Proteolysis of mitochondria in reticulocytes during maturation is ubiquitin-dependent and is accompanied by a high rate of ATP hydrolysis

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The ATP-dependent breakdown of mitochondria-containing stroma proceeds via the ubiquitin-requiring pathway. The proteolysis is linked to a large ATP-cleaved consumption amounting to 1 ATP per peptide bond or more. Proteins of mitochondria-containing stroma are much better substrates of ATP-ubiquitin-dependent proteolysis than heat-denatured ones. Hemin suppresses both proteolysis and ATP hydrolysis.

Ubiquitin *ATP-dependent proteolysis* *Reticulocyte* *Mitochondria* *Proteolysis* *ATP hydrolysis*

1. INTRODUCTION

In former communications we reported that the bulk of proteolysis in reticulocytes is ATP-dependent and directed against their mitochondria [1–3]. A mechanism of ATP-dependent proteolysis which requires ubiquitin has been fully established [4,5]. In that work abnormal or denatured proteins were used as substrates. On the other hand, it has been claimed that a sizeable portion of ATP-dependent proteolysis is independent of ubiquitin [6,7], including the proteolysis in reticulocytes [7]. However, the conclusions of the experiments on reticulocytes appear to be invalidated by the use of phenylhydrazine to produce reticulocytosis, whereby denatured proteins, mitochondrial damage and other deleterious effects are produced [8]. Therefore, it seemed important to find out the mechanism which is operative in the degradation of mitochondria in reticulocytes produced by bleeding.

Another problem concerns the amount of ATP hydrolyzed in relation to the proteolysis. According to the original scheme [4,5,9,10] ATP is required only for the activation of ubiquitin. We found, however, a much higher ratio of ATP split compared with proteolysis [11]. In a recent communication it was reported [12] that the breakdown of ubiquitin–protein conjugates is also an ATP-consuming process. It seemed worthwhile to try to minimize ATP breakdown unrelated to proteolysis to establish more precisely the stoichiometric relation between ATP hydrolysis and proteolysis.

2. MATERIALS AND METHODS

All experiments were carried out on the 7th to the 9th day of bleeding of rabbits by which a reticulocytosis of about 40% was produced [13]. To release ubiquitin from endogenous ubiquitin-conjugates [10] the red blood cells were depleted of ATP by a preincubation at 37°C for 2 h in a mixture containing 150 mM Tris–HCl (pH 7.6), 20 mM 2-deoxyglucose and 0.2 mM 2,4-dinitrophenol. Cells were then hemolysed with 2 vols dithiothreitol (1 mM) and the stroma was separated by centrifugation for 30 min at 40000 × *g*. The sediment was washed twice with a 10-fold volume of Tris–HCl (pH 7.6) and suspended in the same buffer. For some experiments the stroma was denatured by heating at 65°C for 15 min in a water

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bath or pretreated for 15 min at 37°C with *N*-ethylmaleimide (NEM) (5 mM) followed by washing with a 10-fold volume of Tris–HCl (pH 7.6).

The stroma-free lysate was fractionated 1 day before the recombination experiments in fraction I (ubiquitin) and fraction II as described by Ciechanover et al. [14]. A DEAE-cellulose column, 1.3 × 11 cm (Serva DEAE 32) was used. After collecting fraction I (unadsorbed proteins) the column was washed to separate completely fraction I from fraction II (adsorbed proteins) [10].

All cell components used in the recombination experiments corresponded to 0.8 ml packed red cells in the assay. Incubations were carried out for 2 h at 37°C in the presence of 5.7 mM ATP, 5 mM Mg^{2+} and 150 mM Tris–HCl (pH 7.6) in a final volume of 5.6 ml. The final concentration of hemin was 53 μM. Determination of lysine liberation was described in [1]. Proteolysis is expressed in terms of nmol lysine released from the stroma per assay. Inorganic phosphate (P_i) was determined by a standard method [15] and is given in μmol P_i liberated. All data represent the mean ± SEM of products formed.

3. RESULTS AND DISCUSSION

Fig.1 shows the averaged data of experiments on the proteolysis of mitochondria-containing stroma incubated with cytosol or its fractions. Fraction I [16,17] consists of ubiquitin. Fraction II contains the complex proteolytic system. It may be seen that the proteolysis was practically fully dependent on the presence of both fractions combined. It amounted to nil in the presence of fraction I and 17% with fraction II alone as compared with the value with cytosol. The small degree of proteolysis with fraction II is probably due to ubiquitin remaining conjugated to the mitochondria which was not completely removed during the depletion period. Fig.2 shows the corresponding data on ATP hydrolysis. The complete system exhibited a much greater phosphate liberation than stroma alone or with either fraction I or II. ATPase activity in the presence of fraction I alone amounted to only 8%, or to 42% with fraction II. It would seem that the ATPase activity with the combined fractions I + II was smaller than that with cytosol, although the difference falls just short of significance. Figs 1 and 2 also show experiments in which the stroma had been heated to 65°C for 15 min. This treatment led to a drastic diminution of both proteolysis and ATP hydrolysis amounts to 5% of the proteolysis and 9% of the ATP hydrolysis as compared with the values with cytosol.

To reduce the non-specific ATPase activity of the stroma, various procedures were tried. Its pretreatment with NEM proved to be successful. Whereas with untreated stroma, corresponding to 1 ml cells, 26.0 ± 0.7 μmol phosphate were released from ATP in 2 h, only 10.0 ± 0.9 μmol were liberated after treatment with 5 mM NEM (not shown).

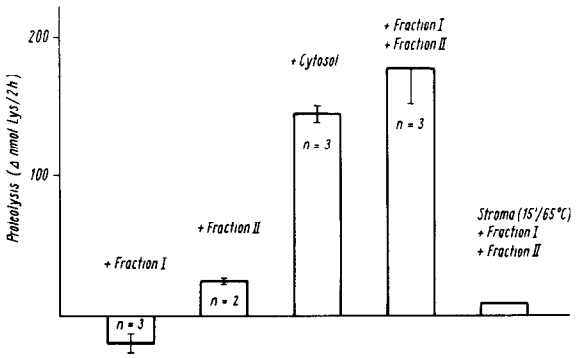

Fig.1. Protein breakdown of reticulocyte stroma (mitochondria) in the presence of ATP (5 mM) and Mg^{2+} (5 mM). Cytosol was fractionated in fraction I (ubiquitin) and fraction II [14]. All components used corresponded to 0.8 ml cells. Data represent the means ± SE.

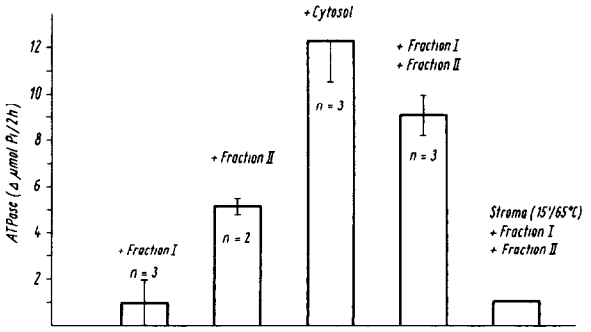

Fig.2. ATPase activity stimulated by stroma in fraction I, fraction II, cytosol and fraction I + fraction II. Conditions as in fig.1. Data represent the means ± SE.

Hemin has been reported to be a selective inhibitor of ATP-dependent proteolysis [18]. Its site of action was deduced to be beyond the ubiquitin-activating reaction steps [19]. It appeared therefore of interest to test its effect on both proteolysis and ATP hydrolysis of reticulocyte mitochondria. Figs 3 and 4 show that hemin indeed completely inhibited proteolysis both in untreated and NEM-treated stroma and also strongly reduced the breakdown of ATP to 39%. After heating stroma proved again to be a poorer substrate with proteolysis amounting to 28% and ATPase activity to 12%. Earlier we reported that native stroma was a better substrate than heat-denatured human serum albumin [3]. The same holds true for the ATPase activity; less than 1 μmol P_i was liberated in the presence of heat-denatured albumin in 2 h in the system usually employed.

In table 1 the ratios are compiled of P_i formed from ATP to the amounts of lysine liberated from the mitochondria-containing stroma in the presence of cytosol or the combined fractions I and II. The difference between the ratio in the presence of the cytosol and the combined fraction I and II is not appreciable while the experiments in the presence of hemin are statistically significant with P values of 0.05. From the values listed, one may estimate that more than one phosphate is liberated per peptide bond cleaved assuming a non-discriminating hydrolysis of the proteins and based on a lysine content of 3% for the stroma [11].

The diminution of the liberation of P_i by the addition of hemin corresponds to the amount of ATP required for the reaction steps beyond the conjugation of ubiquitin. This value amounts to approx. 1 phosphate liberated per peptide bond cleaved. The difference between the ratios with and without hemin is accounted for by the ATP required for the activation of ubiquitin and in addition to the isopeptidase activity [20] which splits the ubiquitin conjugate without proteolysis [12,21]. In this manner a kind of futile cycle results.

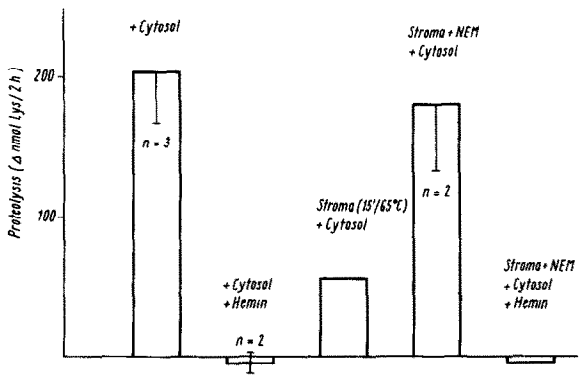

Fig.3. Effect of hemin (50 μM) on the breakdown of native stroma and stroma pretreated with 5 mM *N*-ethylmaleimide (NEM). Data represent the means ± SE.

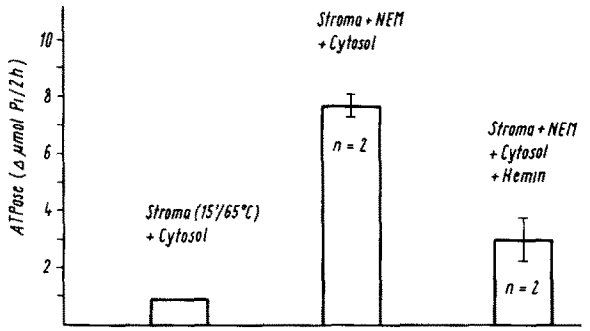

Fig.4. Effect of hemin (50 μM) on the ATPase activity stimulated by stroma pretreated with 5 mM *N*-ethylmaleimide (NEM) or heated (15 min, 65°C). Data represent the means ± SE.

From the results presented the following conclusions can be drawn:

(i) The ATP-dependent breakdown of mitochondria-containing stroma proceeds via the ubiquitin-requiring pathway. Both the experiments with fractionated cytosol and the complete inhibition of proteolysis by hemin support this inference.

Table 1

Relation between P_i- and lysine-liberation in ATP-ubiquitin-dependent proteolysis of mitochondria-containing stroma of reticulocytes

System	*n*	Ratio P_i:lysine
Fraction I		51 ± 4
Fraction II		
+ stroma	3	
Cytosol		
+ NEM-stroma	2	43 ± 4
Cytosol		
+ NEM-stroma		
+ hemin	2	28 ± 3

Data represent the means ± SE

(ii) The proteolysis is linked to a large ATP consumption amounting to 1 ATP per peptide bond cleaved or more. ATP is required both for the activation of ubiquitin and for the reaction steps beyond, probably related to the breakdown of ubiquitin conjugates. These steps account for the bulk of the ATP consumption.

(iii) Proteins of mitochondria-containing stroma are much better substrates of ATP-ubiquitin-dependent proteolysis than heat-denatured ones or denatured serum albumin. It remains to be studied whether the special susceptibility of mitochondrial proteins to the attack by the proteolytic system is due to their quality of being good acceptors for the conjugation with ubiquitin.

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