Protein Degradation in Extracts of Exponential and Stationary Phase *Vibrio* Cells

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The degradation of the foreign protein [^{14}C]methyl apohaemoglobin ([^{14}C -me]globin) was stimulated by ATP in cell-free extracts from exponential phase and shaken and standing stationary phase *Vibrio* cells. A marked stimulation by ATP of the degradation of [^{14}C -me]globin was observed with exponential phase cell extracts which were preincubated for 30 min at 30 °C. Maximum stimulation was obtained with 3 mM-ATP and optimum degradation was at pH 8.0-8.5. Preincubation of extracts from both types of stationary phase cells did not affect the degree of ATP stimulation. The amount of ATP stimulation of [^{14}C -me]globin degradation by exponential phase extracts decreased markedly when the cells were starved in a growth limiting minimal medium before preparation of the cell extracts. In the exponential and both types of stationary phase extracts most of the activity was located in the cytoplasmic fractions. Although the periplasmic preparations contained a minor portion of the total activity, this activity showed a greater percentage stimulation by ATP. In the absence of ATP the specific proteolytic activities of the extracts from exponential and both types of stationary phase cells were similar. The proteolytic activities in all the cell extracts were inhibited to the same extent by phenylmethylsulphonyl fluoride, but the exponential and both types of stationary phase cell extracts were inhibited to different extents by EDTA and *p*-hydroxymercuribenzoate. The results suggest that the proteolytic systems responsible for the degradation of abnormal proteins are different in exponential and stationary phase *Vibrio* cells.

INTRODUCTION

Stationary phase cells of *Vibrio* strain 14 are novel and interesting in that they are able to support phage growth and provide an experimental system for studying regulation in non-sporulating stationary phase cells (Woods, 1976; Robb *et al.*, 1977, 1978). Phage growth on stationary phase cells occurs in standing cultures but not in shaken (aerated) cultures. After shaking has ceased the small, non-flagellated cells present in shaken stationary phase cultures become large, flagellated rods which resemble exponential phase cells (Robb *et al.*, 1980). Robb *et al.* (1980) suggested that the relatively high levels of protein synthesis in the small unhealthy cells from shaken stationary phase cultures may be due to non-specific and erroneous transcription. This could result in a 'sick' uncontrolled protein synthesis similar to that in relaxed mutants (Hall & Gallant, 1972; Travers, 1976), and would result in the production of a high proportion of abnormal proteins in shaken stationary phase cells. Since bacterial and animal cells rapidly degrade proteins with abnormal conformations (Goldberg & Dice, 1974), shaken stationary phase cells may have efficient proteolytic systems which hydrolyse abnormal proteins. In order to determine whether shaken stationary phase cells have an enhanced ability to degrade abnormal

Abbreviations: [^{14}C -me]Globin, [^{14}C]methyl apohaemoglobin; PHMB, *p*-hydroxymercuribenzoate, sodium salt; PMSF, phenylmethylsulphonyl fluoride; TMKD buffer, Tris/HCl (50 mM, pH 8.0), MgCl_2 (10 mM), KCl (10 mM), DTT (0.5 mM).

proteins, we investigated the ability of extracts from shaken and standing stationary phase cells to degrade the foreign protein [^{14}C -me]globin.

Previous studies with reticulocyte lysates have shown that [^{14}C -me]globin is rapidly degraded by the same proteolytic system that hydrolyses analogue-containing or puromycyl polypeptides (Etlinger & Goldberg, 1977). Murakami *et al.* (1979) reported that the degradative systems responsible for the hydrolysis of [^{14}C -me]globin and the disappearance of the small nonsense fragment of β -galactosidase, 545, were similar and concluded that this degradative system is responsible for the energy-dependent degradation of abnormal proteins in *Escherichia coli*. Since [^{14}C -me]globin has been shown to be a suitable substrate for the investigation of abnormal protein degradation in *E. coli* and other cells it was utilized in this study.

In *E. coli* the degradation of proteins is energy-dependent (Kowit & Goldberg, 1977) and protein degradation is stimulated by ATP in extracts of *E. coli* cells (Murakami *et al.*, 1979). This ATP-stimulated proteolytic system in *E. coli* appears to be responsible for the energy-dependent degradation of abnormal proteins. Since ATP concentrations in shaking and standing stationary phase cells are lower than in exponential phase cells (Robb *et al.*, 1980), we also investigated the effect of ATP on the hydrolysis of [^{14}C -me]globin.

METHODS

Media. The tryptone broth of Thomson & Woods (1974) was used for bacterial growth. Incubation was at 30 °C.

Bacteria. The strain previously isolated and classified as an *Achromobacter* strain 14 but recently reclassified as a *Vibrio* strain 14 was used (Robb *et al.*, 1980).

Stationary phase cultures. Stationary phase cultures of *Vibrio* strain 14 were routinely prepared by growth in 1 litre tryptone broth in a 2 litre round flat-bottomed flask on a Gallenkamp rotary shaker at 150 r.p.m. for 3 d at 30 °C (approximately 2×10^9 cells ml^{-1} ; Robb *et al.*, 1978). The culture was then divided: aeration of one half was continued (shaken culture) while the other half was transferred to a 1 litre Schott blue capped bottle and incubated aerobically without shaking (standing culture). The conditions obtained after 24 h standing have been shown to be optimal for stationary phase phage growth (Robb *et al.*, 1978). The levels of RNA and protein synthesis in the shaken and standing stationary phase cultures were determined and shown to be similar to those reported by Robb *et al.* (1980).

Starved exponential phase cells. Cells in exponential phase growth in tryptone broth were harvested and washed by centrifugation, resuspended in a prewarmed growth limiting minimal medium [Tris/HCl, 100 mM, pH 7.6; NaCl, 0.4 M; NH_4SO_4 , 7.6 mM; MgCl_2 , 0.4 mM; sodium citrate, 1.6 mM; glucose, 0.03% (w/v)] and incubated with shaking.

Preparation of cell-free extracts. Exponential and stationary phase cultures were harvested and washed twice by centrifugation at 10000 *g* for 10 min. The pellet of cells was frozen with liquid nitrogen and lysed by a modification of the method of Wickner *et al.* (1972). After thawing the pellet, the cells were suspended in 50 mM-triethanolamine buffer (pH 8.0) containing 10% (w/v) sucrose and 100 μg lysozyme ml^{-1} (2 ml buffer per g wet weight of cells) and incubated on ice for 30 min. KCl (0.5 M) and β -mercaptoethanol (10 mM) were added and the lysed suspension was centrifuged at 30000 *g* for 30 min at 4 °C, and the supernatant was dialysed against TMKD buffer. Dialysed samples (1 ml) were stored frozen in liquid nitrogen. Protein concentrations were determined by the method of Lowry using bovine serum albumin as a standard.

Preparation of radiolabelled substrates. Apohaemoglobin, prepared from bovine haemoglobin (Sigma) by the method of Yonetani (1967), was methylated with [^{14}C]formaldehyde (Rice & Means, 1971). The specific activity of the various preparations varied between 1 and 4×10^6 c.p.m. (mg protein) $^{-1}$.

Protein degradation. The degradation of the labelled substrates was measured by a modification of the method of Murakami *et al.* (1979) at 30 °C by incubating 100 μl extract (15 mg protein ml^{-1}) with 10 μg [^{14}C -me]globin (approximately 10^4 c.p.m.) in the presence or absence of nucleotides or inhibitors. Samples (50 μl) were removed at timed intervals, mixed with 50 μl bovine serum albumin (30 mg ml^{-1}) as a carrier and diluted into 100 μl cold TCA (10%, w/v). After 30 min the samples were centrifuged in an Eppendorf microfuge model 5412 for 1 min and the acid-soluble radioactivity released from the globin was determined. The optimum pH for the proteolytic activity of the extracts was determined in reaction mixtures which contained: cell extract, 50 μl ; [^{14}C -me]globin in saline, 25 μl ; and Tris/saline buffer [Tris/HCl, 200 mM; sodium chloride 0.85% (w/v)], 50 μl . The Tris/saline buffer was adjusted with acetic acid for pH values of 7.0 and below. The effects of the following protease inhibitors were determined; EDTA, *o*-phenanthroline, PHMB and PMSF at the concentrations given in Table 3. An appropriate dilution of the inhibitor (10 μl) was mixed with the cell-free extract (50 μl) for 5 min at 30 °C before the addition of the labelled substrate (65 μl). The acid-soluble radioactivity released after 1 h at 30 °C was assayed. PMSF was dissolved in dimethyl sulphoxide. Control experiments indicated that dimethyl sulphoxide had no effect on the protease activities of the cell extracts.

Cellular localization of proteases. Cytoplasmic and periplasmic proteolytic extracts were prepared by a modification of the method of Kaback (1971). Cells were harvested and washed by centrifugation and then resuspended in buffer [Tris/HCl 30 mM, pH 8.0; sucrose, 20% (w/v); EDTA, 10 mM; lysozyme, 100 $\mu\text{g ml}^{-1}$] and incubated on ice for 1 h. The supernatant obtained after centrifugation at 16000 g for 10 min was the periplasmic fraction. The pellet was resuspended in buffer [triethanolamine, 50 mM, pH 8.0; sucrose, 10% (w/v); mercaptoethanol, 10 mM; MgSO_4 , 5 mM; KCl, 0.5 M; DNAase, 5 $\mu\text{g ml}^{-1}$] and incubated on ice for 30 min, frozen in liquid nitrogen, and then thawed at 10 °C. The supernatant collected after centrifugation at 40000 g for 45 min was the cytoplasmic fraction. The fractions were dialysed for 12 h at 4 °C against TMKD buffer before determination of the proteolytic activities.

RESULTS

Proteolytic activities of cell extracts

The protein concentrations of the dialysed cell-free extracts from exponential and standing stationary phase cells were similar but the equivalent extracts from the shaken stationary phase cells were routinely less than half that of the other extracts. Proteolytic activities of the extracts against [^{14}C -me]globin were always determined at the same protein concentrations and were shown to be very similar without the addition of ATP (Table 1). The proteolytic activities of the extracts were stable for at least one month at -20 °C and for at least 5 d at 4 °C.

Effect of ATP on proteolytic activities

The degradation of [^{14}C -me]globin by dialysed cell-free extracts from exponential phase and shaken and standing stationary phase cells was only slightly stimulated by ATP (Table 2). Preincubation of the extracts from both types of stationary phase cells for 30 min at 30 °C did not markedly affect the degree of ATP stimulation (Table 2). However, preincubation of exponential phase cell extracts resulted in a marked stimulation of proteolytic activity by ATP (approximately 2.5- to 5-fold at 3 mM-ATP; Fig. 1). Although the experiments were repeated at least 20 times under identical conditions, it was not possible to reduce the variation in ATP stimulation between the different preincubated exponential phase extracts. The proteolytic

Table 1. *Specific activities of the dialysed cell-free extracts*

The cell-free extracts were diluted to the same protein concentration (10 mg ml^{-1}) and the activities determined without the addition of ATP. The activities shown are means of 4 experiments, $\pm$ SD.

Cell-free extract	Specific activity [μg globin hydrolysed min^{-1} (mg protein) $^{-1}$]
Exponential	0.57 $\pm$ 0.02
Shaken stationary	0.68 $\pm$ 0.08
Standing stationary	0.62 $\pm$ 0.03

Table 2. *Effect of preincubation on the degradation of [^{14}C -me]globin by extracts from exponential and stationary phase *Vibrio* cells*

Dialysed cell extracts were preincubated without ATP at 30 °C for 30 min before the addition of substrate with or without ATP (3 mM). The results are expressed relative to the dialysed extract assayed without preincubation or the addition of ATP, for which the specific activity was 0.57 μg globin hydrolysed min^{-1} (mg protein) $^{-1}$. The values given are the means of six independent experiments and the amount of variation was <10% from the mean.

Cell-free extract	Dialysed extract, not preincubated		Dialysed extract, preincubated	
	- ATP	+ ATP	- ATP	+ ATP
Exponential	1	1.76	1.07	3.70
Shaken stationary	1	1.73	1.29	1.63
Standing stationary	1	1.70	1.33	1.50

Fig. 1

Fig. 2

Fig. 1. Effect of ATP on the degradation of [^{14}C -me]globin by exponential and stationary phase *Vibrio* cell-free extracts. Preincubated cell extracts from exponential phase cells (a); dialysed cell extracts from shaken (b) and standing (c) stationary phase cells. The points represent means from 20 experiments for the exponential phase extract and 5 experiments for the stationary phase extracts; the bars represent ranges of results.

Fig. 2. Effect of ATP on the degradation of [^{14}C -me]globin by preincubated cell-free extracts from exponential cells resuspended in a growth limiting minimal medium. Cells were starved for 30 (a) and 90 (b) min before preparation of the cell extracts. The points represent means from 3 experiments; the bars represent ranges of results.

activities of the dialysed or preincubated extracts from both types of stationary phase cells and dialysed extracts from exponential phase cells showed less variation in ATP stimulation and were all within 20% of the values given. ATP stimulation of preincubated extracts from exponential phase cells was very sensitive to the method of cell breakage. When less gentle methods of cell lysis were used (several passages through a French pressure cell at 10.3 MPa) ATP stimulation of dialysed or preincubated extracts from exponential phase cells was not observed. Maximum protein degradation was obtained with 3 mM-ATP. The addition of GTP (1 and 2 mM) had no significant effect on the degradation of [^{14}C -me]globin. In exponential cell extracts, GTP (1 and 2 mM) caused a 15 and 13% decrease, respectively, in the proteolytic activities. The shaken and standing stationary phase extracts containing 1 and 2 mM-GTP showed <7% increase in proteolytic activity.

Although the proteolytic activities of the dialysed exponential and stationary phase extracts were stable at -20°C and 4°C for considerable periods, the amount of ATP stimulation decreased on storage.

The amount of ATP stimulation of [^{14}C -me]globin degradation by preincubated exponential phase cell-free extracts decreased markedly when the cells were starved in the growth-limiting minimal medium before preparation of the cell extracts (Fig. 2).

Effect of pH on proteolytic activities

The proteolytic activities of the exponential and the stationary phase extracts had a broad pH optimum between pH 7.0 and 9.5 but with marked inhibition below pH 7.0.

Table 3. Cellular localization of proteolytic activities from exponential and stationary phase *Vibrio* cells

Periplasmic and cytoplasmic cell-free extracts were prepared from exponential and both types of stationary phase cells and assayed for proteolytic activity with and without the addition of ATP (3 mM). The data from one of four independent experiments are given. The results from all the experiments were within 7% of the values given.

Cell-free extract	Activity (c.p.m.)		Stimulation (%)
	- ATP	+ ATP	
Periplasmic			
Exponential	237	349	47
Shaken stationary	430	523	22
Standing stationary	670	769	14
Cytoplasmic			
Exponential	2396	2785	16
Shaken stationary	2175	2516	16
Standing stationary	2261	2311	2

Table 4. Effect of inhibitors on the degradation of [^{14}C -me]globin by exponential and stationary phase *Vibrio* cell extracts

Proteolytic activities were determined without the addition of ATP. The values given are the average of five independent experiments and the amount of variation was <8% from the mean.

Inhibitor	Concn (mM)	Decrease in activity (%) in:		
		Exponential phase	Shaken stationary phase	Standing stationary phase
EDTA	10	94	80	84
	5	91	79	84
	1	0	54	25
<i>o</i> -Phenanthroline	0.5	51	76	66
PMSF	5	80	83	65
PHMB	5	35	69	67
	1	5	70	66
	0.15	0	72	73

Cellular localization of proteolytic activities

The proteolytic activities of periplasmic and cytoplasmic extracts were determined (Table 3). In all the exponential and stationary phase extracts most of the activity was located in the cytoplasmic fractions. Although the periplasmic preparations contained, in each case, a minor proportion of the total activity, they showed a greater percentage stimulation by ATP. Preincubation did not affect the proteolytic activities of the various fractions in the presence or absence of ATP.

Effect of protease inhibitors on proteolytic activities

The effects of various protease inhibitors were studied in the absence of ATP (Table 4). The proteolytic activities of the exponential and stationary phase cell extracts were all markedly inhibited by the metal chelating agents EDTA and *o*-phenanthroline. The extracts were more sensitive to *o*-phenanthroline (which preferentially chelates divalent cations other than Ca^{2+}) than to EDTA. The stationary phase cell extracts were more sensitive to EDTA than the exponential phase cell extracts. The serine protease inhibitor PMSF inhibited all the extracts to approximately the same extent. The thiol group inhibitor PHMB caused a 70% inhibition of the activities of the stationary phase cell extracts over a wide range of PHMB concentrations. The inhibition of the activities of the exponential phase cell extracts was markedly less sensitive to PHMB and the inhibition decreased with a decrease in PHMB concentration.

DISCUSSION

The ability of exponential and stationary phase cell extracts to degrade the foreign protein [^{14}C -me]globin was affected to different extents by ATP and preincubation. Although all the cell extracts were inhibited to the same extent by the serine protease inhibitor PMSF, the exponential and stationary phase extracts were inhibited to different extents by EDTA and PHMB. These results suggest that the proteolytic systems responsible for abnormal protein degradation in exponential and stationary phase *Vibrio* cells are different.

Although maximum stimulation of proteolytic activity by ATP in cell extracts from exponential *Vibrio* and *E. coli* (Murakami *et al.*, 1979) cells was obtained with 3 mM-ATP, the *Vibrio* extracts had to be preincubated before ATP stimulation was observed. In *E. coli* the stimulatory effect of ATP was lost after preincubation (Murakami *et al.*, 1979). The optimum pH for proteolytic activity in extracts from exponential *Vibrio* and *E. coli* cells was similar (pH 8.0 to 8.5). The bulk of *Vibrio* proteolytic activity was cytoplasmic; however, the periplasmic proteolytic activity showed a greater stimulation by ATP.

The amount of ATP stimulation varied considerably between preincubated extracts prepared under identical conditions from exponential phase cells. ATP stimulation was markedly affected by the method of cell breakage and this could play a role in the variation between different experiments. The amount of variation obtained in similar experiments with *E. coli* cell extracts by Murakami *et al.* (1979) was not indicated. In a recent study by Liu *et al.* (1984) on the degradation of abnormal proteins by extracts from yeast, similar variations in proteolytic activities were reported.

The shaken and standing stationary phase proteolytic systems responsible for the degradation of the foreign protein [^{14}C -me]globin were relatively insensitive to ATP stimulation when compared with exponential extracts. This suggests that these stationary phase proteolytic systems in *Vibrio* are different from the ATP-stimulated proteolytic system in *E. coli* which appears to be responsible for the energy-dependent degradation of abnormal proteins (Murakami *et al.*, 1979). Robb *et al.* (1980) reported that stationary phase *Vibrio* cells contain less ATP than exponential phase cells (20 to 57% less). St John & Goldberg (1978) reported that a moderate inhibition of energy metabolism (30 to 50% reduction) accelerated the breakdown of normal cell proteins in a similar fashion to starvation, but a drastic reduction of ATP levels (80% reduction) inhibited the degradation of all cell proteins. Starvation of exponential phase *Vibrio* cells markedly reduced the amount of ATP-stimulated proteolytic activity and the responses of extracts from starved cells to ATP were similar to those obtained with extracts from stationary phase cells.

There was little difference in the rate of proteolysis of the shaken and standing stationary phase extracts when measured against the foreign protein [^{14}C -me]globin. This is interesting as the amount of protein synthesis in the apparently 'healthy' standing stationary phase cells never exceeded 4% of that in the small 'sick' standing stationary phase cells (Robb *et al.*, 1980). Murakami *et al.* (1979) were able to demonstrate considerable differences (two- to threefold) between proteolysis of [^{14}C -me]globin in extracts of *E. coli* Deg⁺ and Deg⁻ strains. Greater proteolysis of this foreign protein in extracts of the Deg⁺ strain correlated with the more rapid breakdown of abnormal proteins in these cells and suggested that proteolysis in these preparations was related to breakdown *in vivo*. The failure to demonstrate differences in the specific activities of the shaken and standing stationary phase extracts suggests there is no general enhancement of proteolytic activities for the degradation of abnormal proteins in 'sick' shaken stationary phase cells.

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