Kinetics of ^{125}I-ubiquitin conjugation with and liberation from rabbit reticulocyte stroma

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Received 16 September 1985

The breakdown of mitochondria-containing stroma of rabbit reticulocytes is an ATP- and ubiquitin-dependent process and there is no evidence for an ATP-dependent but ubiquitin-independent proteolysis in these cells. The ubiquitin conjugate formation with heat-denatured stroma proteins is about one-fifth of that with native stroma. In reticulocytes there exist two mechanisms of ubiquitin liberation from its conjugates with stroma proteins: (i) an ATP-dependent and hemin-resistant release of ubiquitin, which is assumed to be the first step in the degradation of ubiquitin conjugates by the protease system, and (ii) a release of ubiquitin catalyzed by an isopeptidase activity.

Ubiquitin conjugation *Reticulocyte stroma* *ATP-dependent proteolysis*

1. INTRODUCTION

Recently it was shown that the proteolysis of mitochondria in reticulocytes proceeds via the ubiquitin-requiring pathway [1]. Extensive ATP- and ubiquitin-dependent proteolysis was found to be also present in other cells such as mouse mammary carcinoma cells [2] or HeLa cells [3]. However, there are some controversies concerning the obligatory requirement for ubiquitin [4,5]. The present investigation provides evidence that the ubiquitin pathway is the major ATP-dependent proteolytic pathway in reticulocytes.

Denaturation of proteins seems to affect their intracellular breakdown in a more complicated way than it was imagined previously [1,6]. Here we report why heat-denatured stroma is a poorer substrate of the ATP- and ubiquitin-dependent proteolysis in reticulocytes.

At present much is known about the ubiquitin-protein ligase system involving a specific ubiquitin-activating enzyme, E_1 [7,8], ubiquitin-transferring enzymes (E_2s) and protein conjugate formation in the presence of E_3 [9,10] and its interaction with diverse substrates [10–12]. In contrast, the mechanism of the proteolytic breakdown of ubiquitin-conjugated proteins is still obscure. It has been reported [1,13,14] that it is ATP-dependent, consuming 1 ATP per peptide bond. Kinetic studies of the degradation of ubiquitin conjugates [11,14,15] are contradictory with respect to ATP dependence and the existence of an isopeptidase activity which may be important for the regulation of the process.

We chose mitochondria-containing stroma as a substrate of the ubiquitin-protein ligase system to investigate the kinetics of ^{125}I-ubiquitin conjugation with and liberation from stroma proteins.

2. MATERIALS AND METHODS

Reticulocyte-rich blood with a reticulocytosis of about 40% was obtained from rabbits between the 6th and 10th day of bleeding. Preincubation of red blood cells, preparation of washed stroma and its heat denaturation and separation of fraction II from the stroma-free lysate were described in [1]. Fraction II was divided in small aliquots, stored at

Dedicated to Prakash Datta as a token of appreciation

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−20°C and thawed only once for each experiment within 2–3 weeks. Ubiquitin was prepared and purified from crude fraction I or stroma-free lysate by a modification [16] of a previously described procedure [17]. About 20 μg ubiquitin/ml red blood cells were obtained. Extensively dialyzed ubiquitin migrated as a single band at a position corresponding to an M_r of about 9000 in SDS-(12.5%)polyacrylamide gel electrophoresis [18]. Calculations of ubiquitin concentrations were based on an M_r of 8565 [8].

Iodination of purified ubiquitin with 37.3 MBq of carrier-free $Na^{125}I$ (Isocommerz, Dresden) was carried out by the chloramine-T method [11]. After termination of the reaction with sodium metabisulfite (2 mg/ml) the preparation was passed through a column of Sephadex G-25 (0.8 × 22 cm). The ^{125}I-ubiquitin peaks were pooled with an initial specific activity of 7×10^6 cpm/μg (6×10^7 cpm/nmol).

The dilution of the specific activity of ubiquitin was determined in a reaction mixture with a final volume of 5.6 ml containing: 150 mM Tris-HCl (pH 7.6), 5 mg/ml of fraction II, 5.8 nmol ubiquitin (with an initial specific activity between 1.2 and 3.4×10^4 cpm/nmol), washed stroma corresponding to 3.3 ml cells, 5 mM $MgCl_2$ and 5.3 mM ATP. The reaction was started by the addition of the stroma and was carried out at 37°C in a water bath with constant mixing. After 30 min the reaction was stopped by heating of the sample for 15 min at 95°C; ubiquitin conjugates are stable under these conditions [11]. In the zero-time sample $MgCl_2$ was replaced by EDTA (1 mM), ATP was absent and stroma was added during heating. After heating the samples were centrifuged at 30000 × *g* for 15 min and ubiquitin was prepared from the supernatant as described above. The purified protein was dissolved in 1 ml water. The recovery of ubiquitin was about 30–40%. Aliquots of 100 μl were used for the determination of the radioactivity as well as of protein concentration.

The kinetics of ^{125}I-ubiquitin conjugation with native or heat-denatured stroma were followed in an assay mixture corresponding to that described above. In the absence of ATP, $MgCl_2$ was replaced by EDTA (1 mM). The reaction was started by the addition of washed stroma corresponding to 2.5–3.5 ml cells. At various times indicated in the figures aliquots of 1 ml were taken and rapidly centrifuged (2 min at 11000 × *g*). The supernatant fluid was removed and the radioactivity was measured in the supernatant and in the stroma fractions. Results are given in cpm per stroma corresponding to 1 ml red blood cells (cpm values at zero time were subtracted).

P_i liberation was measured by a standard method [19] in aliquots of 100 μl from the same samples.

For the determination of the dissociation kinetics of ^{125}I-ubiquitin, washed stroma was preincubated for 10 min at 37°C in the described reaction mixture in the presence of ATP, Mg^{2+} and hemin. The stroma was then centrifuged, washed twice with a 10-fold volume of Tris-HCl (pH 7.6) and suspended in the same buffer with a final radioactivity of $1.5 \pm 0.2 \times 10^4$ cpm/ml cells in 3 experiments. Thereafter the preincubated stroma was incubated again in the same reaction mixture in the absence of ^{125}I-ubiquitin, with and without ubiquitin, ATP and Mg^{2+} and in the presence of hemin. At indicated times aliquots of 1 ml were centrifuged and the radioactivity was measured in the supernatant. Results are expressed as cpm liberated from stroma corresponding to 1 ml red blood cells. Controls were incubated without fraction II in order to estimate unspecifically bound ^{125}I-ubiquitin. Hemin was used in a final concentration of 70 μM. All measurements of radioactivities were carried out with a γ-counter (Philips PW 4518). Protein concentration was determined by a micro-method [20].

3. RESULTS

In table 1 are summarized the results of 3 ex-

Table 1

Dilution of the specific activity of ubiquitin by unlabeled ubiquitin liberated from the stroma during incubation

Expt	Specific activity (cpm/nmol per 10^4)		Decrease of specific activity (%)
	0 min	30 min	
1	1.65	1.36	17.6
2	1.81	1.47	18.8
3	1.22	1.01	17.2
Mean ± SE			17.9 ± 0.5

periments demonstrating the dilution of the initial specific activity of ubiquitin by 17.9 ± 0.5%. Under our experimental conditions the dilution of the specific activity must be due to a liberation of unlabeled ubiquitin from the stroma. Taking into account the initial specific activity in the experiments of 1.6×10^4 cpm/nmol and a ubiquitin concentration of 5.8 nmol per sample, the measured dilution of the specific activity after 30 min was calculated to be due to a ubiquitin liberation of approx. 1 nmol per sample. With an amount of stroma corresponding to 3.3 ml red blood cells, the calculated residual stroma-bound ubiquitin amounts to 0.3 nmol/ml cells.

In fig.1 is presented the time course of ^{125}I-ubiquitin conjugate formation with native stroma in the presence and absence of ATP and with heat-denatured stroma in the presence of ATP. The half-maximal levels of ubiquitin conjugation and the maximum of conjugate formation of about 2.0×10^4 cpm/ml cells with native stroma in the presence of ATP were reached after about 2 and 10 min, respectively. At 10 min the association of ^{125}I-ubiquitin with native stroma without ATP amounted to only 12% of that measured in the presence of ATP. Less than 25% of ^{125}I-ubiquitin determined was conjugated with heat-denatured stroma in the presence of ATP as compared with native stroma.

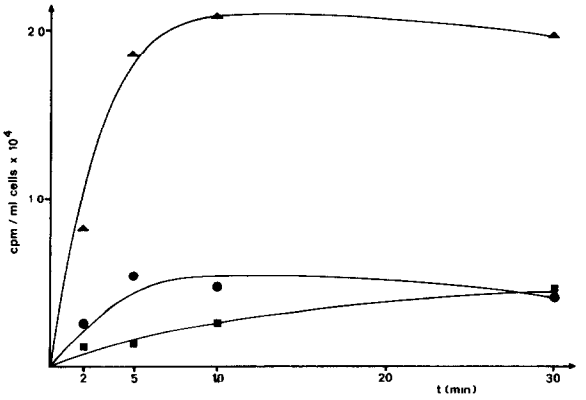

Fig.1. Time course of ^{125}I-ubiquitin conjugation with native and heat-denatured stroma. Native stroma (▲—▲) and heat-denatured stroma (●—●) were incubated in the presence of ATP and Mg^{2+}. (■—■) Native stroma incubated without ATP and Mg^{2+} in the presence of EDTA. Data are representative of 3–6 experiments.

Hemin influenced the time course of ^{125}I-ubiquitin conjugate formation only weakly (fig.2). In its presence half-maximal levels were reached later, after 3–4 min, while the maximum was the same as in the absence of hemin. In contrast, P_i liberation in the presence of hemin was decreased as compared with samples without hemin (table 2). The difference amounted after 30 min on average to 1.52 ± 0.42 μmol/ml cells.

In fig.3 is shown the kinetics of ^{125}I-ubiquitin liberation from the stroma in the presence of hemin with and without ubiquitin, ATP and Mg^{2+}. A rapid dissociation of conjugates is seen during the first 10 min in the presence of ubiquitin, ATP and Mg^{2+} which is retarded after 20–30 min. At an initial average label of the stroma of $1.5 \pm 0.2 \times 10^4$ cpm/ml cells in the case of the addition of

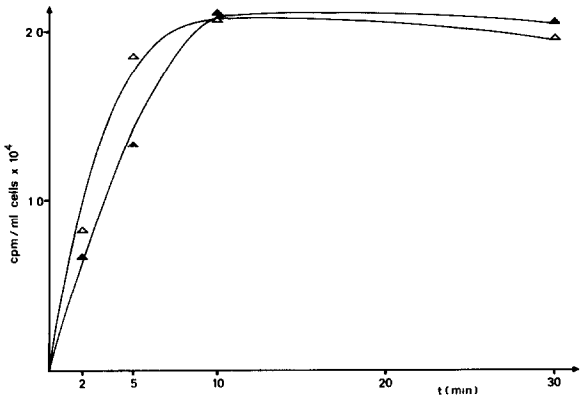

Fig.2. Time course of ^{125}I-ubiquitin conjugation with native stroma in the absence (Δ—Δ) and presence (▲—▲) of hemin (70 μM) with ATP and Mg^{2+}. Data are representative of 3–6 experiments.

Table 2

P_i liberation after 30 min in the presence and absence of hemin (70 μM)

Expt	P_i liberation (μmol/ml cells)		Δ μmol/ml cells
	− hemin	+ hemin	
1	7.80	5.55	2.25
2	7.39	6.61	0.78
3	8.85	8.02	0.83
4	7.46	5.22	2.24
Mean ± SE			1.52 ± 0.42

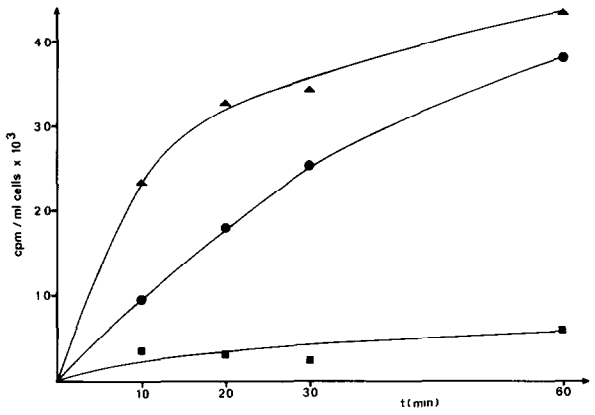

Fig.3. Time course of ^{125}I-ubiquitin liberation from native stroma in the presence of hemin. ^{125}I-ubiquitin-stroma conjugates were incubated in the presence of ubiquitin, ATP and Mg^{2+} (▲—▲) and in the absence of ubiquitin and ATP, with EDTA (●—●). Controls (■—■) were without fraction II. Data are representative of 3 experiments.

unlabeled ubiquitin (1.7–2.3 nmol/ml cells) the release of about 4000 cpm/ml cells could imply a nearly complete exchange of the specific activities so that the processes of ubiquitin conjugation with and liberation from the stroma would already be in a steady state after this time. A slow release of ^{125}I-ubiquitin was observed in the absence of ubiquitin, ATP and Mg^{2+}, conditions under which the ubiquitin-activating enzyme (E_1) and the protease splitting ubiquitin conjugates do not act (fig.3). Less than 30% of the ubiquitin conjugated was liberated from the stroma after 60 min.

4. DISCUSSION

The results presented here support the assumption that ubiquitin conjugate formation is a necessary step in the ATP-dependent proteolysis of mitochondrial proteins [1].

The dilution of the specific activity of ubiquitin by 17.9 ± 0.5% corresponds to about 0.3 nmol ubiquitin/ml cells remaining stroma-bound, even after a previous ATP depletion of red blood cells for 120 min. This remaining ubiquitin could be responsible for the residual proteolysis of mitochondria-containing stroma with fraction II alone, which amounts to 15% as compared with the value in the presence of crude ubiquitin designated as fraction I [1].

Therefore, in our experiments there is no evidence for the assumption of an ATP-dependent, ubiquitin-independent proteolytic pathway in reticulocytes as proposed by Tanaka et al. [4].

Recently it was shown by lysine liberation that heat-denatured stroma is a poorer substrate of the proteolytic system [1]. The main reason for the decreased degradation rates may well be the reduced ^{125}I-ubiquitin conjugation with heat-denatured stroma as demonstrated by the results presented here. Thus, denaturation does not affect the rates of intracellular protein breakdown in a simple predictable way as also shown in cultured hepatoma cells with microinjected proteins [6]. Further complications might be expected for protein denatured in a membrane complex, a process which is still poorly understood. An explanation for the decreased conjugation of heat-denatured stroma proteins with ubiquitin may be the loss of susceptible NH_2 groups which are necessary for protein conjugation and their subsequent breakdown [21]. The circumstance that the residual lysine liberation from heat-denatured stroma was only about one-fourth, while the corresponding formation of ubiquitin conjugates amounted to about one-fifth of the control, indicates that there is no direct stoichiometric relation between the 2 processes.

The kinetics of ^{125}I-ubiquitin conjugation with native stroma observed by us is in good agreement with that found by Ciechanover et al. [11] with endogenous soluble proteins of fraction II as substrates. The maximum of conjugate formation calculated from the initial specific activities of approx. 1 nmol/ml cells is a steady-state level resulting from the rates of ubiquitin conjugation with and liberation from the stroma proteins and depends on the number of susceptible free NH_2 groups. It is not limited by the ubiquitin concentration in the assay mixture since twice its concentration led to nearly the same steady-state level of conjugate formation (not shown). If one compares the initial rate of ubiquitin conjugation with native stroma of about 0.3 nmol/min per ml cells (36 nmol/2 h) and the lysine liberation of about 200 nmol/2 h per ml cells [1], less than 20% of the lysine released by proteolysis was conjugated at the

ϵ-NH_2 groups. It seems therefore that the conjugation of only a limited number of amino groups is necessary for the degradation of one protein molecule. This conclusion is consistent with the suggestion that only the exposure of the free NH_2-terminus of a substrate protein is required for its proteolytic degradation via the ubiquitin pathway [12]. In the case of membrane proteins it should be expected that the bulk of ϵ-NH_2 groups is masked and not available for the ligase system.

Hemin is an inhibitor of the lysine liberation [1]. Its site of action was deduced to be beyond the ubiquitin-protein ligase system [15]. In contrast to earlier observations [15], in our experiments hemin (70 μM) was without effect on the steady-state level of the ubiquitin conjugates (fig.2). This result can be explained by a rapid hemin-resistant liberation of ^{125}I-ubiquitin from the stroma that can be measured in the presence of unlabeled ubiquitin, ATP and Mg^{2+} (fig.3) which cannot be explained by an ATP-independent isopeptidase action and may be due to an early reaction of the protease system. One may assume that hemin does not affect the recycling of ubiquitin, involving its conjugation and liberation, but acts on the subsequent proteolytic degradation step.

The extent of diminution of P_i liberation by the addition of hemin (table 2) supports the assumption that 1 ATP is consumed per peptide bond cleaved [1,13]. With an average lysine content of stroma proteins of about 3% and a hemin-induced diminution of P_i liberation of 1.52 ± 0.42 μmol/ml cells after 30 min, a hemin-sensitive lysine release of about 200 nmol/2 h per ml cells may be estimated. This value is consistent with the hemin-sensitive lysine liberation from reticulocyte stroma measured under nearly the same conditions [1]. From the kinetics of ^{125}I-ubiquitin conjugation with and liberation from the native stroma determined under our conditions the following conclusions can be drawn:

(i) The degradation of the ubiquitin-protein conjugates by the protease system is at least a 2-step process. The first step is an ATP-dependent release of ubiquitin which is resistant to hemin. The subsequent step is characterized by an ATP-dependent degradation of protein substrates, consuming 1 ATP per peptide bond, which is completely inhibited by hemin.

(ii) There exists a second mechanism of ^{125}I-ubiquitin liberation from the stroma which was observed in the absence of unlabeled ubiquitin, ATP and Mg^{2+} (fig.3) and which is due to the existence of an isopeptidase in the cytosol in rabbit reticulocytes confirming earlier investigations [14,22]. The isopeptidase reaction is comparatively slow releasing only about 30% of the previously conjugated ubiquitin after 60 min. The slowness of the release explains the residual stroma-bound ubiquitin under conditions of ATP depletion even after 120 min; the remaining ubiquitin appears to be responsible for the incomplete abolishment of proteolysis.

The system under study is undoubtedly very complex. It involves triggering by lipoxygenase attack [23], the ubiquitin-protein ligase system, the protease system, the isopeptidase and perhaps an acylpeptide hydrolase recently found in red blood cells [24] which could be responsible for the exposure of free NH_2-termini of substrate proteins. Its further characterization and regulation will be the objects of further investigation.

ACKNOWLEDGEMENTS

We thank Dr sc. W. Rohde of the Institut für Experimentelle Endokrinologie, Humboldt-Universität (Berlin, GDR) for the iodination of ubiquitin. The authors are indebted to Dr M.M. Kostić of the Institute of Medical Physiology, Faculty of Medicine Belgrade, Department Kragujevac (Yugoslavia), for his contribution to experiments and discussion.

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