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ATP-dependent peptide release from mitochondria of reticulocytes

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ATP-dependent release of TCA-precipitable peptides from mitochondria-containing stroma (MCS) is described. The process is independent of ubiquitin, but is sensitive to hemin and to heat treatment. Neither chloramphenicol nor EGTA inhibit. 50% of the activity is dependent on charged tRNA. The peptides released from MCS possess a molecular mass of about 1–5 kDa and are degraded to TCA-soluble compounds by a cytosolic protease system (fraction II) without ubiquitin.

Proteolysis, ATP-dependent; Reticulocyte; Mitochondria; Ubiquitin

1. INTRODUCTION

The ATP- and ubiquitin-dependent proteolytic system of reticulocytes has been investigated intensively during the past few years (for review see [1]). It is involved in the degradation of mitochondrial proteins during the maturation process of red blood cells [2,3]. The proteolysis of mitochondria is partially dependent on tRNA, as shown by the reduction of proteolysis to about 50%, if tRNA is degraded by micrococcal nuclease [4]. The requirement for tRNA for conjugation and for the subsequent degradation of certain proteins by the ATP-dependent proteolytic system has been described previously [5]. Ferber and Ciechanover [5] assumed a tRNA-dependent transfer of arginine to acidic amino termini of proteins which is required for their degradation by the ATP- and ubiquitin-dependent system. The portion of proteins with acidic amino termini in the whole cellular protein composition, however, is rather low and cannot explain the large extent of tRNA requirement in the mitochondrial breakdown. Besides the ubiquitin-mediated pathway, soluble [6] and particulate [7] ATP-dependent proteolytic activities which do not require ubiquitin have been described in erythroid cells. Formerly we demonstrated the existence of such an activity in rat liver mitochondria releasing acid-soluble material [8,9]. In the last few years such activities were characterized in mitochondria from different origins [10,11]. Moreover, an ATP-stimulated release of trichloroacetic acid-precipitable polypeptides from rat liver mitochondria was observed [12].

Here we describe a novel membrane-bound, ATP-dependent, but ubiquitin-independent proteolytic system in reticulocytes. It is partially dependent on tRNA and may catalyze the first proteolytic step in the degradation of mitochondrial proteins. It was overlooked up to now since its products are TCA-precipitable peptides.

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Abbreviations: MCS, mitochondria-containing stroma; TNBS, 2,4,6-trinitrobenzene 1-sulfonic acid; EGTA, ethylene glycol bis-(ß-aminoethyl ether)-*N,N, N'*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid; TCA, trichloroacetic acid

2. MATERIALS AND METHODS

Reticulocyte-rich blood from rabbits with a reticulocytosis of about 40% was obtained by bleeding. Preincubation of red blood cells, the separation of the cytosolic proteolytic system (fraction II) from the stroma-free lysate and the preparation of MCS were performed as described [2]. After four washings with about 10 vols of 0.9% NaCl the hemoglobin-free MCS was us-

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ed. The final MCS pellet was resuspended in 150 mM Hepes, pH 7.6, to a concentration of about 4 mg protein/ml, corresponding to approximately 0.5 ml of packed cells. For heat treatment, 1 ml MCS suspension was incubated at 60°C for 30 min.

Ubiquitin was purified to homogeneity according to Haas and Wilkinson [13].

The rate of peptide release was measured in an assay mixture containing 100 mM Hepes, pH 7.6, 0.1 mM dithioerythritol, 5 mM $MgCl_2$, 5 mM ATP, 4.8 mM phospho*enol*pyruvate, 0.1 μkat of pyruvate kinase (Boehringer, Mannheim) and about 0.5 mg native or heat-treated MCS with a final volume of 0.5 ml. In some cases $MgCl_2$, ATP and the ATP-regenerating system were omitted from the assay. In long-term experiments (more than 4 h) 200 μM chloramphenicol were added to the assay mixture. Final concentrations of other additives are indicated. Usually the reaction was started by ATP and stopped by centrifugation at 11 000 × *g* for 5 min. The supernatant fluid was decanted and rapidly frozen. 100 μl-aliquots of the supernatant were used for the TNBS-method [14]. For RNase inhibition and tRNA complementation, the assay mixture in the absence of $MgCl_2$, ATP and the ATP-regenerating systems was preincubated as described [4]. Thereafter the reaction mixture was completed and the incubation was started and stopped as described above. Uncharged tRNA was obtained by a standard method from reticulocyte tRNA [15].

The results were expressed as nmol of primary amines released from MCS per sample using leucine as standard. For the degradation of primary amines released from MCS by fraction II 200 μl-aliquots of the supernatants were incubated in the presence of 100 mM Hepes, pH 7.6, 0.1 mM dithioerythritol and fraction II (2 mg/ml) in a final volume of 0.5 ml. The incubation was carried out for 3 h at 37°C and stopped by TCA-precipitation as outlined in detail [14]. The proteolysis was again measured by the TNBS-method. Protein was determined by the biuret method with albumin as standard.

3. RESULTS AND DISCUSSION

There is no release of TCA-soluble compounds from MCS alone [2]. ATP-dependent release of TNBS-reactive material from MCS was only found if TCA-precipitation was avoided. It was concluded that an ATP-dependent release of peptides from MCS exists, which are precipitable by TCA. Fig.1 demonstrates the time-dependence of the peptide release with and without MgATP and in the presence of hemin. Stimulation by MgATP, as high as 5 fold was observed. The ATP-stimulated process came to an end only after 8 h. Hemin inhibited the ATP-dependent peptide release nearly completely. The process did not require the addition of cytosolic proteases.

As shown in fig.2 ubiquitin had no effect on the peptide release from native MCS, in either the absence or presence of MgATP. Heat-treated MCS

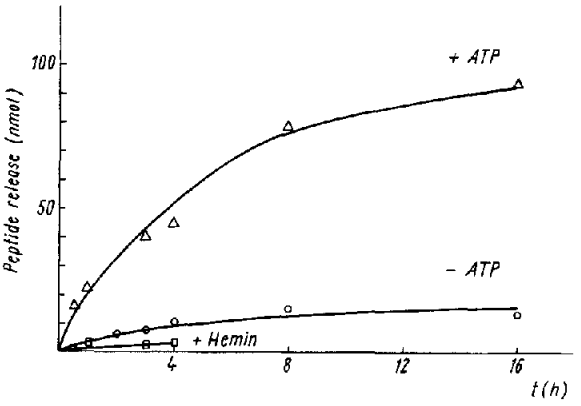

Fig.1. Time-dependence of peptide release from MCS with and without MgATP and in the presence of hemin (500 μM). Primary amines were measured in the supernatant by the TNBS-method as described in section 2. Data are averages of 2–3 experiments.

is a poorer substrate of the whole proteolytic system in reticulocytes [2]. The same holds true for the release of peptides. Less than 15% of the peptides were released from heat-treated MCS as compared with the native one (fig.2).The process cannot be detected in stroma obtained from erythrocytes (not shown) attesting the necessity of mitochondria. Chloramphenicol (200 μM), an inhibitor of mitochondrial protein synthesis, did not affect the ATP-dependent release of TCA-insoluble material from native MCS of reticulocytes. A peptide release of 37.4 ± 3.1 nmol/3 h ($n=4$) was determined as compared to the average value

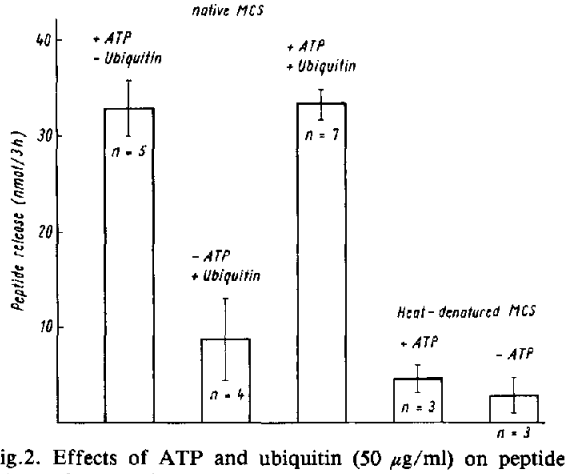

Fig.2. Effects of ATP and ubiquitin (50 μg/ml) on peptide release from native and heat-treated MCS. Data represent means ± SE.

without chloramphenicol of 33.2 ± 1.6 nmol/3 h ($n = 7$). Therefore, chloramphenicol could be used for inhibition of bacterial growth in long-term experiments.

In the presence of EGTA (2 mM) an ATP-dependent peptide release of 31.4 ± 9 nmol/3 h ($n = 5$) was measured. Ca^{2+} (1 mM) did not stimulate (not shown). The complete degradation of RNA in the reaction mixture by micrococcal nuclease reduced the peptide release from MCS by about 50% (fig.3). tRNA, isolated from rabbit reticulocytes, restored the original ATP-dependent activity completely. It is noteworthy, that uncharged tRNA was ineffective.

200 μl-aliquots of supernatant (500 μl) obtained from preincubation of MCS (+ MgATP) subsequently incubated in the presence of fraction II produced TCA-soluble TNBS-reactive compounds (57.2 × 5.0 nmol/3 h, $n = 20$) without requirement for ubiquitin. All effects observed by direct measurements of TCA-insoluble material in the MCS-supernatants were adequately reproducible by determination of TCA-soluble TNBS-reactive compounds after the action of fraction II. The nature of the peptides released from MCS is as yet uncertain. Assuming complete breakdown to free amino acids by fraction II their amount would correspond to hexapeptides.

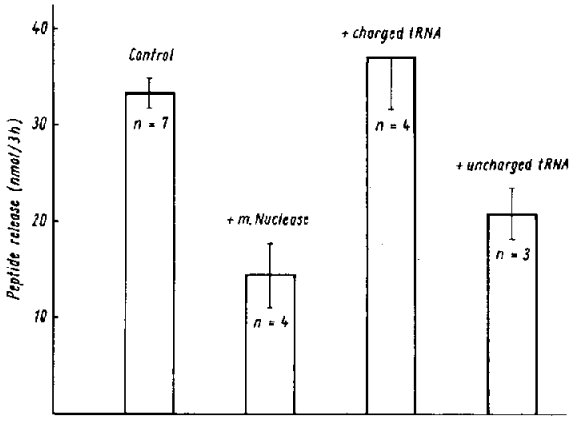

Fig.3. Effects of preincubation with micrococcal nuclease and subsequent complementation with charged or uncharged tRNA on the peptide release from MCS. Preincubations of reaction mixtures containing MCS (about 0.5 mg/ml), Hepes (100 mM) and dithioerythritol (0.1 mM) for 30 min at 37°C with 1 mM $CaCl_2$ and 5 μg/ml of micrococcal nuclease (Boehringer, Mannheim) were carried out, followed by the addition of 1.5 mM EGTA and incubation for an additional 15 min. The peptide release was initiated by addition of the ATP-regenerating system, ubiquitin (50 μg/ml) and charged or uncharged tRNA (20 μg/ml). Data represents means ± SE.

A preliminary assessment was provided by gel filtration experiments. MCS was incubated in the presence and in the absence of MgATP for 8 h and the supernatants were put on a Sephadex-G25 column. In fig.4, a typical pattern of the TNBS-signals in the collected 1 ml-fractions is demonstrated. Only in the supernatants obtained from incubation with MgATP were significant amounts of TNBS-reactive material found in the fractionation range of the column used (1–5 kDa). Two peaks may be seen, one of which was eluted just before free TNBS.

Summing up, the characteristics of the activity studied, its ATP-dependence, its inhibition by

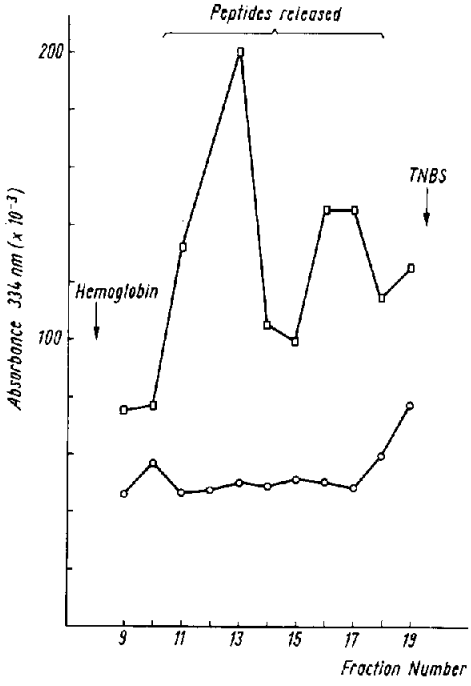

Fig.4. Sephadex G25-gel filtration of MCS-supernatants obtained from incubations for 8 h in the presence (□—□) or absence (○—○) of MgATP. The column (0.8 × 38 cm) was equilibrated with destilled water and calibrated with hemoglobin, eluting at the void volume and TNBS (MW 347.2, Serva), eluting at an elution volume approximately equal to the bed volume. About 500 μl of MCS supernatants were put on the column. The column was eluted with water and 1 ml-fractions were collected. 200 ml-aliquots of the 1 ml-fractions were mixed with 200 μl of a TNBS solution (1 mg TNBS/3 ml of 0.1 M borate buffer, pH 9.0) and incubated for 20 min at 40°C. The reaction was terminated by the addition of 1 ml of 1 M HCl and the absorbance was measured at 334 nm.

hemin and heat treatment and the low molecular mass of the peptides released suggest that their appearance is due to an enzymatic cleavage rather than to a disaggregation or disassociation process. Thus, a novel proteolytic system which releases TCA-precipitable peptides from mitochondria of reticulocytes has been identified and partially characterized. It may catalyze the first proteolytic step in the degradation of mitochondrial proteins. It seems to be tightly bound to MCS and possesses characteristics common to the whole proteolytic system of reticulocytes: (i) the ATP-dependence, (ii) the hemin-sensitivity, (iii) the large dependence on charged tRNA.

It differs from the classic ubiquitin-dependent system by its independence from ubiquitin. Moreover, the activity is independent of Ca^{2+} and thus is not related to Ca^{2+} proteinases [16]. Thus, the degradation of mitochondrial proteins proceeds in at least two steps: first, the ATP-dependent release of TCA-precipitable peptides, and second, the degradation of the peptides to acid-soluble compounds by a cytosolic protease system which is also independent from ubiquitin. Considering the presented results the low susceptibility of heat-denatured MCS to degradation by the ATP-dependent and ubiquitin-mediated system may not be due to a lower ubiquitination as assumed before but rather due to the denaturation of the membrane-bound protease.

Since ubiquitin is required for maximal activity of the whole proteolytic system of reticulocytes [2,17], the question arises as to the ubiquitin-requiring step in the whole proteolytic system. Apparantly ubiquitin exerts its effect if incorporated during the incubation of MCS (see fig.1), [2]), presumably by ubiquitination of amino groups favoring proteolysis. The peptide release from MCS could expose amino acids of membrane protein fragments for a subsequent ubiquitination. This assumption is supported by the fact that native MCS is ubiquitinated to a much higher extent than the heat-treated one [3].

One may speculate that both ubiquitin-dependent and ubiquitin-independent pathways are operative in the degradation of mitochondria.

The characterization of the membrane-bound ATP-dependent proteolytic activity will be investigated further.

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