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2,3-Bisphosphoglycerate inhibits ATP-stimulated proteolysis

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Intracellular protein breakdown could be regulated at the substrate level by changes in the environment. Under in vitro conditions, ATP increases the proteolytic susceptibility of several mitochondrial and cytosolic proteins, while 2,3-bisphosphoglycerate not only has the opposite effect but also prevents the ATP-stimulated proteolysis. ATP and 2,3-bisphosphoglycerate, present at relatively high levels in many tissues, provide a good model of environmental components that may influence intracellular proteolysis.

ATP; 2,3-Bisphosphoglycerate; Proteolysis; Glyceraldehyde-3-phosphate dehydrogenase; Protein degradation; Protease

1. INTRODUCTION

We have recently demonstrated that several low-M_r phosphorylated compounds (particularly 2,3-BPG) protect OTC (EC 2.1.3.3) against proteolytic inactivation by broken lysosomes [1]. It has also been shown that the proteolytic susceptibility of this enzyme to broken lysosomes is increased by ATP [2]. Because ATP and 2,3-BPG are present at relatively high concentrations in many tissues, they appeared suitable as models of environmental components that might influence intracellular proteolysis, in opposite directions. Since OTC is a mitochondrial enzyme, it seemed of interest to test the effect of these metabolites on a cytosolic enzyme. We chose GAPDH (EC 1.2.1.12) because it is very abundant (19% of the soluble protein in yeast [3] and 10% in rabbit muscle [4]) and plays a key role in glycolysis. GAPDH is also one of the most abundant proteins in erythrocytes [5], where there are high concentrations of 2,3-BPG (5 mM) and ATP (2 mM) [5]. Here, we show that GAPDH, due to its proteolytic susceptibility to broken lysosomes, can also be used as a model in that 2,3-BPG protects against the enhanced proteolysis which occurs in the presence of ATP. Other cytosolic and mitochondrial proteins behave in a similar way.

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Abbreviations: 2,3-BPG, 2,3-bisphosphoglycerate; DTT, dithiothreitol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OTC, ornithine carbamoyltransferase; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

2.1. *Chemicals*

GAPDH (from rabbit muscle) was purchased from Sigma (St. Louis, MO) as a crystalline suspension in ammonium sulfate; the salt was removed by gel filtration as in [6]. Elastase (EC 3.4.21.36, from porcine pancreas, 100 U/mg), 2,3-BPG, ATP (magnesium salt), 2-(*N*-morpholino)ethanesulfonic acid (Mes) and Tris were also purchased from Sigma. Proteinase K (EC 3.4.21.14, 20 U/mg) and ATP (disodium salt) were from Boehringer (Mannheim, FRG). Metrizamide was from Nyegard (Oslo). Other reagents were of analytical grade.

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2.2. *Preparation of subcellular fractions*

Lysosomes from male Wistar rats, starved overnight prior to decapitation, were isolated using a discontinuous metrizamide gradient [7]. Mitochondria from rat liver were obtained as in [8]. The mitochondrial and lysosomal pellets were suspended in 5 and 3 vols, respectively, of 5 mM DTT, and disrupted by 10 cycles of freeze-thawing. The resulting suspensions were used as such. Cytoplasmic extracts were obtained by centrifuging the supernatants of the mitochondrial fractions at 100000 × *g* in a Beckman L5-65 centrifuge for 60 min, to eliminate the microsomal fraction. Both mitochondrial and cytosolic extracts were dialyzed for 24 h against 5 mM DTT, changed every 4 h, or subjected to gel filtration in PD-10 columns (Pharmacia, Uppsala) and then concentrated to ~50 mg protein/ml in a Centricon microconcentrator from Amicon (Danvers, MA).

2.3. *General*

Incubations were carried out as described in the figures. At the times indicated, portions were taken and assayed for activity [9] or subjected to SDS-PAGE [10]. The bands obtained were quantified by densitometric analysis with an LKB densitometer. Azocasein and azoalbumin hydrolysis were measured by monitoring at 340 nm the release of the trichloroacetic acid soluble dye. *β-N*-Acetylglucosaminidase activity was measured as in [11]. Protein was measured by standard procedures [12,13]. [^{14}C]GAPDH was prepared as in [14] and proteolysis was estimated by liquid scintillation counting as described [1]. All values represent means of five or more independent experiments with duplicated samples.

3. RESULTS

3.1. *Effect of ATP and 2,3-BPG on the proteolysis of GAPDH by broken lysosomes*

Since lysosomes constitute a well-known proteolytic system in eukaryotic cells [15–17], experiments were initially carried out using broken lysosomes, at acid pH (optimum for most lysosomal proteases). Proteolysis of GAPDH was followed by loss of enzymatic activity (fig.1A), partial disappearance of the GAPDH band in SDS-PAGE gels (fig.1B) and release of trichloroacetic acid-soluble radioactivity from

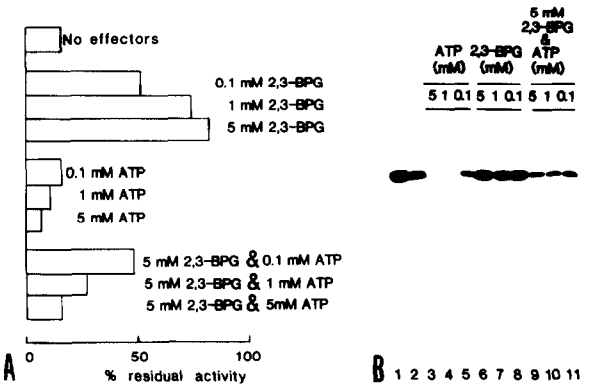

Fig.1. Effect of 2,3-BPG and ATP on the proteolytic inactivation of GAPDH by broken lysosomes. (A) 20 μg GAPDH were incubated at 37°C for 1 h with broken lysosomes (the equivalent of 0.008 U *β-N*-acetylglucosaminidase) in 50 mM Mes-NaOH buffer, 5 mM DTT, in a final volume of 100 μl and with the indicated amounts of ATP and 2,3-BPG. At the end of the incubation, GAPDH activity was assayed as described in section 2 and expressed as % of activity of GAPDH incubated without lysosomes. (B) GAPDH (20 μg) was incubated as in A, in the presence of 5, 1 and 0.1 mM ATP (lanes 3,4,5), 5, 1 and 0.1 mM 2,3-BPG (lanes 6,7,8) or 5 mM 2,3-BPG plus 5, 1 and 0.1 mM ATP (lanes 9,10,11) in 50 mM Mes-NaOH buffer, pH 5.5, 5 mM DTT, and subjected to SDS-PAGE (13.5% acrylamide). As controls, GAPDH was incubated with (lane 2) or without lysosomes (lane 1) and no additions.

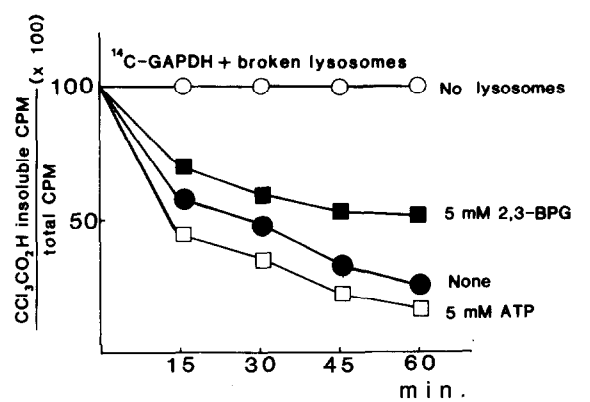

Fig.2. Effect of 2,3-BPG and ATP on the proteolysis of [^{14}C]GAPDH. [^{14}C]GAPDH (35000 cpm, 5 μg) was incubated at 37°C with broken lysosomes [0.008 U *β-N*-acetylglucosaminidase (●)] plus 5 mM 2,3-BPG (■) or 5 mM ATP (□), in a final volume of 100 μl. As a control, [^{14}C]GAPDH was incubated without lysosomes (○).

[^{14}C]GAPDH (fig.2). At the concentrations used, 2,3-BPG greatly protected GAPDH from proteolytic inactivation by broken lysosomes. ATP alone inactivated the enzyme (not shown), in agreement with previous results [18]. When ATP was added together with broken lysosomes the proteolytic inactivation increased. The ATP effect was observed with both the Mg^{2+} and the Na^{+} salt. As illustrated in fig.1B, 2,3-BPG protects not only against the proteolysis by broken lysosomes, but also against the increased proteolytic inactivation produced by ATP.

3.2. *Effect of ATP and 2,3-BPG on the proteolysis of GAPDH by the neutral proteases elastase and proteinase K*

Since the optimum pH of most lysosomal proteases is acidic [19], no significant proteolysis was observed, as expected, when incubations with broken lysosomes were carried out at neutral pH (not shown). To determine whether the observed effects also occur with neutral proteases, we tested the effect of ATP and 2,3-BPG on the proteolysis of GAPDH by elastase and proteinase K. Essentially the same results were obtained with both neutral proteases (fig.3): ATP increased the proteolytic susceptibility of GAPDH and 2,3-BPG had the opposite effect and also protected against the increased proteolysis produced by ATP. These results suggest that ATP and 2,3-BPG act at the substrate level rather than on the proteolytic system, since the effects of both metabolites are observed with different proteases and at different pH values. These results are in agreement with experiments with azocasein and azoalbumin; 2,3-BPG and ATP had no effect on the proteolysis

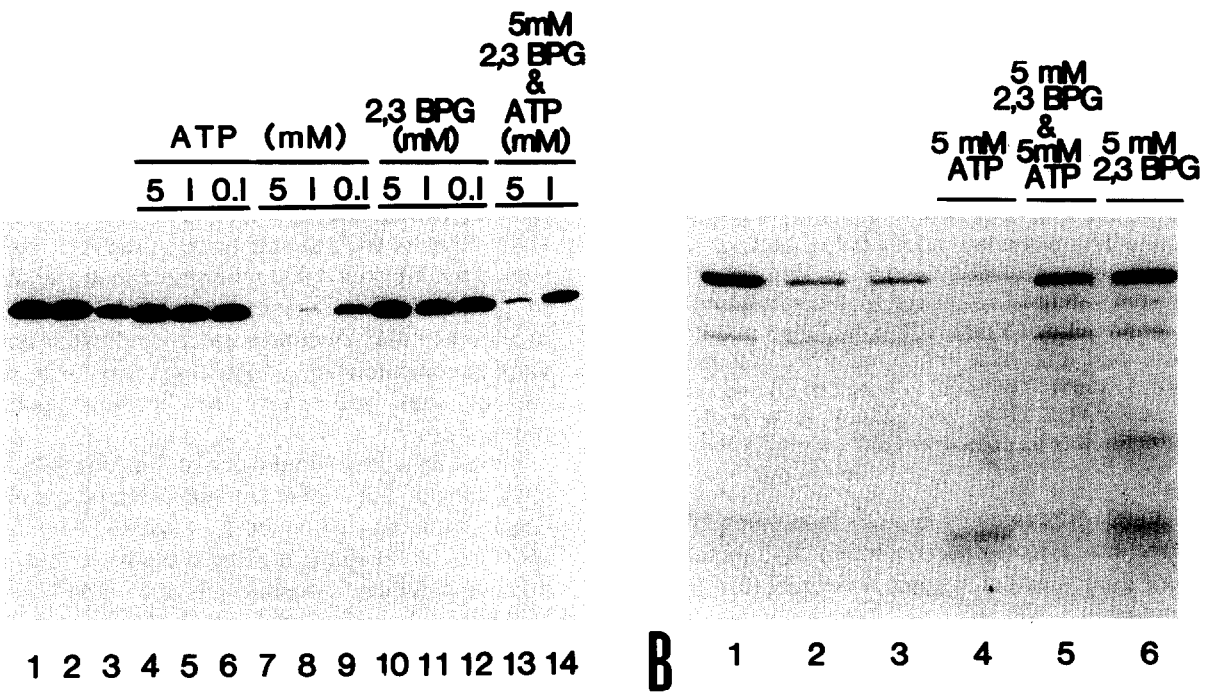

Fig.3. Effect of 2,3-BPG and ATP on the proteolysis of GAPDH by neutral proteases elastase and proteinase K. (A) 100 μg GAPDH were incubated (final volume 100 μl) at 37°C for 1 h with 1 μg elastase (lane 3) in the presence of 5, 1 and 0.1 mM 2,3-BPG (lanes 10,11,12), 5, 1 and 0.1 mM ATP (lanes 7,8,9) or 5 mM 2,3-BPG plus 5 and 1 mM ATP (lanes 13,14) in 0.1 M triethanolamine buffer, pH 7.6, 5 mM DTT, and subjected to SDS-PAGE (13.5% acrylamide). As controls, GAPDH was incubated without elastase (lane 2) and in the presence of 5, 1 and 0.1 mM ATP (lanes 4,5,6). Lane 1 denotes zero time. (B) GAPDH (30 μg) was incubated (final volume 100 μl) at 25°C for 3 min with 0.24 μg proteinase K (lanes 2,3) in the presence of 5 mM ATP (lane 4), 5 mM 2,3-BPG (lane 6) or 5 mM 2,3-BPG plus 5 mM ATP (lane 5) in 20 mM Tris-Cl buffer, pH 7.6, 10 mM magnesium acetate, 50 mM NaCl, and subjected to SDS-PAGE (13.5% acrylamide). As a control GAPDH was incubated without proteinase K (lane 1).

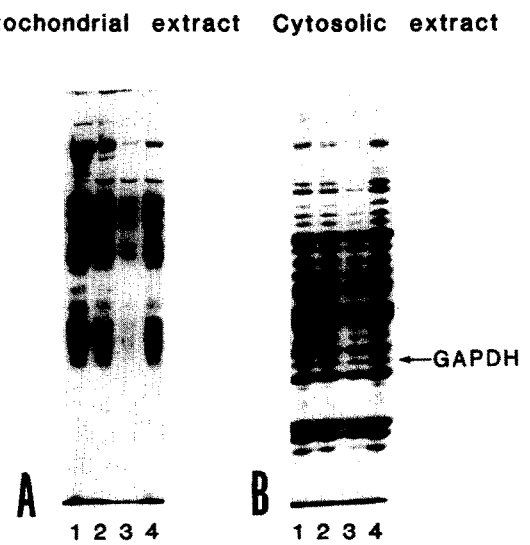

Fig.4. Effect of 2,3-BPG and ATP on the proteolysis of mitochondrial and cytosolic extracts. (A) 100 μg dialyzed mitochondrial extract were incubated for 1 h at 37°C with broken lysosomes (0.11 U β-*N*-acetylglucosaminidase, lane 2) in the presence of 5 mM ATP (lane 3) or 5 mM 2,3-BPG plus 5 mM ATP (lane 4) in 50 mM Mes-NaOH buffer, pH 5.5, 5 mM DTT (final volume 100 μl) and subjected to SDS-PAGE (10% acrylamide). As a control, the extract was incubated without lysosomes (lane 1). (B) 150 μg dialyzed cytosolic extract were incubated for 1 h at 37°C with broken lysosomes (0.055 U β-*N*-acetylglucosaminidase, lane 2) in the presence of 5 mM ATP (lane 3) or 5 mM 2,3-BPG plus 5 mM ATP (lane 4) in 50 mM Mes-NaOH buffer, pH 5.5, 5 mM DTT (final volume 100 μl) and subjected to SDS-PAGE (10% acrylamide). As a control, the extract was incubated without lysosomes (lane 1).

of these substrates by broken lysosomes. The intermediate effects observed when both metabolites were used together suggest a competition in their binding to GAPDH.

3.3. *Effect of ATP and 2,3-BPG on the proteolysis of mitochondrial and cytosolic proteins*

To test if the stimulation of proteolysis by ATP and the protection thereof by 2,3-BPG were general effects, we incubated mitochondrial and cytosolic extracts with broken lysosomes in the presence or absence of ATP and/or 2,3-BPG (fig.4).

We have shown earlier that 2,3-BPG protects mitochondrial and cytosolic extracts against several proteases [1]. Here we show that ATP accelerated the proteolysis of a number of proteins, particularly mitochondrial, and that when 2,3-BPG was added together with ATP, this stimulating effect diminished. As shown, not all the proteins were equally affected by these metabolites and many proteins remained apparently unaffected (fig.4).

4. DISCUSSION

Cellular proteins are in a dynamic state of constant and extensive turnover and specific proteins are degraded within cells at widely different rates. In mammalian cells there are distinct lysosomal and nonlysosomal mechanisms [20,21] implicated in protein breakdown. However, the molecular basis for the selectivity which determines the different half-lives remains unknown. It has been suggested [22–24], among many other hypotheses (see, for example [25,26]; review [16]), that degradative rates of some proteins under in vivo conditions are largely controlled by environmental components, thus explaining variations in half-lives of intracellular proteins with the metabolic state. Factors such as substrates, products, cofactors and inhibitors could modulate the degradation of proteins. Since the in vivo levels of low-M_r metabolites may fluctuate widely and often under varying conditions (e.g. following meals or in starvation) they are likely to be physiological modulators.

Following the original work of Simpson [27], the requirement for ATP in intracellular protein degradation has often been reported. Here, we show that ATP stimulates the proteolytic inactivation of a typical cytosolic enzyme, GAPDH, at acid (figs 1,2) and neutral (fig.3) pH values as well as the proteolysis of several mitochondrial and cytosolic proteins (fig.4). The degradation of GAPDH appears to be influenced by conformational changes, induced by the binding of ATP on the protein substrate, since the effects are seen with a variety of proteases. This represents a new role for ATP, different from those already proposed to explain the requirement for ATP in intracellular protein degradation (e.g. in autophagy [28], in the ubiquitin-dependent pathway of reticulocytes [21], or in stimulation of ATP-dependent proteases

[29]). Interestingly, 2,3-BPG, which at physiological concentrations protects OTC and other cytosolic and mitochondrial proteins from proteolytic inactivation by lysosomes [1], also inhibits the ATP-stimulated proteolysis of GAPDH. Since the levels of ATP and 2,3-BPG (see [30] and references therein) can be altered in response to a number of physiological conditions, these fluctuations could regulate the in vivo degradation of some proteins.

We have found in recent preliminary experiments that when isolated hepatocytes and L-132 cells were combined with ATP and 2,3-BPG, degradation of intracellular proteins was also increased or decreased respectively (not shown). However, the mechanism of these effects in intact cells remains to be clarified. It should be noted that a stimulation by ATP of proteolysis in hepatocytes labeled in vivo [31] has been reported and that a very good correlation has been shown between ATP levels and intracellular proteolytic rate in hepatocytes and cultured cells [28,32].

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