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A HEAT-STABLE POLYPEPTIDE COMPONENT OF AN ATP-DEPENDENT PROTEOLYTIC SYSTEM FROM RETICULOCYTES

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SUMMARY: The degradation of denatured globin in reticulocyte lysates is markedly stimulated by ATP. This system has now been resolved into two components, designated fractions I and II, in the order of their elution from DEAE-cellulose. Fraction II has a neutral protease activity but is stimulated only slightly by ATP, whereas fraction I has no proteolytic activity but restores ATP-dependent proteolysis when combined with fraction II. The active principle of fraction I is remarkably heat-stable, but it is non-dialysable, precipitable with ammonium sulfate and it is destroyed by treatment with proteolytic enzymes. In gel filtration on Sephadex-G-75, it behaves as a single component with a molecular weight of approximately 9,000.

INTRODUCTION: Intracellular protein degradation in various tissues depends upon the continuous supply of cellular energy (for reviews, see 1, 2). We have been studying the energy-dependence of protein degradation in reticulocytes, as a convenient model system (3). The major protein synthesized in these cells, hemoglobin, is normally stable, but abnormal globin molecules containing certain amino acid analogs (4), mutant globin chains (5) or puromycin peptides (6) are rapidly degraded. The degradation of abnormal globin molecules is energy-dependent (3, 7), and we have shown that energy is required at an early stage of the process (3).

Recently, Etlinger and Goldberg have reported that the degradation of analog-containing protein in reticulocyte lysates is stimulated by ATP (7). Crude lysates also carry out the ATP-dependent degradation of heme-free denatured globin (3). The elucidation of the mode of action of ATP obviously requires the separation and characterization of the enzyme(s) involved. Goldberg and coworkers have purified an endopeptidase from reticulocytes, but the stimulation by ATP was lost on purification (8). We now report that the ATP-dependent cell-free system is composed of complementing species, and describe the properties of one of the components.

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METHODS: Reticulocyte Lysate. Reticulocytosis of 70-80% was induced in rabbits by injections of phenylhydrazine (4). The cells were washed twice with ice-cold saline and incubated (37°C, 2 h) with 0.2 mM 2,4-dinitrophenol and 20 mM 2-deoxyglucose, to deplete cellular ATP (3). Following additional washing, the cells were lysed with 1.6 volumes of water containing 1 mM dithiothreitol and particulate material was removed by centrifugation at 80,000 x g for 90 min.

Fractionation on DEAE-Cellulose: All operations were at 0-4°C. 30 ml lysate was applied to a 1.5 x 12 cm column of DEAE-cellulose (Whatman, DE-52) equilibrated with 3 mM potassium phosphate, pH 7.0. Nonadsorbed protein was eluted with 50 ml of the above buffer containing 1 mM dithiothreitol. The last two-thirds of the nonadsorbed material containing hemoglobin were collected - designated fraction I. Adsorbed protein was then eluted with 70 ml of a solution containing 10 mM Tris-Cl (pH 7.1), 0.5 M KCl and 1 mM dithiothreitol. Solid ammonium sulfate was added to 90% saturation (65 g per 100 ml of solution) and the suspension was stirred for 30 min. Precipitated protein was collected by centrifugation, dissolved in 4-5 ml of 10 mM Tris-Cl (pH 7.1), 1 mM dithiothreitol, and dialyzed for 20 h against 2 lit of the same buffer; this preparation was designated fraction II.

Partial Purification of Fraction I: The nonadsorbed material of the DEAE-cellulose column was heated at 90°C for 10 min, stirred up and chilled on ice. Denatured protein was removed by 2 repeated centrifugations at 35,000 x g for 20 min. Solid ammonium sulfate was added to 90% saturation and after stirring for 30 min, the precipitate was collected by centrifugation, dissolved in 1.5% of the original volume and dialyzed overnight against 10 mM Tris-Cl, pH 7.6. If residual hemoglobin color remained, the preparation was heated again (90°C, 15 min) and centrifuged as before; the final preparation was practically colorless. In a typical preparation, the partially purified material contained 0.1% of the initial protein, the recovery of activity was around 40%, and thus the purification was approximately 400-fold. Protein was determined by the method of Lowry et al. (9).

Preparation of [^{3}H]-Labeled Globin: A 50% suspension of reticulocytes in Krebs-Ringer solution (3) was incubated at 37°C for 60 min with L-[4,5-^{3}H]leucine (38 Ci/mmole, 60 μCi/ml) and 0.25 mM each of the other 19 unlabeled amino acids. Hemoglobin was purified by DEAE-cellulose chromatography and heme-free globin was precipitated with HCl-acetone, as described (10). The purified globin had a specific radioactivity of 50,000 dpm/mg of protein, and was devoid of any proteolytic activity. Before use, it was denatured by heating in 0.01 N NaOH at 60°C for 30 min, to increase its susceptibility to ATP-dependent proteolysis (3).

Assay of ATP-Dependent Degradation of [^{3}H]Globin: The reaction mixture contained in a final volume of 0.5 ml: 100 mM Tris-Cl (pH 7.6), 5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 80 μg/ml [^{3}H]globin, 0.5 mM ATP, 10 mM phosphocreatine, 100 μg/ml creatine phosphokinase (Boehringer) and the enzyme fractions as indicated. The ATP-generating system was added to prevent the formation of AMP that inhibits the reaction (3); control experiments showed that creatine phosphate and kinase had no influence on proteolysis in the absence of ATP. Following incubation at 30°C for 60 min, the reaction was stopped with 1 ml of 5% trichloroacetic acid and following centrifugation, 10 mg of bovine serum albumin was added to the supernatants and the samples were centrifuged again. Radioactivity in the clear supernatants was estimated and the percentage of the degradation of [^{3}H]globin to acid-soluble products was computed.

RESULTS AND DISCUSSION: In our initial attempts to purify the ATP-dependent proteolytic activity from reticulocyte lysates, we have employed the widely used

TABLE 1: Resolution of the ATP-Dependent Cell-Free Proteolytic System Into Complementing Activities

Enzyme fraction	Degradation of [^{3}H]globin percent/h	
	-ATP	+ATP
lysate	1.5	10.0
fraction I	0	0
fraction II	1.5	2.7
fraction I and fraction II	1.6	10.6

Enzyme fractions were separated by DEAE-cellulose as described under "Methods" and supplemented at the following amounts (mg of protein/ml reaction volume): lysate, 28; fraction I, 45; and fraction II, 3.5. Where indicated, ATP was added together with phosphocreatine and creatine phosphokinase.

method of stepwise separation on DEAE-cellulose (11): at neutral pH and at low ionic strength, most non-hemoglobin proteins are adsorbed on the resin, while hemoglobin and a few more basic proteins are not retained. As shown in Table 1, the protein fraction adsorbed on DEAE-cellulose and eluted with 0.5 M KCl (fraction II) contained a neutral proteolytic activity, but it was stimulated only slightly by ATP, as compared to the effect of ATP in the whole lysate. The unadsorbed fraction (fraction I) had no proteolytic activity either in the presence or absence of ATP. However, the supplementation of fraction I to fraction II restored to a large extent the stimulation of [^{3}H]-globin degradation by ATP. This action of fraction I appeared to be specific for the ATP-dependent system, since it had no influence on the ATP-independent proteolytic activity in fraction II. It should be noted that in different preparations, considerable variations were observed in the amount of ATP-independent proteolytic activity in fraction II (up until 10% [^{3}H]globin degraded/h). However, the extent of the additional proteolysis stimulated by fraction I and ATP was much more constant.

Figure 1 shows the dependence of ATP-stimulated protein degradation on the

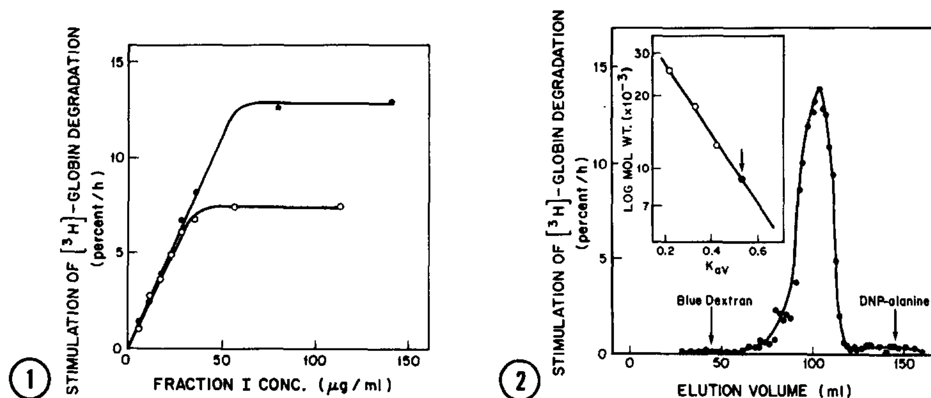

Fig. 1. Concentration dependence of the effect of fraction I on ATP-dependent proteolysis. The degradation of [^{3}H]globin was assayed with increasing amounts of partially purified fraction I, in the presence of the following concentrations of fraction II (mg/ml): open circles, 2.9; closed circles, 5.8. The stimulation of [^{3}H]globin degradation was computed by subtracting the breakdown values obtained without fraction I (fraction II with ATP); these were 3.9 and 5.6 percent/h for the low and high concentrations of fraction II, respectively.

Fig. 2. Gel filtration analysis of the heat-stable polypeptide. A 7.0 mg sample of partially purified fraction I was introduced into a column (1.5 x 86 cm) of Sephadex-G-75 which had been previously equilibrated with 10 mM Tris-Cl (pH 7.1). Elution was with the same buffer, fractions of 2 ml were collected and aliquots of 150 μl of each fraction were assayed for the stimulation of [^{3}H]globin degradation with 5.8 mg/ml of fraction II. The results are expressed as the increase in [^{3}H]globin degradation above that obtained with fraction II and ATP, that was 6.2%/hr. Insert: Estimation of molecular weight. The Sephadex-G-75 column was calibrated with the following marker proteins (open circles): α-chymotrypsinogen (mol. wt. 25,000); myoglobin (mol. wt. 17,800); and cytochrome C (mol. wt. 12,100). The closed circle indicates the elution position of heat-stable polypeptide activity.

mutual concentrations of fractions I and II. Proteolysis increased in a linear fashion with increasing concentrations of fraction I until it attained a constant maximal level. At this stage, the response to fraction I was clearly limited by the level of fraction II, since increasing the concentration of fraction II allowed a further stimulation by high concentrations of fraction I.

Preliminary characterization of the activity in fraction I revealed some rather unusual features. The active factor is remarkably stable at high temperatures that would inactivate most proteins, and retains considerable activity even following heating at 96°C for 60 min (Table 2). However, it appears to

TABLE 2: Properties of Fraction I

Treatment of fraction I	Increase of ATP-dependent proteolysis	
	[^{3}H]globin degraded (percent/h)	% of control
Experiment 1		
Control	6.6	100
Heated 96°C, 15 min	6.3	95
Heated 96°C, 60 min	4.6	70
Dialyzed (20 h)	6.7	102
Pronase-treated	0	0
Chymotrypsin-treated	0	0
Experiment 2		
Control	4.7	100
$(NH_4)_2$ SO_4 precipitate	4.1	87
$(NH_4)_2$ SO_4 supernatant	0	0

Fraction I was heated at 87°C for 10 min and centrifuged as described in "Methods". Portions of this preparation ("control", 0.38 mg protein/ml) were then further heated in closed tubes at 96°C for the time periods indicated and centrifuged at 100,000 x g for 30 min. Other portions were subjected to dialysis for 24 h or precipitated with ammonium sulfate (90% saturation) and then dialyzed. Treatments with pronase (Sigma, 25 μg/ml) or α-chymotrypsin (Boehringer, 25 μg/ml) were at 37°C for 90 min at pH 7.6 or 7.8, respectively. The incubation was terminated by boiling for 15 min; control experiments showed that this was sufficient to destroy completely added protease activities, whereas the activity of fraction I decreased only slightly. The reaction mixture contained 3.5 mg/ml fraction II, 80 μg/ml fraction I (or equivalent amounts after the respective treatments) and other ingredients as described in "Methods". The increase of ATP-dependent proteolysis was calculated by subtraction of the values of [^{3}H]globin degradation without fraction I (fraction II with ATP); these were 4.6%/h for experiment 1 and 9.9%/h for experiment 2.

be a polypeptide by a number of criteria: it is not dialysable, it is precipitated by ammonium sulfate and furthermore, the activity is completely destroyed by treatment with proteolytic enzymes such as pronase or chymotrypsin (Table 2). By the combination of drastic heat-treatment and ammonium sulfate precipitation, an approximately 400-fold partial purification of the heat-stable polypeptide was achieved (see "Methods"). Gel filtration analysis of the partially purified factor on Sephadex-G-75 column showed a single component with a molecular weight of approximately 9,000 (Fig. 2).

The heat-stable polypeptide does not contain any peptide bond cleaving activity, tested not only by the formation of acid-soluble products (Table 1), but also by

the possible cleavage of [^{3}H]globin to large fragments, analyzed by polyacrylamide gel electrophoresis. Furthermore, a number of reticulocyte aminopeptidase activities were also absent in partially purified fraction I, but were completely recovered in fraction II (H. Heller and A. Hershko, unpublished results).

As opposed to the properties of fraction I, the activity in fraction II is remarkably unstable and is destroyed by relatively mild heat-treatment (42°C, 30 min). The function of fraction I does not appear to be to stabilize fraction II, since it does not protect the latter against heat inactivation (data not shown).

The elucidation of the roles of the heat-stable polypeptide, or of ATP, depends upon the characterization of further component(s) in fraction II. This relatively small polypeptide might be an easily dissociable subunit of a larger enzyme complex, an activator of an enzyme present in fraction II, an inhibitor of an antagonistic factor, or it may interact directly with the substrate or ATP. Our previous studies on the degradation of analog-containing globin chains in intact reticulocytes indicated that ATP is required at or before the initial cleavage of the complete globin molecule (3). It might well be that the heat-stable polypeptide participates in such an early event that preceeds the actual proteolytic reactions.

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REFERENCES:

1. Goldberg, A.L. and St. John, A.C. (1976) Ann. Rev. Biochem. 45, 747-803.
2. Hershko, A. (1974) Regulation of Gene Expression in Eukaryotic Cells. (Harris, M. and Thompson, B., eds.), Fogarty International Center Proceedings No. 25, pp. 85-96, U.S. Printing Office, Washington, D.C.
3. Hershko, A., Heller, H., Ganoth, D. and Ciehanover, A. (1978) Protein Turnover and Lysosomal Function (Segal, H.L. and Doyle, D.J., eds.), in press.
4. Rabinowitz, M. and Fisher, J.M. (1964) Biochim. Biophys. Acta 91, 313-322.
5. Rieder, R.F., Wolf, D.J., Clegg, J.B. and Lee, S.L. (1975) Nature 254, 725-727.
6. McIlhinney, A. and Hogan, B.L.M. (1974) FEBS Lett. 40, 297-301.
7. Etlinger, J.D. and Goldberg, A.L. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 54-58.
8. Goldberg, A.L., Etlinger, J.D., Murakami, K. and Klemes, Y. (1978) Protein Turnover and Lysosomal Function (Segal, H.L. and Doyle, D.J., eds.), in press.
9. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
10. Schapira, G., Rosa, J., Maleknia, N. and Padieu, P. (1968) Methods in Enzymology, Vol. 12B, pp. 747-769, Academic Press, New York.
11. Henessey, M.A., Waltersdorph, A.M., Huennekens, F.M. and Gabrio, B.W. (1962) J. Clin. Invest. 41, 1257-1262.