ATP-dependent and -independent Protein Degradation in Extracts of the Psychrotrophic Bacterium *Arthrobacter* sp. S₁55

By PATRICK POTIER,¹* PASCAL DREVET,¹
ANNE-MONIQUE GOUNOT,¹ AND ALAN R. HIPKISS²

¹Laboratoire de Microbiologie Physiologique et Appliquée, UA CNRS 697, Université Claude-Bernard-Lyon 1, 43 boulevard du 11 Novembre 1918, 69622 Villeurbanne Cedex, France

²Department of Biochemistry, King's College London, Strand, London WC2R 2LS, UK

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Proteolysis of endogenous and exogenous substrates in cell-free extracts of the psychrotrophic bacterium *Arthrobacter* sp. S₁55 has been compared. Endogenous proteins were degraded only after treatment with cyanogen bromide. The hydrolysis of exogenous proteins of high M_r (i.e. casein) was optimum at alkaline pH and was stimulated by Ca^{2+} , Mg^{2+} , Mn^{2+} and ATP. The serine protease inhibitor phenylmethylsulphonyl fluoride had no effect on ATP stimulation. Small peptides (i.e. insulin) were degraded at very high rates. This activity was optimum at slightly acidic pH and was stimulated by Ca^{2+} , strongly inhibited by Mn^{2+} , but not affected by ATP. Degradation of cyanogen bromide-treated cellular proteins displayed two pH optima which corresponded to the optimum pH for the degradation of insulin and casein. The characteristics of these acidic and alkaline activities were identical to those active against insulin and casein respectively. The proteases which degraded casein were much more heat resistant than those which degraded insulin.

INTRODUCTION

Until recently, intracellular protein degradation in micro-organisms has been mainly studied in 'standard' bacteria such as *Escherichia coli* or *Bacillus megaterium*. Under optimum growth conditions, the overall rates of protein breakdown within these cells are low (Calandruccio & Larrabee, 1981; Chaloupka & Strnadova, 1982; Willets, 1967), and most of the proteins synthesized during exponential growth are stable (Goldberg & St John, 1976; Larrabee *et al.*, 1980; Pine, 1972). However, changes in growth conditions can cause a rapid and selective degradation of certain enzymes (Rivett *et al.*, 1985). Rapid rates of protein breakdown occur in various conditions such as starvation (Golberg & St John, 1976; Mandelstam, 1960; Nath & Koch, 1970; Pine, 1972), growth in the presence of agents that induce the production of abnormal proteins (Carr & Woods, 1984; Goldberg & Dice, 1974; Goldberg & St John, 1976; Kemshead & Hipkiss, 1974; Pine, 1972), or during a shift to high temperatures (Baker *et al.*, 1984; Goff *et al.*, 1984; Lindquist, 1986). It is now recognized that intracellular protein degradation is precisely regulated, and increased proteolysis appears to be an important physiological response of most organisms to adverse conditions.

In nature, many bacteria can grow harmoniously in very hostile environments such as polar regions and cold water, acidic hot springs, salterns, dry rock surfaces, deserts, or depths of the sea (Kushner, 1978). Such organisms are more able to withstand harsher conditions than typical mesophilic bacteria, e.g. *E. coli*, and they are often submitted to rapid variations of the environment. Several reports showed that the basal rate of protein turnover in micro-organisms that live in extreme environments, e.g. thermophiles (Amelunxen & Murdock, 1978) and the

Abbreviations: PHMB, *p*-hydroxymercuribenzoate; PMSF, phenylmethylsulphonyl fluoride.

moderate halophile *Vibrio costicola* (Hipkiss *et al.*, 1980), is much greater than that in *E. coli*. It is therefore possible that rapid protein breakdown may provide these organisms with some selective advantage, and proteolysis could be an important regulatory system in the process of adaptation of the cells to unstable harsh conditions.

Psychrophilic bacteria must contend with temperatures close to freezing. Recent investigations showed that the rate of protein degradation in the facultative psychrophile (psychrotroph) *Arthrobacter* sp. S₁₅₅ (which can grow between 0 and 32 °C) is high (Potier *et al.*, 1985). As in other cells, this process is selective and it is stimulated when the cells are grown in the presence of miscoding antibiotics, or inhibited by inhibitors of ATP synthesis. An interesting observation was that the rate of proteolysis in *Arthrobacter* S₁₅₅ is especially high at the maximum growth temperature (i.e. 32 °C). *In vitro* experiments revealed that there is considerably more protease activity in cells grown at 32 °C than in those grown at 10 °C (Potier *et al.*, 1987). Furthermore, this activity differs qualitatively in cells grown at different temperatures. The physiological reasons for this modulation are still unknown. Thus, it is important to gain an understanding of the activity of the proteolytic apparatus, its specificity and its regulation. The experiments reported here were designed to investigate proteolytic activities in extracts of *Arthrobacter* sp. S₁₅₅.

METHODS

Bacterial strain and growth conditions. *Arthrobacter* sp. S₁₅₅ was grown at 20 °C (optimum growth temperature) in a synthetic medium as previously described (Potier *et al.*, 1985).

Chemicals. Reagents whose source is not mentioned were of the highest purity available, and were purchased from standard commercial sources. U-¹⁴C-labelled L-amino acids [specific activity 60 mCi (mmol carbon)⁻¹, 2.22 GBq (mmol carbon)⁻¹] were obtained from the Commissariat à l'Energie Atomique. [³H]Formaldehyde (specific activity 75 mCi mmol⁻¹) was purchased from Du Pont de Nemours. Aqualyte scintillation cocktail was obtained from J. T. Baker. CNBr, EDTA, iodoacetamide and phenylmethylsulphonyl fluoride (PMSF) were from Merck. *p*-Hydroxymercuribenzoate (PHMB) was purchased from Fluka. Insulin, α -casein, BSA, ATP (dipotassium salt), DTT, phosphocreatine (di-sodium salt) and creatine phosphokinase [160 units (mg protein)⁻¹] were obtained from Sigma.

Preparation of cell-free extracts. Cells in exponential growth (500 ml) were harvested by centrifugation at 12000 *g* for 10 min and the pellet was washed twice with fresh medium. Two methods for the preparation of cell-free extracts were used.

(i) **Lysozyme treatment and sonication.** The pelleted cells were resuspended in lysis buffer [100 mM-Tris/HCl pH 8, 10 mM-EDTA, 10% (w/v) sucrose and 5 mg lysozyme ml⁻¹] in the proportion of 2 ml buffer (g wet cells)⁻¹. After 30 min incubation at 20 °C, cells were collected by centrifugation at 12000 *g* for 10 min and the pellet was resuspended in either buffer C (50 mM-Tris/HCl pH 8, 10 mM-CaCl₂, 10 mM-MgCl₂, 0.5 mM-DTT), or in buffer I (50 mM-Tris/HCl pH 6.5, 10 mM-CaCl₂, 0.5 mM-DTT). Cell walls were then disrupted by sonication (Braun Labsonic, 50 W) for six periods of 2 min with cooling in an ice bath. Unbroken cells were removed by centrifugation at 12000 *g* for 10 min, and the supernatants were used immediately to examine protein breakdown.

(ii) **Grinding with glass beads.** The pelleted cells were resuspended in a small volume of either buffer C or I, and broken in a Braun-MKS grinder for 2 min at maximum speed with 0.10–0.11 mm diameter glass beads. Overheating was prevented by cooling with liquid CO₂ for 10 s intervals every 20 s. Unbroken cells and glass beads were removed by centrifugation at 12000 *g* for 10 min, and the supernatant was used immediately to test protein degradation.

Preparation of radiolabelled substrates. Radiolabelled proteins of *Arthrobacter* S₁₅₅ were obtained by adding a mixture of U-¹⁴C-labelled amino acids (final concentration 0.025 μ Ci ml⁻¹) to exponentially growing cells, and growth was continued for 15 h. Cells were harvested by centrifugation at 12000 *g* for 10 min. Labelled proteins were collected after mechanical disruption of the cells with glass beads as described above and stored at –20 °C.

Insulin, BSA and casein were methylated with [³H]formaldehyde using the method of Rice & Means (1970). The specific activity of the various preparations was approximately 2×10^6 c.p.m. (mg protein)⁻¹. The products were used immediately after preparation.

Chemical cleavage of *Arthrobacter* S₁₅₅ proteins. ¹⁴C-Labelled proteins of *Arthrobacter* S₁₅₅ were cleaved by CNBr treatment according to the method of Kelley *et al.* (1973). The formic acid/CNBr solution was removed by lyophilization, the peptides dissolved in water, re-lyophilized, and the products stored at –20 °C.

Determination of protein degradation. The degradation of [³H]casein was assayed in *Arthrobacter* S₁₅₅ cell-free extracts prepared in buffer C (above) and that of [³H]insulin in buffer I (above). *In vitro* protein degradation was estimated by measuring the conversion of TCA-precipitable radioactivity into TCA-soluble radioactivity.

Table 1. Rates of degradation of various substrates in cell-free extracts of *Arthrobacter* S₁₅₅ obtained after sonication of exponentially growing cells

Reaction mixtures (200 µl) containing 100 µl extract, 70 µl buffer (50 mM-Tris/HCl pH 7.6, 50 mM-KCl, 10 mM-MgCl₂) were incubated for 15 min at 20 °C before the addition of the labelled substrates. Incubations were done at 20 °C for 60 min. The values represent the means $\pm$ SE of four independent experiments.

Origin of substrate	Substrate	Approximate M_r	Hydrolytic activity [h ⁻¹ (mg protein in extract) ⁻¹]
Endogenous (<i>Arthrobacter</i> S ₁₅₅)	¹⁴ C-Labelled total cellular proteins	Mixture	0.0 $\pm$ 0.0
	CNBr-cleaved ¹⁴ C-labelled cellular peptides	Mixture of short peptides	28.6 $\pm$ 2.8
Exogenous	[³ H]BSA	66000	2.5 $\pm$ 0.4
	[³ H]Casein	90000	5.5 $\pm$ 0.9
	[³ H]Insulin	6000	59.0 $\pm$ 5.2

Extracts (100 µl, containing 10–20 mg protein ml⁻¹) were mixed with 10 µl radioactive substrates (approximately 10⁴ c.p.m.) and incubated at 20 °C. Samples (50 µl) were removed at timed intervals, mixed with 5 µl 5% (w/v) BSA as a carrier and diluted into 50 µl cold TCA (10% w/v). After 60 min, the samples were centrifuged at maximum speed in an Eppendorf-type microfuge (MLW TH 21) for 2 min. Samples (50 µl) of the supernatant were assayed for radioactivity in the presence of 2 ml Aqualyte cocktail.

The optimum pH for proteolytic activity was determined in cell-free extracts obtained after sonication of the cells in 50 mM-potassium acetate buffer (pH 4–6.5) or in 50 mM-Tris/HCl buffer (pH 6–10).

Protein determination. Protein concentration was determined by the Lowry procedure and calibrated using BSA as standard.

RESULTS

Determination of proteolytic activity in cell-free extracts of *Arthrobacter* S₁₅₅

Table 1 presents the rates of degradation of various substrates in cell-free extracts of *Arthrobacter* S₁₅₅. When total ¹⁴C-labelled endogenous proteins were mixed with extracts obtained by sonication of the cells, no significant protein degradation was detected. The same result was observed with extracts obtained by mechanical grinding of the cells (data not shown). Several reports indicate that foreign proteins, abnormal molecules or small peptides are more sensitive *in vivo* and *in vitro* to proteolytic attack within various cells (Carrier *et al.*, 1984; Etlinger & Goldberg, 1977; George *et al.*, 1980; Grinde & Seglen, 1981; Kemshead & Hipkiss, 1974, 1976). We therefore tested the ability of cell-free extracts of *Arthrobacter* S₁₅₅ to degrade ¹⁴C-labelled peptides obtained after CNBr cleavage of labelled endogenous proteins. High rates of protein degradation were observed with this type of substrate. In addition, the degradation of three exogenous proteins of differing M_r was compared. The smallest, insulin, was rapidly degraded whereas the larger proteins (i.e. BSA and casein) were degraded at much lower rates. The method of cell breakage (sonication or mechanical grinding) did not affect the rates of degradation of the various substrates tested. Since ultrasonic treatment seemed to have no effect on the activity of proteases under the conditions used, this method was preferred for practical reasons (e.g. more concentrated extracts).

Effect of pH on the degradation of casein and insulin

To characterize the *in vitro* degradative system further, we examined the hydrolysis of proteins of various sizes as a function of pH: [³H]casein, [³H]insulin and ¹⁴C-labelled CNBr peptides were tested over pH values ranging from 4 to 10. Preliminary experiments showed that the release of TCA-soluble radioactivity from the substrates was linear for 20 min in the case of [³H]insulin, and for as long as 90 min in the case of [³H]casein (data not shown). Thus, the rate of degradation of insulin was determined after 10 min incubation periods, and that of [³H]casein

Table 2. *Effects of metal-chelating agents and ions on the degradation of [³H]insulin and [³H]casein in cell-free extracts of Arthrobacter S₁55*

Reaction mixtures (200 µl), containing 100 µl extract, 70 µl of either buffer I (pH 6.5) or C (pH 8), and the indicated inhibitors or ions, were incubated for 15 min at 20 °C before the addition of the labelled substrates. When metal-chelating agents were tested, ions were omitted from both buffers. Incubations were done at 20 °C for 10 min in the case of [³H]insulin, and for 60 min in the case of [³H]casein. Substrate hydrolysis in the absence of added inhibitors or ions is expressed as 100% activity, which for [³H]insulin and [³H]casein degradation was 3.0 ± 0.8 and 0.32 ± 0.04 µg hydrolysed h⁻¹ (mg protein in extract)⁻¹ respectively. Means from five independent experiments are reported; the SE values were less than 10% of the recorded values.

Addition	Concn (mM)	Percentage degradation of	
		[³ H]Insulin	[³ H]Casein
None	—	100	100
EDTA	5	—	98
	10	35	59
<i>o</i> -Phenanthroline	1	—	100
	5	25	82
Mg ²⁺	5	104	161
Ca ²⁺	5	145	270
Mn ²⁺	5	44	165
Co ²⁺	5	13	69
Cu ²⁺	5	2	15
Zn ²⁺	5	3	17

and ¹⁴C-labelled CNBr peptides after 60 min. The degradation of [³H]casein was stimulated under alkaline conditions with a maximum rate at pH 8, while degradation of [³H]insulin presented a narrow optimum at pH 6.5 (Fig. 1*a*). When total CNBr-treated ¹⁴C-labelled proteins of *Arthrobacter* S₁55 were used as a substrate, both peaks were observed (Fig. 1*b*). This was probably due to the presence of a mixture of peptides of various sizes in the substrate. In the remaining experiments, therefore, the degradation of [³H]insulin was assayed at pH 6.5, and that of [³H]casein at pH 8, unless otherwise indicated.

Metal ion requirement

The degradation of [³H]insulin and [³H]casein in cell-free extracts of *Arthrobacter* S₁55 was strongly inhibited by 10 mM-EDTA (Table 2). The hydrolysis of casein was only slightly affected by 5 mM-*o*-phenanthroline (which chelates divalent cations more readily), whereas that of insulin decreased dramatically.

At 5 mM, both Mg²⁺ and Ca²⁺ stimulated the hydrolysis of [³H]insulin and [³H]casein in cell-free extracts of *Arthrobacter* S₁55. At the same concentration, Co²⁺, Zn²⁺ and Cu²⁺ inhibited the degradation of both molecules, whereas Mn²⁺ stimulated the degradation of [³H]casein, but had a strong inhibitory effect on the hydrolysis of [³H]insulin. The effects of Ca²⁺, Mg²⁺ and Mn²⁺ on the degradation of ¹⁴C-labelled CNBr peptides were also tested as a function of pH. The addition of Ca²⁺ or Mg²⁺ to the extracts increased the rate of degradation of this substrate, but the profile observed in Fig. 1(*b*) was unchanged (data not shown). However, Mn²⁺ stimulated the degradation of CNBr peptides at alkaline pH, but strongly inhibited the activity at acidic pH (Fig. 2).

Sensitivity to protease inhibitors and effects of group specific reagents

The effects of various protease inhibitors and group specific reagents on the degradation of [³H]casein and [³H]insulin in cell-free extracts of *Arthrobacter* S₁55 are shown in Table 3. 2-Iodoacetamide (5 mM) inhibited the degradation of [³H]casein, and had no effect on the hydrolysis of [³H]insulin. The hydrolysis of both substrates was inhibited by 1 mM-PHMB, which also reacts with thiol groups. Furthermore, the addition of 0.5 mM-DTT to reaction buffers significantly increased the degradation of both substrates. The hydrolysis of both

Fig. 1

Fig. 2

Fig. 1. Effect of pH on the hydrolysis of $[^3\text{H}]$ casein, $[^3\text{H}]$ insulin and ^{14}C -labelled CNBr peptides. (a) The degradation of $[^3\text{H}]$ casein (●) and $[^3\text{H}]$ insulin (□) was assayed as described in Table 2, except that 50 mM-potassium acetate buffer (pH 4–6.5) or 50 mM-Tris/HCl buffer (pH 6–10) were used instead of buffers C and I. Maximum degradation of casein and insulin was 2.8 ± 0.6 and $0.25 \pm 0.03\text{ }\mu\text{g}$ hydrolysed h^{-1} (mg proteins in extract) $^{-1}$ respectively. (b) Hydrolysis of the ^{14}C -labelled CNBr peptides (○) was assayed in the same conditions after 60 min incubation at 20 °C.

Fig. 2. Influence of Mn^{2+} on the degradation of ^{14}C -labelled CNBr peptides in cell-free extracts of *Arthrobacter* S₁₅₅ as a function of pH. Reaction mixtures (200 μl) contained 20 μl extract, 140 μl buffer at the appropriate pH with (●) or without (□) 5 mM- Mn^{2+} . Samples were incubated for 15 min at 20 °C before the addition of the radioactive substrate, and the reaction was carried out for 60 min at the same temperature.

Table 3. Effects of protease inhibitors and group specific reagents on the degradation of $[^3\text{H}]$ insulin and $[^3\text{H}]$ casein in cell-free extracts of *Arthrobacter* S₁₅₅

Experiments were done as described in Table 2. Water-insoluble inhibitors were dissolved in ethanol before addition to the reaction mixtures; the control reaction mixture contained equal volumes of the solvent. The 100% activities for $[^3\text{H}]$ insulin and $[^3\text{H}]$ casein degradation were 3.3 ± 0.8 and $0.39 \pm 0.04\text{ }\mu\text{g}$ hydrolysed h^{-1} (mg protein in extract) $^{-1}$ respectively. The values represent the means $\pm$ SE of four independent experiments.

Addition	Concn (mM)	Percentage degradation of	
		$[^3\text{H}]$ Insulin	$[^3\text{H}]$ Casein
None	–	100	100
PMSF	1	100 ± 4	100 ± 5
	5	95 ± 6	94 ± 9
	10	90 ± 5	92 ± 5
Iodoacetamide	5	90 ± 8	77 ± 6
PHMB	1	61 ± 13	71 ± 11
DTT	0.5	139 ± 4	145 ± 15

Fig. 3

Fig. 4

Fig. 3. Effect of ATP on protein degradation in cell-free extracts of *Arthrobacter* S₁₅₅. Experiments were done as described in Table 2. Extracts were incubated in the presence of ATP for 15 min at 20 °C prior to the addition of labelled substrates: [³H]casein (●) and [³H]insulin (□). Results are expressed as percentage stimulation of the proteolytic activity obtained in extracts incubated without ATP [$0.37 \pm 0.03 \mu\text{g hydrolysed h}^{-1} (\text{mg protein in extract})^{-1}$].

Fig. 4. Effects of ATP on the degradation of ¹⁴C-labelled CNBr peptides as a function of pH. Reaction mixtures (200 μl) containing 20 μl extract and 140 μl buffer at the appropriate pH (see Methods) were pre-incubated for 15 min at 20 °C in the presence (●) or absence (□) of an ATP-regenerating system before the addition of the labelled substrate. The ATP-regenerating system consisted of 3 mM-ATP, 12.5 mM-phosphocreatine and 0.02 mg creatine phosphokinase ml^{-1} . Incubations were done at 20 °C for 60 min.

[³H]casein and [³H]insulin was not affected by the serine protease inhibitor PMSF at the three concentrations tested.

Effect of ATP on proteolytic activities

The rate of degradation of [³H]insulin by cell-free extracts of *Arthrobacter* S₁₅₅ was not affected by ATP at the concentrations tested (Fig. 3). However, incubation of the extracts with ATP markedly stimulated [³H]casein hydrolysis. The amount of stimulation increased with ATP concentration and was maximum at 3 mM. At this concentration, the rate of degradation of [³H]casein was twice that observed in extracts incubated without ATP. We also tested the effect of ATP on the degradation of ¹⁴C-labelled CNBr peptides as a function of pH (Fig. 4). ATP had no effect on the proteolytic activity observed in Fig. 1(b) at acidic pH, but markedly stimulated the activity found under alkaline conditions. This stimulation was maximum at pH 8.5. The addition of increasing amounts of PMSF (up to 10 mM) to extracts of *Arthrobacter* S₁₅₅ did not prevent the stimulatory effect of ATP on the degradation of [³H]casein.

Heat inactivation of casein and insulin degradation

The degradation of [³H]insulin and [³H]casein was assayed at 20 °C after pre-incubating the extracts for 30 min at various temperatures (Fig. 5). Thermoinactivation of the hydrolysis of [³H]insulin occurred when the pre-incubation temperature was higher than 30 °C. The degradative system responsible for the degradation of [³H]casein was found to be more heat resistant since inactivation was achieved only above 50 °C. In fact, the activity increased dramatically after incubation at temperatures between 40 and 50 °C. This difference in the thermostability of the hydrolytic activity towards the two substrates tested strongly suggests the existence of distinct proteolytic systems in extracts of *Arthrobacter* S₁₅₅.

Fig. 5. Heat inactivation of protein degradation in cell-free extracts of *Arthrobacter* S₁₅₅. Experiments were done at 20 °C as described in Table 2, except that the extracts were pre-incubated at various temperatures for 30 min and allowed to cool before the addition of the labelled substrates: [³H]insulin (□) and [³H]casein (●). Degradation of insulin and casein with untreated extracts was 4.0 ± 0.6 and 0.37 ± 0.03 μg hydrolysed h^{-1} (mg protein in extract)⁻¹ respectively.

DISCUSSION

The present experiments indicate that the efficiency of protein degradation in extracts of exponentially growing cells of *Arthrobacter* S₁₅₅ depends upon the nature of the substrate: intracellular proteases seemed to have no or very little affinity for native proteins *in vitro*, unless this substrate was submitted to a chemical cleavage prior to enzymic attack. By exposing bacterial cells to radioactive amino acids for varying periods of time, Nath & Koch (1970) revealed the existence of two general classes of proteins of different *in vivo* stability in bacteria: a small fraction that is degraded rapidly and another fraction that is more stable. The same observation has been made with *Arthrobacter* S₁₅₅ (Potier *et al.*, 1985). In the experiments reported here, the labelled native proteins were obtained by exposing the cells to radioactive amino acids for 15 h. This would allow only the more stable fraction of the cellular proteins to be labelled, which could account for the lack of degradation of native proteins by cell-free extracts of *Arthrobacter* S₁₅₅. However, appreciable proteolytic activity was detected in the extracts when CNBr-treated proteins or exogenous peptides were used as substrates (Table 1). As observed in other cells (Carr & Woods, 1984; Etlinger & Goldberg, 1977), greater proteolysis of foreign proteins in the extracts correlated with the more rapid breakdown of abnormal proteins that has been described in exponentially growing *Arthrobacter* S₁₅₅ cells (Potier *et al.*, 1985) and suggested that proteolysis in these preparations was related to breakdown *in vivo*. Therefore, it appears likely that endogenous proteins of *Arthrobacter* S₁₅₅ were specifically protected *in vivo* and *in vitro* against attack by intracellular proteases. This would correlate with the general observation that proteins with abnormal structures, including shortened chain length, are rapidly and specifically degraded in *Arthrobacter* S₁₅₅ (Potier *et al.*, 1985), and a wide range of exponentially growing cells (Carrier *et al.*, 1984; Chaloupka & Strnadova, 1982; Goldberg & St John, 1976; Hipkiss *et al.*, 1980; Kemshead & Hipkiss, 1974; Pine, 1972), while the bulk of endogenous proteins remain stable.

We found that CNBr pre-treatment (which cleaves proteins at methionine residues) triggered the degradation of this normally stable substrate. This result compares with the observations of Kemshead & Hipkiss (1976), who found that native alkaline phosphatase is stable in cell-free

extracts of *E. coli*, whereas its CNBr products of low oligomeric M_r (<29000) are rapidly degraded to amino acids. In addition, we found that insulin (an exogenous protein of low M_r) was degraded at very high rates by extracts of *Arthrobacter* S₁₅₅, while larger molecules, i.e. BSA or casein, were more stable (Table 1). These variations in the rates of degradation of large and small proteins suggest that the degradative system in *Arthrobacter* S₁₅₅ probably consists of several proteolytic enzymes whose activities depend, at least in part, on the size of the substrate.

In order to test the validity of this hypothesis, we examined the biochemical characteristics of the mechanisms involved in the degradation of exogenous proteins of different M_r (i.e. insulin and casein) by cell-free extracts of *Arthrobacter* S₁₅₅. The data were compared to the degradation of CNBr-treated endogenous proteins, which consist of a complex range of peptides of varying sizes. Our results indicate the existence of at least two distinct proteolytic systems. (i) Maximal proteolytic activity was obtained under alkaline conditions with casein as substrate, and at pH 6.5 with insulin (Fig. 1). Furthermore, two peaks of activity were observed at these pH values when CNBr peptides were used as substrate. (ii) The degradation of these two substrates was inhibited to different extents by metal-chelating agents, thus indicating different ionic requirements for maximum activity. The rate of degradation of casein increased significantly when Mg^{2+} , Ca^{2+} or Mn^{2+} was added to the reaction mixtures. The hydrolysis of insulin was stimulated only by Ca^{2+} , and was strongly inhibited by Mn^{2+} (Table 2). The degradation of CNBr peptides was activated by Mg^{2+} and Ca^{2+} over the complete range of pH, whereas Mn^{2+} inhibited the peak of activity observed at pH 6.5, and activated that at pH 8 (Fig. 2). (iii) The degradation of both insulin and casein was activated by reducing conditions, but the proteolytic enzymes involved in the degradation of these two substrates presented different sensitivities to thiol group inhibitors (Table 3). (iv) ATP had no effect on the degradation of insulin, but stimulated that of casein up to twofold (Fig. 3). ATP stimulated the degradation of CNBr peptides only in alkaline conditions (Fig. 4). (v) The hydrolytic systems responsible for the degradation of insulin and casein were inactivated at different temperatures (Fig. 5). Casein hydrolysis was much more resistant to heat than was insulin degradation, thus indicating that at least one rate-limiting thermosensitive protease was involved in the degradative pathway of insulin, but not in that of casein.

Since the characteristics of the degradation of insulin and casein by cell-free extracts of *Arthrobacter* S₁₅₅ were respectively identical to that of CNBr peptides at pH 6.5 and 8, it appears very likely that the differences observed for these two exogenous substrates were not due to some specific degradative reactions towards either molecule. Previous work (Potier *et al.*, 1985) indicated that the initial endoproteolytic steps in protein breakdown in growing cells of *Arthrobacter* S₁₅₅ require metabolic energy, and it is therefore likely that these steps are catalysed by the ATP-stimulated system responsible for the degradation of casein that we describe here. Because insulin is a small polypeptide, it may be degraded by enzymes involved only in the later steps in the complete degradation of cell proteins. Therefore, we suggest that intracellular protein degradation in *Arthrobacter* S₁₅₅ could be a two-step mechanism. (i) An initial event of the proteolytic pathway may involve the specific recognition and partial cleavage of 'unprotected' molecules (e.g. altered endogenous proteins or exogenous peptides) by a class of proteases, one of them at least being stimulated by ATP. (ii) The shortened polypeptides released during this interaction may become the preferential substrate for a second set of proteolytic enzymes that would catalyse their rapid and complete degradation to amino acids. The nature of the discrimination between the two types of substrate could depend on substrate size, availability of terminal groups, exposure of internal amino acid residues, specific amino acid sequences, or any combination of these and other determinants of proteolytic susceptibility.

It is now well established that *E. coli* cells contain at least eight distinct soluble proteolytic enzymes, and their subcellular distribution has been determined (Goldberg *et al.*, 1982; Swamy & Goldberg, 1981, 1982). Two of them degrade insulin but not larger peptides, whereas the six others (including the ATP-dependent protease, protease La) degrade globin, casein and some peptides, but not insulin. Several observations indicate that an initial step in intracellular protein breakdown in these cells is catalysed by the ATP-stimulated protease La (Kowit & Goldberg, 1977; Voellmy & Goldberg, 1981). The characteristics of the proteolytic system

responsible for the degradation of casein that we describe here resemble those reported for *E. coli* (Murakami *et al.*, 1979) or rabbit reticulocytes (Etlinger & Goldberg, 1977): optimum activity around pH 8, stimulation by ATP, inhibition by EDTA, *o*-phenanthroline and iodoacetamide. However, the ATP-dependent activity observed in *Arthrobacter* S,55 extracts was not affected by the serine protease inhibitor PMSF, as opposed to protease La in *E. coli*. It is therefore possible that the initial event of proteolysis in *Arthrobacter* S,55 may involve at least one ATP-stimulated protease that is probably different from that found in *E. coli*. In order to gain an understanding of the intracellular signals involved in protein degradation, it should therefore be of interest to compare this enzyme(s) with protease La, and we have already undertaken the purification of this enzyme(s). Another interesting development of this research would be the characterization in *Arthrobacter* S,55 of the thermosensitive protease(s) involved in the degradation of small proteins. A primary task for further studies will be to determine whether similar proteases exist in other psychrotrophic micro-organisms, their physiological significance, regulation and role in adaptation of the cells to their environment.

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