

Regulation of Carbamylphosphate Synthesis in *Serratia marcescens*

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Serratia marcescens HY possessed a single carbamylphosphate synthase (CPSase) which was subject to cumulative repression by arginine and a pyrimidine. CPSase did not appear to be a part of a multifunctional enzyme complex as is the case for other enzymes of pyrimidine biosynthesis in this organism. CPSase was purified to homogeneity. The molecular weight of the enzyme was estimated to be 167,000 by sucrose density gradient ultracentrifugation. The double-reciprocal plot for magnesium adenosine triphosphate was linear, yielding a K_m value of 2.5 mM. The enzyme utilized either glutamine (K_m , 0.1 mM) or NH_3 (K_m , 10.5 mM) as a nitrogen donor in the reaction. CPSase activity was subject to activation by ornithine and feedback inhibition by uridine monophosphate, as is the case for other enteric bacteria. Carbamate kinase activity, detected in crude extracts of *S. marcescens*, was shown to be due to a constitutive acetate kinase. The absence of carbamate kinase from *S. marcescens* HY is consistent with the inability of this organism to utilize arginine as a source of energy under anaerobic conditions.

Carbamylphosphate is an intermediate in the biosynthesis of arginine and pyrimidines (Fig. 1). In *Escherichia coli* (20) and *Salmonella typhimurium* (2, 3), it is synthesized from glutamine, bicarbonate, and ATP by a single enzyme, carbamylphosphate synthase (CPSase; EC 2.7.2.9), which is subject to cumulative repression by arginine and a pyrimidine compound and to feedback control: UMP inhibits activity and ornithine stimulates it. The combined effects provide an efficient mechanism of controlling the supply of carbamylphosphate for arginine and pyrimidine biosynthesis. In contrast to the single CPSase of enteric bacteria, *Saccharomyces cerevisiae* (13, 21) and *Neurospora crassa* (7) contain two CPSases, each specific to either arginine or pyrimidine biosynthesis and each under separate control. The pyrimidine-specific CPSase and the succeeding enzyme in pyrimidine biosynthesis, aspartic transcarbamylase, are associated in an enzyme complex. These two enzymes are repressible by pyrimidines, and their activities are subject to feedback inhibition by UTP (7, 13, 21). The arginine-specific CPSase is subject only to repression and appears insensitive to feedback control (21, 22). Recent reports (19) indicate that *Bacillus subtilis* also possess two CPSases, thus resembling eucaryotic microorganisms with respect to carbamylphosphate synthesis.

Examination of carbamylphosphate synthesis in *Serratia marcescens* is of particular interest in view of the reported differences in regulation of pyrimidine biosynthesis between this organism and other members of the *Enterobacteri-*

aceae. Aspartic transcarbamylase from *S. marcescens* was found to be insensitive to repression or feedback inhibition (28), in sharp contrast to the case in other enteric bacteria (17). Furthermore, a multifunctional complex of three enzymes of pyrimidine biosynthesis, dihydroorotase, orotidine monophosphate pyrophosphorylase, and orotidylate decarboxylase, has been reported for *S. marcescens* HY (26, 27). Enzyme complexes have been frequently encountered in the pyrimidine pathway in eucaryotes (10) but not previously reported in procaryotes. These differences, together with an early report (9) on the purification of a carbamate kinase (ATP: carbamate phosphotransferase, EC 2.7.2.2) from *Serratia marcescens*, suggested that carbamylphosphate metabolism in this organism might differ significantly from that in *E. coli* and *Salmonella typhimurium*. The present paper shows that this is not the case; CPSase from *Serratia marcescens* is very similar in regulatory properties to the well-characterized enzymes from *E. coli* and *S. typhimurium* and does not appear to be associated with other enzymes of pyrimidine biosynthesis. Carbamate kinase activity observed in crude extracts of *S. marcescens* is due to a constitutive acetate kinase (ATP:acetate phosphotransferase, EC 2.7.2.1) and not to a distinct carbamate kinase as was previously reported (9).

MATERIALS AND METHODS

Materials. All amino acids, pyrimidines, glutamine, streptomycin sulfate, lithium carbamylphosphate, ADP, and ATP were obtained from Sigma Chemical

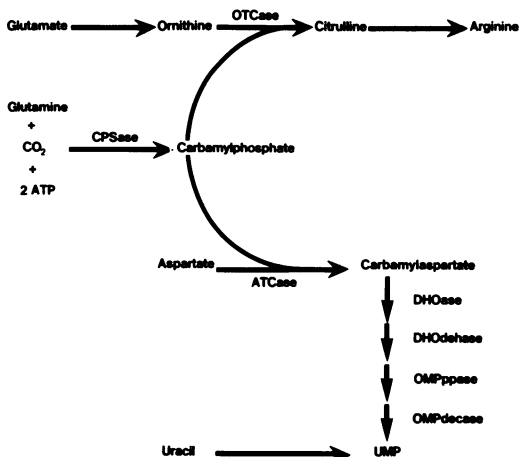


FIG. 1. Pathways of arginine and pyrimidine biosynthesis in *S. marcescens*. OTCase, Ornithine carbamyltransferase; CPSase, carbamylphosphate synthase; ATCase, aspartic carbamyltransferase; DHOase, dihydroorotase; DHOdehase, dihydroorotate dehydrogenase; OMPppase, orotidine 5'-monophosphate pyrophosphorylase.

Co. Sodium [^{14}C]carbonate was purchased from New England Nuclear Corp. Yeast alcohol dehydrogenase was obtained from Boehringer-Mannheim.

Strains. *S. marcescens* HY was obtained from J. R. Wild, Texas A & M University, College Station, Tex.

Media and growth conditions. The basal salts medium described by Vogel and Bonner (25), supplemented with glucose (0.2%, wt/vol) and FeCl_3 (0.02 g/liter), was used. The specific growth rate and CPSase titer for cells grown in this medium were comparable to those for cells grown in the ammonium citrate-glycerol medium (12) used by other investigators for *S. marcescens*. For examination of carbamate kinase activity in cells grown with arginine as the sole source of nitrogen, the growth medium had the following composition (grams per liter): dibasic potassium phosphate, 13.6; calcium chloride, 0.01; ferrous sodium sulfate, 2; arginine, 4 (final pH, 7.4).

Preparation of cell extracts. Cells were harvested during exponential growth, washed once with water, and suspended at 0.5 g (wet weight) per ml in 0.1 M potassium phosphate buffer, pH 7.6, containing 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM ATP, and 4 mM MgCl_2 . Cell extracts were obtained by a single passage through a continuous-flow Aminco French pressure cell with pressure maintained at 15,000 lb/in 2 . The crude-cell extract was centrifuged at 20,000 $\times g$ for 20 min. Protein concentration was determined by the method of Lowry et al. (14), with crystalline bovine serum albumin as a standard.

Enzyme assays. CPSase activity was determined in reaction mixtures (final volume, 0.5 ml) containing enzyme, 100 mM triethanolamine buffer (pH 8.0), 12 mM ATP, 16 mM MgCl_2 , 10 mM $\text{NaH}^{14}\text{CO}_3$ (20,000 to 100,000 cpm/ μmol), 100 mM KCl, and 10 mM glutamine or 100 mM NH_4Cl . The [^{14}C]carbamylphosphate formed was determined as previously described (3).

Carbamate kinase and acetate kinase activities were determined by the conversion of [^3H]ADP to ATP, using carbamylphosphate and acetylphosphate, respectively, as phosphoryl donors. The reaction mixture (final volume, 0.15 ml) contained enzyme, 100 mM sodium citrate (pH 5.4), 15 mM ADP (3×10^5 to 5×10^5 cpm/ μmol), 20 mM MgCl_2 , and 15 mM carbamylphosphate or acetylphosphate. The reaction was started by the addition of the phosphoryl donor, and at appropriate times samples were spotted on polyethyleneimine-Avicel cellulose thin-layer plates and dried with hot air to stop the reaction. Plates were developed in 0.5 M potassium phosphate (pH 3.4) to 15 cm above the origin. Dried plates were exposed overnight to X-ray films. The spots corresponding to ADP and ATP were cut out, extracted with 0.25 ml of 2 M NH_4OH for 20 min, and counted in toluene-based scintillation mixture containing 10% BioSolv (Beckman Instruments, Inc.). For each assay, a parallel reaction was run in the absence of carbamylphosphate, and the amount of conversion obtained under these conditions was subtracted to obtain the reported values for carbamate kinase or acetate kinase.

Aspartic carbamyltransferase and ornithine carbamyltransferase were measured colorimetrically as previously described (4, 28).

Purification of CPSase. Preliminary experiments showed that CPSase from *S. marcescens* aggregates in the presence of the activator ornithine and is monomeric in the presence of the inhibitor UMP, as is the case for the enzymes from *E. coli* (24) and *Salmonella typhimurium* (3). This change in molecular size in response to various ligands was used in purification of CPSase from *S. marcescens* by the procedure previously described for the enzyme from *Salmonella typhimurium* (3). The final enzyme preparation appeared homogeneous after electrophoresis on 7.0% polyacrylamide gel at pH 9.5 (8).

Sucrose gradient ultracentrifugation. Density gradient centrifugation was carried out as described by Martin and Ames (16), using an SW-40Ti rotor in a Beckman L2-65B ultracentrifuge. Samples (0.2 ml) of enzyme preparation were layered on 13-ml linear sucrose gradients in cellulose nitrate tubes. After centrifugation for 12 to 24 h at 100,000 $\times g$, fractions of 0.3 ml were collected from the bottom of each tube and assayed for enzyme activity.

RESULTS

Absence of a specific carbamate kinase from *S. marcescens*. Glasziou reported the partial purification of a carbamate kinase from a strain of *S. marcescens* (9). Adequate criteria, however, were not used to distinguish between carbamate kinase activity and activities of acetate kinase and CPSase (for a review, see reference 1). Carbamate kinases from streptococci (1) as well as acetate kinase (29) and CPSase (5) purified from *E. coli* all catalyze the phosphorylation of ADP by carbamylphosphate. Accordingly, ADP kinase activities using carbamylphosphate or acetylphosphate as the phosphoryl donor were determined in extracts of cells grown with ammonia or arginine as a sole nitrogen

source. The results (Table 1) show that carbamate kinase activity was not induced by growth on arginine and that the ratio of acetate kinase to carbamate kinase activities remained constant (25:1). Extracts from *S. marcescens* cells grown with arginine as a nitrogen source were applied to sucrose gradients, and fractions were assayed for ammonia- and glutamine-dependent CPSase, carbamate kinase, and acetate kinase activities. The results (Fig. 2) show the co-sedimentation of acetate kinase and carbamate kinase activities with a ratio of activities of 25:1. When extracts of *S. marcescens* were applied to DEAE-cellulose or Sephadex-G-200, carbamate kinase activity eluted similarly to acetate kinase activity. These results indicate that carbamate kinase activity detected in crude extracts of *S. marcescens* is due to a constitutive acetate kinase.

The results in Fig. 2 also show the presence of a single CPSase (molecular weight, 167,000) that could utilize glutamine or ammonia as an amide donor in the synthesis of carbamylphosphate. This CPSase had no detectable carbamate kinase activity under the assay conditions used here.

Absence of association of CPSase with other enzymes of pyrimidine biosynthesis. Cell extracts were applied to sucrose gradients (5 to 30% in 0.1 M potassium phosphate buffer, pH 7.6, containing 5% glycerol and 0.5 mM EDTA) in the absence and presence of various combinations of substrates and effectors (glutamine, ATP, Mg^{2+} , ornithine, and UMP each, when present, at a final concentration of 5 mM). Since dimethyl sulfoxide stabilized a complex involving CPSase in mammalian cells (23), additional gradients (5 to 20% sucrose) containing 30% dimethyl sulfoxide were also included. The gradients were assayed for CPSase and for aspartic and ornithine carbamyltransferases. CPSase was not associated with either, and a sedimentation coefficient of 9.0 corresponding to a molecular weight of 167,000 was obtained in all gradients. The sedimentation coefficient for purified CPSase was similar to that obtained in crude extracts, indicating that CPSase was not associated with other enzymes of the pyrimidine

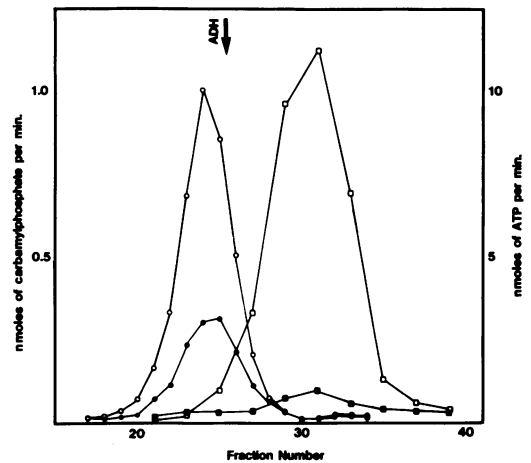


FIG. 2. Sucrose density gradient ultracentrifugation of extracts of *S. marcescens* HY; 0.3 ml of extract was layered on a 13-ml linear sucrose gradient (5 to 20%) in 0.1 M potassium phosphate buffer, pH 7.6, containing 5% glycerol, 1 mM EDTA, 4 mM $MgCl_2$, and 1 mM phenylmethylsulfonyl fluoride and centrifuged at 39,000 rpm for 24 h at 4°C. Fractions of 8 drops were collected. Yeast alcohol dehydrogenase (ADH) was used as a marker. Symbols: ○, glutamine-dependent CPSase; ●, ammonia-dependent CPSase; □, acetate kinase; ■, carbamate kinase.

biosynthetic pathway not assayed in the gradients. Gel filtration of Sephadex G-200 also yielded the same molecular weight value (160,000) for CPSase in crude extracts and in purified preparations.

Properties of purified CPSase. The MgATP saturation curve was determined in the presence of excess $MgCl_2$. Based on a stability constant for MgATP of 77,000 M^{-1} in triethanolamine buffer at pH 8.0 (18), the concentration of free Mg^{2+} was 4 mM. Under these conditions, the double-reciprocal plot was linear (Fig. 3a), yielding a K_m value of 2.5 mM. A Hill plot of the data (Fig. 3b) yielded an interaction coefficient of 1.0, indicating the absence of cooperativity in binding of MgATP. CPSase activity was also determined as a function of glutamine and ammonia concentrations. In both cases, the double-reciprocal plots were linear, yielding K_m values of 0.1 and 10.5 mM for glutamine and NH_3 , respectively.

Possible allosteric effectors of CPSase were tested for modulation of glutamine-dependent activity at nonsaturating concentrations of ATP. CPSase activity was stimulated by ornithine and inhibited by UMP (Fig. 4). Both compounds were effective at physiological concentrations; $A_{0.5}$ (the concentration required for half-maximal activation) was 0.05 mM for ornithine, and $I_{0.5}$ (concentration required for half-maximal in-

TABLE 1. *Titers of ADP kinase in S. marcescens*

Nitrogen source for growth	Sp act ($\mu\text{mol}/\text{min}$ per mg of protein) assayed with:	
	Acetyl-phosphate	Carbamyl-phosphate
Ammonia	67.5	2.5
Arginine	65.6	2.5

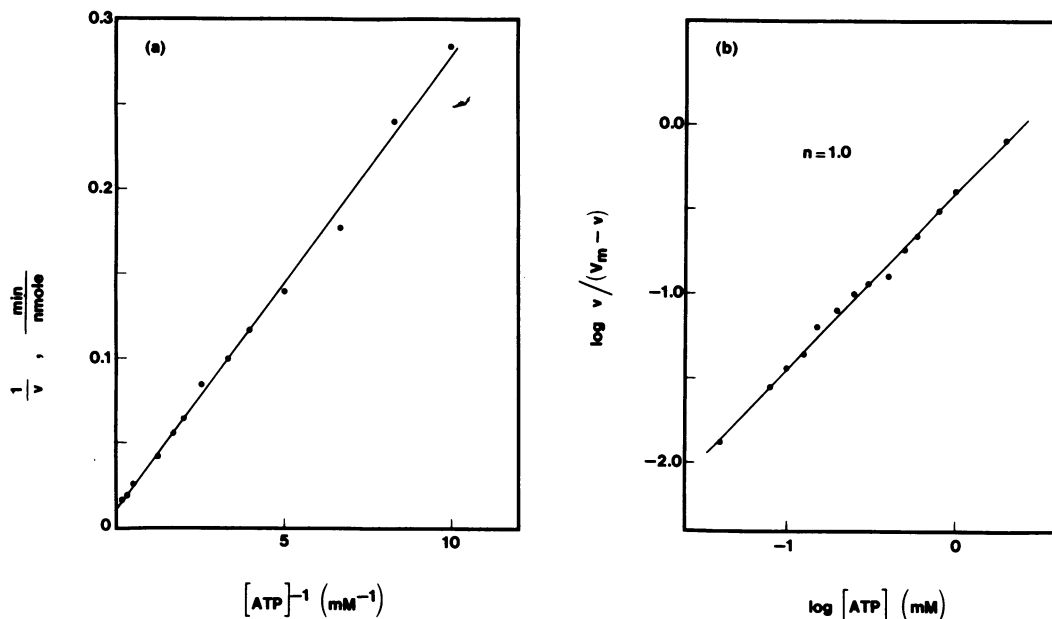


FIG. 3. CPSase activity as a function of ATP concentration. (a) Double-reciprocal plot; (b) Hill plot. The enzyme was assayed in triethanolamine buffer at pH 8.0 as described in the text, except that the concentration of free MgCl_2 was kept constant at 4 mM.

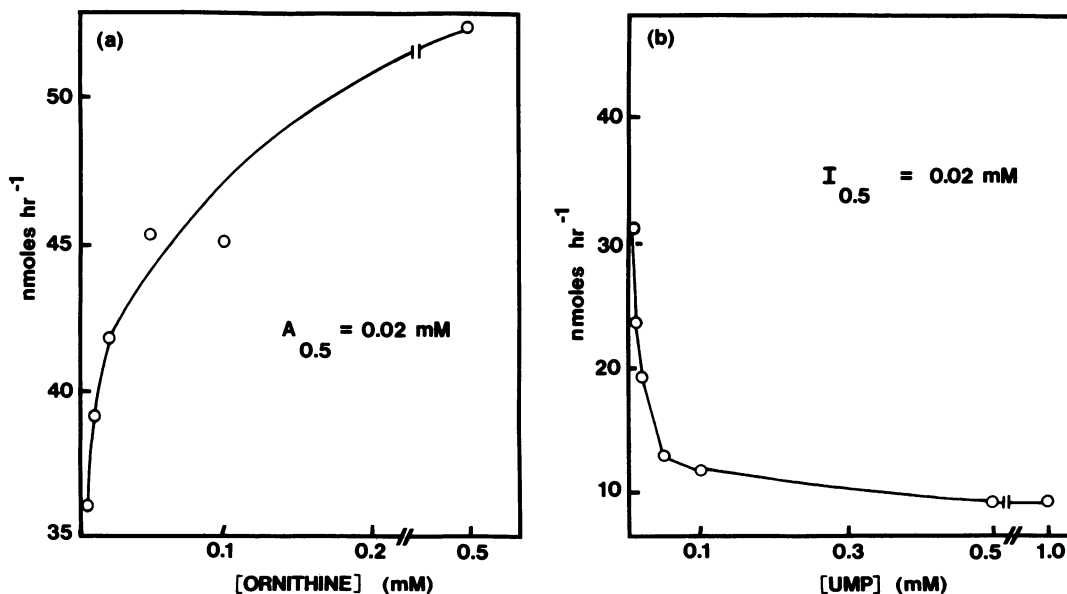


FIG. 4. Effect of ornithine and UMP. Activity was determined as described in the text, except that the concentrations of ATP and MgCl_2 were 2 and 6 mM, respectively. $I_{0.5}$ (inhibitor concentration required for half-maximal inhibition) and $A_{0.5}$ (activator concentration required for half-maximal activation) were determined from Hill plots of the data (not shown).

hibition) was 0.02 mM for UMP. Arginine and cytidine triphosphate had no effect on CPSase activity.

Repression of CPSase synthesis. The ti-

ters of CPSase were determined in extracts of cells grown in the absence and presence of arginine and uridine. The results (Table 2) indicate that both compounds could repress the synthesis

TABLE 2. *Repression of CPSase activity in S. marcescens HY*

Addition to growth medium	Sp act ^a (nmol/h per mg of protein)	Relative sp act (%)
None	11.5	100
Arginine ^b	6.9	60
Uridine ^b	5.6	49
Arginine and uridine	2.8	24

^a Extracts were dialyzed in 0.1 M potassium phosphate buffer, pH 7.6, for 3 h at 4°C before assay.

^b Arginine and uridine were added to the growth medium at a final concentration of 200 µg/ml.

of the enzyme and that these effects were cumulative.

DISCUSSION

Carbamate kinase, together with ornithine carbamyltransferase and arginine deiminase, comprise the deiminase pathway which catalyzes the conversion of arginine to ornithine, carbon dioxide, and ammonia with the formation of 1 mol of ATP per mol of arginine utilized. The arginine deiminase pathway provides a significant source of energy for fermentative organisms and a minor one for aerobes (1). The results reported here indicate that carbamate kinase activity detected in extracts of *S. marcescens* is due to a constitutive acetate kinase. Acetate kinases from *E. coli* (29) and *Pseudomonas aeruginosa* (1) also catalyze the phosphorylation of ADP by carbamylphosphate. The absence of a distinct carbamate kinase was also shown for *E. coli* (11) and *Salmonella typhimurium* (3; A. T. Abdelal, unpublished data). In all these cases, the absence of a separate carbamate kinase is consistent with the fact that these enteric organisms cannot utilize arginine as a source of energy under anaerobic conditions (1).

The presence of a specific carbamate kinase as well as a CPSase in certain organisms (e.g., *P. aeruginosa*) raises the interesting question about the mechanisms by which an energy-wasteful cycle involving carbamylphosphate is avoided (1). Two moles of ATP is utilized in the reaction catalyzed by CPSase, whereas only 1 mol of ATP is generated in the reaction catalyzed by carbamate kinase. Accordingly, simultaneous operation of both enzymes would result in loss of 1 mol of ATP per mol of carbamylphosphate processed. In the case of *S. marcescens*, the acetate kinase present can also phosphorylate ADP, using carbamylphosphate as a donor in the reaction. This activity, however, may not be physiologically significant in view of the low affinity of acetate kinases for carbamylphosphate (1, 24, 30) in comparison with the

high affinity exhibited by the biosynthetic enzymes which utilize carbamylphosphate, namely, aspartic and ornithine carbamyltransferases (4, 17).

Studies by Wild and Belser (26, 27) of the enzymes of pyrimidine biosynthesis in *S. marcescens* HY indicated the presence of a multifunctional enzyme complex composed of three enzymes: orotidine 5'-monophosphate pyrophosphorylase, orotidylate decarboxylase, and dihydroorotase. These authors have suggested that this association of three nonsequential enzymes may represent a larger biosynthetic complex which provides a channeling or regulatory unit for pyrimidine biosynthesis. The results presented here show that CPSase is not associated with such a complex or with other pyrimidine biosynthetic enzymes.

CPSase from *S. marcescens* is similar in molecular weight and many of its kinetic parameters to the enzymes from *E. coli* (20, 24) and *Salmonella typhimurium* (2, 3). The three enzymes are also regulated similarly; they are subject to cumulative repression by arginine and a pyrimidine as well as to activation by ornithine and feedback inhibition by UMP. These similarities are particularly significant in view of the reports (6, 15) that *Serratia* is the most divergent genus among the *Enterobacteriaceae*. Finally, in view of the finding that aspartic carbamyltransferase in *S. marcescens* HY is insensitive to feedback inhibition (28), it is clear that modulation of CPSase titer and activity plays an important role in the control of pyrimidine biosynthesis in this organism.

ACKNOWLEDGMENTS

This work was supported by a research grant from the National Science Foundation (PCM 78-10039).

We are grateful to Omana Nainan and Su-Yu Lin Ruo for excellent technical assistance.

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