Temperature-dependent changes in proteolytic activities and protein composition in the psychrotrophic bacterium *Arthrobacter globiformis* S₁₅₅

PATRICK POTIER,^{1†} PASCAL DREVET,¹ ANNE-MONIQUE GOUNOT¹ and ALAN R. HIPKISS^{2*}

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

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

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Proteolytic activities, active against casein and insulin are present in cell-free extracts of the psychrotrophic bacterium *Arthrobacter globiformis* S₁₅₅. These activities were compared (i) following growth of the bacterium at different temperatures (10, 20 and 32 °C) and (ii) after a temperature shift from 10 to 32 °C. Both activities (measured at 20 °C) were greater in cells grown at 32 °C and after a temperature shift. Chloramphenicol prevented the increases in activities. The proteolysis of casein was stimulated by ATP; the stimulation was greater following a temperature shift. In addition, proteolysis of casein (measured at 20 °C) was activated by pre-incubation at temperatures between 45 and 60 °C; the proportion of proteolytic activity activated by such treatment also increased with growth temperature. Comparison by two-dimensional electrophoresis of the polypeptides synthesized at 10 and 32 °C suggests that *A. globiformis* S₁₅₅ produces proteins specific to both temperatures (13 species were either exclusive to or present in increased amounts during growth at 10 °C, while at least 21 polypeptides were preferentially synthesized at 32 °C). A temperature shift from 10 to 32 °C promoted the synthesis of at least 16 polypeptides, many with *M_r* values similar to those of heat shock proteins synthesized at higher temperatures in mesophiles. A temperature up-shift of *A. globiformis* S₁₅₅ from 10 to 20 °C did not provoke the synthesis of new proteins; heat shock proteins were produced only at 28 °C and higher. The similarity in kinetics of the appearance of heat shock proteins and the increase in proteolytic activities suggest that in *A. globiformis* S₁₅₅ some heat shock proteins might also possess protein catabolic function.

Introduction

According to their ability to grow at high, intermediate or low temperatures, micro-organisms have been divided into three broad categories: thermophiles, mesophiles and psychrophiles respectively. The last category has been further subdivided into psychrophiles *sensu stricto*, which have optimal growth temperatures below 16 °C and an upper temperature limit of 20 °C, and psychrotrophs, which are able to divide at 0 °C or below and grow optimally at temperatures around 20–25 °C (Morita, 1975). In their natural habitat, psychrotrophic micro-organisms are frequently submitted to large and rapid temperature changes and they can develop over a wide temperature range. This ability must be accompanied by adaptative changes in response to alterations of a large number of physical and chemical parameters, including

solubility, reaction kinetics, membrane fluidity and protein conformation. Therefore, the biochemical effects following large temperature changes are likely to be complex, involving a number of interacting phenomena. For example, high and low temperatures may effect structural and functional changes in proteins to modify biological behaviour by altering rates of enzyme activities, which may have important consequences for the integration of biochemical pathways; denaturation events could unfold proteins or cause subunit dissociation. The conformational stability of proteins results from a complex balance of individual forces (hydrogen-bonding, electrostatic or hydrophobic interactions) which vary in different ways with temperature. Therefore, all proteins are stable only within a limited temperature range. Intracellular protein catabolism is an interesting example because not only do changes in temperature affect the rate of protein breakdown for kinetic reasons, but the susceptibility of the substrate to proteolytic attack is also determined by its conforma-

† Present address: Unité de Microbiologie, Station de Recherches sur la Viande, INRA de Theix, 63 122 Ceyrat, France.

Abbreviation: IEF, isoelectric focusing.

tional state. Of course, extremes of temperatures may inactivate the intracellular proteases or their inhibitors. Indeed a general response to supra-optimal temperatures (sometimes only a few degrees above 'optimum') is the synthesis of heat shock or stress proteins (Craig, 1985; Neidhardt *et al.*, 1984; Yamamori *et al.*, 1978). Proteins of altered conformation (aberrant proteins) can also induce the synthesis of stress proteins (Anathan *et al.*, 1986; Goff & Goldberg, 1985; Kelly & Schlesinger, 1978; Munro & Pelham, 1985). In *Escherichia coli*, mutations in some heat shock genes (*dnaK*, *dnaJ*, *grpE*, *groES*, *groEL*, *lon* and *rpoH*) result in defective *in vivo* catabolism of puromycin peptides and of a nonsense fragment of β -galactosidase (Straus *et al.*, 1988). Conversely, over-expression of these genes results in enhanced rates of proteolysis. Straus *et al.* (1988) suggest that at least one general function of heat shock proteins is to contribute to the ability of the cells to degrade abnormal peptides.

McCallum *et al.* (1986) reported that the synthesis of heat shock proteins is induced at relatively low temperatures in a psychrophilic and a psychrotrophic bacterium. *Arthrobacter globiformis* S₁₅₅ is a psychrotrophic bacterium capable of growth between -5°C and $+32^{\circ}\text{C}$ (Gounot, 1976). This organism was isolated from a Scandinavian arctic glacial region where surface temperature varied considerably. Our previous studies have shown that *in vivo* proteolysis and *in vitro* degradation of protein fragments correlate with growth temperature: cells grown at the highest permissible temperature showed the greatest *in vivo* and *in vitro* proteolytic activities (Potier *et al.*, 1985, 1987a). Qualitative differences in degradative activity towards protein fragments were also detected in cells grown at 10, 20 and 32°C . Partial characterization of the proteolytic activities using insulin and casein as substrates revealed the presence of proteolytic enzymes with differences in metal-ion requirements, pH optima, ATP stimulation and heat lability (Potier *et al.*, 1987b, c). The higher rates of protein degradation observed when *A. globiformis* S₁₅₅ cells are cultured above their optimal growth temperature could be due to direct effects of temperature on some key protease(s) or on some controlling element(s), and/or to the induction of the synthesis of new enzymes or activators. In this communication, we report our studies on the comparison of the protein pattern and intracellular proteolytic activities present in this bacterium when grown at different temperatures and following temperature changes. The data show that temperature shifts from 10°C to supra-optimal growth temperatures result in rapid increases in the rate of both insulin and casein degradation, together with the synthesis of heat shock proteins.

Methods

Bacterial strain and growth conditions. *Arthrobacter globiformis* S₁₅₅ was grown aerobically at 10, 20 or 32°C in a synthetic medium to mid-exponential phase (OD_{570} 0.3–0.5) as previously described (Potier *et al.*, 1985). The generation times at 10°C and 20°C were 19 h and 4 h 40 min respectively. At 32°C , growth rate was apparently exponential during the first 7 h, with a generation time of 3 h 20 min. The length of bacteria increased progressively with time: after 7 h at this temperature, cells formed filaments and there was an increase in the apparent generation time to 7 h 50 min (Potier *et al.*, 1985).

Preparation of cell-free extracts. Cells in exponential growth (500 ml) at 10, 20 or 32°C were harvested by centrifugation at 10000 *g* for 10 min and the pellets were washed twice with fresh medium. Cells were broken by ultrasonic treatment either in buffer C (50 mM-Tris/HCl, pH 8, 10 mM- CaCl_2 , 10 mM- MgCl_2 , 0.5 mM-DTT) or in buffer I (50 mM-Tris/HCl, pH 6.5, 10 mM- CaCl_2 , 0.5 mM-DTT) as previously described (Potier *et al.*, 1987b). Unbroken cells were removed by centrifugation at 12000 *g* for 10 min and the supernatants were used immediately to test proteolytic activities.

Extracts of heat shocked cells were obtained by transferring 25 ml of bacteria growing exponentially at 10°C into prewarmed 500 ml flasks and incubating at 20, 25, 28, 30 or 32°C . With shaking at 100 r.p.m., the 25 ml cultures took less than 3 min to reach the higher temperatures. Lysozyme (2 mg ml⁻¹) was added to the temperature-shifted cells 10 min before harvesting. After centrifugation (12000 *g* for 1 min) cells were resuspended in 1 ml of either buffer C or buffer I containing 400 μg chloramphenicol. Cell walls were immediately disrupted by sonication (Braun Labsonic; 60 W) for three periods of 2 min with cooling in an ice-bath. After centrifugation at 12000 *g* for 10 min, the supernatants were used immediately to test protein degradation.

Preparation of radiolabelled substrates. Insulin and α -casein (Sigma) were methylated with [³H]formaldehyde [NEN; 75 mCi mmol⁻¹ (2.78 GBq mmol⁻¹)] 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.

Determination of protein degradation. The degradation of [³H]casein was assayed in *A. globiformis* S₁₅₅ cell-free extracts prepared in buffer C, and that of [³H]insulin in buffer I. *In vitro* protein degradation was estimated by measuring the conversion of TCA-precipitable radioactivity into TCA-soluble radioactivity. Extracts (40 μl) containing an equal quantity of DNA (0.3 mg ml⁻¹) were mixed with 140 μl of either buffer C or buffer I, and incubated at the indicated temperatures in presence of 20 μl of the appropriate substrates. Unless otherwise indicated, degradation of [³H]casein and [³H]insulin was stopped after incubation with the cell-free extracts for 50 min and 10 min, respectively (Potier *et al.*, 1987b), by mixing samples (50 μl) with 5 μl 5% (w/v) BSA as a carrier and diluting into 50 μl cold TCA (10%, w/v). After 60 min at room temperature, the samples were centrifuged at 12000 *g* for 10 min. Samples (50 μl) of the supernatant were assayed for radioactivity in the presence of 2 ml Aqualyte scintillation cocktail (J. T. Baker Chemicals, the Netherlands).

Radiolabelling during heat-shock and sample preparation. Heat shock was accomplished by shifting 5 ml of cells from 10°C to prewarmed test tubes (13 $\times$ 100 mm) at the temperatures indicated. For the periods of protein synthesis examined, radiolabelling of cellular proteins for SDS-PAGE was done by incubating 250 μl of cells in prewarmed 1.5 ml microcentrifuge tubes containing 30 μCi (1.11 MBq) L-[³⁵S]methionine (Amersham; specific activity > 37 TBq mmol⁻¹). Growth was allowed to continue for 10 min; radiolabel incorporation was then

halted by addition of 1 ml acetone at -20°C . Cells were washed twice in 1 ml cold acetone and dried under vacuum. Dried cells were resuspended in 1 ml of lysis buffer (10 mM-Tris/HCl, pH 8, 2 mg lysozyme) and incubated at 4°C for 30 min. Cells were washed twice with 62.5 mM-Tris/HCl (pH 6.8) and resuspended in 100 μl of the SDS sample buffer of Laemmli (1970). Cell walls were disrupted by sonication (Braun Labsonic; 50 W) for four periods of 2 min. Unbroken cells were removed by centrifugation at 12000 g for 5 min. Samples were stored at -80°C .

Acid-insoluble radioactivity was determined by spotting 5 μl of solubilized cell extract onto glassfibre filters (Whatman GF/C), which were then rinsed with 5 ml cold 25% TCA and 10 ml cold 10% TCA, followed by 10 ml 96% (v/v) ethanol. Filters were placed in 4 ml of Aqualyte scintillation cocktail and precipitated radioactivity was determined.

SDS-PAGE. SDS-solubilized protein samples were heated in a boiling water-bath for 2 min before electrophoresis on a 1.5-mm-thick, 12% (w/v) polyacrylamide gel overlaid with an SDS/4.5% polyacrylamide stacking gel using the discontinuous buffer system of Laemmli (1970). Equal amounts of TCA-precipitable radioactivity (10^6 c.p.m.) in equal volumes of SDS sample buffer were loaded onto each lane of a gel. Electrophoresis was done at 15°C and a constant 15 mA current per slab gel until the tracking dye entered the resolving gel, at which time the current was increased to 30 mA. The M_r standards (Sigma) were α -lactalbumin (M_r 14200), trypsin inhibitor (20100), trypsinogen (24000), carbonic anhydrase (29000), glyceraldehyde-3-phosphate-dehydrogenase (36000), egg albumin (45000) and bovine albumin (66000). Gels were stained with 0.1% Coomassie blue R250 in 50% (v/v) methanol/7.5% (v/v) acetic acid, and destained with 30% (v/v) ethanol/10% (v/v) acetic acid. Fluorograms were made by exposing fluor-treated (Amplify; Amersham) dried gels to pre-flashed Fuji RX films.

Two-dimensional electrophoresis. Exponentially growing cells (250 μl) at 10 or 32°C were labelled for 1 h with L-[^{35}S]methionine [$120 \mu\text{Ci ml}^{-1}$ (4.44 MBq ml^{-1})]. Radiolabel incorporation was halted by addition of cold acetone. After washing with acetone and drying as described above, dried cells were resuspended in 50 μl of the lysis buffer of O'Farrell (1975). Isoelectric focusing (IEF) was done in 12×1.5 mm gels following the procedure described by O'Farrell (1975). The gel mixture was 4% acrylamide, 9.2 M-urea, 2% Nonidet P40, 5% carrier ampholytes (LKB) ampholines; 1 part pH 3.5–10, 2 parts pH 5–7). Samples (10 μl) containing 10^6 c.p.m. were loaded on the basic side of the gels and IEF was done for 10000 V $\cdot$ h. Gels were then incubated for 30 min in the denaturing buffer of Laemmli (1970), and placed on top of a slab gel. Conventional electrophoresis in second dimension was done as described above.

Protein and DNA determination. Protein was determined by the Lowry method, with BSA as the standard. The DNA content of the cell-free extracts was estimated according to Burton (1951) using calf thymus DNA (Sigma) as the standard.

Results

Effects of growth temperature on proteolytic activity in *A. globiformis* S₁55

Growth-temperature-related differences in degradation of protein fragments (cyanogen bromide peptides) in cell-free extracts of *A. globiformis* S₁55 have previously been detected (Potier *et al.*, 1987a). Proteolysis of casein

Fig. 1. (a) Effect of incubation temperature on the initial rates of proteolysis of [^3H]insulin ($\square$) and [^3H]casein ($\bullet$) in cell-free extracts of *A. globiformis* S₁55 grown at 20°C . Proteolysis of insulin is expressed as the percentage of [^3H]insulin degraded in 10 min per mg extract protein; proteolysis of casein is expressed as the percentage of [^3H]casein degraded in 30 min per mg extract protein. (b) Relationship between rates of proteolysis and incubation temperature: deviation from the $Q_{10} = 2$ law. The values express the deviation between the observed and the expected proteolytic rates at each temperature based on the data obtained at 10°C , assuming that $Q_{10} = 2$: 100% correspondence between the experimental and the theoretical value is equivalent to zero deviation.

Table 1. Effect of growth temperature on proteolytic activities in extracts of *A. globiformis* S₁55

Initial rates of [^3H]insulin degradation were determined after 5, 10 and 15 min, and those of [^3H]casein degradation after 30, 45 and 60 min. The assay temperature was 20°C .

Growth temperature	Initial rate of degradation [μg substrate hydrolysed h^{-1} (mg DNA in extract) $^{-1}$]	
	[^3H]Insulin	[^3H]Casein
10°C	27 ± 3	3.8 ± 0.4
20°C	29 ± 2	4.0 ± 0.2
32°C	42 ± 3	7.3 ± 0.2

and insulin in cell-free extracts of *A. globiformis* S₁55 is effected by enzymes with differences in pH optima, and in metal-ion requirements and sensitivities (Potier *et al.*, 1987b). The proteolytic activities at 20°C of extracts of cells grown at 10, 20 and 32°C were compared (Table 1). Cells grown at 32°C possessed the greatest activities

Fig. 2. Effects of pre-incubation temperature on proteolytic activities in extracts of *A. globiformis* S₁₅₅ grown at 20 °C. Extracts were pre-incubated at the indicated temperatures for 30 min, then allowed to return to 20 °C before addition of [³H]insulin (□) or [³H]casein (●). Results are expressed as a percentage of the activities ($\equiv 100\%$) detected in extracts pre-incubated at 20 °C.

towards insulin and casein and cells grown at 10 °C the least.

Heat stability of proteolytic activities in extracts of *A. globiformis* S₁₅₅

Using extracts obtained from cells grown at 20 °C (optimal growth temperature), we found that the kinetics of casein and insulin degradation differed in their response to the assay temperature (Fig. 1*a*). Up to 30 °C, the two activities showed similar and expected increases in activity which appeared to obey the simple $Q_{10} = 2$ law (Fig. 1*b*). Both activities deviated in a similar way from the theoretical values at temperatures between 30 and 40 °C. Above 40 °C, proteolysis of insulin declined with increasing temperature, whereas the degradation of casein showed a second and higher peak of activity at 60 °C (Fig. 1*a*). However, this stimulation was not sufficient to produce activities which approached the theoretical maximum (Fig. 1*b*). Similar activity profiles were obtained with extracts of cells grown at 10 and 32 °C (results not shown). The stimulation of casein degradation observed between 45 and 60 °C was surprising. It is possible that the conformation of casein changes at the higher temperatures, thereby increasing its susceptibility to proteolysis. Additionally or alternatively, it is possible that the caseinolytic activity is itself activated above 45 °C. Evidence for this proposition was obtained by pre-incubation of cell-free extracts at

Fig. 3. Effects of pre-incubation temperature on casein-degrading activity in extracts of *A. globiformis* S₁₅₅. Experiments were done as in Fig. 2, except that cells were grown at 10 (□), 20 (●) and 32 °C (▽).

Fig. 4. Variation of insulin-degrading (*a*) and casein-degrading (*b*) activities present in extracts of *A. globiformis* S₁₅₅ following a temperature shift of the cells from 10 to 32 °C. Proteolytic activities were measured at 20 °C. Results are expressed as a percentage of the initial activity (100%) measured immediately before transfer of cells to the higher temperature. Similar results were obtained in four independent experiments. □, ○, Cells grown at 10 °C; ■, ●, cells transferred to 32 °C.

temperatures up to 70 °C for 30 min. After cooling, the substrates were added and the proteolytic activities were assayed at 20 °C. The activity responsible for casein degradation was stimulated above 45 °C with a maximum at 55 °C, whereas the component responsible for insulin degradation was completely inactivated by heating at 45 °C (Fig. 2).

Growth temperature affected the relative proportion of heat-activated casein-degrading activity (Fig. 3). Cells grown at 10 °C possessed the smallest proportion of heat-stimulated activity, which gave a 3-fold increase in the

Fig. 5. Two-dimensional gel electrophoresis of polypeptides of *A. globiformis* S₁₅₅ labelled with [³H]methionine during exponential growth at 10 (a) or 32 °C (b). Arrows (► and →) indicate peptides synthesized only or preferentially at 10 and 32 °C, respectively.

rate of casein degradation, whereas a 12-fold stimulation was observed in extracts of cells grown at 32 °C. In contrast, the heat stability pattern was identical in all extracts when insulin was used as substrate, and was similar to that reported in Fig. 2.

Changes in proteolytic activities in A. globiformis S₁₅₅ following temperature shifts

The results described in the previous section clearly demonstrate quantitative differences in proteolytic activities when *A. globiformis* S₁₅₅ is grown at low and high temperatures. We therefore investigated the response of *A. globiformis* S₁₅₅ to a temperature shift from 10 to 32 °C. Parallel changes (measured at 20 °C) of casein and insulin degradation in cell-free extracts occurred, with maximal stimulation within 1 h following transfer (Fig. 4). Addition of chloramphenicol (400 µg ml⁻¹) or rifampicin (200 µg ml⁻¹) to the cells 15 min before the temperature shift prevented the increases in both proteolytic activities.

In previous studies, we demonstrated that casein hydrolysis in extracts of *A. globiformis* S₁₅₅ was stimulated by ATP (Potier *et al.*, 1987*b, c*). The caseinolytic activity in extracts of cells grown at 10 °C was increased by up to 60% by addition of an ATP-regenerating system. The temperature shift from 10 to 32 °C resulted in increased amounts of ATP-stimulated proteolysis (up to 80%), such that within 1 h, the cells possessed about twice the initial ATP-stimulated activity

Table 2. Variation in proteolytic activities in extracts of *A. globiformis* S₁₅₅ after a temperature up-shift

Cells were transferred from 10 °C to temperatures from 20 to 32 °C. Proteolytic activities of cell extracts were determined as in Table 1. Values are means of three independent experiments.

Transfer temperature	Initial rate of degradation [µg substrate hydrolysed h ⁻¹ (mg DNA in extract) ⁻¹]	
	[³ H]Insulin	[³ H]Casein
10 °C (control)	25	3.5
20 °C	27	3.6
25 °C	27	3.8
28 °C	38	6.7
30 °C	40	7.0
32 °C	41	7.2

present prior to the temperature change. For the activities measured, the temperature shift increased degradation by between 60 and 100% of that originally present in cells grown at 10 °C. When the extracts were pre-incubated at 55 °C for 30 min, degradation of casein was increased as in Fig. 2, but the stimulation by ATP was no longer detectable (result not shown).

We also did temperature up-shift experiments designed to determine whether temperatures below the maximal permissible growth temperature could similarly trigger the changes in cell-free proteolysis measured at 20 °C. Table 2 shows that the stimulation in degradation of both insulin and casein occurred only when cells grown at 10 °C were transferred to 28 °C or higher.

Fig. 6. Time-course of temperature-induced protein synthesis in *A. globiformis* S₁₅₅. Cells were shifted from 10 to 32 °C for the periods of time indicated below and labelled with [³⁵S]methionine for the last 10 min of incubation. Sample preparation and SDS-PAGE were as described in Methods. Each lane received 10⁶ c.p.m. of ³⁵S-labelled TCA-insoluble material. Arrows (►) indicate the heat shock polypeptides; pointers indicate mobilities of protein standards. Lane A, cells grown at 10 °C. Other lanes: cells shifted from 10 to 32 °C for 15 (B), 30 (C), 60 (D), 90 (E) and 120 min (F).

Again, the increases in both activities were no longer observed in extracts obtained from cells incubated with 400 µg chloramphenicol ml⁻¹ or 200 µg rifampicin ml⁻¹ during the temperature shift. Lower temperatures did not provoke these responses; the activities in extracts of cells transferred to 20 and 25 °C were similar to those initially present prior to transfer.

Effects of temperature on protein composition of A. globiformis S₁₅₅

We were also interested to determine whether growth at low and high temperature is accompanied by differences in protein synthesis. Fig. 5 presents an analysis by two-dimensional gel electrophoresis of the proteins present in cells labelled with [³⁵S]methionine for 1 h during

Fig. 7. Synthesis of heat shock proteins by *A. globiformis* S₁₅₅ at various temperatures. Cells were shifted from 10 °C to the temperatures indicated below for 1 h. [³⁵S]Methionine was added for the last 10 min of incubation. Cell lysis, electrophoresis and fluorography were as described in Methods. Lane A, cells grown at 10 °C. Other lanes: cells shifted for 1 h from 10 °C to 20 (B), 28 (C), 30 (D) and 32 °C (E). ●, Location of heat shock proteins; from top to bottom of the gel the *M_r* values of the proteins are 70000, 62000, 48000, 45000, 34000, 30000, 26000, 23000, 22000, 21500, 16000, 14500 and 13500.

exponential growth at 10 or 32 °C. At least 13 polypeptides appeared to be either exclusive or present in increased quantities following growth at 10 °C (Fig. 5a). At 32 °C, at least 21 peptides were preferentially synthesized (Fig. 5b).

Effects of temperature up-shifts on protein synthesis

To determine if a 10 to 32 °C temperature change induces any immediate effects on protein biosynthesis, cells were radiolabelled for 10 min with [³⁵S]methionine at various

times after such a shift. Analysis by SDS-PAGE revealed that the cells responded to the heat shock by synthesizing a set of proteins which either were not produced before the temperature shift or were produced at lower levels (Fig. 6). At least 16 polypeptides with apparent M_r values of 13000 to 83000 could be seen. Synthesis of at least eight peptides (70, 48, 45, 22, 21, 16, 14.5 and 13 K) was stimulated in the first 15 min following the temperature change. Eight other polypeptides (83, 62, 34, 30, 26, 24, 23 and 19K) appeared within 30 min. Synthesis of the 83, 70 and 45K species appeared to then decline after 60 min, while others were synthesized for at least 2 h following transfer. Conversely, synthesis of at least three polypeptides (42, 37 and 14.2K) was transiently inhibited within 15 min. When the cells grown at 10 °C were transferred to 20 °C (Fig. 7), no new proteins were detected, whereas at 28 °C and above, synthesis of most of the new proteins occurred. It was particularly evident, however, that synthesis of the 14.5K polypeptide was much greater at 32 °C than at 28 °C.

Discussion

The results presented in this communication both extend and clarify our previous observations on the effects of temperature on intracellular protein degradation in the psychrotrophic bacterium *A. globiformis* S₁₅₅. We show that growth temperature determines the activity of the two proteolytic systems detected in this organism. It is noticeable that the simultaneous fall between 30 and 40 °C in the *in vitro* rate of insulin and casein degradation occurred at temperatures that also limit growth of *A. globiformis* S₁₅₅. It is now well-established that intracellular protein breakdown is a fundamental process that participates in a number of important physiological functions in cells, including protein maturation and secretion (Davis & Tal, 1980), regulation of the level of intracellular proteins (Goldberg & St John, 1976; Mosteller *et al.*, 1980), elimination of abnormal peptides (Goff & Goldberg, 1985; Goldberg, 1972) and provision of amino acids for the synthesis of proteins required for adaptation (Mandelstam, 1963). Therefore the diminution above 30 °C of the efficiency of the proteolytic system present in *A. globiformis* S₁₅₅ could contribute to the cessation of growth at these temperatures.

The heat-induced activation of casein degradation is intriguing and has yet to be explored. Unfolding of one or more of the enzymes involved to widen its specificity, binding of activating ligands or heat-denatured proteins, or the denaturation or dissociation of an inhibitor are areas worthy of consideration. Our previous findings, where we employed a heterogeneous polypeptide substrate (cyanogen bromide peptides), also indicated that

growth temperature has major effects on the total proteolytic activity present in *A. globiformis* S₁₅₅, and on the proportion which is activated by heat (Potier *et al.*, 1987a). The physiological function of this apparently latent proteolytic activity is also unknown. Circumstances which require rapid increases in proteolytic activity, in the absence of protein synthesis, might be considered likely. This may be especially important when one considers the natural environment (cold and nutritionally poor) of *A. globiformis* S₁₅₅, where rates of protein synthesis might be expected to be slow, but when a rapid increase in proteolytic activity might be necessary to cope with sudden changes in the environment.

It is also striking that the proteolytic activity of cells grown at 32 °C is greater than that of cells grown at 10 or 20 °C. Such an increase might be expected because the protein content of the cells grown at this temperature is up to 45% greater than that of cells grown at 10 or 20 °C (protein:DNA ratios cells grown at for 10, 20 and 32 °C were 8.7 ± 0.2 , 8.5 ± 0.4 , and 11.4 ± 0.3 , respectively). Therefore, one might expect the cells to produce more enzymes to deal with the increased amount of substrate should the necessity arise. In addition, cells growing close to their maximal permitted temperature are reported to produce continuously some heat shock proteins, some of which are proteases (Goff *et al.*, 1984; Goff & Goldberg, 1987) or enhance protein degradation (Straus *et al.*, 1988). Therefore, it is likely that *A. globiformis* S₁₅₅, when cultured at 32 °C, similarly synthesizes increased amounts of heat shock proteins which could contribute to the temperature-dependent increases in proteolytic activities that we detected. Furthermore, we speculatively suggest that the increased level of casein-degrading activity measured at higher temperatures may be a particular example of such a stress-related enzyme.

Of special interest is the ATP-stimulated caseinolytic activity, which increases proportionately more than the ATP-independent enzymes following the 10 to 32 °C temperature shift. In *E. coli*, protease La is an ATP-stimulated protease and also heat shock protein (Chung & Goldberg, 1981; Phillips *et al.*, 1984). It is possible, therefore that one or more analogous enzymes exist in *A. globiformis* S₁₅₅, and that such enzymes are responsible for the increase in caseinolytic activity at higher temperatures. After heat-activation of the caseinolytic activity, the ATP stimulation was no longer detected, either because heat-provoked effects eliminated the ATP-mediated response, or the ATP effects became masked by the elevated activity.

The temperature-shift experiments also revealed rapid changes in protein synthesis in *A. globiformis* S₁₅₅. It may be coincidental that the changes in protein synthesis

(Fig. 6) and proteolytic activities (Fig. 4) were similarly rapid, as were the temperatures which elicited these responses (Table 2, Fig. 7). It is tempting to suggest that some of the proteins specifically synthesized following a temperature shift may indeed represent proteolytic enzymes and/or activators. Furthermore, the increase in protease activity detected when the temperature of the culture was raised is not likely to result from heat-activation of the pre-existing caseinolytic activity, because the latter phenomenon was only observed above 45 °C in the *in vitro* studies.

Growth of *A. globiformis* S₁₅₅ at low and high temperatures was associated with the appearance of a number of unique polypeptide species particular to 10 or 32 °C. While the functions of these peptides are unknown, some might be concerned with proteolytic activity and its regulation as discussed above. It is possible that some of these proteins are necessary for growth at these temperatures, thereby reflecting adaptive responses of the cells. Alternatively, the appearance of temperature-specific proteins may be secondary responses to other temperature-regulated activities which alter the metabolism or expression of these polypeptides. The fact that protease activities differ at 10 and 32 °C increases the likelihood that changes in protein processing might occur.

McCallum *et al.* (1986) demonstrated that increases in culture temperature of the strictly psychrophilic Arctic bacterium Res-10 and the psychrotroph *Bacillus psychrophilus* induce the synthesis of a number of proteins (heat shock proteins) with *M_r* values similar to those of proteins detected in *A. globiformis* S₁₅₅, and which are also detected in *E. coli* at higher temperatures (Neidhardt & VanBogelen, 1987). Hence we suggest that expression of the heat shock proteins is not regulated by the absolute temperature. The important event in the induction of the heat shock genes might involve the effects of heat on those controlling elements which determine the activation of the relevant regulon(s). Temperatures considered to be extreme for one organism might be tolerable by another. In fact, the same biochemical event may occur at a relatively high temperature for a mesophile, and at a much lower temperature for a psychrophile. The molecular bases of the relevant protein thermocouples would make an interesting evolutionary study: eventually the application of protein engineering techniques might permit the design of a range of molecules, each of which may respond to a specific temperature.

Finally, psychrophiles growing at low temperatures and possessing low proteolytic activities might be considered suitable tools for the production of commercially and medically useful molecules which are very susceptible to proteolysis at higher temperatures in other organisms. For example, interferon α -2 is rapidly

degraded at 37 °C in *E. coli* and *Methylophilus methylotrophus*, but is stable in both organisms at 29 °C and below (Chesshyre & Hipkiss, 1989). Other polypeptides of interest might undergo similar stabilization, but at much lower temperatures.

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