Fe-dependent formation of a protein that makes mitochondria lipoxygenase-susceptible during maturation of reticulocytes

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During in vitro incubation the mitochondria of immature reticulocytes fail to be degraded by lipoxygenase and subsequent ATP-dependent proteolysis. Susceptibility is conferred on the mitochondria by a protein, the 'mitochondria susceptibility factor' which is synthesized during maturation of the reticulocytes in an iron-containing medium.

Reticulocyte maturation *Lipoxygenase* *Mitochondria*

1. INTRODUCTION

It has been shown previously that mitochondria of immature rabbit reticulocytes fail to be degraded by lipoxygenase and subsequent ATP-dependent proteolysis during in vitro incubation [1]. The conclusion was drawn that the susceptibility of the mitochondria is determined by the maturational state of the reticulocytes. The question emerged as to why the cells did not mature under the conditions employed.

In this work it is demonstrated that the susceptibility is conferred on the mitochondria by a protein, named 'mitochondria susceptibility factor (MSF)', which is synthesized during maturation of the reticulocytes. Its formation depends on the supply of iron in the incubation medium.

2. MATERIALS AND METHODS

The fractionation of reticulocytes as well as the preparation of osmotic hemolysates have been described before [1]. Cells (hematocrit 5%) were incubated in a combined Eagle-Borsook medium [2], pH 7.6, without ascorbate and $FeSO_4$ unless indicated otherwise. The solutions were protected against microbial contamination by the addition of 10 mg penicillin and 10 μg streptomycin per l.

Fe^{3+}-transferrin was prepared according to Bates and Schlabach [3] and employed at a final concentration of 0.1 mM. Pulse labelling and determination of proteolysis on the basis of specific activity and concentration of lysine were performed as described [4]. Cell suspensions (hematocrit 40%) were incubated for 15 min at 37°C in 0.15 M Tris-HCl buffer, pH 7.6, containing 2.6 mM [U-^{14}C]lysine (5.8 mCi/mmol). The degree of proteolysis was expressed in percent of lysine released from hemoglobin-free stroma in a given period of time.

Lipoxygenase activity was measured spectrophotometrically as described previously [5].

3. RESULTS AND DISCUSSION

In fig.1 it is shown that immature reticulocytes, i.e. fraction I, exhibit extensive proteolysis when their incubation medium is supplied with iron, while they fail to do so in its absence. To ensure that the iron did not act in an physiological manner, the iron supplement was added in the form of the iron-transferrin complex. It is well established

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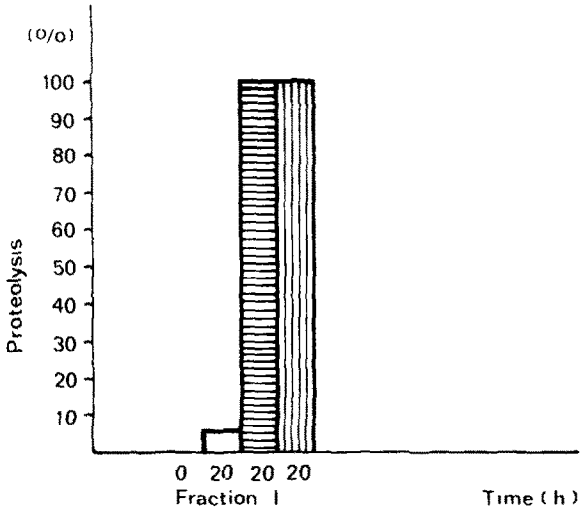

Fig.1. Effect of iron on proteolysis in intact immature reticulocytes. Fraction I (see [1]) was used. (□) Borsook-Eagle medium without ascorbate and ferrous sulfate. (■) The same with ferrous sulfate. (■) The same with Fe^{3+}-transferrin complex.

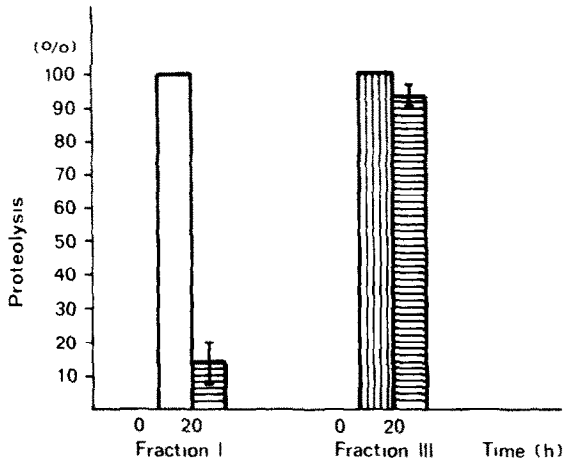

Fig.2. Effect of cycloheximide on proteolysis in reticulocytes. Fraction I, immature reticulocytes; fraction III, mature reticulocytes. Cycloheximide was used at 20 μM final concentration. All incubations were carried out in the presence of Fe^{3+}-transferrin. (□) Fraction I, control; (■) fraction III, control; (■) incubation with cycloheximide.

that physiological uptake of iron is mediated in a regulated manner by specific receptors, by which the iron-transferrin complex is internalized [6].

In fig.2 it is demonstrated that cycloheximide, an inhibitor of protein synthesis, suppressed the effect of supplementation with iron-transferrin on immature reticulocytes; proteolysis of mature reticulocytes, represented by fraction III of the cell population, was unaffected by cycloheximide. The possibility that cycloheximide might have acted by inhibiting the synthesis of lipoxygenase is discounted by the data in fig.3; they show that the cytosol of the immature reticulocyte fraction contained high activities of lipoxygenase, which increased somewhat during incubation in the absence of cycloheximide and decreased moderately in its presence.

The presence of a factor which is formed in the cytosol of immature reticulocytes and is still present in that of mature reticulocytes was demonstrated in experiments in which the mitochondria-containing stroma of immature reticulocytes was incubated with the cytosol of cells of the same fraction after 10 h incubation with the iron-supplemented medium. A representative result is shown in fig.4. The data in fig. 5 indicate that the amount of MSF in the cytosol of mature reticulocytes (fraction III) is well in excess of that needed for maximal rate of breakdown of mitochondria.

The next series of experiments were designed to

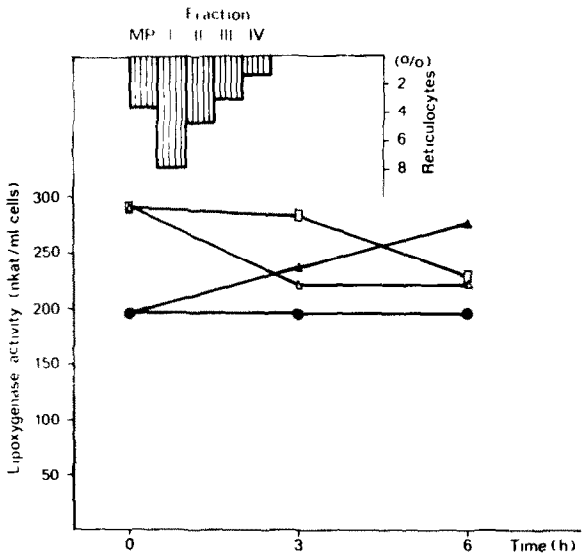

Fig.3. Activity of lipoxygenase in various reticulocyte fractions. (▲) Fraction I; (●) fraction I + cycloheximide; (□) fraction III; (△) fraction III + cycloheximide.

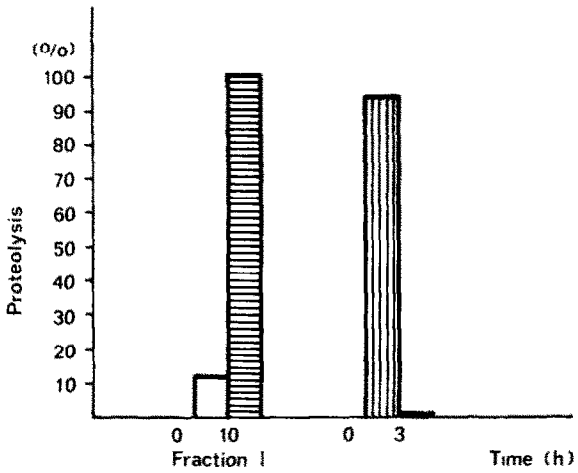

Fig.4. Effect of salicyl hydroxamate on the degradation of lipoxygenase-susceptible mitochondria-rich stroma of reticulocytes. (□) Immature reticulocytes in Fe^{3+}-transferrin-enriched Borsook-Eagle medium incubated for 10 h at 0°C. (■) The same at 37°C. (■) Stroma from immature reticulocytes incubated with cytosol from the same fraction of cells preincubated in the presence of Fe^{3+}-transferrin. (■) The same in the presence of salicyl hydroxamate.

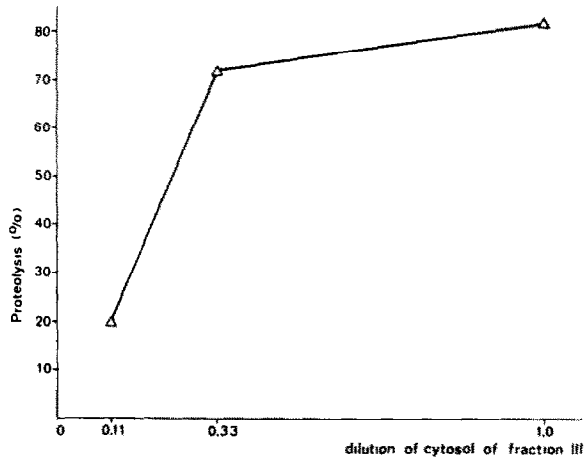

Fig.5. Proteolysis of stroma of fraction I with different amounts of cytosol III. Dilution was carried out with cytosol of fraction I to maintain the concentration of lipoxygenase and of the ATP-dependent proteolysis system. Incubation conditions: 3 h, 37°C, pH 7.6.

answer the following questions: (i) Does the newly found factor act by conditioning the mitochondria to the subsequent attack by lipoxygenase, to be followed by proteolysis? (ii) Is this sequence obligatory? (iii) Do the immature mitochondria require this factor under all conditions before they can be attacked by the ATP-ubiquitin-dependent proteolytic system?

The answers to these questions are documented in fig.4 and tables 1 and 2. In the experiment presented in fig.4, it is shown that in the presence of salicyl hydroxamate, a selective inhibitor of lipoxygenase, proteolysis did not occur, owing to the interruption of the sequence MSF–lipoxygenase–proteolysis. The data in table 1 serve to support this conclusion. It is shown that pure lipoxygenase failed to attack the stroma of the immature cell fraction. In the presence of an MSF-containing cytosol of the mature cell fraction, in which the lipoxygenase had been irreversibly inactivated by a suicide substrate, proteolysis was similarly practically absent, but appeared if supplemented by lipoxygenase.

Thus the physiological system consists of 3 factors acting in sequence, in which each predecessor acts as a trigger in a permissive manner. The whole system hinges on a subtle balance of interactions which determine in an as yet undefined manner distinct physiological states of the mitochondria: (i) a state of non-susceptibility to lipoxygenase; (ii) one of susceptibility to lipoxygenase but not to

Table 1

The cytosol of the most mature reticulocytes contains the 'susceptibility protein' and causes degradation of the mitochondria of the youngest reticulocytes

Experimental conditions	LOX[a]	ETYA cytosol[b] of fraction III	Cytosol of fraction I	Proteolysis (%)
Stroma of fraction I	+	−	−	1
	−	+	−	4
	+	+	−	51
	−	+	+	36
	−	−	+	1

[a] Pure reticulocyte: lipoxygenase (LOX) (preparation [5]) was added at a final concentration of 290 kcat/ml cells

[b] Cytosol of fraction III was treated for 3 h at 37°C with 10^{-4} M ETYA (5,8,11,14-eicosatetraynoic acid). After this time no ETYA was detectable (not shown)

Incubation conditions: 2 h, 37°C, pH 7.6

Table 2

Ca^{2+} ionophore and hypotonicity lead to an increase of proteolysis in the youngest reticulocytes, which is not affected by inhibitors of lipoxygenase

Experimental conditions		Ca^{2+} ionophore	SHAM	Hypotonicity	Proteolysis (%)
Cells	control	–	–	–	10
of		+	–	–	70
frac-		+	+	–	66
tion		–	–	+	68
I		–	+	+	64

Incubation conditions: 4 h, 37°C, pH 7.6. Final concentrations: Ca^{2+} ionophore A23187, 0.134 μM; salicyl hydroxamate (SHAM), 3×10^{-3} M; hypotonicity – the osmolarity of this medium was 200 mosmol/l

ATP-ubiquitin proteolysis; (iii) one of susceptibility to proteolysis.

The subtlety and vulnerability of the interactions were indicated previously by the fact that influx of Ca^{2+} in the cell permitted the proteolysis of mitochondria, short-circuiting the preceding steps. To test the assumption that the Ca^{2+} effect consisted in swelling of mitochondria a further experiment was performed in which the swelling was produced in a direct unequivocal manner. The results of such an experiment are shown in table 2 in which the effects of incubation in the presence of a Ca^{2+} ionophore and in a mildly hypotonic medium, in which hemolysis did not occur, were determined; it may be seen that under both conditions proteolysis occurred and was not inhibited by salicyl hydroxamate, thus indicating a short-circuiting of the physiological regulated sequence.

At present, one can only speculate about the nature and mechanism of action of the MSF, the action of which must precede the attack of lipoxygenase. Here again subtle changes in lipid-protein interactions may be involved. It is conceivable that the MSF affects the conformation of the mitochondria, be it by an attack on the mitochondrial membrane or on the system causing their condensed state, or possibly on their energy state. Another possibility might be that MSF has a specific affinity for mitochondrial lipids, thus rendering the phospholipids accessible to the lipoxygenase. The subtle nature of the changes involved is indicated by the observations that in model experiments on liver mitochondria swelling of the mitochondria, either as a result of the hypotonicity in salt medium, or caused by Ca^{2+}, sufficed to make the mitochondria susceptible to the attack by lipoxygenase or to ATP-dependent proteolysis [7].

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