic buffer to swell mitochondria. Their results could be explained by our observations that Ca^{2+} promotes 15-lipoxygenase binding to mitochondria. Washing with high-salt buffers (1 M KCl) removes most of the mitochondrially associated 15-lipoxygenase (results not shown), suggesting that the interaction may be ionic. After solubilization of mitochondrial membranes with 1% (w/v) Triton X100, previously bound 15-lipoxygenase is also solubilized and no longer sedimentable (results not shown), suggesting that sedimentability of lipoxygenase resulting from incubation with Ca^{2+} and mitochondria is indeed due to association with lipid membranes. However, reticulocyte 15-lipoxygenase binds trypsin-treated rat liver mitochondria (results not shown); therefore the interaction may not require a 'docking protein', or such a protein may be inaccessible to trypsin. Stimulation of 15-lipoxygenase activity against linoleic acid by Ca^{2+} in the absence of detergents (Figure 5) suggests that interaction of the enzyme with lipid substrates (fatty acid micelles, membranes) in a certain physical form requires Ca^{2+} .

If reticulocyte mitochondria must be primed for lipoxygenase attack, how can we explain the association of reticulocyte 15-lipoxygenase with freshly isolated rat liver mitochondria? A 12 kDa protein had tentatively been identified as the mitochondrial susceptibility factor which may act as an uncoupler [19], and is presumably present and active in the reticulocyte cytosol preparations used in our experiments, which are prepared from a mixed population of reticulocytes, most of which will probably contain the susceptibility factor. Incubation of isolated mitochondria at 37 °C by itself may lead to sufficient damage to permit 15-lipoxygenase association in the presence of Ca^{2+} . Freeze-thawed mitochondria isolated from a population of

young reticulocytes with no membrane-associated lipoxygenase will bind lipoxygenase *in vitro* (Figure 4). This may be a consequence of the extensive membrane damage resulting from freeze-thawing, which may explain the binding of lipoxygenase seen in the absence of Ca^{2+} , although Ca^{2+} still promotes a much greater association of stroma with lipoxygenase (Figure 4). However, binding in the absence of Ca^{2+} could be due to inadequate chelation of mitochondrial-associated Ca^{2+} by EGTA. Also, the stromal fraction contains plasma membrane, which may bind lipoxygenase in a Ca^{2+} -independent fashion. Indeed, we have found that erythrocyte ghosts bind lipoxygenase, although to a lesser extent than do mitochondria or stroma, and half of this association is Ca^{2+} -independent. In addition, the reticulocyte cytosol employed in this experiment will include mitochondrial susceptibility factor.

It has been reported that energized mitochondria *in vitro* are resistant to the action of reticulocyte 15-lipoxygenase [20]. Our experiments were carried out in the absence of substrates for mitochondrial respiration. Attempts to produce mitochondria which were fully coupled and with no uncoupled mitochondria present were unsuccessful (results not shown). However, initial experiments indicate that the presence of substrates (ADP and succinate) does decrease the binding of reticulocyte 15-lipoxygenase to rat liver mitochondria, even in the presence of Ca^{2+} (results not shown). Inhibition of binding is not complete, which could be due to uncoupled mitochondria providing sufficient targets for the 15-lipoxygenase to obscure the effect of energizing mitochondria on lipoxygenase binding. This is an attractive hypothesis, as synthesis of a protein during reticulocyte maturation (mitochondrial susceptibility factor) which uncouples mitochondria could lead to an efflux of Ca^{2+} from mitochondria, leading to local high concentrations, which in turn would promote 15-lipoxygenase binding.

The low proportion of 15-lipoxygenase associated with the membrane fraction of reticulocytes (approx. 10–15%) suggests that 15-lipoxygenase is synthesized in excess of the cell's requirement to inactivate mitochondria. Apparent over-production of 15-lipoxygenase in stress-induced (i.e. bleeding-induced anaemia) reticulocytes has been observed by others [3]. We have not examined the distribution of 15-lipoxygenase in reticulocytes normally resident in the circulation, where the level of 15-lipoxygenase may be lower and the proportion of bound enzyme higher. The Ca^{2+} -promoted 15-lipoxygenase association with mitochondria and generation of lipid products strongly suggest that Ca^{2+} may play an important role *in vivo* in regulating reticulocyte 15-lipoxygenase attack on mitochondria during maturation, and therefore the subsequent proteolytic elimination of mitochondria.

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