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Role of Membrane-Bound 5'-Nucleotidase in Nucleotide Uptake by the Moderate Halophile *Vibrio costicola*

CARMELA BENGIS-GARBER†* AND DONN J. KUSHNER

Department of Biology, University of Ottawa, Ottawa, Ontario, Canada K1N 6N5

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Intact cells of *Vibrio costicola* hydrolyzed ATP, ADP, and AMP. The membrane-bound 5'-nucleotidase (C. Bengis-Garber and D. J. Kushner, J. Bacteriol. **146**:24–32, 1981) was solely responsible for these activities, as shown by experiments with anti-5'-nucleotidase serum and with the ATP analog, adenosine 5'-(βγ-imido)-diphosphate. Fresh cell suspensions rapidly accumulated 8-^{14}C-labeled adenine 5'-nucleotides and adenosine. The uptake of ATP, ADP, and AMP (but not the adenosine uptake) was inhibited by adenosine 5'-(βγ-imido)-diphosphate similarly to the inhibition of the 5'-nucleotidase. Furthermore, the uptake of nucleotides had Mg^{2+} requirements similar to those of the 5'-nucleotidase. The uptake of ATP was competitively inhibited by unlabeled adenosine and vice versa; inhibition of the adenosine uptake by ATP occurred only in the presence of Mg^{2+}. These experiments indicated that nucleotides were dephosphorylated to adenosine before uptake. The hydrolysis of [α-^{32}P]ATP as well as the uptake of free adenosine followed Michaelis-Menten kinetics. The kinetics of uptake of ATP, ADP, and AMP also each appeared to be a saturable carrier-mediated transport. The kinetic properties of the uptake of ATP were compared with those of the ATP hydrolysis and the uptake of adenosine. It was concluded that the adenosine moiety of ATP was taken up via a specific adenosine transport system after dephosphorylation by the 5'-nucleotidase.

We have recently purified a specific 5'-nucleotidase (EC 3.1.3.5) from the membrane of a moderate halophile, *Vibrio costicola* (3). The enzyme hydrolyzed ATP, ADP, and AMP with adenosine and phosphate as end products and required high concentrations of Mg^{2+} for optimal activity. We have been very interested in a possible physiological role of this novel enzyme. Preliminary experimental evidence suggested that, in the intact cell, this nucleotidase was oriented in the cytoplasmic membrane toward exogenous nucleotides. Since bacterial plasma membranes are considered impermeable to nucleotides (5), we thought that the physiological function of this enzyme might reside in cleaving extracellular nucleotides for uptake.

Experiments described here showed that in whole cells of *V. costicola* the membrane-bound 5'-nucleotidase dephosphorylates exogenous adenine 5'-nucleotides to adenosine, which is then taken up by a mediated transport mechanism.

MATERIALS AND METHODS

Materials. 8-^{14}C-labeled ATP, adenosine, and adenine were the products of New England Nuclear Corp.; [8-^{14}C]ADP, [8-^{14}C] AMP, [α-^{32}P]ATP, and [γ-^{32}P]ATP were obtained from Amersham Corp. Unlabeled adenine 5'-nucleotides, adenosine, adenine, and sugar phosphates were purchased from Sigma Chemical Co. Adenosine 5'-(βγ-imido)-diphosphate (AMP-PNP) was obtained from Boeringer-Mannheim Corp. *N,N*-Dicyclohexylcarbodiimide (DCCD) was the product of Sigma. All chemicals used were of analytical grade.

Cell growth. *V. costicola* NRC 37001 was grown in proteose peptone-tryptone broth containing 1 M NaCl as previously described (3). For all assays of the 5'-nucleotidase activities and uptake of radioactively labeled substrates, the cells were grown in the above medium to an optical density at 660 nm of 0.9 to 1.3 and washed once with a buffer containing 50 mM Tris-hydrochloride (pH 8), 2 mM $MgCl_2$, and 1.1 M NaCl. No differences in the uptake were observed when phosphate buffer (pH 8) was used instead of Tris-hydrochloride or when 1.1 M NaCl was replaced by 1 M NaCl plus 10% sucrose. The pellet was suspended in the washing solution at a protein concentration of 1.5 mg/ml and used for the assays at once.

For experiments testing growth on different carbon sources, the cells were grown in a minimal medium in the presence of 1 M NaCl (20), in which glucose was replaced by an appropriate substrate. Each substrate was added to a final concentration of 1% (wt/vol).

Uptake of radioactively labeled adenine 5'-nucleotides, adenosine, and adenine. The reaction mixture for the uptake experiments contained (in a final volume of 1 ml) 50 mM Tris-hydrochloride (pH 8), 2 mM $MgCl_2$, 5 mM K_2HPO_4, 1 M NaCl, and one of the following 8-

† Present address: Department of Biology, The Technion-Israel Institute of Technology, Haifa 32000, Israel.

^{14}C-labeled substrates in concentrations of 0.1 to 4 μM: ATP (55.4 Ci/mol), ADP (55 Ci/mol), AMP (59 Ci/mol), adenosine (45.5 Ci/mol), or adenine (55.6 Ci/mol).

The uptake was started by the addition of 50 μl of fresh cell suspensions and stopped, after 90 s of incubation at 30°C, by rapid filtration of 0.5-ml samples through a 0.45-μm membrane filter (type HA; Millipore Corp.) in a Millipore 1225 sampling manifold. The filters were washed three times with 1-, 3-, and 3-ml samples of the buffer used for suspending the cells, and their radioactivity was measured in a liquid scintillation counter. Uptake values at zero time were determined for each sample and subtracted in all cases. In all kinetic experiments the concentrations of substrates after 45-s incubations were calculated and used in the determination of kinetic parameters whenever initial concentrations decreased at least 5%.

Assays of the 5'-nucleotide phosphohydrolyzing activities. For kinetic studies on the 5'-nucleotidase in whole cells, [α-^{32}P]ATP (41 Ci/mmol) or [γ-^{32}P]ATP (4.02 Ci/mmol) was used in concentrations of 0.1 to 4 μM. The reaction mixtures were identical to those of the uptake experiments. The reaction was started by adding cells and stopped after 30 s of incubation at 30°C by adding 0.1 ml of 60% trichloroacetic acid. The precipitated protein was removed by centrifugation, and 50- to 200-μl samples were added to 4 ml of 1.2% ammonium molybdate in 1.2 N HCl. The released P_i was separated from ATP by one extraction with isobutanol-benzene-acetone as described by Nelson et al. (12) and counted for radioactivity in a liquid scintillation counter. Zero time values for each sample were obtained by adding cells to the reaction mixtures containing 0.1 ml of 60% trichloroacetic acid. Assays of the hydrolysis of adenine 5'-nucleotides with unlabeled substrates were performed by the method of Taussky and Shorr (22) as previously described (3).

Protein determination. The concentrations of protein were determined by the method of Lowry et al. (9) as previously described (3).

Antibody preparation. The 5'-nucleotidase was purified from the membranes of *V. costicola* as previously described (3), and the rabbit antibody against the purified enzyme was prepared by the method of Nelson et al. (13). Immunodiffusion experiments were performed by a published procedure (15); agarose gel plates contained 0.5% Triton X-100.

RESULTS

Hydrolysis of adenine 5'-nucleotides by whole cells of *V. costicola*. To establish the orientation of the 5'-nucleotidase in the cytoplasmic membrane, its activities were assayed, in comparison with the DCCD-sensitive F_0F_1 ATPase, in intact and lysed cells. The F_0F_1 ATPase in bacteria is located on the inside of the cytoplasmic membrane (24). We had previously shown that the DCCD-sensitive ATPase and the 5'-nucleotidase could be selectively assayed in membrane preparations from *V. costicola*, using 2 and 20 mM $MgCl_2$ respectively, with 4 mM ATP as a substrate (3). In the experiment depicted in Fig. 1, cells grown at 1 M NaCl were diluted 20-fold into the reaction mixtures containing 0 to 1 M NaCl and assayed for the 5'-nucleotidase and DCCD-sensitive ATPase. Electron micrographs showed that such dilutions into the assay mixtures containing less than 0.5 M NaCl caused local disruptions of the envelope or complete lysis depending on the concentrations of NaCl; the lysis was accompanied by a drastic fall in turbidity (data not shown). It is evident from Fig. 1B that the DCCD-sensitive ATPase was maximally detectable in lysed cells and negligible in intact cells (0.5 M NaCl and above). On the other hand, the rates of the ATP hydrolysis in the presence of 20 mM $MgCl_2$ were similar in

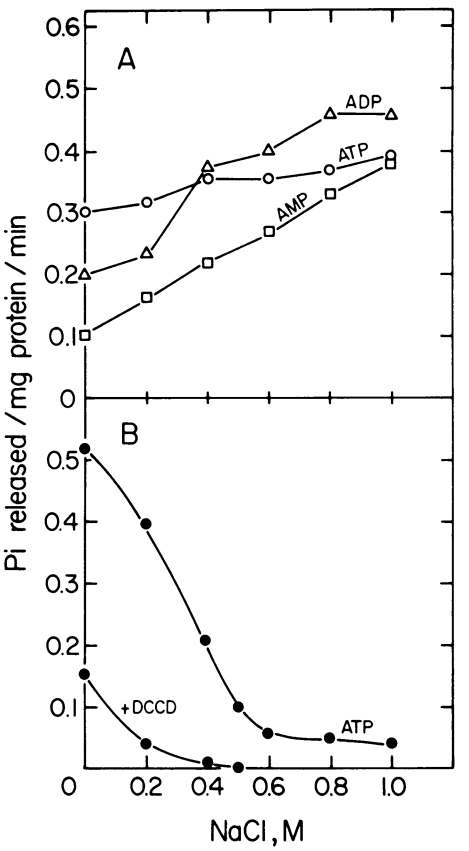

FIG. 1. Activities of the 5'-nucleotidase and the DCCD-sensitive ATPase in whole cells of *V. costicola* in the presence of various NaCl concentrations in the reaction mixture. Washed cells of *V. costicola*, suspended in a solution of 1 M NaCl, 10% sucrose, and 50 mM Tris-hydrochloride (pH 8) at a concentration of 2 mg of protein per ml, were diluted 20-fold into reaction mixtures containing NaCl in concentrations varying from 0 to 1 M and were assayed for 5'-nucleotidase (A) and DCCD-sensitive ATPase (B). The assays were performed as described in the text. All nucleotides were added to 4 mM. The 5'-nucleotidase and DCCD-sensitive ATPase activities were assayed in the presence of 20 and 2 mM $MgCl_2$, respectively.

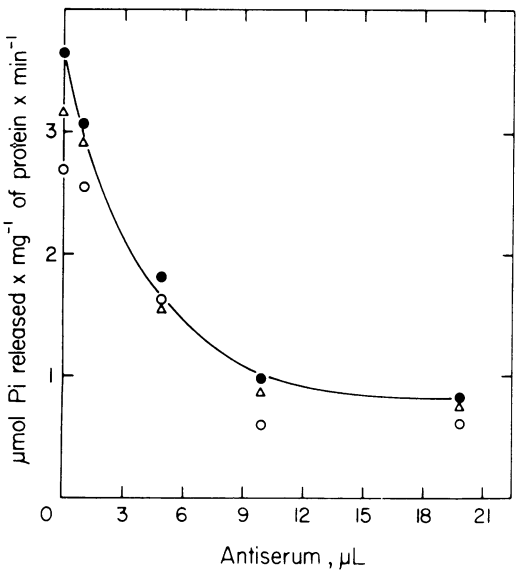

FIG. 2. Antibody inhibition of the purified 5′-nucleotidase of *V. costicola*. The 5′-nucleotidase was purified as previously described (3). The reaction mixture for the assay of the ATP, ADP, and AMP hydrolysis contained (in a final volume of 1 ml) 50 mM Tris-hydrochloride (pH 8), 1 M NaCl, 20 mM $MgCl_2$, 10 μg of the purified enzyme, and the volume of the antiserum indicated in the figure. After 10 min of incubation at room temperature the reaction was started with 4 mM either ATP, ADP, or AMP (final concentrations). The release of P_i from ATP (●), ADP (△), and AMP (○) is illustrated.

intact and lysed cells (Fig. 1A). The apparent higher activity of ADP and AMP hydrolysis in intact cells than in the lysed cells was probably a reflection of the higher salt requirements of the ADP and AMP hydrolyzing activities of the membrane-bound 5′-nucleotidase (3). ATP hydrolysis at 20 mM $MgCl_2$ was DCCD insensitive (data not shown).

The anti-5′-nucleotidase serum obtained by injecting a rabbit with the purified 5′-nucleotidase as described by Nelson et al. (13) inhibited ATP, ADP, and AMP hydrolyzing activities of the purified enzyme (Fig. 2). In immunodiffusion experiments, the antibody formed precipitin bands without any spur with the purified 5′-nucleotidase, membrane preparations, or cells solubilized with Triton X-100 (data not shown). The antibody did not interfere with the 5′-nucleotidase activities in intact cells (Table 1) or membrane preparations (data not shown), suggesting that the antigenic site was not accessible to its antibody. However, after whole cells (Table 1) or membranes (data not shown) were solubilized with Triton X-100, the antibody inhibited hydrolysis of ATP, ADP, and AMP in the presence of 20 mM $MgCl_2$ in the assay medium. The serum obtained from the same rabbit before immunization caused no inhibition of the 5′-nucleotidase in any of the preparations containing this enzyme. As expected, the anti-5′-nucleotidase serum did not inhibit the DCCD-sensitive ATPase in Triton X-100-treated cells. As shown in Table 1, the specific activities of the nucleotide hydrolysis in Triton X-100-treated cells were similar to those in intact cells. Furthermore, the extent of the antibody inhibition in Triton X-100-treated cells (Table 1) was similar to that in the membrane preparations (data not shown) or in the preparations of the purified 5′-nucleotidase. In addition, the activities of other phosphoric monoester hydrolases (EC 3.1.3.5) in whole cells or in Triton X-100-solubilized cells were negligible. Thus, the antibody experiments, although performed in vitro, indicated that the hydrolysis of 5′-nucleotides in intact cells in the presence of 20 mM $MgCl_2$ was due to the activity of the 5′-nucleotidase.

Uptake of labeled adenine 5′-nucleotides, adenosine, and adenine by fresh cell suspensions of *V. costicola*. We denote "uptake" as the accumulation (within the cell) of radioactivity derived from extracellular labeled substrate regardless of enzymatic transformations outside or inside the cytoplasmic membrane.

(i) Mg^{2+} requirements for the uptake of ATP. As described in the preceding paper (3), ATP (and ADP) hydrolysis by the membrane-bound or purified 5′-nucleotidase had an absolute requirement for Mg^{2+}. In the presence of 2 to 4 mM ATP the Mg^{2+} activation of this hydrolysis

TABLE 1. Inhibition of 5′-nucleotidase in Triton-X-100-treated cells of *V. costicola* by anti-5′-nucleotidase serum

Prepn	μmol of P_i released/mg of protein/min with 5′-nucleotidase[a]		
	ATP	ADP	AMP
Intact cells	0.53	0.58	0.44
Intact cells + 20 μl of antiserum[b]	0.56	0.60	0.48
Triton X-100-treated cells[c]	0.57	0.62	0.50
Triton X-100-treated cells + 20 μl of antiserum[b]	0.14	0.12	0.07

[a] The 5′-nucleotidase activities were assayed as described in the text in the presence of 1 M NaCl in the reaction mixture.

[b] The cells were incubated with antibodies in the assay medium for 10 min at room temperature, and the reaction was started with the substrate.

[c] The Triton X-100 treatment involved incubation of the cell suspension (2 mg of protein per ml) with 2% Triton X-100 for 30 min on ice before the assay.

TABLE 2. Mg^{2+} requirements for the uptake of ATP by cell suspensions of *V. costicola*

Substrate (μM)	$MgCl_2$ (mM)	Uptake[a] (nmol/0.1 mg of protein/90 s)
[8-^{14}C]ATP (0.37)	None[b]	0.0
	0.1	0.030
	2.0	0.089
	20.0	0.090
[8-^{14}C]adenosine (0.44)	None[b]	0.105
	20	0.106

[a] The uptake experiments were performed as described in the text.

[b] To ensure complete absence of Mg^{2+}, EDTA (1 mM) was added to reaction tubes where $MgCl_2$ was not added.

reached a plateau at about 20 mM $MgCl_2$ and did not change up to at least 100 mM Mg^{2+}. In whole cells, the 5'-nucleotidase in the presence of 2 to 4 mM ATP had similar Mg^{2+} activation profile, whereas at the micromolar concentrations of ATP used in kinetic studies, the rate of ATP hydrolysis was already saturated at 2 mM $MgCl_2$ and was constant up to at least 20 mM $MgCl_2$ (data not shown). The Mg^{2+} requirements of the uptake of [8-^{14}C]ATP (or ADP) were similar to those of the ATP hydrolysis, whereas the uptake of free adenosine did not require and was not stimulated by the Mg ion (Table 2).

(ii) Inhibition of the uptake of ATP, ADP, and AMP by AMP-PNP. The uptake of ^{14}C-labeled adenine 5'-nucleotides was inhibited by an ATP analog, AMP-PNP (Table 3), unlike the uptake of free adenosine, which was not affected by AMP-PNP (data not shown). The hydrolysis of adenine-5'-nucleotides was also inhibited by AMP-PNP in intact cells as well as in the preparations of the purified 5'-nucleotidase (Table 4). Thus, at an eightfold excess of the analog (4 mM) over the substrate (0.5 mM), AMP-PNP inhibited the rate of ATP hydrolysis by the purified 5'-nucleotidase to about 20% of the rate in the absence of the inhibitor (Table 4). Similarly, the rate of uptake of labeled ATP (Table 3) was inhibited to the same extent by an 11-fold excess of the inhibitor (10 μM) over the substrate (0.9 μM). The hydrolysis of ADP and AMP by the purified 5'-nucleotidase (Table 4) and the respective rates of uptake of these substrates (Table 3) were also similarly inhibited by AMP-PNP. The extent of the AMP-PNP inhibition of the 5'-nucleotidase activities in whole cells was similar to that in the purified enzyme preparations, except for the inhibition of AMP hydrolysis, which was somewhat greater in intact cells (Table 4). It is noteworthy that the K_m value for ATP hydrolysis by the purified 5'-nucleotidase was 2 orders of magnitude higher (data not shown) than that value for ATP hydrolysis by intact cells (1.3 μM, see below). Therefore, millimolar concentrations of nucleotides and of AMP-PNP were used in the experiment depicted in Table 4 as compared with micromolar concentrations used in the uptake experiment (Table 3).

TABLE 3. Inhibition of the uptake of [8-^{14}C]adenine nucleotides by an ATP analog, AMP-PNP, in cell suspensions of *V. costicola*

Substrate (μM)	AMP-PNP (μM)	Uptake[a] (nmol/0.1 mg of protein/90 s)	% of control[b]
ATP (0.9)		0.100	100
	10	0.027	27
	20	0.012	12
ADP (0.9)		0.120	100
	4	0.060	50
	10	0.037	30
	20	0.024	20
AMP (0.85)		0.123	100
	4	0.073	60
	10	0.054	44
	20	0.033	27

[a] Uptake experiments were performed as described in the text.

[b] Control uptake rates were those measured in the absence of AMP-PNP.

(iii) Kinetics of the uptake of ATP, ADP, AMP, adenosine, and adenine. The uptake of radioactively labeled adenine- 5'-nucleotides and adenosine each appeared as saturable transport

TABLE 4. Inhibition of the hydrolysis of adenine 5'-nucleotides in whole cells and in purified preparations of the 5'-nucleotidase by an ATP analog, AMP-PNP

Substrate (mM)	AMP-PNP (mM)	P_i released, % of control[a]	
		Whole cells	Purified 5'-nucleotidase[b]
ATP (0.5)		100	100
	2	37	
	4	20	17
ADP (0.5)		100	100
	2	42	
	4	29	35
AMP (0.5)		100	100
	2	39	
	4	27	50

[a] The assays of the nucleotide hydrolysis were performed as described in the text. The specific activities of the hydrolysis in the absence of AMP-PNP were taken as controls.

[b] The 5'-nucleotidase was purified as previously described (3).

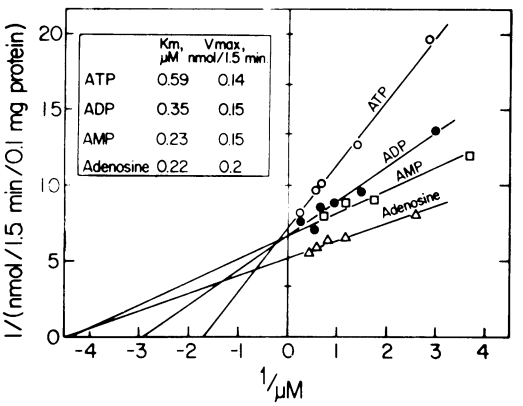

FIG. 3. Effect of the concentrations of ATP, ADP, AMP, or adenosine on their respective uptake into intact cells of *V. costicola*. Uptake of all substrates was assayed in one experiment, with the same cell suspension. The assays were performed with fresh cells as described in the text; 8-^{14}C-labeled substrates were diluted into the reaction mixtures to final concentrations specified in the figure, and the reaction was initiated by the addition of cells.

which followed Michaelis-Menten kinetics. Lineweaver-Burk plots of the rates of uptake of ATP, ADP, AMP, and adenosine per 90 s per 0.1 mg of cell protein as a function of substrate concentrations are shown in Fig. 3. In all kinetic experiments precautions were taken to use freshly grown cells immediately after washing and resuspension in the buffer. Under such conditions glucose did not stimulate uptake and therefore was not added to the reaction mixtures. However, we observed that the addition of glucose did stimulate the uptake by 20 to 50% in cells which were kept in ice for 1 to 3 h before the assay. P_i (5 to 10 mM) caused twofold stimulation of uptake of all substrates and was therefore added to all assays.

The experiments described above gave substantial evidence in favor of the dephosphorylation of adenine 5'-nucleotides by the membrane-bound 5-nucleotidase before uptake. The uptake of [8-^{14}C]adenine (data not shown) also followed Michaelis-Menten kinetics, with K_m and V_{max} values of 0.6 μM and 0.05 nmol/0.1 mg of protein per 90 s, respectively. Thus, the V_{max} value of adenine uptake was 3 to 4 times lower than the respective values for the uptake of nucleotides or adenosine (Fig. 3) measured under the same conditions and usually in the same experiment. Therefore, it was assumed that the adenosine moieties of ATP, ADP, or AMP after hydrolysis were taken up directly without prior catabolism to adenine. The data on substrate inhibition of the uptake of ATP, adenosine, and adenine (described below) supported this hypothesis. The uptake of [8-^{14}C]ATP was competitively inhibited by unlabeled adenosine, with a K_i value of 0.2 μM. The uptake of adenosine was competitively inhibited by unlabeled ATP (K_i, 1.67 μM); as expected, this inhibition occurred only in the presence of Mg^{2+}. Although the uptake of labeled adenine was inhibited competitively by adenosine (K_i, 0.14 μM), adenine at 11-fold excess over adenosine was able to inhibit uptake of the latter only by a maximum of 20%. Thus, the adenosine moiety of ATP apparently used the adenosine transport system, whereas adenine seemed to be transported via a separate system.

(iv) Kinetics of the 5'-nucleotidase activity in whole cells of *V. costicola*. Figure 4 shows the Lineweaver-Burk plot of the rate of hydrolysis of [α-^{32}P]ATP and [γ-^{32}P]ATP, as a function of substrate concentrations, by whole cells of *V. costicola*. As described above, the assays of the ATP hydrolysis were performed with strictly fresh cells under the conditions identical to those of uptake. As expected, the hydrolysis of both substrates had the same K_m value, whereas the velocity of the release of γ-labeled phosphate was higher than that of the α-labeled phosphate release. These results supported our previous assumption that the γ-, β-, and α-phosphates of ATP were cleaved sequentially by

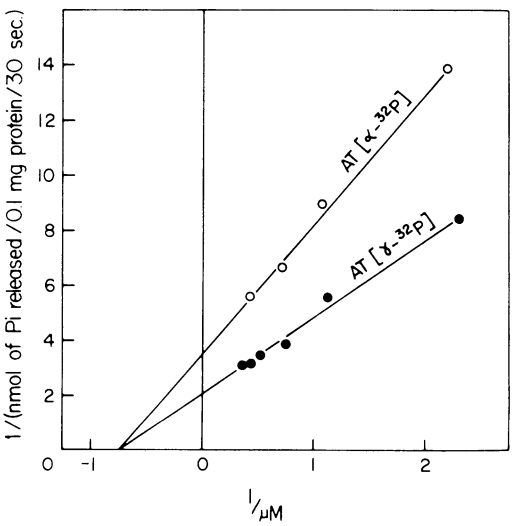

FIG. 4. Effect of ATP concentration on the rates of release of the γ- and α-labeled phosphates of the ATP molecule in intact cells of *V. costicola*. The assays of the hydrolysis of [α-^{32}P]ATP and [γ-^{32}P]ATP were performed in one experiment. Freshly harvested cells were washed, suspended in the washing solution (see text), and used at once; the assay conditions and the reactions mixture were the same as those used in uptake experiments; the reaction was started by the addition of cells and stopped by the addition of 0.1 ml of 60% trichloroacetic acid.

the 5'-nucleotidase (3). The rate of release of the α-labeled phosphate from ATP was taken as a rate of release of adenosine, since the presence of 10^3- to 10^4-fold excess of unlabeled P_i in the reaction mixture virtually eliminated the possibility of metabolism of the phosphate released from ATP.

(v) Theoretical analysis of the mechanism of ATP uptake by whole cells of *V. costicola*. As can be concluded from the experiments shown above, the uptake of ATP consisted of two processes, both of which followed Michaelis-Menten kinetics: dephosphorylation of ATP to adenosine by the 5'-nucleotidase

$$\frac{1}{V_1} = \frac{K_{m_1}}{V_{\max_1}} \frac{1}{[\text{ATP}]} + \frac{1}{V_{\max_1}} \quad (1)$$

and uptake of adenosine (Ado)

$$\frac{1}{V_2} = \frac{K_{m_2}}{V_{\max_2}} \frac{1}{[\text{Ado}]} + \frac{1}{V_{\max_2}} \quad (2)$$

where [ATP] and [Ado] are substrate concentrations, and V_1 and V_2 are their respective rates of hydrolysis and uptake. If adenosine in equation 2 is derived from the hydrolysis of ATP (equation 1), then we can substitute 1/[Ado] by $1/(V_1 t)$, since under our experimental conditions both the hydrolysis of [α-^{32}P]ATP (Fig. 4) and the uptake of adenosine were linear with time up to 3 min (data not shown). Furthermore, substitution of $1/V_1$ from equation 1 into equation 2 gives a theoretical relationship for the uptake of ATP if it consisted of two sequential processes

$$\frac{1}{V_3} = \left\{ \frac{1}{t} \cdot \frac{K_{m_2}}{V_{\max_2}} \cdot \left[\left(\frac{K_{m_1}}{V_{\max_1}} \cdot \frac{1}{[\text{ATP}]} \right) + \frac{1}{V_{\max_1}} \right] \right\} + \frac{1}{V_{\max_2}} \quad (3)$$

The values of the kinetic parameters are K_{m_1} = 1.3 μM and $V_{\max_1}$ = 0.5 nmol/0.1 mg of protein per min for the hydrolysis of [α-^{32}P]ATP (Fig. 4), and K_{m_2} = 0.24 μM and $V_{\max_2}$ = 0.14 nmol/0.1 mg of protein per min for the uptake of adenosine (Fig. 3).

The theoretical relationship (equation 3) was compared with the experimental curve obtained for the uptake of ^{14}C-labeled ATP, for the reaction time t = 1.5 min and ATP concentrations of 0.4 to 4 μM, which were the conditions of the experiment shown in Fig. 3; the experimental curve had a form of Michaelis-Menten relationship with apparent kinetic parameters, K_m = 0.6 μM and $V_{\max_1}$ = 0.094 nmol/0.1 mg of protein per min.

The calculated K_m and $V_{\max}$ values obtained from equation 3 were 0.3 μM and 0.11 nmol/0.1 mg of protein per min, respectively. The experimental velocities of the ATP uptake were within 65 to 80% of those calculated from equation 3, in the range of ATP concentrations from 0.4 to 4 μM, respectively. This treatment assumes that at least under these experimental conditions, the dephosphorylation of ATP and the uptake of the released adenosine proceeded sequentially; in other words, the 5'-nucleotidase functioned only in the hydrolysis of the nucleotides and not in the transport of their nucleoside moieties.

Growth of *V. costicola* on AMP or adenosine as a sole carbon source. *V. costicola* was able to use adenosine or AMP as the only carbon and energy source for growth (Fig. 5). Growth on adenosine was almost as fast as on glucose. However, the growth curve with AMP as a substrate had a longer lag and doubling time. This indicated that dephosphorylation of AMP was the rate-limiting step under the conditions of the growth experiments, whereas this was not the case in the uptake experiments (see above). It is noteworthy here, to resolve this seeming contradiction,

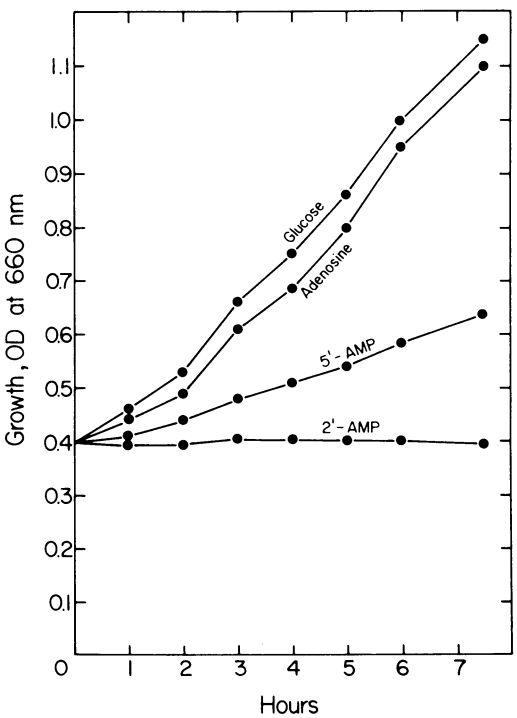

FIG. 5. Growth of *V. costicola* on AMP or adenosine as a carbon source. *V. costicola* was grown on glucose in a minimal medium as described in the text. The cells were washed in the late-logarithmic stage and resuspended in the minimal medium, in which glucose was replaced by a carbon source specified in the figure. Each substrate was added to a final concentration of 1%.

that the substrate concentrations in the growth experiments were millimolar, and not micromolar as those used in all kinetic experiments. Preliminary kinetic experiments not shown here indicated that a different transport system with higher K_m and V_{max} values might be responsible for the uptake of higher adenosine concentrations.

The cells did not grow on adenine, ribose, ribose 5-phosphate, ribose 1-phosphate, or a combination of adenine with ribose phosphates. These results indicated that the ribose moiety of adenosine had to be transported as a part of adenosine molecule to be used as a growth substrate. This was another line of evidence supporting our conclusion that adenosine was taken up by a specific transport system without prior catabolism.

All experiments described above were repeated several times, with close agreement when freshly harvested cells were used.

DISCUSSION

We present here direct evidence of a biochemical mechanism by which bacteria can efficiently utilize low concentrations of extracellular adenine nucleoside 5′-tri-, -di-, and -monophosphates. This mechanism involves two consecutive processes: dephosphorylation of nucleotides by membrane-bound 5′-nucleotidase (3) and uptake of the released adenosine by a carrier-mediated transport system.

It is generally believed that in bacteria external nucleotides must be cleaved to nucleosides or bases outside the cytoplasmic membrane before uptake into the cell. However, no experiments have as yet been reported which would show directly the biochemical pathway for the utilization of the nucleotides by a bacterial cell. In *Escherichia coli* a soluble periplasmic 5′-nucleotidase (14) has been linked with the uptake of AMP (25). However, the evidence supporting this conclusion was based only on a study of the AMP uptake by a mutant deficient in this nucleotidase.

Although numerous studies of nucleoside uptake by bacteria have appeared, the views as to the mechanisms of uptake are conflicting. One group of investigators reached a conclusion that nucleosides are transported without prior catabolism (8, 10, 11, 19), whereas other workers postulated that adenosine is cleaved by phosphorolysis before uptake (7, 17, 25). The results presented in this paper suggest that in *V. costicola* adenosine is taken up without prior catabolism. Furthermore, as was also found for *E. coli* (19), adenosine and adenine apparently use separate although possibly overlapping transport systems, since the uptake of adenine was competitively inhibited by adenosine, whereas adenine at 11-fold excess inhibited the uptake of adenosine only by 20%.

As to the possible molecular mechanism of the nucleotide uptake, the experiments described here showed that at least under our experimental conditions the uptake of the adenosine moiety of the ATP molecule and the uptake of free adenosine appeared as kinetically similar processes. In agreement with these data, the competitive inhibition studies (see above) suggested that the adenosine formed as a result of dephosphorylation and the free adenosine used the same transport system. In addition, an ATP analog, AMP-PNP, which inhibited the uptake of nucleotides, did not inhibit the adenosine uptake (Table 3 and above). On the basis of the above considerations it seems unlikely that the adenosine transport system is a part of the 5′-nucleotidase complex. It is noteworthy here that the 5′-nucleotidase of lymphocyte membranes (4, 18) does facilitate the uptake of adenosine formed as a result of ATP hydrolysis (4). It must be kept in mind, however, that the uptake of adenosine in mammalian cells occurs by a mechanism different from that of bacteria (16), and that the structures of the two enzymes may be completely different although their physiological functions appear quite similar.

It has been recently reported that seawater contains dissolved ATP in significant (nanomolar) concentrations, and furthermore, that marine bacteria could rapidly accumulate radioactivity from added ATP, ADP, and AMP (2). Interestingly, several marine strains (1, 23) and the slightly halophilic *Vibrio alginolyticus* found in marine fish (6) possessed membrane-bound 5′-nucleotidase activities with properties similar to those of the 5′-nucleotidase of *V. costicola*. Although the 5′-nucleotidase activities of marine bacteria were not assayed in whole cells and no purification of these activities was attempted, it is tempting to suggest that marine bacteria and moderately halophilic *V. costicola* may possess a similar 5′-nucleotidase. *V. costicola*, which had been originally isolated from bacon-curing brines in Australia (21), could well have had marine origin, possibly entering these brines as a contaminant of solar salt.

Transport of nucleosides and bases in bacteria is considered to be energy dependent (8, 10, 11, 16, 19). In fresh cells of *V. costicola*, as mentioned above, glucose did not stimulate the uptake of either adenosine or adenine. Similarly, transport of the nonmetabolizable amino acid analog, α-aminoisobutyric acid, into fresh cells of *V. costicola* was not stimulated by glucose, but was inhibited by KCN (D. J. Kushner and R. A. McLeod, unpublished data). In line with these data, recent experiments in our laboratory

have shown that *V. costicola* was able to maintain high rates of endogenous respiration for as long as 2 h in the absence of an added energy source (F. Hamaide and D. J. Kushner, unpublished data).

Finally, the uptake of only adenine 5'-nucleotides was studied in this work. It would seem worthwhile, however, to look at the uptake of other purine and pyrimidine 5'-nucleotides since the 5'-nucleotidase hydrolyzed all purine and pyrimidine 5'-nucleotides, except CTP, at comparable rates (3).

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