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# Polyamines inhibit the ATP-dependent proteolytic pathway in rabbit reticulocyte lysates

Ewa F. Wajnberg and Julie M. Fagan

*Department of Animal Sciences, Rutgers University, New Brunswick, NJ 08903, USA*

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Reticulocytes contain a soluble nonlysosomal proteolytic pathway that requires ATP and ubiquitin. Polyamines at physiological concentrations were found to inhibit rapidly the ATP-dependent proteolytic system in reticulocyte lysates; spermidine and putrescine inhibited this process by 26–72% and spermine by 71–96%. Spermine had little effect on the ATP-independent breakdown of oxidant-treated hemoglobin. By fractionating the ATP-dependent system, we show that polyamines inhibit the ATP-dependent degradation of ubiquitin-protein conjugates.

Polyamine; Protein degradation; ATP; (Reticulocyte)

## 1. INTRODUCTION

The polyamines, putrescine, spermidine and spermine, are present in all mammalian cells [1] and are thought to be essential for cellular growth [2]. Polyamines stimulate growth by increasing rates of DNA and protein synthesis [1,3,4] but little is known about their possible effects on intracellular proteolysis. Polyamines have been reported to inhibit acidic and neutral proteases in oat leaves [5] and neutral proteases in alfalfa leaf extracts [6]. In isolated hepatocytes, polyamines did not affect the rate of degradation of proteins within the lysosome [7]. In reticulocytes, many cell proteins are degraded during maturation by a nonlysosomal proteolytic pathway that requires metabolic energy [8]. ATP is required both for the conjugation of ubiquitin (Ub) to proteins and for the degradation of Ub-protein conjugates [9]. In this communication we demonstrate that, in rabbit reticulocyte lysates, polyamines are potent inhibitors of the ATP + Ub-dependent proteolytic system.

*Correspondence address:* J.M. Fagan, Department of Animal Sciences, Rutgers University, PO Box 231, New Brunswick, NJ 08903, USA

## 2. MATERIALS AND METHODS

Reticulocytes were collected in saline containing heparin from rabbits treated with phenylhydrazine [8]. Reticulocyte extracts were prepared as described previously [10]. In some experiments, extracts were fractionated by chromatography on DEAE-cellulose (Whatman DE-52) and Sephacryl S-300 [10]. Fractions containing proteins >450 kDa (containing high molecular mass proteases) and between 30–300 kDa (containing the enzymes which conjugate Ub to proteins [conjugating fraction or CF]) [10] were separately pooled and concentrated.

Proteolytic activity was assayed in a final volume of 200 $\mu$l containing 25–75 $\mu$l of reticulocyte lysate, 50 mM Tris-Cl (pH 8), 10 mM $MgCl_2$, 1 mM DTT, 1–10 mM spermine or other compounds, 5 mM ATP, 5 $\mu$g [$^3$H]globin (11000 cpm/$\mu$g) or [$^3$H]lysozyme (3000 cpm/$\mu$g). ATP content, determined by the firefly-luciferase assay [11], was not changed by the addition of polyamines. After a 1.5–2 h incubation at 37°C, the protein was acid-precipitated and the acid-soluble radioactivity determined. The breakdown of endogenous cell proteins and of oxidant-treated hemoglobin [12] was estimated by monitoring the release of free alanine using alanine dehydrogenase [13].

## 3. RESULTS AND DISCUSSION

As seen previously by others [8–10,12], readdition of ATP to dialyzed reticulocyte extracts stimulates proteolysis of radiolabelled and endogenous protein substrates 3- to 11-fold. Addition of polyamines to such extracts at physiological concentration (up to 5 mM in erythroid cells

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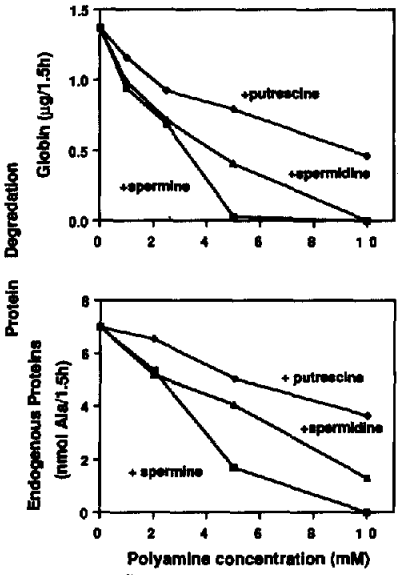


Fig.1. Effect of polyamine concentration on the ATP-dependent degradation of proteins in reticulocyte lysate.

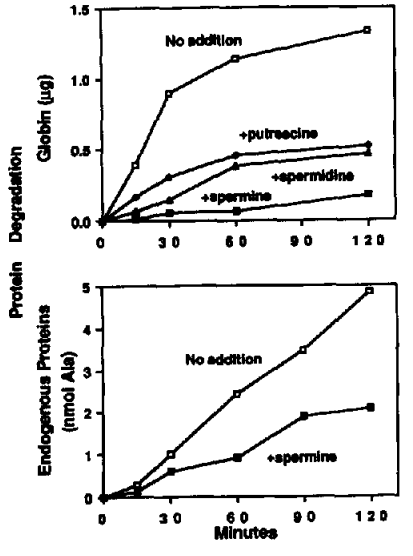


Fig.2. Time course of the inhibition of polyamines of ATP-dependent proteolysis in reticulocyte lysates.

[4,14]) significantly inhibited the ATP-dependent degradation of [$^3$H]globin and of endogenous reticulocyte proteins (fig.1). At concentrations greater than 2 mM, spermine inhibited degradation more effectively than spermidine or putrescine (fig.1). Inhibition of the ATP-dependent degradation of both exogenously added and endogenous protein substrates was apparent within 15–30 min after exposure of reticulocyte lysates to polyamines (fig.2).

To examine whether compounds structurally related to polyamines also inhibit ATP-dependent proteolysis, L-ornithine, a precursor of putrescine synthesis, and *N'*-acetylspermidine and *N'*-acetylspermine, metabolites of spermidine and spermine respectively, were added to reticulocyte lysates and incubated in the presence and absence of ATP. *N'*-Acetylspermine inhibited the ATP-dependent degradation of [$^3$H]globin, [$^3$H]lysozyme and of endogenous reticulocyte proteins less well than spermine but was more effective than *N'*-acetylspermidine and L-ornithine, which had the least inhibitory effect (table 1). Since *N'*-acetylspermine differs from spermine by acetylation of one of the four amino groups, the positive charges on the amino groups of spermine may be important for its ability to inhibit the ATP-dependent proteolytic system.

In addition to the ATP-dependent proteolytic system, reticulocytes also contain a proteolytic system that does not require ATP or Ub which degrades oxidatively damaged proteins [12]. To examine whether polyamines also inhibit the ATP-independent proteolytic system, spermine was added to reticulocyte lysates containing phenylhydrazine-treated hemoglobin. Spermine had little effect on the degradation of oxidatively damaged hemoglobin by the ATP-independent proteolytic pathway (table 2). Thus spermine inhibits specifically

Table 1

Effect of polyamine-related compounds on ATP-dependent proteolysis in reticulocyte lysates

| Addition | % inhibition of ATP-dependent degradation of | | |
|---|---|---|---|
| | [$^3$H]Globin | [$^3$H]Lysozyme | Endogenous proteins |
| Spermine (10 mM) | 93 | 83 | 75 |
| *N'*-Acetylspermine (10 mM) | 68 | 20 | 44 |
| *N'*-Acetylspermidine (10 mM) | 39 | ND | 27 |
| L-Ornithine (10 mM) | 27 | ND | 16 |

ND, not determined

Table 2

Effect of spermine on the ATP-independent degradation of oxidant-treated hemoglobin (Ox-Hb)

| Addition | Protein degradation (nmol Ala/2 h) | |
|---|---|---|
| | ATP-independent | ATP-dependent |
| None | 1.33 | 9.33 |
| Spermine (10 mM) | 1.67 | 1.84 |
| Ox-Hb | 4.90 | 0 |
| Ox-Hb + spermine (10 mM) | 4.35 | 0.52 |

128 μg of phenylhydrazine-treated hemoglobin was added to the assay mixture where indicated. ATP-dependent protein degradation was calculated as alanine produced/2 h in the presence of ATP minus that produced/2 h in the absence of ATP

Table 3

Effect of spermine on the ATP-dependent degradation of Ub-lysozyme conjugates

| Addition to proteolytic fraction | ATP + Ub-dependent proteolysis (ng lysozyme/2 h) | | |
|---|---|---|---|
| | − spermine | + spermine | Inhibition |
| Ub-lysozyme conjugates | 84 | 0 | 100% |
| Lysozyme + CF + Ub | 435 | 13 | 97% |

Ub-lysozyme conjugates were prepared as described previously [15] except that [$^3$H]lysozyme was incubated with reticulocyte CF. ATP and the high molecular mass fraction containing proteases was then added to Ub-lysozyme conjugates or to Ub, [$^3$H]lysozyme and CF in the presence or absence of 10 mM spermine and incubated for 2 h

the breakdown of proteins degraded by the ATP-dependent proteolytic system in reticulocytes.

In order to determine whether spermine inhibits the ATP-dependent proteolytic system by inhibiting the ATP-dependent protease [10,15], the high molecular mass protease-containing fraction was isolated. This fraction, when recombined with CF, ATP and Ub, degrades lysozyme in an ATP-dependent fashion (table 3 and [10]. In the presence of spermine, degradation of lysozyme by this reconstituted ATP-dependent system was inhibited by 97% (table 3). To examine whether polyamines inhibit the degradation of Ub-protein conjugates, Ub-[$^3$H]lysozyme conjugates were prepared by incubating 8 μg [$^3$H]lysozyme and 12.5 μg Ub in the presence of 30 μg CF, 5 mM ATP and 5 mM $MgCl_2$ for 15 min at 37°C. When conjugates were added to 150 μg of the high molecular mass protease-containing fraction with ATP in the presence of spermine, their breakdown was completely inhibited (table 3). These results demonstrate that spermine inhibits the degradation of Ub-lysozyme conjugates. It is possible that this cellular polycation binds electrostatically, or covalently in a reaction catalyzed by transglutaminase [16], to the protease preventing it from functioning. In addition, polyamines may bind to the Ub-protein conjugates making them inaccessible to proteolytic attack.

The physiological importance of the finding that polyamines inhibit the ATP-dependent proteolytic system in reticulocytes, remains to be investigated. Based on these observations, we suggest that polyamines may promote cellular growth not only by stimulating macromolecular synthesis, but also by inhibiting protein degradation.

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