An analysis of the effect of changes in growth temperature on proteolysis *in vivo* in the psychrophilic bacterium *Vibrio* sp. strain ANT-300

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In the psychrophilic bacterium *Vibrio* sp. strain ANT-300, the rate of protein degradation *in vivo*, measured at fixed temperatures, increased with elevation of the growth temperature. A shift in growth temperature induced a marked increase in this rate. Dialysed cell-free extracts hydrolysed exogenous insulin, globin and casein (in decreasing order of activity) but did not hydrolyse exogenous cytochrome *c*. Cells contained at least seven proteases separated by DEAE-Sephacel chromatography, one of which was an ATP-dependent serine protease. The ATP-dependent proteolytic activity in extracts of cells incubated for 3 h at 16 °C after a shift-up from 0 °C increased to a level 36% and 17% higher than that of cells grown at 0 °C and 13 °C, respectively. A shift-down to 0 °C from 13 °C induced only a slight increase in the proteolytic activity. Extracts of all cells, whether exposed to temperature shifts or not, showed the same temperature dependence with respect to both ATP-dependent and ATP-independent protease activity. In all the extracts these proteases also exhibited the same heat lability. The ATP-dependent protease was inactivated by incubation at temperatures above 25 °C. There was an increase in ATP-independent protease activity during incubation at temperatures between 25 and 30 °C, but a decrease at 35 °C and higher. These results suggest that the marked increases in proteolysis *in vivo*, caused by a shift in temperature, may result not only from increases in levels of ATP-dependent serine protease(s) but also from increases in the susceptibility of proteins to degradation.

Introduction

The rate of protein turnover in exponentially growing *Escherichia coli* is usually low, and the degradation of intracellular proteins is selective and responsive to the nutritional state of the cells (Pine, 1972; St John & Goldberg, 1978). Thus, it appears that the degradation of proteins is metabolically regulated. While most proteins in *E. coli* are stable (Larrabee *et al.*, 1980; Mosteller *et al.*, 1980), a number of proteins, such as Sula, RcsA and σ^{32} , are degraded rapidly by energy-dependent proteases (Gottesman, 1987). In micro-organisms that live in extreme environments, such as thermophiles, psychrotrophs and halophiles, the rates of degradation of proteins are much higher than in mesophiles (Amelunxen & Murdoch, 1983; Hipkiss *et al.*, 1980; Potier *et al.*, 1985). However, very little information is available about the stability of cellular proteins and the properties of the enzymes that are

involved in the turnover of proteins in such micro-organisms.

Potier *et al.* (1987*a, b*, 1990) reported that the proteolytic activity in extracts of the psychrotrophic *Arthrobacter* sp. strain S₁₅₅ grown at 10 °C was significantly more heat-labile than that in extracts of cells grown at 20 °C and 32 °C. I recently showed that the levels of individual proteins in the psychrophilic *Vibrio* sp. strain ANT-300 changed in response to the growth temperature (Araki, 1991*a, b*). Moreover, temperature shifts caused transient changes in the levels of a number of proteins. The results suggested that the levels of individual proteins may be regulated by degradation as well as by synthesis. The studies described here were undertaken to clarify whether, during the response of cells to changes in temperature, changes in rates of degradation of proteins *in vivo* are closely correlated with levels of proteases or of substrates that are susceptible to degradation.

Methods

Bacterial strain and growth conditions. The psychrophilic *Vibrio* sp. strain ANT-300 was grown in an artificial seawater medium,

Abbreviations: [¹⁴C-Me], ¹⁴C-methylated; PMSF, phenylmethylsulphonyl fluoride; HMPs, *p*-hydroxymercuriphenyl sulphonic acid; BSA, bovine serum albumin; TCA, trichloroacetic acid.

supplemented with nutrients, as previously described (Araki, 1991a). Cells were grown to the mid-exponential phase at either 0 °C or 13 °C. In the experiments that involved temperature shifts, portions of each culture were mixed with two volumes of fresh medium, which had been pre-equilibrated to the desired temperature, and held at this temperature for the periods indicated.

Preparation of cell-free extracts. Cells were harvested by centrifugation at 10000 *g* for 10 min at the growth temperature, washed with artificial seawater and then resuspended in 50 mM-Tris/HCl buffer (pH 7.8) containing 10 mM-MgCl₂, 2 mM-EDTA, 2 mM-Na₂S₂O₃, 50 mM-mercaptoethanol and 10% (v/v) glycerol, to give a final cell concentration of 250 mg wet weight ml⁻¹. Each suspension of cells was gently sonicated with an Artek sonic dismembrator for 2 × 60 s at 0 °C. After centrifugation at 20000 *g* for 30 min, the supernatant was dialysed for 20 h at 4 °C against 10 mM-Tris/HCl buffer (pH 7.8) containing 5 mM-MgCl₂, 1 mM-EDTA, 1 mM-Na₂S₂O₃, 50 mM-mercaptoethanol and 10% (v/v) glycerol (buffer A), and the insoluble material was removed by centrifugation. The concentration of protein in these preparations ranged between 50 and 60 mg ml⁻¹.

Fractionation of cell-free extracts on DEAE-Sephacel. Dialysed cell-free extracts (1 g protein) were applied to a DEAE-Sephacel column (1.5 × 15 cm) equilibrated with buffer A. The column was washed with two column volumes of the same buffer. Bound proteins were then eluted with a linear gradient of KCl from 0 to 0.4 M in a total volume of 300 ml. The flow rate was 20 ml h⁻¹, and fractions of 100 drops (about 3.5 ml) were collected. Absorbance at 280 nm was used to monitor eluted material.

Preparation of radiolabelled substrates. Bovine globin and insulin (Sigma) were methylated with [¹⁴C]formaldehyde (New England Nuclear) and sodium cyanoborohydride by the method of Dottavio-Martin & Ravel (1978). The specific activities of [¹⁴C-Me]globin and [¹⁴C-Me]insulin were 0.10 and 0.33 MBq (mg protein)⁻¹, respectively. [¹⁴C-Me]Casein (89 kBq mg⁻¹) and [¹⁴C-Me]cytochrome *c* (1 MBq mg⁻¹) were obtained from New England Nuclear.

Determination of rates of degradation of proteins in vivo. Cells grown to the mid-exponential phase at either 0 °C or 13 °C were separately transferred to the temperatures indicated and then incubated for 30 min at that temperature. The cells were then labelled with L-[U-¹⁴C]leucine (12.9 GBq mmol⁻¹, Amersham) at 148 kBq ml⁻¹ for 30 min at the temperature indicated. They were collected by centrifugation at 10000 *g* for 10 min, and resuspended in artificial seawater medium containing 1 mg non-radioactive leucine ml⁻¹ and 400 µg chloramphenicol ml⁻¹. Portions (5 ml) of each cell suspension were separately incubated at 0, 13 and 21 °C, and 0.5 ml aliquots were removed at appropriate intervals and mixed with 2 ml cold 10% (w/v) TCA. The precipitation of proteins was initiated by addition of 10 µl of 5% (w/v) BSA as a carrier to each tube. After centrifugation at 3000 *g* for 15 min, aliquots (0.5 ml) of the supernatant were monitored for radioactivity in the presence of 4.5 ml Aquasol-2 (New England Nuclear) in a liquid scintillation counter. The extent of protein degradation was estimated from the radioactivity liberated from cellular proteins into the TCA-soluble fraction and the parameter 'percentage proteolysis' was defined as the amount of radioactivity liberated expressed as a percentage of that present initially in cells.

Assay of proteolytic activity in vitro. The reaction mixture contained 50 mM-Tris/HCl buffer (pH 8.0), 10 mM-MgCl₂, 100 µg of radiolabelled proteins, with ATP and inhibitors added as indicated, in a final volume of 1.0 ml. The reaction was initiated by the addition of either cell extract (3 mg protein) or enzyme fraction (200 µl). Samples (100 µl) were removed at appropriate intervals and mixed with 10 µl 5% (w/v) BSA and 390 µl of 10% (w/v) TCA. After 30 min the samples were centrifuged at 3000 *g* for 15 min, and then the radioactivity in TCA-soluble fractions was determined as described above.

Fig. 1. Effects of temperature shifts and antibiotics on growth of *Vibrio* sp. strain ANT-300. Portions of a culture incubated at 0 °C were transferred to 16 °C at zero time (arrow), and incubated with no addition (○), with rifamicin (100 µg ml⁻¹) (△), or with chloramphenicol (400 µg ml⁻¹) (□). Other portions of the culture were maintained at 0 °C with no addition (●) or with chloramphenicol (400 µg ml⁻¹) (■).

Results

Effects of temperature shifts and antibiotics on growth

The psychrophilic *Vibrio* sp. strain ANT-300 grows exponentially at temperatures below 13 °C but cannot grow at temperatures above 18 °C. A shift-up from 0 °C to 16 °C resulted in an increased rate of growth (Fig. 1). An initial stimulation of growth was followed by a more gradual increase in growth rate. At 16 °C, *Vibrio* sp. strain ANT-300 showed apparently exponential growth during the first few hours, and numbers of filamentous cells increased progressively with time (data not shown). After a shift-up in the presence of antibiotics, chloramphenicol markedly inhibited growth after 2 h, and rifampicin completely inhibited growth after 45 min (Fig. 1). Chloramphenicol also inhibited the growth of cells incubated at 0 °C, with a similar time-course to that at 16 °C.

Degradation of cellular proteins after temperature shifts

When cells grown at 0 °C were labelled for 30 min with [¹⁴C]leucine at 0 °C and at 16 °C the radioactivity in TCA-soluble fractions was about 45% and 25%, respectively, of the total [¹⁴C]leucine incorporated into cells (Fig. 2a, b). During a subsequent chase, the radioactivity in TCA-soluble fractions of cells labelled at 0 °C decreased during the first 2 h at 0 °C and 45 min at 16 °C and then increased progressively with time. Even a high concentration of unlabelled leucine and chloramphenicol failed to prevent the reincorporation of [¹⁴C]leucine into cellular proteins in cells labelled and chased at low temperatures. Cells labelled at 16 °C showed no decrease

Fig. 2. Changes in TCA-soluble radioactivity liberated from ^{14}C -labelled cellular proteins during a chase. Cells grown at 0°C were labelled with [^{14}C]leucine in the absence ($\circ$, $\bullet$) or in the presence of rifampicin ($100\text{ }\mu\text{g ml}^{-1}$) (Δ) for 30 min at 0°C (a), for 30 min at 16°C (b), and for 30 min at 16°C after incubation for 2.5 h at 16°C (c), and then chased at 0°C ($\bullet$) or 16°C ($\circ$, Δ) in the presence of chloramphenicol ($400\text{ }\mu\text{g ml}^{-1}$). Each point represents the mean of duplicate assays in one of four separate experiments. All the experiments showed similar patterns; individual results were within 15% of one another.

Fig. 3. Effects of temperature shifts on the rate of protein degradation in *Vibrio* sp. strain ANT-300. Cells grown at 0°C (a) or at 13°C (b) were incubated for 30 min at the temperatures indicated, labelled with [^{14}C]leucine for 30 min at that temperature, and then chased in the presence of chloramphenicol ($400\text{ }\mu\text{g ml}^{-1}$) at 0°C ($\bullet$), 13°C ($\circ$), and 21°C ($\bullet$). The rates of protein degradation are expressed as the percentage of the total radioactivity present initially in the cells that was liberated in 60 min.

in the TCA-soluble radioactivity during the chase at either 0°C or 16°C , and the rate of increase in TCA-soluble radioactivity was slightly greater than that in cells labelled at 0°C . During the chase at 16°C , the rate of degradation of proteins in cells labelled at 16°C was less if the cells had been labelled in the presence of rifampicin. However, the rate was still greater than that in cells labelled at 0°C .

When cells that had been shifted to 16°C for 2.5 h were labelled with [^{14}C]leucine at 16°C for 30 min, the radioactivity in TCA-soluble fractions was about 30% of the total radioactivity (Fig. 2c). The rate of degradation

of proteins in these cells at either 0°C or 16°C was apparently increased over that in cells labelled immediately after the shift-up. The rate of degradation of proteins in cells labelled in the absence of rifampicin was slightly greater than that in cells labelled in the presence of rifampicin.

In order to clarify the effect of a temperature shift on the degradation of proteins, cells grown at either 0°C or 13°C were incubated for 30 min at various temperatures, labelled with [^{14}C]leucine for 30 min, and the subsequent rates of protein degradation at 0, 13, or 21°C were examined. The rate of protein degradation in cells grown at 0°C clearly increased as the labelling temperature increased (Fig. 3a). In contrast, the rate of protein degradation in cells grown at 13°C increased if the cells were labelled at temperatures either lower or higher than 13°C (Fig. 3b). When the cells were labelled at temperatures higher than 13°C , the increase in the rate of protein degradation in cells grown at 13°C was lower than that in cells grown at 0°C .

Proteolytic activity of cell-free extracts

The degradation of radiolabelled proteins by dialysed cell-free extracts was examined in the presence and absence of 3 mM-ATP (Table 1). Extracts from cells grown at 0°C and 13°C hydrolysed [^{14}C -Me]casein, [^{14}C -Me]globin and [^{14}C -Me]insulin to TCA-soluble material, but they had hardly any effect on [^{14}C -Me]cytochrome *c*, from which no TCA-soluble radioactivity could be detected after incubation at 30°C for 3 h.

The proteolytic activity of the extracts was dependent on the protein substrate. The extracts of cells grown at either 0°C or 13°C degraded insulin extensively in the absence of ATP, whereas their ability to hydrolyse insulin was somewhat reduced by the addition of ATP.

Table 1. *Effects of ATP on the degradation of various proteins by extracts of cells grown at 0 °C and at 13 °C*

Dialysed cell-free extracts were prepared from cells grown at either 0 °C or 13 °C and assayed for proteolytic activity at 30 °C in the presence or absence of 3 mM-ATP. All substrates were [¹⁴C-Me] derivatives. The proteolytic activities are expressed as the amount of TCA-soluble radioactivity, given as a percentage of that present initially in cells. The values given are means of five experiments ($\pm$ SD).

Growth temp.	Substrate	Proteolytic activity [% hydrolysed h ⁻¹ (mg protein) ⁻¹]		Activity with ATP (% of activity with no ATP)
		Without ATP	With ATP	
0 °C	Casein	1.7 $\pm$ 0.1	7.4 $\pm$ 0.6	435 $\pm$ 37
	Globin	3.5 $\pm$ 0.3	7.6 $\pm$ 0.6	217 $\pm$ 24
	Insulin	33.9 $\pm$ 2.1	30.2 $\pm$ 1.6	89 $\pm$ 5
	Cytochrome c	<0.01	<0.01	—
13 °C	Casein	1.6 $\pm$ 0.1	8.1 $\pm$ 0.5	506 $\pm$ 44
	Globin	3.5 $\pm$ 0.2	8.2 $\pm$ 0.6	234 $\pm$ 25
	Insulin	35.5 $\pm$ 2.0	31.6 $\pm$ 1.5	89 $\pm$ 5
	Cytochrome c	<0.01	<0.01	—

Table 2. *Effects of temperature shifts on the proteolytic activity in cells of Vibrio sp. strain ANT-300*

Dialysed cell-free extracts were prepared from cells either maintained at a constant temperature or exposed to temperature shifts. Degradation of [¹⁴C-Me]casein by cell-free extracts was assayed at 30 °C in the absence or presence of 3 mM-ATP. The proteolytic activities are expressed as the amount of TCA-soluble radioactivity released. The values given are means of five independent experiments ($\pm$ SD).

Growth temp.	Temp. shift	Proteolytic activity [d.p.m. h ⁻¹ (mg protein) ⁻¹]		Activity with ATP (% of activity with no ATP)
		Without ATP	With ATP	
0 °C	None	909 $\pm$ 54	3956 $\pm$ 328	435 $\pm$ 37
	16 °C, 3 h	879 $\pm$ 61	5212 $\pm$ 342	593 $\pm$ 48
13 °C	None	855 $\pm$ 57	4325 $\pm$ 268	506 $\pm$ 44
	0 °C, 3 h	859 $\pm$ 59	4464 $\pm$ 319	520 $\pm$ 47

In the absence of ATP, the rates of degradation of casein and globin were very low. The addition of ATP stimulated the degradation of these proteins by extracts of cells grown at either 0 °C or 13 °C. Maximal stimulation of proteolytic activity in both preparations was obtained at 3 mM-ATP (data not shown). In extracts of cells grown at 13 °C, the degree of stimulation by ATP was slightly higher than that in cells grown at 0 °C.

To determine the effects of temperature shifts on proteolytic activity, extracts were prepared from cells grown at 0 °C and from parallel batches of cells incubated for 3 h after a shift-up to 16 °C. The ability to degrade [¹⁴C-Me]casein was then measured. In the absence of ATP, both extracts degraded casein at almost the same rate. The proteolytic activity of both extracts was again markedly stimulated by the addition of ATP to 3 mM (Table 2): 4.4-fold and 5.9-fold stimulation for extracts from control and up-shifted cells, respectively.

Extracts were also prepared from cells grown at 13 °C and from cells incubated for 3 h after a shift-down from

13 °C to 0 °C. The proteolytic activity of both extracts was again markedly stimulated by the addition of ATP: 5.1-fold and 5.2-fold stimulation in extracts of control and down-shifted cells, respectively (Table 2). ATP had a much greater stimulatory effect upon the extracts of cells grown at 13 °C than upon those of cells grown at 0 °C, presumably because there were higher levels of ATP-dependent proteases. However, the change in the activity of ATP-dependent proteolysis after a shift-down was quite small.

Temperature dependence of proteolytic activity in the extracts

The effects of temperature on the degradation of [¹⁴C-Me]casein and [¹⁴C-Me]insulin were examined in dialysed cell-free extracts of cells grown at either 0 °C or 13 °C. In both types of extract, the rate of degradation of casein in the absence or presence of ATP displayed a similar temperature dependence (Fig. 4a). Similarly, in

Fig. 4. Temperature dependence of the degradation of [^{14}C -Me]casein (a) and [^{14}C -Me]insulin (b) by extracts of *Vibrio* sp. strain ANT-300. Dialysed cell-free extracts were prepared from cells grown at 0 °C (circles) and at 13 °C (triangles). The degradation of proteins was assayed at the temperatures indicated in the absence (●, ▲) or presence (○, △) of 3 mM-ATP. The points and bars represent means from five experiments and ranges of results, respectively.

the case of insulin degradation, the activity in both extracts decreased in parallel with a reduction in the reaction temperature (Fig. 4b). No change in the temperature characteristics, as estimated from slopes of Arrhenius plots, was observed in extracts prepared from cells subjected to a temperature shift (0 °C to 13 °C or vice versa) (data not shown).

Separation of proteolytic enzymes in cell-free extracts

When a dialysed cell-free extract prepared from cells grown at 0 °C was passed through a DEAE-Sephacel column, seven different peaks of proteolytic activity against casein and insulin were found (Fig. 5). These were denoted peaks I–VII, according to their order of elution from the column. Enzymes of peak I did not adsorb to DEAE-Sephacel and showed high activity against casein and insulin in the absence of ATP. Enzymes in peak II showed casein-degrading activity in the absence of ATP and showed some insulin-degrading activity, which overlapped with enzymes of peak III that contained both insulin- and casein-degrading activities. Enzymes of peak IV showed high activity against insulin (Fig. 5b). Enzymes of peak V, which eluted at 0.12–0.20 M-KCl, showed appreciable casein-degrading activity in the presence of 3 mM-ATP but not in its absence. Stimulation by ATP was 8–12-fold (Fig. 5a). Enzymes of peaks VI and VII showed activity against casein and insulin, respectively, although their separation with the DEAE-Sephacel column was incomplete.

When dialysed cell-free extracts prepared from cells grown at 13 °C, or from cells grown at either 0 °C or 13 °C and then exposed to temperature shifts, were applied to a DEAE-Sephacel column, the proteolytic enzymes showed qualitatively the same elution profile as that in Fig. 5.

Fig. 5. DEAE-Sephacel chromatography of proteolytic enzymes from *Vibrio* sp. strain ANT-300. Dialysed cell-free extract of cells grown at 0 °C was passed through a DEAE-Sephacel column and the bound proteins were eluted as described in Methods. The seven activity peaks are designated I–VII, according to their order of elution from the column. (a) Casein-degrading activity in the absence (○) or presence (●) of 3 mM-ATP was assayed at 20 °C for 120 min. —, Gradient of KCl concentration. (b) Insulin-degrading activity in the absence of ATP was assayed at 20 °C for 60 min (○). ●—●, A_{280} of eluted material.

Table 3. Effects of various protease inhibitors on the hydrolysis of casein by enzyme(s) of peak V

Reaction mixtures (100 μl) containing 50 mM-Tris/HCl (pH 8.0), 10 mM-MgCl₂, 3 mM-ATP, 20 μl of enzyme solution, and the indicated inhibitors were incubated for 5 min at 20 °C prior to the addition of [^{14}C -Me]casein (10 μg). Enzyme solution was prepared from cells grown at 0 °C. Incubations were done at 20 °C for 60 min. The relative activity is expressed as a percentage of the activity in the absence of inhibitors. The values given are means of four independent experiments.

Addition	Concn (mM)	Relative activity (%)
None	—	100
PMSF	2	85
	5	66
	10	34
HMPS	2	87
	5	74
N-Ethylmaleimide	5	84
	10	70
o-Phenanthroline	1	84
	2	65

Fig. 6. Thermostability of the proteolytic enzymes in *Vibrio* sp. strain ANT-300. Dialysed cell-free extracts (a), peak V (b) and peak IV (c) were pre-incubated for 30 min at the indicated temperatures and allowed to cool before the addition of substrate. The degradation of [^{14}C -Me]casein in the absence ($\circ$) or presence ($\bullet$) of 3 mM-ATP and of [^{14}C -Me]insulin (Δ) at 20 °C was measured. The results are expressed as percentages of the initial activity. The points and bars represent means from five experiments and ranges of results, respectively.

Characterization of ATP-dependent protease(s)

The casein-degrading activity of peak V was dependent on the presence of ATP and Mg^{2+} ions. Maximal stimulation of proteolytic activity was obtained at 3 mM-ATP. Other nucleoside triphosphates and ADP were less effective than ATP (data not shown). To characterize the enzyme(s) in peak V further, several protease inhibitors were tested (Table 3). The enzyme(s) appeared to be a serine protease since it was strongly inhibited by 10 mM-PMSF, although total inhibition was not achieved. In addition, this enzyme appeared to require free thiol groups for function, as *N*-ethylmaleimide and HMPS were also inhibitory. The ATP-dependent hydrolysis of casein was also affected by *o*-phenanthroline, which chelates divalent cations.

Thermostability of proteolytic enzymes

Dialysed cell-free extracts, and peaks IV and V, were pre-incubated for 30 min at various temperatures above 20 °C and their ability to hydrolyse casein and insulin was then examined at 20 °C in the absence or presence of 3 mM-ATP (Fig. 6). In the extracts of cells grown at 0 °C, the hydrolysis of insulin and the ATP-dependent hydrolysis of casein were both decreased to some extent by pre-incubation at temperatures above 32 °C. The ATP-independent hydrolysis of casein was increased by pre-incubation at temperatures between 30 and 35 °C, but was decreased if the pre-incubation temperature was above 38 °C (Fig. 6a). The enzymes in extracts prepared either from cells grown at 13 °C or from cells exposed to temperature shifts had the same thermostability as the enzymes in extracts of cells grown at 0 °C (data not shown).

The enzyme(s) responsible for ATP-dependent hydrolysis of casein in peak V was inactivated to some extent by pre-incubation at 25 °C and completely inactivated by

pre-incubation at temperatures above 40 °C, whereas the activity of enzymes responsible for ATP-independent hydrolysis of casein was increased by pre-incubation at temperatures between 25 and 30 °C (Fig. 6b). After pre-incubation at temperatures above 30 °C, the activity decreased, and the enzyme was completely inactivated by 30 min pre-incubation at temperatures above 40 °C. The insulin-degrading activity in peak V, which also contained some of the enzyme of peak IV, decreased slightly after pre-incubation at 30 °C and all activity was lost after heating at 60 °C for 30 min (Fig. 6b). The protease which hydrolysed insulin but not casein (peak IV) was inactivated by heating at temperatures above 30 °C, and its heat-induced inactivation profile was the same as that of insulin-hydrolysing enzyme in peak V (Fig. 6b, c).

Discussion

In the psychrophilic bacterium *Vibrio* sp. strain ANT-300, ATP-dependent proteolysis, measured in cell extracts, varied slightly with growth temperature. ATP-independent activity was not affected. ATP-dependent activity in the extracts of cells exposed to temperature shifts increased by about 36% and 17% as compared with the activity in extracts of cells grown solely at 0 °C and 13 °C, respectively (Tables 1 and 2). Similarly, ATP-stimulated proteolysis in *Arthrobacter globiformis* S₁55 has been demonstrated to be increased by a shift from 10 °C to 32 °C (Potier *et al.*, 1990). The proteolytic enzymes detected in cells of *Vibrio* sp. strain ANT-300 grown at different temperatures and exposed to temperature shifts showed the same temperature dependency and heat lability (Figs 4 and 6a). These results suggest that the level of ATP-dependent protease(s), rather than the type of enzyme, may be regulated in response to environmental temperature.

E. coli contains at least 15 different endoproteases, two of which are ATP-dependent serine proteases La and Ti (Clp) (Charette *et al.*, 1981; Chung & Goldberg, 1981; Hwang *et al.*, 1987; Katayama *et al.*, 1988; Miller, 1987). In *htpR* mutants the intracellular level of protease La and the cell's capacity to degrade abnormal proteins are reduced (Baker *et al.*, 1984; Goff *et al.*, 1984; Goff & Goldberg, 1987).

An ATP-dependent proteolytic system resembling that in *E. coli* (Murakami *et al.*, 1979) has been observed in unfractionated cell-free extracts of *Vibrio* sp. strain 14 (Car & Woods, 1984). In the present study, we found that *Vibrio* sp. strain ANT-300 contained at least seven distinct proteases and that the casein-degrading activity of one of these was strongly dependent on the presence of ATP and Mg^{2+} ions (Fig. 5). The enzyme(s) with ATP-dependent activity was sensitive to a serine protease inhibitor (PMSF) and thiol-group inhibitors (Table 3). Therefore, it seems likely that *Vibrio* sp. strain ANT-300 contains ATP-dependent serine protease(s), similar to those in *E. coli*. The ATP-dependent serine protease(s) might thus play an important role in the selective degradation of proteins in response to changes in temperature.

In the present study, the marked increase in the rate of degradation of proteins *in vivo* caused by a shift in temperature exceeded the increase in proteolytic activity detected in corresponding cell extracts (Table 2, Fig. 3). Moreover, the increase in the protein degradation rate *in vivo* caused by a shift-up in temperature was repressed by the addition of rifampicin (Fig. 2). These results suggest that the increased rate of protein degradation *in vivo* in cells exposed to temperature shifts may result not only from an increase in the level of protease(s) but also from increased synthesis of cellular proteins that are more susceptible to degradation.

The induction of the synthesis of proteins in response to temperature shifts has been reported in various cold-adapted micro-organisms (Araki, 1991*a, b*; Julseth & Inniss, 1990; Maniak & Nellen, 1988; McCallum *et al.*, 1986, 1989; McCallum & Inniss, 1990; Muller-Taubenberg *et al.*, 1988). The cellular response to changes in temperature could be associated with an increase in the synthesis of short-lived proteins that are programmed to bring about the reorganization of metabolic networks. Moreover, the accumulation of susceptible proteins would be a direct result of changes in temperature, since such changes would lead to miscoding during protein synthesis and to conformational changes in proteins present before the changes in temperature.

The thermostability of ATP-dependent proteases in cold-adapted micro-organisms is of particular interest. The ATP-dependent serine protease in *Vibrio* sp. strain ANT-300 was much more heat-labile than those reported

in *E. coli* (Goldberg *et al.*, 1981). The ATP-dependent protease activity was decreased by pre-incubation at temperatures above 25 °C, whereas the ATP-independent activity was increased by heating at temperatures between 25 and 30 °C, and then decreased above 30 °C (Fig. 6*b*). In crude extracts of *Arthrobacter globiformis* S₁₅₅ casein degradation is similarly increased by heating at temperatures between 40 and 60 °C (Potier *et al.*, 1990). The degree of the heat-induced activation in *A. globiformis* was much greater than that reported here. The physiological function of this apparently latent proteolytic activity, and the mechanisms by which the heating elicits activation, are still unknown.

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