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Adenine Phosphoribosyltransferase in *Mycoplasma mycoides* and *Escherichia coli*

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Some kinetic properties of the adenine phosphoribosyltransferases from *Escherichia coli* and *Mycoplasma mycoides* have been studied. For the *E. coli* enzyme, Michaelis constants for adenine and 5-phosphoribosyl-1-pyrophosphate (PRPP) are 1.3 and 10 μM, respectively. Adenosine monophosphate, the most effective nucleotide inhibitor, inhibits competitively with respect to PRPP, the inhibition constant being 26 μM. The *M. mycoides* enzyme has more complex kinetics. The response to increasing PRPP concentration is sigmoidal, the degree of sigmoidality depending on both the concentration of adenine and the *p*H. At low PRPP levels, high concentrations of adenine are inhibitory. Guanosine monophosphate is the most effective inhibitor, being inhibitory at all *p*H values, but other nucleotides have been found to activate at *p*H 7 and inhibit at *p*H 8. The elution profile of the *M. mycoides* enzyme from Sephadex suggests an association of enzyme subunits in the presence of PRPP. This is consistent with the observed kinetics if the associated form has increased stability and activity.

Adenine phosphoribosyltransferase (adenylate:pyrophosphate phosphoribosyl-transferase, EC 2.4.2.7) catalyzes a reaction between adenine and 5-phosphoribosyl-1-pyrophosphate (PRPP) to produce adenosine monophosphate (AMP) and pyrophosphate. It is widely distributed in both mammals and bacteria (2, 7, 11-14, 20). In *Escherichia coli* and other organisms which can synthesize purine nucleotides de novo, this reaction provides an alternative way of making AMP when there is a supply of free adenine. *Mycoplasma mycoides* strain V5 is presumably lacking in the pathways of de novo synthesis and is incapable of many interconversions of nucleotides since it requires guanine, uracil, and thymine for growth (17), and growth is further stimulated by adenine. Presumably it utilizes adenine phosphoribosyltransferase for adenine nucleotide synthesis.

In view of the possible difference in the importance of the transferase reaction to *E. coli* and *M. mycoides*, we have studied the kinetic properties and effects of nucleotides for the enzymes of both organisms to determine whether they are different in characteristics of potential regulatory significance.

MATERIALS AND METHODS

Organisms and culture media. *E. coli* strain ABLA, which requires leucine and thiamine, was grown aerobically overnight in the glucose minimal salts medium of Vogel and Bonner (19) supplemented with 40 μg of leucine per ml and 0.5 μg of thiamine per ml.

Cultures of *M. mycoides* strain V5, grown in medium C_2 of Rodwell (18) and containing 20 μg each of adenine, guanine, and uracil per ml and 10 μg of thymine per ml, were kindly supplied by A. Rodwell, C.S.I.R.O., Division of Animal Health.

Preparation of extract. *E. coli* cells were washed in 0.9% NaCl and suspended in cold 100 mM tris(hydroxymethyl)aminomethane (Tris) buffer, *p*H 7.8. Cells were disrupted by two passages through an Aminco French pressure cell at 7 tons/in.^{2}, and the suspension was centrifuged at 20,000 × *g* for 20 min. The supernatant fluid was dialyzed against Tris buffer for 3 hr at 4 C.

M. mycoides cells were harvested from the growth medium by centrifuging at 10,000 × *g* for 20 min and then were washed in a solution of 0.25 M NaCl, 0.02 M $Na_2HPO_4 \cdot NaH_2PO_4$ (*p*H 6.95), and 0.01 M $MgCl_2$ to prevent osmotic lysis. Cells were disrupted either in a French pressure cell (with approximately 10 mg of cells in 5 ml of 100 mM Tris, *p*H 7.8, containing 2 mg of bovine serum albumin per ml) or by osmotic lysis (suspending the cells in 0.8 ml of 5 mM Tris, *p*H 7.8, for 15 min and then adding Tris and bovine serum albumin to a final concentration of 100 mM Tris and 2 mg of bovine serum albumin per ml). In both methods, cell debris was removed by centrifugation at 20,000 × *g* for 20 min, and the supernatant fluid was dialyzed against Tris buffer for 2 to 3 hr at 4 C.

Enzyme assay. Unless otherwise specified, assay mixtures contained PRPP (P.L. Biochemicals Inc., Milwaukee, Wis.), adenine-*8*-^{14}C (Radiochemical Centre, Amersham, England), 5 mM $MgCl_2$, and 0.2 mg of bovine serum albumin per ml, with an appropriate amount of protein, in 50 μliters of Tris-hydrochloride buffer at *p*H 7.7 at 37 C. The mixtures were incubated at 37 C for 5 min, and then 20-μliter samples were spotted onto cellulose-phosphate paper (Whatman P81) for chromatography. Spotting onto the chromatogram was effective in terminating the reaction.

The cellulose-phosphate paper was prewashed with distilled water to remove material which caused uneven running and displacement of AMP from the solvent front. A number of samples were applied per sheet. To prevent overlap of adjacent samples, the paper (8 cm long by 20 cm wide) was divided into strips 1.5 cm wide and 6 cm long, separated by 1-mm slots, which terminated 1 cm from the top and the bottom of the sheet. Samples were spotted within the strips, 2 cm from the bottom of the paper.

After development of the chromatograms for 4 cm in water, they were dried. The solvent fronts (containing the product AMP) and, when required, the origins (containing unchanged adenine) were cut out and placed in scintillation vials with 5 ml of scintillation mixture (5 g of 2,5-diphenyloxazole [PPO] and 0.3 g of dimethyl POPOP per liter of toluene) for counting in a liquid scintillation spectrometer.

Other nucleotides (e.g., adenosine diphosphate and adenosine triphosphate) run with AMP at the solvent front; hypoxanthine and inosine run about half-way up the chromatograms; and adenine and adenosine remain near the origin. Thus the assay system would detect the formation of AMP even if it were subsequently converted to other nucleotides, but would be free of interference from non-nucleotide products. Under the assay conditions used for adenine phosphoribosyltransferase, no hypoxanthine or inosine formation was detected, nor was there any detectable breakdown of AMP during a 2-hr incubation period.

Identification of AMP as the product of reaction. A sample of 100 μliters from an incubation was run on cellulose-phosphate with added marker AMP and eluted onto a polyethyleneimine-cellulose thin-layer for two-dimensional chromatography (16). The solvent system used was a modification of that described by Neuhard (15).

For both *E. coli* and *M. mycoides*, radioautography showed a single distinct spot, identical in size, position, and shape with the AMP marker which was observed under a Mineralight ultraviolet lamp.

Determination of true PRPP concentration. PRPP was dissolved in 5 mM ethylenediaminetetraacetic acid at *p*H 7.5 to give a 4 mM solution by weight. The actual concentration of PRPP in solution was determined by its capacity to convert [^{14}C]adenine to [^{14}C]AMP in the reaction catalyzed by *E. coli* adenine phosphoribosyltransferase.

RESULTS

For the adenine phosphoribosyltransferase from *E. coli* and *M. mycoides*, activity is proportional to the amount of extract added when the incubation mixture contains 0.2 mg of bovine serum albumin per ml. A rate which is constant for 10 min, or until substrates become limiting, is obtained by preincubating the enzymes with PRPP for 5 min and starting the reaction by adding [^{14}C]adenine. The enzyme from *E. coli* is quite stable and can be stored at −15 C for many months without noticeable loss of activity. Freezing and thawing have little effect on the enzyme. Under assay conditions, the activity falls off only slowly. Adenine phosphoribosyltransferase from *M. mycoides* loses activity on freezing and thawing. Under assay conditions in the absence of PRPP, the enzymatic activity is halved in about 15 min. Bovine serum albumin and adenine stabilize the enzyme slightly; PRPP increases the stability markedly. Heating the enzyme at 50 C for 40 min in buffer with 5 mM $MgCl_2$ decreases its activity below 10%, whereas 70% of the activity is retained after similar heating in buffer with 5 mM $MgCl_2$ plus 0.4 mM PRPP. β-Mercaptoethanol and dithiothreitol have little effect on either stability or activity.

Kinetic studies. The enzyme from *E. coli* shows simple Michaelis-Menten kinetics between 0.25 and 22 μM adenine and between 2.8 and 65 μM PRPP. The double-reciprocal plots obtained for each substrate, at various concentrations of the other substrate, obviously intersected, indicating an ordered bi-bi mechanism (3–5). Michaelis constants for adenine and PRPP are 1.3 and 10 μM, respectively. The enzyme is most strongly inhibited by AMP. Inhibition is competitive with respect to PRPP, the inhibition constant being 26 μM.

Figures 1a and b show the variations of initial velocity with concentration of the substrates adenine and PRPP at *p*H 7.7 for the adenine phosphoribosyltransferase from *M. mycoides*. The enzyme is readily saturated by adenine. At low concentration of PRPP, it is inhibited by high concentrations of adenine. At high concentrations of PRPP, the concentration of adenine for half-maximal rate is approximately 0.3 μM. The response to increasing concentration of PRPP is sigmoidal. Hill plots of the curves for varying PRPP concentrations show slopes of about 2.1 at all concentrations of adenine (6).

The variation of activity of adenine phosphoribosyltransferase from *M. mycoides* with *p*H is shown in Fig. 2. The *p*H optimum depends on the PRPP concentration used. The *p*H dependence of the relationship of the ac-

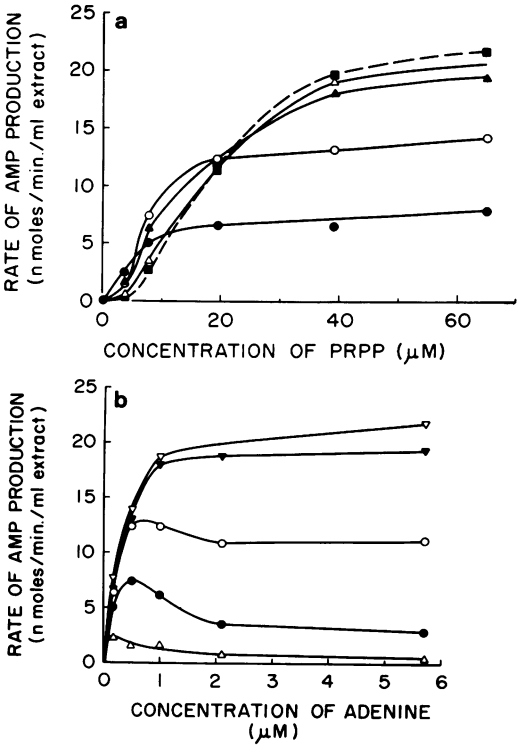

FIG. 1. *Effect of substrate concentrations on adenine phosphoribosyltransferase from M. mycoides. Incubations contained 5 mM $MgCl_2$ and 0.2 mg of bovine serum albumin per ml in 40 mM Tris-hydrochloride at pH 7.7. The concentrations of adenine for Fig. 1a were 0.18 μM (●), 0.5 μM (○), 1.0 μM (▲), 2.1 μM (△), 5.7 μM (■). For b, PRPP concentrations were 3.8 μM (△), 7.8 μM (●), 19 μM (○), 39 μM (▼), 65 μM (▽). Assays were performed as described in the text.*

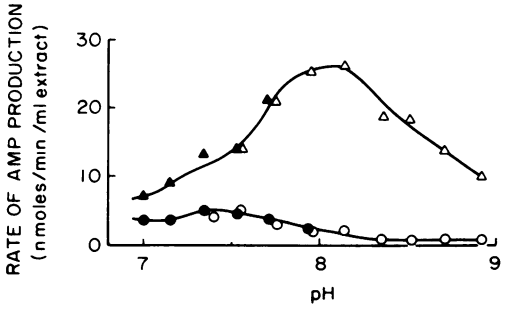

FIG. 2. *Effect of pH on activity of adenine phosphoribosyltransferase from M. mycoides. The concentrations of PRPP used were 65 μM (▲) and (△), and 7.8 μM (●) and (○). The concentration of adenine was 5 μM. N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer was used at the lower pH values (▲ and ●), Tris buffer at the higher pH values (△ and ○). The pH values are those given by the buffers at 37 C.*

tivities at the two PRPP concentrations suggested that the response of rate to PRPP concentration might be less sigmoidal at lower *p*H. Figure 3 shows the variation of initial velocity with concentration of PRPP at *p*H 7.0 and 8.15. The maximal velocity is lower at *p*H 7.0 than at *p*H 8.15, and at the lower *p*H value the curve appears to have lost most of its sigmoidal character. Hill plots for varying PRPP concentrations have slopes of 1 at *p*H 7.0 and 2 at *p*H 8.15.

The adenine phosphoribosyltransferase from *E. coli* has a fairly broad *p*H optimum (Fig. 4). It obeys Michaelis-Menten kinetics at *p*H 7.7 and 9.0.

The effect of nucleotides on *M. mycoides* enzyme is also strongly influenced by *p*H, as shown in Table 1 which also presents the effect of nucleotides on *E. coli* enzyme at *p*H 7.7. At *p*H 7.1, pyrimidine nucleotides activate the *M.*

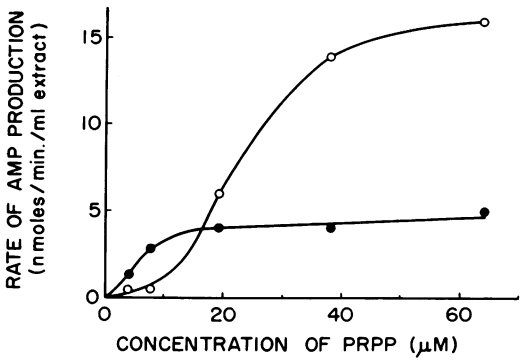

FIG. 3. *Effect of PRPP concentration on adenine phosphoribosyltransferase from M. mycoides at two pH values. Symbols: ○, Tris-hydrochloride, 40 mM, pH 8.14; ●, HEPES 40 mM, pH 7.0. Incubations contained 5 μM adenine, 5 mM $MgCl_2$, and 0.2 mg of bovine serum albumin per ml. The pH values are those given the buffers at 37 C.*

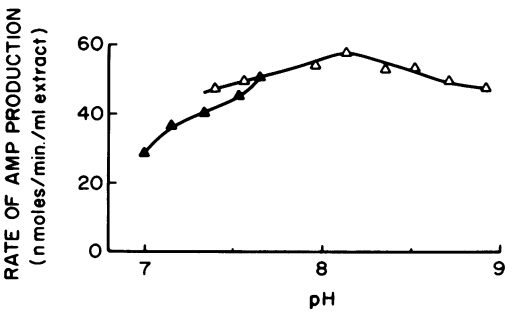

FIG. 4. *Effect of pH on activity of adenine phosphoribosyltransferase from E. coli. Incubations contained 5 μM adenine, 65 μM PRPP, 5 mM $MgCl_2$, and 0.2 mg of bovine serum albumin per ml in 40 mM HEPES (▲) or 40 mM Tris-hydrochloride (△).*

mycoides enzyme, as do guanosine triphosphate and inosine monophosphate. Guanosine monophosphate (GMP) causes the strongest inhibition at all *p*H values. At *p*H 8.0, all nucleotides, except uridine monophosphate and cytosine monophosphate, are inhibitory. Only one nucleotide, AMP, has been tested for its effect on the enzyme from *M. mycoides* over a range of PRPP concentrations. Figure 5 shows

TABLE 1. *Effect of nucleotides on adenine phosphoribosyltransferase*[a]

Nucleotide (2 mM)	*E. coli* rate of reaction (% of control) at *p*H 7.7, 5 mM Mg^{2+}	*M. mycoides* rate of reaction (% of control) at		
		*p*H 7.1, 15 mM Mg^{2+}	*p*H 7.7, 5 mM Mg^{2+}	*p*H 8.0, 15 mM Mg^{2+}
Nil	100	100	100	100
AMP	11	100	23	4
ATP	12	89	34	15
GMP	62	50	7	1
GTP	41[b]	215	41	27
UMP	94	365	131	97
UTP	58	285	95	51
CMP	98	375	124	97
CTP	74	235	63	54
IMP	100	190	43	10

[a] Incubations contained 300 μM PRPP, 20 μM adenine, and 0.2 mg of bovine serum albumin per ml in 40 mM Tris-hydrochloride (*p*H 7.7 and 8.0) or in 20 mM HEPES, 20 mM Tris (*p*H 7.1). Nucleotides were added as specified. Abbreviations: AMP, adenosine monophosphate; ATP, adenosine triphosphate; GMP, guanosine monophosphate; GTP, guanosine triphosphate; UMP, uridine monophosphate; UTP, uridine triphosphate; CMP, cytidine monophosphate; CTP, cytidine triphosphate; IMP, inosine monophosphate.

[b] One millimolar GTP.

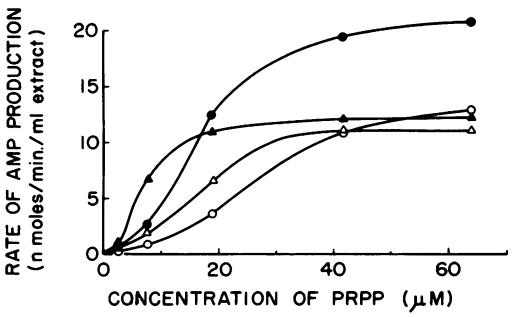

FIG. 5. *Effect of AMP on adenine phosphoribosyltransferase from M. mycoides at various PRPP concentrations. Incubations contained 0.2 mg of bovine serum albumin per ml and 5 mM $MgCl_2$ in 40 mM Tris-hydrochloride at pH 7.7. Symbols: ○ and △, 20 μM AMP; ● and ▲, controls with no added nucleotide; ○ and ●, 5 μM adenine; △ and ▲, 0.5 μM adenine.*

how initial velocity varies with PRPP concentration at two concentrations of adenine, in the presence of 20 μM AMP, at *p*H 7.7. Double-reciprocal plots of these data give curves which are concave upwards, whereas the intercepts on the $1/V$ axis are unaffected by the presence of AMP. Slopes of Hill plots of the results at both adenine concentrations are close to 2 and are virtually unchanged on addition of 20 μM AMP.

Sephadex column chromatography. When an extract of *M. mycoides* was chromatographed on Sephadex G-150 at *p*H 8.0, a double peak of adenine phosphoribosyltransferase activity was obtained. Two peaks of activity were also obtained when extract was chromatographed in the same buffer containing 5 mM $MgCl_2$, but in this case the elution volumes were decreased. In the presence of 50 μM PRPP and 5 mM $MgCl_2$ a very broad peak of enzymatic activity was obtained, with most appearing at lower elution volumes, suggestive of an aggregation of enzyme molecules in the presence of PRPP. In accord with the stabilizing effect of PRPP on the enzyme, the recovery of enzymatic activity from the column developed with PRPP was more than fourfold that from the other columns (Fig. 6).

DISCUSSION

The aim of this investigation was to compare the kinetic properties of the adenine phosphoribosyltransferases from *E. coli* and *M. mycoides* and, in particular, to observe what differences might exist in the mechanism regulating enzymatic activity in the two organisms.

Both enzymes have very low Michaelis constants for adenine, consistent with the efficient use of even very low concentrations of adenine by both organisms. Differences are observed in the response to PRPP, for which the enzyme from *E. coli* shows Michaelis-Menten kinetics with a K_m at 10 μM, whereas the one from *M. mycoides* has more complex sigmoidal kinetics, at least at higher *p*H values. The enzyme from *E. coli* should thus utilize PRPP at near maximal rate down to very low concentrations. In vivo observation (A. Bagnara, *personal communication*) has shown that the addition of adenine to cultures of *E. coli* causes a drastic decrease in intracellular concentration of PRPP with a concurrent rise in concentration of ATP and decrease in concentrations of uridine triphosphate and cytosine triphosphate. The enzyme from *M. mycoides* should be much less effective in competing for the utilization of PRPP, and it has been found

that addition of adenine to cultures of *M. mycoides* causes smaller changes in nucleotide concentrations (A. Mitchell, *personal communication*).

A study of the effects of nucleotides on the *E. coli* adenine phosphoribosyltransferase showed that AMP, the product of the reaction, is the most effective inhibitor, with a K_i of 26 μM. The other adenine nucleotides also inhibit strongly, whereas guanine nucleotides, which can serve as precursors of AMP in *E. coli* (1), inhibit to a lesser extent. The pyrimidine triphosphates are inhibitory, but the monophosphates are not.

The response of the adenine phosphoribosyltransferase from *M. mycoides* to nucleotides depends on *p*H. Pyrimidine monophosphates are strongly activating at *p*H 7.1 but have no effect at *p*H 8.0. AMP inhibits strongly at *p*H 8.0 but has virtually no effect at *p*H 7.1. GMP, which is the most effective inhibitor, inhibits much more strongly at *p*H 8.0 than at *p*H 7.1. This apparent regulatory role of GMP may be related to the capacity of guanine to serve as a sole purine source in *M. mycoides*. However, attempts to assess the physiological significance of these kinetic properties must be limited without more information on the effects of nucleotides, at varying concentrations of both nucleotides and substrates, and also on the levels of PRPP and nucleotides normally encountered in the cell.

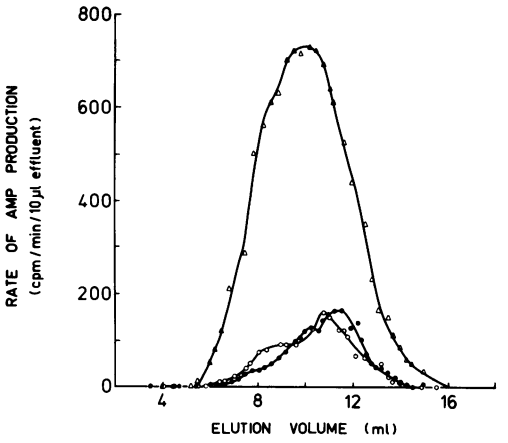

FIG. 6. *Elution of M. mycoides adenine phosphoribosyltransferase from a column of Sephadex G-150 with various eluents. Samples (50 μliters) of M. mycoides extract, with 0.5 mg of cytochrome c added, were applied to the column (1 by 20 cm) which had been previously equilibrated with eluent. The eluate was continuously monitored for cytochrome c by percent transmission at 410 nm. For each eluate, the mean elution volume for cytochrome c was 12.3 ml. Fractions (0.3 ml) were collected between 4.0 and 16.0 ml elution volume and assayed for adenine phosphoribosyltransferase activity. Symbols: ●, 100 mM Tris (pH 8.0); ○, 100 mM Tris (pH 8.0) and 5 mM $MgCl_2$; △, 100 mM Tris (pH 8.0) and 50 μM PRPP and 5 mM $MgCl_2$.*

The observations on the kinetics of the adenine phosphoribosyltransferase from *M. mycoides* and on its elution from Sephadex would be consistent with the existence of association-dissociation reactions of enzyme subunits, with increased activity in the associated form. Thus the sigmoidal kinetics observed in response to increasing PRPP concentration at higher *p*H could be the result of an association of subunits induced by the binding of PRPP.

Subsequent to completion of this investigation, extensive data on the enzyme of *E. coli* have become available (8–10). These data differ from our results in indicating much higher K_m values for both adenine and PRPP. Possibly the differences stems from some difference in the strains of *E. coli* used or in the differing treatments to which the enzymes had been subjected. Hochstadt-Ozer and Stadtman used enzyme purified to apparent homogeneity and constant specific activity, whereas the results presented here were obtained with freshly prepared crude extracts. We have, however, obtained very similar results with an enzyme extract purified 25-fold by ammonium sulfate fractionation, followed by chromatography on diethylaminoethyl-cellulose and on Sephadex G-150. The apparent molecular weight of the enzyme, as estimated by Sephadex G-150 chromatography, is about 30,000 which is in agreement with the average molecular weight obtainable from the elution data presented by Hochstadt-Ozer and Stadtman (8–10).

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