Properties of the Membrane-bound 5'-Nucleotidase and Utilization of Extracellular ATP in *Vibrio parahaemolyticus*

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Vibrio parahaemolyticus utilized ATP, ADP or AMP as the sole source of carbon. About three times higher activity of membrane-bound 5'-nucleotidase was observed in cells grown in the presence of these nucleotides than in their absence: and therefore the enzyme seems to be inducible. Since the 5'-nucleotidase activity could be measured with whole cells, the active site of this enzyme appears to be outwardly oriented. Both Mg^{2+} and Cl^- were required for activity. Among the divalent cations tested, Mn^{2+} and Co^{2+} could replace Mg^{2+} to some extent, whereas Zn^{2+} strongly inhibited activity. Among the anions tested, Br^- , I^- and NO_3^- could replace Cl^- , but SO_4^{2-} and CH_3COO^- could not. When cells were grown with ATP, Cl^- was indispensable and Zn^{2+} strongly inhibited growth. Therefore, it is concluded that extracellular ATP and other 5'-nucleotides are cleaved by the membrane-bound 5'-nucleotidase outside the cells and that the adenosine produced is then utilized.

INTRODUCTION

5'-Nucleotidase is widely distributed in nature. In animal cells this enzyme is a marker for cytoplasmic membranes. The main contribution of 5'-nucleotidase in membranes to cellular metabolism is hydrolysis of extracellular nucleoside 5'-monophosphates (Frick & Lowenstein, 1978). The properties and roles of this enzyme in animal cells have been extensively investigated (Ahmed & Reis, 1958; De Pierre & Karnovsky, 1974; Naito & Lowenstein, 1981; Harb *et al.*, 1983). 5'-Nucleotidase is also present in many bacteria (Kohn & Reis, 1963), where it occurs in the periplasm as well as in the cytoplasm and cytoplasmic membrane. The periplasmic 5'-nucleotidase of *Escherichia coli* has been purified and extensively characterized (Neu, 1967).

Several marine bacteria possess strong activity of membrane-bound 5'-nucleotidase (Drapeau & MacLeod, 1963; Thompson *et al.*, 1969; Hayashi *et al.*, 1970; Bengis-Garber, 1985). Bengis-Garber & Kushner (1981) purified the 5'-nucleotidase from the cytoplasmic membrane of a moderately halophilic bacterium, *Vibrio costicola*. This enzyme was an intrinsic membrane protein that was solubilized with detergent; the activity of the purified preparation was markedly stimulated by high concentrations of NaCl and required Mg^{2+} . The hydrolysis of ATP, ADP and AMP by whole cells of *V. costicola* was also stimulated by NaCl; in addition this organism could grow with adenosine or AMP as sole carbon source (Bengis-Garber & Kushner, 1982).

We have been studying membrane-related phenomena in the marine bacterium *Vibrio parahaemolyticus* (Tsuchiya & Shinoda, 1985), a major cause of food-poisoning in Japan and we are interested in the membrane-bound 5'-nucleotidase of this organism. Here we report the properties and roles of this enzyme.

METHODS

Bacterium and growth. *V. parahaemolyticus* AQ3334 cells (Tsuchiya & Shinoda, 1985) were grown aerobically at 37 °C in medium S consisting of 50 mM-Tris/ H_2SO_4 (pH 7.5), 25 mM- $MgSO_4$, 10 mM-KCl, 0.33 mM- K_2HPO_4 , 1 mM- $CaCl_2$, 0.01 mM- $FeSO_4$, 10 mM- $(NH_4)_2SO_4$ and 0.2 M-NaCl supplemented with 0.5% (w/v) polypeptone unless otherwise stated. For testing the effect of Cl^- , the cells were grown in SO_4^{2-} -medium, which had the same

composition as medium S except that SO_4^{2-} salts replaced Cl^- salts. Growth was monitored as optical density at 650 nm. Cells were harvested in the late exponential phase of growth, washed twice with buffer containing 20 mM-Tricine (adjusted to pH 8.0 with Tris), 0.2 M-NaCl (or 0.2 M- Na_2SO_4) and 20 mM- MgSO_4 (unless otherwise indicated), and resuspended in the same buffer.

Assay of 5'-nucleotidase. The standard assay mixture (0.6 ml) consisted of 20 mM-Tricine/Tris pH 8.0, 20 mM- MgSO_4 , 0.2 M-NaCl, 4 mM-AMP or ATP as the substrate and about 4 μg cell protein. Assay mixtures were incubated at 37 °C for 15 min, and the released inorganic phosphate was determined by the method of Fiske & SubbaRow (1925). One unit of activity is defined as the release of 1 μmol inorganic phosphate min^{-1} . Protein was determined by the Lowry method with bovine serum albumin as standard.

RESULTS

Growth of cells with nucleotides

The growth of *V. parahaemolyticus* with ATP, ADP, AMP or adenosine as the sole source of carbon was as fast as that with glucose (Fig. 1). Cells could not grow with adenine as the sole source of carbon. *Vibrio* possesses membrane-bound 5'-nucleotidase (Bengis-Garber, 1985) and as shown below, the 5'-nucleotidase activity of *V. parahaemolyticus* could be measured with whole cells as in *V. costicola* (Bengis-Garber & Kushner, 1982).

Properties of the membrane-bound 5'-nucleotidase

The 5'-nucleotidase activity of cells grown in the presence of 5'-nucleotides was much higher than that of cells grown in their absence, suggesting that the enzyme might be induced by the substrates. Since 5'-nucleotides seem to be cleaved by 5'-nucleotidase outside the cells, the direct inducer of the enzyme is presumably adenosine. During the hydrolysis of ATP and ADP we could not detect ADP, AMP or pyrophosphate, but only adenosine and inorganic phosphate as products (data not shown), suggesting that there is no significant accumulation of an intermediate. Therefore, we tested whether adenosine could induce the 5'-nucleotidase. The 5'-nucleotidase activity of cells grown in the absence of adenosine was about 0.8 units (mg cell protein) $^{-1}$ and increased to a maximum of about 3 units (mg cell protein) $^{-1}$ about 2 h after the addition of adenosine to the culture medium (Fig. 2). The addition of adenosine did not significantly affect cell growth. Similar induction profiles were obtained with ATP, ADP and AMP (data not shown), the enzyme activity being about three times that in uninduced cells (data not shown).

Since the membrane-bound 5'-nucleotidases of *V. alginolyticus* and *V. costicola* have been reported to be stimulated by Cl^- (Hayashi *et al.*, 1970; Bengis-Garber & Kushner, 1981), we tested the effect of Cl^- on the 5'-nucleotidase in cells of *V. parahaemolyticus* grown in the absence of added Cl^- . With AMP as substrate, a relatively low concentration of Cl^- (about 10 mM) gave a maximum stimulation of the enzyme, but with ATP higher concentrations of Cl^- (50–200 mM) were necessary for full stimulation (Fig. 3). The time course of enzyme induction was similar in the absence and presence of Cl^- (data not shown), although the 5'-nucleotidase activity of cells grown in the absence of Cl^- was considerably lower than that of cells grown in its presence. Thus the 5'-nucleotidase may be partly inactivated in the absence of Cl^- or alternatively, Cl^- may be necessary for full induction of the enzyme. The stimulatory effect of NaCl on the 5'-nucleotidase of *V. costicola*, a moderately halophilic bacterium, was reported to be 2–3 times that of KCl, but we found no significant difference between the effects of NaCl and KCl on the 5'-nucleotidase of *V. parahaemolyticus*, a slightly halophilic bacterium.

Of the anions tested, Br^- had as great a stimulatory effect as Cl^- , I^- and NO_3^- had considerable stimulatory effects, but CH_3COO^- and SO_4^{2-} were not stimulatory (Table 1). Monovalent cations (Na^+ , K^+ , Li^+ and NH_4^+) did not influence the effects of these anions. Activity was stimulated by Mg^{2+} and high concentrations of Mg^{2+} (10–20 mM) were necessary for maximum activity with ATP as substrate, while lower concentrations (about 1 mM) were sufficient with AMP as substrate (Fig. 4). Of the other divalent cations tested, Mn^{2+} and Co^{2+} could replace Mg^{2+} to some extent, but Ca^{2+} , Fe^{2+} , Ni^{2+} and Zn^{2+} could not with AMP as substrate (data not shown). Zn^{2+} was reported to inhibit the 5'-nucleotidase of animal cells (Ahmed & Reis, 1958) and strongly inhibited the 5'-nucleotidase of *V. parahaemolyticus* (Fig. 5).

Fig. 1

Fig. 2

Fig. 1. Growth of *V. parahaemolyticus* with nucleotides. Cells were cultured in medium S supplemented with 12 mM-ATP (○), 12 mM-ADP (●), 12 mM-AMP (□), 12 mM-adenosine (■), 20 mM-glucose (▲) or 24 mM-adenine (△) at 37 °C.

Fig. 2. Induction of 5'-nucleotidase by adenosine. Two cultures were grown in medium S supplemented with 0.5% polypeptone as carbon source with shaking at 37 °C. Adenosine (3 mM) was added to one culture at zero time (●); no addition was made to the other culture (○). Samples were taken at intervals, for measurement of their OD₆₅₀ values (a) and 5'-nucleotidase activity with AMP as substrate (b).

Fig. 3

Fig. 4

Fig. 3. Effect of Cl⁻ concentration on the 5'-nucleotidase activity of *V. parahaemolyticus*. Cells were grown in medium S (in which Cl⁻ salts were replaced with SO₄²⁻ salts) supplemented with 0.5% polypeptone and 5 mM-adenosine, and washed with buffer containing 20 mM-Tricine/Tris pH 8.0, 0.2 M-Na₂SO₄ and 20 mM-MgSO₄. The 5'-nucleotidase activity in cells was measured with ATP (●) or AMP (○) as substrate. The total concentration of NaCl plus Na₂SO₄ in the assay mixtures was kept at 0.2 M.

Fig. 4. Effect of Mg²⁺ on the 5'-nucleotidase activity of *V. parahaemolyticus*. Cells were grown in the presence of 0.5% polypeptone plus 5 mM-adenosine and washed with buffer containing 20 mM-Tricine/Tris pH 8.0 and 0.2 M-NaCl. The indicated concentrations of MgSO₄ were present in the assay mixtures. ATP (●) or AMP (○) was added as substrate.

However, inhibition by Zn²⁺ was greater with ATP than with AMP as substrate. For example, 1 mM-Zn²⁺ inhibited ATP hydrolysis strongly, but AMP hydrolysis only partially, while 10 mM-Zn²⁺ inhibited the hydrolysis of both substrates almost completely; 50% inhibition was observed at 0.1 mM-Zn²⁺ with ATP, and 0.5 mM with AMP.

All the 5'-nucleotides tested were hydrolysed: ATP, ADP, AMP and IMP were hydrolysed at comparable rates, whereas GMP, CMP and UMP were hydrolysed more slowly (Table 2).

Fig. 5

Fig. 5. Inhibition of the 5'-nucleotidase by Zn^{2+} . The 5'-nucleotidase activity in cells was measured in the presence of various concentrations of ZnSO_4 . Assay mixtures contained either ATP (●) or AMP (○) as substrate.

Fig. 6

Fig. 6. Effect of Cl^- on ATP hydrolysis and on growth rate. Hydrolysis of ATP by cells was measured in the presence of the indicated concentrations of Cl^- (○). The effect of the Cl^- concentration in medium S on growth of the cells with 20 mM-ATP as carbon source was also tested (●). The total concentration of NaCl plus Na_2SO_4 in the media was kept at 0.2 M.

Table 1. *Effects of monovalent cations and anions on the 5'-nucleotidase*

Salt	Salt concn (M)	5'-Nucleotidase activity* [units (mg protein) ⁻¹]
NaCl	0.2	2.61
NaBr	0.2	2.61
NaI	0.2	2.01
NaNO_3	0.2	1.97
NaOCOCH_3	0.2	0.48
Na_2SO_4	0.2	0.30
Na_2SO_4	0.1	0.35
KCl	0.2	2.64
LiCl	0.2	2.75
NH_4Cl	0.2	2.43

* Activity of whole cells was measured with AMP as substrate.

Table 2. *Substrate specificity of the 5'-nucleotidase*

Substrate*	Dephosphorylation activity† [units (mg protein) ⁻¹]
5'-ATP	3.39
5'-ADP	3.46
5'-AMP	3.00
5'-IMP	3.26
5'-GMP	2.20
5'-CMP	1.34
5'-UMP	2.24
3'-AMP	0.06
3'-GMP	0.09
Ribose 5-Phosphate	0.14

* Substrates were added at 4 mM.

† Activity of whole cells was measured.

Fig. 7. Effect of Zn^{2+} on growth. *V. parahaemolyticus* cells were grown in medium S supplemented with 20 mM-ATP (●) or 20 mM-adenosine (○) in the absence (a) or presence (b) of 0.5 mM- ZnSO_4 .

3'-Nucleotides and ribose 5-phosphate were not hydrolysed. Three mols of inorganic phosphates are released from one mol of ATP, and only one from AMP. For assay of enzyme activity we determined the release of inorganic phosphate, and observed similar release from both substrates indicating that AMP is a better substrate than ATP with respect to production of adenosine.

The purified 5'-nucleotidase of *V. costicola* consisted of one major polypeptide of 70000 Da as shown by SDS-PAGE (Bengis-Garber & Kushner, 1981). We partially purified the 5'-nucleotidase of *V. parahaemolyticus* after its solubilization from the membranes with a detergent (unpublished results). The apparent molecular mass of the enzyme was similar to that of the enzyme of *V. costicola*.

Effects of Cl^- and Zn^{2+} on cell growth

The 5'-nucleotidase activity with ATP as substrate was totally dependent on Cl^- and was strongly inhibited by a relatively low concentration of Zn^{2+} (Figs 3 and 5). *V. parahaemolyticus* grew with ATP as carbon source only when sufficient Cl^- was present, and there was a good correlation between the effects of Cl^- concentration on hydrolysis of ATP and on cell growth with ATP (Fig. 6). With adenosine as the sole carbon source, Cl^- was not required for growth (data not shown). These results imply that hydrolysis of ATP by the 5'-nucleotidase is rate-limiting for growth with ATP as the sole carbon source.

In the presence of 0.5 mM- Zn^{2+} , cell growth was completely inhibited with ATP as the carbon source but was not inhibited significantly with adenosine as the carbon source (Fig. 7) indicating that Zn^{2+} did not have any significant effect on adenosine transport, adenosine metabolism or other essential metabolic pathways.

These results are consistent with the view that external ATP (and perhaps other 5'-nucleotides) was dephosphorylated by the membrane-bound 5'-nucleotidase, and that the adenosine produced was taken up and utilized by the cells. We have found an adenosine transport system(s) in *V. parahaemolyticus* (unpublished results).

DISCUSSION

The properties of the 5'-nucleotidase in whole cells of *V. parahaemolyticus* were very similar to those of the enzyme in the membrane fraction of *V. alginolyticus* (Hayashi *et al.*, 1970), and to those of the 5'-nucleotidase purified from the membranes of *V. costicola* (Bengis-Garber & Kushner, 1981). The 5'-nucleotidase described in this paper is presumably the same as that reported to be a cation-stimulated enzyme in *V. parahaemolyticus* (Hayashi *et al.*, 1965). Since

extracellular nucleotides are thought to be cleaved to nucleosides or bases outside the cytoplasmic membrane before uptake into cells (Bengis-Garber & Kushner, 1982), the catalytic site of the membrane bound 5'-nucleotidase of *V. parahaemolyticus* seemed to be outwardly oriented in the cytoplasmic membrane, and so extracellular nucleotides could reach the enzyme. Therefore, ATP, ADP and AMP were probably hydrolysed by the 5'-nucleotidase of *V. parahaemolyticus* to adenosine, and the adenosine then taken up by cells for use as a carbon source.

We observed some differences in the effects of Mg^{2+} , Cl^{-} and Zn^{2+} on the hydrolysis of ATP and AMP by the 5'-nucleotidase. There are two possible explanations for these differences: the effects of ions on the enzyme may differ to some extent depending on the substrate or two similar enzymes (ATPase and AMPase) may be present. The former explanation seems to be correct, because adenosine induced ATP and AMP hydrolysing activity simultaneously and the purified 5'-nucleotidase of *V. costicola* hydrolysed both ATP and AMP (Bengis-Garber & Kushner, 1981). Since we observed similar growth rates with ATP and AMP, hydrolysis of the nucleotides by the 5'-nucleotidase did not seem to be a rate-limiting step for cell growth when sufficient Cl^{-} was present. However, with insufficient (less than 50 mM) Cl^{-} the supply of adenosine from ATP by the 5'-nucleotidase did not seem rapid enough for maximum cell growth, and thus hydrolysis of ATP must have been rate-limiting.

Requirement for Cl^{-} is a unique characteristic of the 5'-nucleotidase of *Vibrio*. Since *Vibrio* lives in Cl^{-} -rich conditions, this property should be advantageous for its function. No nucleotide transport system is known in the cell membrane of any micro-organism except the intracellular parasites *Bdellovibrio bacteriovorus* (Ruby *et al.*, 1985), *Rickettsia prowazeki* (Winkler, 1976), *Chlamydia psittaci* (Hatch *et al.*, 1982) and *Mycoplasma mycoides* (Neale *et al.*, 1984), which utilize nucleotides of the host cell. In general, nucleotides seem to be hydrolysed to nucleosides before uptake by bacterial cells. Thus the Cl^{-} -stimulated 5'-nucleotidase of *V. parahaemolyticus* seems to be indispensable for utilization of 5'-nucleotides, which have been reported to be present at low levels in sea water (Azam & Hodson, 1977).

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