Maturation of rabbit reticulocytes: susceptibility of mitochondria to ATP-dependent proteolysis is determined by the maturational state of reticulocyte

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(1) A simple procedure is described to separate reticulocytes of different maturity in high yield. (2) It is shown that exhaustion of supply of mitochondria susceptible to degradation by the lipoxygenase-ATP-dependent proteolysis system limits the extent of breakdown of mitochondria during in vitro maturation. The susceptibility of mitochondria depends on the maturity of the reticulocytes. (3) Incubation in the presence of calcium ions and calcium ionophore leads to full susceptibility of mitochondria in immature reticulocytes but has no effect on those in mature reticulocytes which are already fully susceptible to degradation. (4) Conditions which lead to rapid degradation of mitochondria do not affect the behaviour of the reticulocyte count. There appears to be no obligatory connection between the breakdown of mitochondria and of ribosomes.

Reticulocyte *ATP-dependent proteolysis* *Mitochondria* *Maturation*

1. INTRODUCTION

The reticulocyte is a well-defined intermediate stage in the differentiation of erythroid cells [1]. It is characterized by the presence of mitochondria and ribosomes with concomitant active respiration and protein synthesis on the one hand and on the other by the absence of the nucleus in mammals or its inactivity in lower vertebrates. There are also only vestigial remnants of endoplasmatic reticulum and few if any lysosomes left. The transition to the mature erythrocyte involves the loss of mitochondria and consequently of the respiratory capacity of the cell as well as of ribosomes and most of the receptors and transport proteins of the cell membrane.

In former studies it was demonstrated that the degradation of the mitochondria proceeds via a cascade of events beginning with the unmasking of the lipoxygenase – mRNA, followed by massive synthesis of the enzyme which then lyses the mitochondrial membranes and exerts specific inhibitory effects on the respiratory chain [2,3]. This attack precedes and triggers ubiquitin-ATP-dependent proteolysis of the mitochondria [4]. Finally other proteases and phospholipases complete their degradation.

The differentiation processes from stem cell to mature erythrocyte require about 6 days; half of this time is spent in the reticulocyte stage. Therefore one should be able to distinguish more and less mature reticulocytes. Their maturity can be gauged by their hemoglobin content which also permits the separation of reticulocytes by density-gradient centrifugation according to age.

There are in the main 3 factors, the interplay of which should determine the extent and time course of the degradation of mitochondria. First, the presence and amount of lipoxygenase; the mRNA for the enzyme is masked in the most immature reticulocytes in form of mRNP [5]. Secondly, the susceptibility of the mitochondria to the attack of lipoxygenase; in model experiments with liver mitochondria wide differences had been found

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depending on their functional state [6]. Thirdly, the activity of the ATP-dependent proteolytic system; its drastic decline during maturation of reticulocytes has been described by several authors [7–9].

The aim of the present investigation was the elucidation of these interrelations under conditions of maturation in vitro. For this purpose a 3-step procedure based on density differences was developed to subfractionate the reticulocytes of the peripheral blood of anemic rabbits into 3 fractions, which differ among themselves in a reproducible manner with respect to their properties.

2. MATERIALS AND METHODS

Fractionation of reticulocytes was according to density. Reticulocytosis of 20–40% was produced in rabbits of 3–4 kg body weight by daily bleeding for 3 days followed by an interval of 2 days. Blood in a quantity of 100 ml was obtained from the femoral artery on the 6th day. The cells were washed 3 times with 5-fold volumes of 0.9% NaCl.

The cells, usually about 20 ml, were separated and after thorough mixing resuspended in an equal volume of 0.9% NaCl and distributed in portions of 10 ml in narrow graduated centrifuge tubes, which were spun for 30 min at 2000 × *g* at 4°C. The supernatant fluid was discarded and the upper 10% of the cell mass was collected. This constitutes fraction I, the youngest reticulocytes. The next 30% of the cells were taken from each centrifuge tube, suspended in an equal volume of 0.9% NaCl and subjected to a second centrifugation for 30 min at 2000 × *g* at 4°C. After discarding the supernatant fluid the upper half of the cell mass from each centrifuge tube was collected; this portion constitutes fraction II, the intermediate reticulocytes. The remainder of the cell mass of the first and second centrifugations were resuspended in an equal volume of 0.9% NaCl, pooled and again subjected to the centrifugation at 2000 × *g*. The upper 20% of the cell mass from each tube was collected, and constituted fraction III, the mature reticulocytes. The remainder, used only for balancing the yield of reticulocytes, was the reticulocyte-poor fraction IV. Starting with a reticulocyte count of 30% for the whole blood, that of fraction I was more than 80%, fraction II more than 50%, and fraction III more than 35%, whereas it was well below 10% in fraction IV. The yield of reticulocytes in the fractions approached 100%.

2.1. *Conditions for the in vitro maturation and handling of cell suspensions*

Suspensions with a hematocrit of 5% were incubated in a basal medium according to Eagle (see [10]) to which were added 5 mM $NaHCO_3$ and 5.5 mM glucose, final concentration; in addition, 5 mg each of penicillin and streptomycin were added. In the experiments with the Ca^{2+} ionophore A23187, this compound was added in a final concentration of 0.0025 mM. The incubations were performed in flasks with gentle shaking. At appropriate times the samples were removed for analysis. Haemolysis was always monitored but was negligible.

Hemolysates were prepared by adding 2 vols water to one vol. cells. The hemolysates were centrifuged at 22000 × *g* for 15 min at 4°C and the supernatant fluids were collected carefully. The remaining stroma was washed 2 times with 10-fold volumes of 0.9% NaCl. For the incubations ATP and Mg^{2+} were added in final concentrations of 5 mM.

2.2. *Analytical methods*

The method described for pulse labelling and the determination of proteolysis on the basis of specific activity and concentration of lysine was followed [11]. For this purpose cells were collected by centrifugation and suspended in 0.15 Tris-HCl (pH 7.4) to a hematocrit value of 40%. For pulse labelling the cell suspensions were incubated for 15 min at 37°C in the presence of 2.6 mM [U-^{14}C]lysine (5.8 mCi/mol). All experiments were performed at least in duplicate.

3. RESULTS AND DISCUSSION

3.1. *The quantity of susceptible mitochondria limits their proteolysis*

ATP-dependent proteolysis is a time-limited process which stops after 2–3 h [11]. There remains the question as to the limiting factor: is it exhausted of substrate, i.e., of susceptible mitochondria or the decay of the proteolytic system?

A representative experiment is depicted in fig.1.

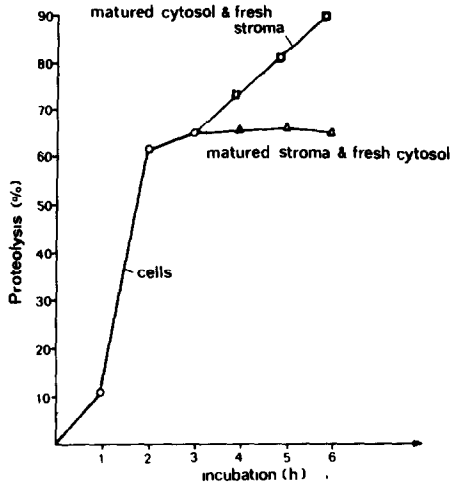

Fig.1. The stop of proteolysis of reticulocytes during in vitro maturation is caused by exhaustion of susceptible substrate. After a preincubation for 3 h at 37°C the cells were separated in supernatant fluid and stroma, and each was complemented by a portion of the corresponding fraction that had been kept at 0°C.

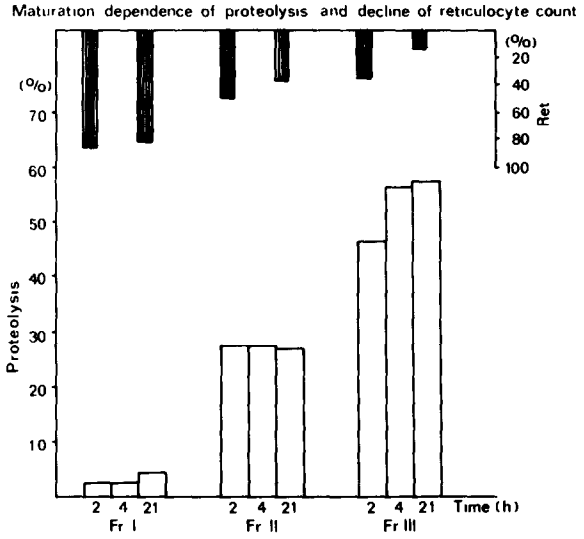

Fig.2. Maturation dependence of proteolysis, and changes in reticulocyte count during in vitro maturation of red cells of anemic rabbits. On the abscissa is listed the extent of proteolysis of the stroma of fractions I, II and III after 2, 4 and 21 h periods. The upper right scale indicates the reticulocyte count at the beginning and after 21 h.

It may be seen that after an incubation for 3 h at 37°C the proteolysis had stopped. The cytosol from the incubated cells was still able to attack added unincubated mitochondria – containing stroma, albeit at a rate reduced to about one-third. On the other hand, unincubated fresh cytosol could not degrade the stroma from pre-incubated cells any further. The conclusion may be drawn that the stopping of proteolysis is determined by the exhaustion of available substrate.

3.2. *Proteolysis of mitochondria is strongly dependent on the maturational state of the reticulocytes*

In experiments as depicted in fig.2 the differences in proteolysis between reticulocytes of varying maturity, which had been prepared by fractional density separation were tested. It may be seen that the youngest fraction (I) appeared to be highly resistant to proteolysis. It also showed only an insignificant decrease of the reticulocyte count which reflects primarily the number of ribosomes. In contrast, the oldest fraction (III) exhibited massive proteolysis which was about complete within 4 h. This fraction also showed a drastic decrease of the reticulocyte count to one-third. The intermediate fraction (II) exhibited an intermediate behaviour. From these experiments one may assume that the mitochondria of immature mitochondria are probably resistant to the attack of lipoxygenase, which should have been synthesized during the incubation period [2,3].

3.3. *Swelling of mitochondria increases the susceptibility to proteolytic attack*

The resistance of the mitochondria to proteolysis may be caused by a specific type of protein-lipid interaction. In line with this suggestion are the observations that swelling induced by hypotonicity may greatly increase the susceptibility of liver mitochondria to the attack by lipoxygenase [6]. It is well known that mitochondria may be induced to swell by exposure to elevated calcium concentrations in the cell. Accordingly, experiments were performed in which incubations in the presence of low concentrations of calcium ionophore and calcium ions were compared with controls.

The results in fig.3 indicate clearly the differential effect of this type of manipulation. Mitochondria from mature reticulocytes exhibit without ad-

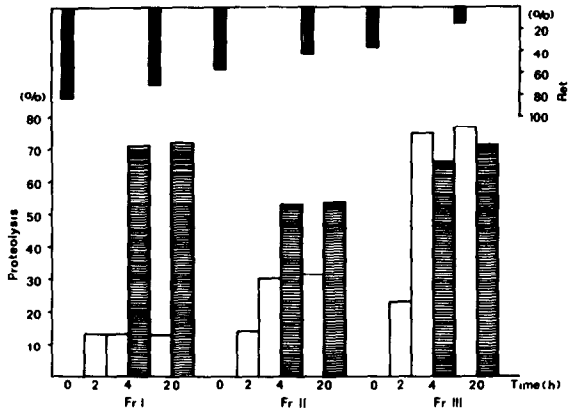

Fig.3. The effect of elevation of intracellular Ca^{2+} on the proteolysis during in vitro maturation. Parallel samples with and without Ca^{2+} ionophore were incubated for 20 h at 37°C. The shaded columns repressed the ionophore-containing samples, other details as in fig.2.

ditions massive proteolysis, which was not further enhanced in the presence of calcium ionophore and calcium ions. Obviously their mitochondria had been in a maximally susceptible state. On the other hand, the youngest fraction, which is a control incubation, showed only a little proteolysis, there was a dramatic effect of the treatment. Its proteolysis equalled, if not exceeded, that of the most mature fraction. It may be noticed that the manipulations had no effect on the behaviour of the reticulocyte count, indicating that mitochondrial breakdown and decay of ribosomes or ribosomal RNA are not obligatorily connected and may be dissociated from each other.

Conceivable indirect effects such as activation of phospholipases by Ca^{2+} whereby the supply of substrate for the lipoxygenase may be increased have been ruled and in experiments (not shown) which indicated that the proteolytic breakdown of calcium-exposed mitochondria is not very dependent on the preceding action of lipoxygenase. It is not affected by inhibitors of lipoxygenase, therefore the mitochondria of immature reticulocytes treated with Ca^{2+} behave like those of mature reticulocytes.

3.4. *The effects of inhibition of lipoxygenase on proteolysis*

The attack of lipoxygenase precedes and triggers ATP-dependent proteolysis which is therefore

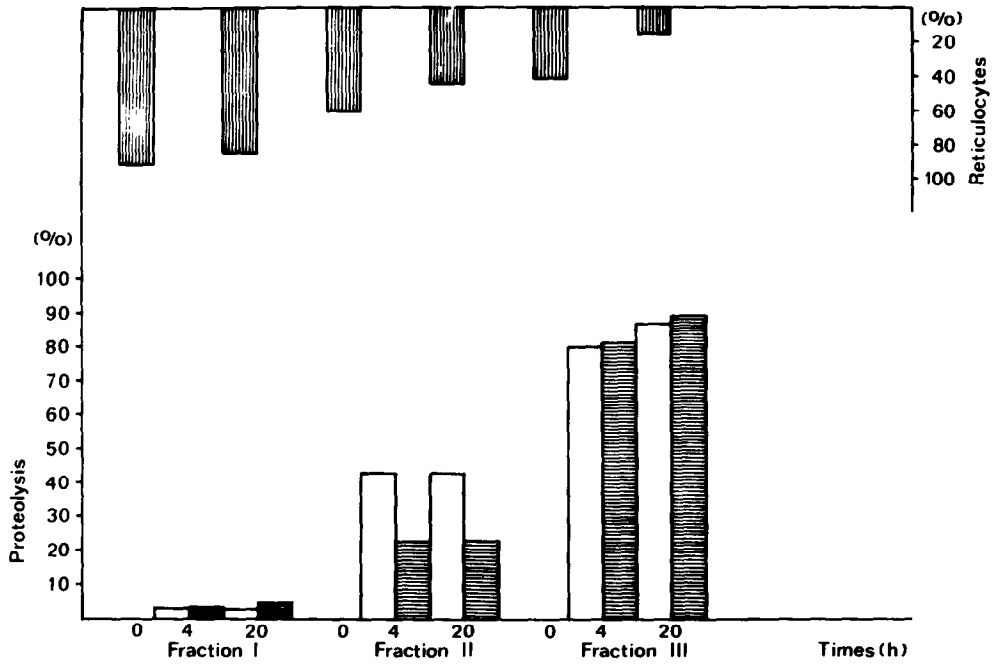

Fig.4. The effect of SHAM on the proteolysis of maturity-fractionated reticulocytes during in vitro maturation. Parallel samples with and without SHAM were incubated for 20 h at 37°C. The shaded columns indicate the SHAM-containing samples.

largely suppressed by inhibitors of lipoxygenase such as salicylhydroxamic acid (SHAM). It was of interest to study the effects of this inhibitor on the 3 reticulocyte fractions during in vitro maturation. A representative experiment is shown in fig.4. It may be seen that SHAM failed to influence the extent of proteolysis of either the youngest or the most mature reticulocytes; the first fraction since it was not yet susceptible to the attack by lipoxygenase and the mature reticulocytes because their mitochondria had already been subjected to the action of the enzyme. Their proteolysis was therefore no longer dependent on the triggering by lipoxygenase. The intermediate fraction, on the other hand, showed a strong inhibitory effect of SHAM, in confirmation of earlier work [12], in which short periods of incubation were employed.

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