PYRIMIDINE METABOLISM IN *STREPTOMYCES GRISEUS*

THESIS

Presented to the Graduate Council of the University of North Texas in Partial Fulfillment of the Requirements For the Degree of

MASTER OF SCIENCE

by

Lee E. Hughes, B.A.

Denton, Texas

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Salvage of pyrimidine nucleosides and bases by S. griseus and the regulation of aspartate transcarbamoylase (ATCase) were studied. The velocity-substrate curve for S. griseus ATCase was hyperbolic for both aspartate and carbamoylphosphate. The enzyme activity was diminished in the presence of ATP, CTP, or UTP. The synthesis of ATCase was repressed in cells grown in the presence of exogenous uracil. The specific activity of cells grown with uracil was 43 percent of that for cells grown in minimal medium only. Maximal ATCase and dihydroorotase activities were found in the same column fraction after size-exclusion chromatography, suggesting that both activities could reside in the same polypeptide. The pyrimidine salvage enzymes cytosine deaminase and uridine phosphorylase were identified in S. griseus using HPLC reversed-phase chromatography.
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INTRODUCTION

The study of pyrimidine metabolism is important due to the role of pyrimidines as building blocks for the macromolecules, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). Along with purines, the pyrimidines are essential for cellular growth and for the passing of genetic information to subsequent generations. The synthesis of pyrimidine nucleotides de novo has been studied extensively in bacteria (Yates & Pardee, 1956a, 1956b, 1957; Beckwith et al., 1962; Hayward & Belser, 1965; Yan & Demerec, 1965; Hutson & Downing, 1968; Foltermann et al., 1981; Grogan & Gunsalus, 1993), fungi (Lacroute, 1968; Caroline, 1969) and mammals (Hager & Jones, 1967; Nakanishi et al., 1968). The pathway appears to be universal and follows the same sequence in all organisms thus far studied (Ó'Donovan & Neuhard, 1970; Grogan & Gunsalus, 1993). Although the sequence is the same, regulation of the pathway varies with different organisms.

Another important aspect of pyrimidine metabolism is the salvage pathway. Recycling of pyrimidine compounds, released primarily from the degradation of messenger RNA, conserves the initial biosynthetic energy investment.
Pyrimidine biosynthetic pathway

There are six enzymatic steps in the biosynthesis of uridine-5'-monophosphate (UMP) (Fig. 1), which serves as a precursor for all the pyrimidine nucleoside triphosphates.

The first step in the synthesis of pyrimidines is catalyzed by the enzyme carbamoylphosphate synthetase (CPSase). The reaction utilizes bicarbonate, ammonium ions or glutamine, and two molecules of ATP in the formation of one molecule of carbamoylphosphate and ADP (Anderson & Meister, 1965; Kalman et al., 1966). Carbamoylphosphate is required for both arginine and pyrimidine synthesis (Abdelal et al., 1969).

The production of carbamoylaspartate by aspartate transcarbamoylase (ATCase) is the first committed step in pyrimidine biosynthesis. Aspartate is carbamoylated at the amino group, producing carbamoylaspartate and releasing inorganic phosphate. The structure and regulation of ATCase is discussed in detail elsewhere in this paper.

The next reaction involves the cyclization of carbamoylaspartate, with the release of a molecule of water, to produce dihydroorotate. This step is catalyzed by the enzyme dihydroorotase (DHOase). Following this, dihydroorotate is oxidized to orotate in a reaction catalyzed by dihydroorotate dehydrogenase. The first pyrimidine nucleotide is then produced by the transfer of
ribose-5'-phosphate from 5'-phosphoribosyl-1'-pyrophosphate (PRPP) to orotate to form orotidine-5'-monophosphate (OMP), a reaction catalyzed by orotate phosphoribosyltransferase (OPRTase). OMP is decarboxylated by the enzyme OMP decarboxylase (OMPdeCase) in the final step in the production of UMP.

The pyrimidine nucleoside triphosphates, uridine-5'-triphosphate (UTP) and cytidine-5'-triphosphate (CTP), are ultimately produced from UMP. In sequential steps, UMP is first phosphorylated to uridine-5'-diphosphate (UDP) by the highly specific UMP kinase. UDP is further phosphorylated by a non-specific enzyme, nucleoside diphosphate kinase, to form UTP. UTP is converted to CTP by the enzyme CTP synthetase, which transfers an amino group from glutamine.

Organization of enzyme activities

In the bacterial systems except Bacillus studied thus far, it appears that the six enzymatic steps of pyrimidine biosynthesis are encoded by unlinked genes. This is not the case for fungi and animals.

For fungi, Lacroute et al. (1965) showed the presence of two CPSases in yeast, one for the arginine pathway (CPSase-arg) and the other for the uracil pathway (CPSase-ur). The same was found for Neurospora crassa (Davis, 1963, 1965) and Coprinus radiatus (Prevost, 1966). The carbamoylphosphate produced by these enzymes is
preferentially channeled to their respective pathways. Additionally, CPSase-ur and ATCase are coded for in a single locus \((\text{ura}2)\) in yeast and are transcribed into a single messenger RNA \((\text{Denis-Dupil} \& \text{Lacroute}, 1971)\). The first two enzymatic activities of the pyrimidine biosynthetic pathway in fungi are combined in a single, multienzyme complex \((\text{Lue} \& \text{Kaplan}, 1969)\). The remainder of the genes for UMP biosynthesis are unlinked \((\text{Jones}, 1972)\). Thus, in simple eukaryotes, there are only five structural genes.

Two main CPSase enzymes have also been found in mammalian systems. CPS-I is mitochondrial and is found in high levels in the liver, along with the first enzyme in the arginine biosynthetic pathway, ornithine transcarbamoylase \((\text{OTCase})\). CPS-II is cytoplasmic and is associated with ATCase \((\text{Makoff} \& \text{Radford}, 1978)\). Jones \((1971)\) suggested that the first three enzymatic activities, CPSase, ATCase, and DHOase, and the final two, OPRTase and OMPdeCase, are likely to be multienzymic proteins like the yeast \(\text{ura}2\) gene product. More recent evidence \((\text{Jones}, 1980)\) supports the hypothesis of a single multienzymic polypeptide for CPSase-ATCase-DHOase activities, as well as for a multienzyme OPRTase-OMPdeCase. Therefore, in mammals, the six enzymatic steps of pyrimidine biosynthesis are encoded in only 3 structural genes.
Bacterial aspartate transcarbamoylase

As the enzyme catalyzing the first committed step in bacterial pyrimidine biosynthesis, the study of the structure and regulation of ATCase is important in understanding the catalytic mechanism and evolutionary history of this enzyme in different bacterial species. Three classes of bacterial ATCase have been described, with varying molecular weights of the holoenzyme, quaternary structure, and enzyme kinetics (Bethell & Jones, 1969; Wild et al., 1980) (Fig. 2).

The largest ATCases are found in Class A. One example, Pseudomonas fluorescens ATCase, has a complex with a molecular weight of 464 kDa. This complex appears to consist of a 1:1 ratio of 34 kDa and 45 kDa polypeptides (Bergh & Evans, 1993). The presence of two polypeptides is consistent with recent sequencing studies in P. aeruginosa and P. putida which show two open reading frames encoding polypeptides of approximately these sizes (J.F. Vickrey and M.J. Schurr, personal communication). Based on the mass of the constituent subunits and the stoichiometry of the complex, the protein must be a dodecamer (Bergh & Evans, 1993). Characteristics include hyperbolic substrate saturation curves and inhibition by ATP, UTP, and CTP which is competitive with carbamoylphosphate and noncompetitive with aspartate (Neumann & Jones, 1964; Bethell & Jones, 1969; A.J. Linscott, personal communication).
Figure 2. Classes of bacterial ATCase. Adapted from Bergh & Evans (1993).
Class A ATCases

Catalytic Trimer

45 kDa

34 kDa

Class B ATCases

Catalytic Trimer

17 kDa

34 kDa

Regulatory Dimer

Class C ATCases

Catalytic Trimer

34 kDa
The Class B ATCases are smaller and are distinguished by sigmoidal substrate saturation curves. The classical example of this class is in *Escherichia coli*, whose ATCase is one of the most studied regulatory enzymes in nature. The enzyme consists of six larger, catalytic polypeptides with a molecular weight of 34 kDa each, which are grouped into two trimers, and of six smaller, regulatory polypeptides with a molecular weight each of 17 kDa, which are organized in three dimers (Kantrowitz & Lipscomb, 1988). The holoenzyme has a molecular weight of 300 kDa (Fig. 2). *E. coli* ATCase is inhibited by physiological levels of CTP (Yates & Pardee, 1956b; Gerhart & Pardee, 1962, 1964) and activated by ATP. Subunit interactions are responsible for sigmoidal concentration curves for both aspartate (Gerhart & Pardee, 1962) and carbamoylphosphate (Bethell et al., 1968).

The Class C ATCases are characterized by their small size, insensitivity to pyrimidine nucleotide effectors, and typical Michaelis-Menten kinetics for the carbamoylphosphate and the aspartate saturation curves. The *Bacillus subtilis* enzyme represents a typical Class C ATCase. The native enzyme is a trimeric protein with a molecular weight of 102 kDa, consisting of three 33.5 kDa subunits (Brabson & Switzer, 1975) (Fig. 2).
Pyrimidine salvage pathways

The recycling pathways of pyrimidines are important in the conservation of the initial energy investment made in pyrimidine biosynthesis, for the scavenging of exogenous pyrimidine compounds, and for the survival of organisms blocked in the biosynthetic pathway.

The utilization of exogenous pyrimidine compounds in recycling pathways is widely variable. For example, Pseudomonas species are unable to use thymine or thymidine (Potter et al., 1982) while Neisseria cannot use cytosine, cytidine, thymine, or thymidine (Jyssum & Jyssum, 1979). Likewise, some organisms are unable to directly utilize intracellular pyrimidine bases or nucleosides. Pseudomonas cannot directly deaminate cytidine to uridine, but can circumvent this by converting cytidine to cytosine which is then deaminated to uracil (West, 1988). Perhaps the best example of the importance of pyrimidine salvage pathway is found in Mycoplasma. These parasitic bacteria lack the pyrimidine de novo pathway completely and fulfill their pyrimidine requirements using exogenously supplied cytidine, uridine, uracil and thymine (Mitchell & Finch, 1977).

Because of the diversity among organisms in their pyrimidine salvage pathways, it should be possible to use the salvage pathways as taxonomic probes for different families or to trace the evolutionary history of a genus.
Figures 3-6 illustrate some of the different salvage schemes utilized by organisms (D.E. Beck, Ph.D proposal).

Streptomyces

Pyrimidine metabolism has not been studied in one important order, the Actinomycetes. This order contains a wide range of genera and species, generally divided into two broad morphological groups, nocardioform-actinomycetes and sporo-actinomycetes. The latter group, which is more morphologically complex and has the ability to form spores in or on the mycelium, contains a number of genera, one of the most widely known being Streptomyces.

The members of the genus Streptomyces are common, filamentous soil bacteria belonging to the order Actinomycetales. They are most notable for their large genome size, $10.5 \times 10^6$bp (Benigni et al., 1975), their high G+C content of 69-78%, and the wide range of antibiotics, vitamins, enzymes and enzyme inhibitors they have been found to produce (Goodfellow & Cross, 1983). As a result, thousands of strains have been isolated from soils and sediments around the world. While much work has been done to identify the medically and industrially important secondary metabolites, there are many basic questions of primary metabolism which have yet to be answered.
Figure 4. Pyrimidine salvage pathway utilized by Rhizobium. cdd = cytidine deaminase, cod = cytosine deaminase, cmk = CMP kinase, ndk = nucleoside diphosphate kinase, nmg = nucleoside monophosphate glycosylase, nuh = nucleoside hydrolase, pyrG = CTP synthetase, udk = uridine kinase, umk = UMP kinase, upp = uracil phosphoribosyltransferase.
Figure 5. Pyrimidine salvage pathway utilized by *Pseudomonas* and *Saccharomyces*. **cod** = cytosine deaminase, **cmk** = CMP kinase, **ndk** = nucleoside diphosphate kinase, **nuh** = nucleoside hydrolase, **pyrG** = CTP synthetase, **umk** = UMP kinase, **upp** = uracil phosphoribosyltransferase.
A representative species was chosen for study. Since *Streptomyces griseus* was one of the original antibiotic-producing organisms discovered by Waksman, it was the choice.

In order to understand the aspects of pyrimidine metabolism within the genus *Streptomyces* and possible evolutionary significance, study of the pyrimidine biosynthetic and salvage pathways of *S. griseus* was undertaken.

**METHODS**

Organism

*Streptomyces griseus* (ATCC 10137) was obtained from the American Type Culture Collection.

Media and growth conditions

*S. griseus* spore suspensions were obtained from growth on sporulation agar (Hopwood et al., 1985). This medium contained 1 g yeast extract, 1 g beef extract, 2 g tryptose, trace FeSO₄, 10 g glucose, and 15 g agar per liter.

Two different defined media were used to grow *S. griseus*. The solid medium used was *Streptomyces* minimal agar medium (Hopwood, 1967), modified for alternate carbon source. This medium contained 2 g (NH₄)₂SO₄, 0.5 g K₂HPO₄, 0.2 g MgSO₄·7H₂O, 0.01 g FeSO₄·7H₂O, and 15 g agar per liter.
Succinate, pH 7.0, was added after autoclaving to a final concentration of 20 mM. Liquid growth was done in Streptomyces minimal liquid medium (Hopwood et al., 1985), which contained 2 g (NH₄)₂SO₄, 5 g Difco Casamino acids, 0.6 g MgSO₄·7H₂O, 50 g polyethyleneglycol 8000, and 1 ml minor elements solution (per liter: 1 g ZnSO₄·7H₂O, 1 g FeSO₄·7H₂O, 1 g MnCl₂·4H₂O, and 1 g CaCl₂, anhydrous) in 800 ml distilled water. After autoclaving, 150 ml of 0.1 M NaH₂PO₄/K₂HPO₄ buffer, pH 6.8, and carbon source, succinate, pH 7.0, to 20 mM final concentration were added. Required growth factors were added at the time of inoculation.

Streptomyces cultures were grown at room temperature (20-25°C). Liquid cultures were shaken vigorously on an orbital shaker, in a baffled flask when available.

Streptomyces spore suspension

Spores were harvested from plate cultures of *S. griseus* according to a modified version of the procedure of Hopwood et al. (1985). All solutions and apparati were sterilized by autoclaving prior to use. Water, typically 9 ml, was added to a well-sporulating plate. The surface of the plate was scraped with a loop to suspend the spores. The liquid was then removed with a pipette and transferred to a tube. The suspension was mixed vigorously on a vortexer for 1-2 minutes, then filtered by vacuum through fiberglass wool to remove mycelial fragments. The filtrate was centrifuged for
10 minutes at 1800 x g in a Sorvall H1000B rotor. The supernatant was immediately poured off. After resuspending the pellet in the remaining drop, 1.5 ml of water was added, mixed well, and transferred to a screw-top vial containing 0.5 ml 80% glycerol. The suspension was mixed and stored at -20°C until use.

**Aspartate transcarbamoylase assay**

Cells for ATCase assays were prepared by inoculating a flask containing 100 ml of *Streptomyces* minimal liquid medium with 100 μl of a dense spore suspension of *S. griseus*. After 3 days incubation, 2 ml of this starter culture were transferred to one liter of the same medium. After 3 days, the cells were harvested by centrifugation at 10,000 x g for 20 minutes at 4°C with the centrifuge brake turned off. Most of the supernatant was poured off and the pellet was resuspended in the remaining amount (10-15 ml). The cells from 500 ml of the culture were transferred to 50 ml, disposable conical tubes. The centrifuge tubes were washed with 10 ml of distilled water to remove residual cells and the wash added to the cells in the conical tubes. The conical tubes were then centrifuged in a Sorvall H1000B rotor for 15 minutes at 1800 x g at 4°C. The supernatant was poured off. The cell pellet was either frozen at -20°C for storage or used immediately. Just prior to use, the
cells were resuspended in ATCase breaking buffer (3 grams of cells, wet weight, to 2 ml buffer). ATCase breaking buffer consists of 2 mM β-mercaptoethanol, 20 μM ZnSO₄, and 50 mM Tris-HCl, pH 8.0.

Cell extract was prepared by breaking the cells suspended in ATCase breaking buffer using sonication. The cell suspension was sonicated for 5 minutes while the tube was in an ethanol-ice water slurry to control heating of the sample. The sonicated suspension was transferred to an SA600 centrifuge tube and spun at 4°C for 1 hour at 37,000 x g. The resulting supernatant was transferred to a sterile 15 ml, disposable conical tube and stored at 4°C until the cell extract was used in an assay. All assays were performed within 48 hours of cell extract preparation.

ATCase activity was assayed by measuring the amount of carbamoylaspartate (CAA) produced in 20 minutes at 30°C according to the method of Gerhart & Pardee (1962), with modifications using the color development procedure of Prescott & Jones (1969). The optimal pH for ATCase in S. griseus was first determined by assaying ATCase activity with pH varied from 7.0 to 10.0. All the subsequent assays were done at the optimal pH.

Assays were performed to determine the Km for aspartate (Kmₘₐₛₚ) and for carbamoylphosphate (Kmₜₚ) at pH 9.5. For determination of Kmₘₐₛₚ, the assay tubes
contained the following reagents in a final volume of 2 ml: 1 mM to 30 mM potassium aspartate at pH 9.5, 40 µl cell extract, 200 µl dilithium carbamoylphosphate (8 mg/ml), 80 µl Tri-buffer (stock solution contains 0.051 M diethanolamine, 0.051 M N-ethylmorpholine, and 0.1 M MES) adjusted to pH 9.5 (Ellis & Morrison, 1982), and ddH$_2$O to volume. For determination of $K_m^{cp}$, the same reagents were used with the exception that potassium aspartate concentration was 20 mM for all tubes and the carbamoylphosphate concentration varied from 0.081 mM to 5.2 mM. Additionally, in both determinations, effector response was examined by the addition of ATP, CTP or UTP to the assay tubes at a final concentration of 1 mM.

All assay tubes were prepared in advance, without addition of carbamoylphosphate, and preincubated at 30°C for 4 minutes. The reaction was initiated by the addition of 200 µl of carbamoylphosphate (8 mg/ml) for the aspartate curve and, for the carbamoylphosphate curve, by 200 µl of carbamoylphosphate of varying concentrations (0.081 mM - 5.2 mM). At 10 minute intervals up to 30 minutes, 0.5 ml of the reaction mixture was pipetted to stop tubes which were on ice. These tubes contained 0.5 ml ddH$_2$O and 1 ml color mix (2 parts 5 mg/ml antipyrine in 50 percent sulfuric acid and 1 part 8 mg/ml monoxime in 5 percent acetic acid). These tubes remained on ice until all samples were collected.
Color was developed by incubating the tubes in a 60°C water bath with the tubes exposed to the light of the room and capped by marbles to limit evaporation. After 2 hours of incubation, the absorbance was read in a Beckman DU-40 spectrophotometer at a wavelength of 466 nm. Controls were utilized to determine background readings in tubes containing all reactants but no cell extract and in tubes with cell extract but lacking substrate. All readings were blanked to these controls. The kinetic curves were generated by plotting specific activity of the enzyme (nmol carbamoylaspartate/min/mg protein) versus the concentration of aspartate or carbamoylphosphate. The $K_{m_{asp}}$ and $K_{m_{cp}}$ were determined from the intercepts of the x-axis on Lineweaver-Burk plots (1/substrate versus 1/velocity).

Repression of ATCase in cells grown in the presence of exogenous uracil (50 $\mu$g/ml) was also studied. ATCase activity was measured as previously described, in this case using a potassium aspartate concentration of 20 mM, and the specific activity of ATCase in cells grown with uracil was compared to the specific activity of cells grown in minimal medium without uracil.

**Protein concentration determination**

Protein was quantified by the method of Lowry et al. (1951). Bovine serum albumin (BSA) at 0-100 $\mu$g was used to
produce a standard curve. The BSA was divided into 11 tubes at 10 \( \mu g \) increments as follows: the 0 \( \mu g \) tube contained only 200 \( \mu l \) ddH\(_2\)O, while the 10 \( \mu g \) tube contained 190 \( \mu l \) ddH\(_2\)O + 10 \( \mu l \) 0.1 percent BSA (100 \( \mu l \) of 1 percent dessicated BSA in water diluted in 900 \( \mu l \) ddH\(_2\)O yields a 1 \( \mu g/\mu l \) solution of BSA). The rest of the tubes used in the standard curve were set up accordingly. Three different volumes (5 \( \mu l \), 10 \( \mu l \), and 20 \( \mu l \)) of the unknown sample cell extracts, as well as 1:10 dilutions in ddH\(_2\)O of each sample, were taken and brought up to 200 \( \mu l \) total volume with ddH\(_2\)O. To the standard and unknown tubes, 800 \( \mu l \) alkaline copper reagent (0.5 ml 2 percent sodium-potassium tartrate, 0.5 ml 1 percent copper sulfate, mixed before adding 49 ml of 2 percent sodium carbonate in 0.1 N sodium hydroxide) was added. The tubes were allowed to stand at room temperature for 10 minutes. Folin reagent (100 \( \mu l \), 1 N commercial preparation diluted 1:1 with ddH\(_2\)O) was added to all tubes while mixing. After incubating at room temperature for 30 minutes, the absorbance was read at 660 nm. The absorbance was plotted against the concentration of BSA to establish a standard curve.

**Nondenaturing polyacrylamide activity gels**

Cell extract was prepared as described previously for the ATCase activity assay. Samples of 18 \( \mu l \) of cell extract
were mixed with 2 μl of 10X loading buffer (1 ml separating
gel buffer, 10 ml glycerol, 1 ml ddH₂O containing 2 mg
bromophenol blue). The total volume of 20 μl was loaded
onto a 6 percent nondenaturing polyacrylamide gel and run at
5 watts for 12 hours at room temperature. The stock
solution of acrylamide contained 40 percent w/v acrylamide
and 1 percent w/v N,N’-bis-methylene acrylamide in ddH₂O.
The separating gel buffer contained 36.3 g Tris in 100 ml
ddH₂O adjusted to pH 8.9 with concentrated HCl. A 6 percent
acrylamide gel contained: 9.64 ml of 40 percent acrylamide,
7.5 ml separating gel buffer, 4.48 ml 40 percent sucrose and
38.38 ml ddH₂O. Ammonium persulfate, in the amount of 50
mg, was added to this mixture and then degassed for 10
minutes. Prior to pouring the gel, 20 μl of TEMED was added
to the solution. The polyacrylamide gel was poured between
20.5 cm × 20.5 cm siliconized glass plates. The gel was
allowed to set for 2 hours before use and was covered with
plastic wrap to prevent dehydration. The running buffer
contained 10 mM Tris and 40 mM glycine at a pH of 8.3.

The gel was stained specifically for ATCase activity by
a procedure developed by Bothwell (Bothwell, 1975,
Dissertation, University of California, Berkeley) and
further modified by K. Kedzie (1987, Ph.D. dissertation,
Texas A&M University, College Station), who replaced
histidine for imidazole. When the bromophenol blue had
reached the bottom of the gel, the plates were separated, and the gel was placed in 250 ml ice-cold 50 mM histidine, pH 7.0, for 5 minutes while gently shaking. After this equilibration, 5 ml of 1 M aspartate and 10 ml of 0.1 M carbamoylphosphate were added to the gel and allowed to react for 10 minutes. The gel was rinsed 3 times with ice-cold distilled water to remove the reactants. Orthophosphate released enzymatically and trapped in the gel was allowed to precipitate by the addition of 3 mM lead nitrate in ice-cold 50 mM histidine, pH 7.0. Lead nitrate was removed after 10 minutes with 3 changes of ice-cold water. ATCase activity was visible as white bands at the site of lead phosphate precipitation.

**Sephacryl S-400 column chromatography**

Cell extract was prepared from the cell pellet of 1 liter of culture resuspended in 5 ml ATCase buffer. To this extract, 0.02 g of blue dextran was added and mixed by repeated pipetting with a Pasteur pipette.

This mixture was loaded onto a 50 x 3 cm BioRad column containing a Sephacryl S-400 matrix. Size-exclusion buffer (16 ml 250 mM EDTA, 80 μl 1 M zinc acetate, 2 ml β-mercaptoethanol, and 40 ml Tris-HCl, pH 8.2, in ddH₂O to a final volume of 4 liters) was used as the elution buffer. The front buffer of 70 ml was removed and discarded, after
which 80 tubes of 2 ml fractions were collected. Samples were stored at 4°C.

Each fraction was assayed for both ATCase and DHOase activity. The ATCase assay was used as described previously with the exception that 200 μl of each fraction sample was used where the protocol calls for 40 μl of cell extract. The volume of ddH₂O was adjusted to compensate. The DHOase assay reaction tubes contained 200 μl 10 mM EDTA, 200 μl fraction sample, 200 μl 1 M Tris, pH 8.6, and 1200 μl ddH₂O to which 200 μl of dihydroorotate (0.0316 g to 10 ml 0.1 M phosphate buffer, pH 7.5) were added to start the reaction. After a 20 minute incubation at 30°C, 0.5 ml of the reaction mix was removed and placed in 1.5 ml of stop mix (same as used for ATCase assay). These tubes were incubated at 60°C for 2 hours and then read at 466 nm.

Characterization of salvage pathways

Cell extract was obtained by sonication and centrifugation of S. griseus grown in 500 ml Streptomyces minimal medium plus cytidine (50 μg/ml). The cells were washed twice in 5 ml of HPLC breaking buffer (4 mM MgCl₂, 10 mM Tris-HCl, pH 7.0) before being resuspended in 1 ml HPLC breaking buffer per 0.5 g cells for sonication. Cell debris was removed by centrifuging at 10,000 x g for 10 minutes.
Salvage enzyme activity was observed by the addition of 50 μl of cell extract to 450 μl of 1 mM solutions of cytosine, cytidine or uridine. The tubes were incubated for 1 hour at room temperature, then the solutions were drawn into a 3 cc sterile syringe using a 19 1/2 gauge needle. The solutions were filtered using Gelman Acrodisc LC13 PVDF 0.2 μm filters and divided into two tubes each. One tube was frozen at -20°C to stop the reaction, and the other was left at room temperature to incubate an additional hour. A control containing 50 μl cell extract in 450 μl HPLC breaking buffer was also prepared in this manner.

After incubation, the samples were analyzed by high performance liquid chromatography (HPLC). Samples of 20 μl of the assay mix were injected onto a reversed-phase column (Beckman Ultrasphere ODS 5 μm, 25 cm x 4.6 mm I.D.; particle size 5 μm, pore size 100 Å). Utilizing a Waters Model 510 pump, a U6K injector, and a variable wave-length SpectroMonitor 5000 photodiode array detector (LDC Analytical), peak heights were quantified on a Waters Model 740 Data Module and the LCTalk Software Package (LDC Analytical) in conjunction with a personal computer. Compounds were detected by monitoring the column effluent at 254 nm with a sensitivity set at 0.06 absorbance units full scale (a.u.f.s.). Individual nucleosides and bases were identified using retention times established by monitoring
known standards and their retention times on the reversed-phase column.

RESULTS AND DISCUSSION

Kinetic analysis of ATCase

The ATCase of Streptomyces griseus was characterized by performing assays to study the enzyme activity. It was first necessary to determine the pH for optimal activity. This was found to be pH 9.5. Then, the range of aspartate concentrations to be used to generate velocity-substrate curves for aspartate was determined. Concentrations from 1 mM to 30 mM aspartate were used.

Two primary difficulties manifested themselves immediately in the study of ATCase in S. griseus. First, the enzyme appeared to lose activity rapidly in the cell extracts. In most cases, ATCase activity fell to unmeasurable levels within 24 to 72 hours after breakage of the cells. A second factor, high background reading at 466 nm, exacerbated the enzyme activity problem since this background appeared to increase from the time the cells were freshly broken. As a consequence of these factors, all assays were performed within 48 hours of the preparation of the cell extract. When possible, the experiments were carried out immediately.
Data points for the ATCase assays represent the average of three measurements. The velocity-substrate curve for aspartate for *S. griseus* was hyperbolic in nature (Fig. 7). This is typical of an enzyme which has no cooperativity between subunits. The enzyme gave a $K_m_{asp}$ of 4.17 mM and a $V_{max}_{asp}$ which is reached at 15 mM (Fig. 8). The velocity-substrate curve for carbamoylphosphate was also hyperbolic (Fig. 9). The enzyme was found to have a $K_m_{cp}$ of 1.25 mM (Fig. 10). When the effector molecules ATP, CTP or UTP were present, activity of ATCase in *S. griseus* was diminished (Table 1).

**Table 1. Effector response of *S. griseus* ATCase at $V_{max}$**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>No effector</th>
<th>ATP</th>
<th>CTP</th>
<th>UTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartate</td>
<td>100</td>
<td>39</td>
<td>40</td>
<td>48</td>
</tr>
<tr>
<td>Carbamoylphosphate</td>
<td>100</td>
<td>25</td>
<td>15</td>
<td>17</td>
</tr>
</tbody>
</table>

The synthesis of ATCase was repressed in cells grown in the presence of exogenous uracil. These cells had a specific activity which was 43 percent of that found for cells grown in minimal only. This suggests that there is control at the gene expression level. Repression of the gene for ATCase would prevent the cell from unnecessarily expending energy to produce a gene product which is not needed. Since ample uracil was available in the medium, the
Figure 7. *S. griseus* ATCase activity as a function of aspartate concentration.
Figure 8. Lineweaver-Burk plot of *S. griseus* ATCase activity as a function of aspartate concentration.
Figure 9. *S. griseus* ATCase activity as a function of carbamoylphosphate concentration.
Figure 10. Lineweaver-Burk plot of *S. griseus* ATCase activity as a function of carbamoylphosphate concentration.
cells did not need to synthesize pyrimidines but could make use of salvage pathways to satisfy pyrimidine requirements.

**Nondenaturing polyacrylamide activity gels**

A single band of ATCase activity was seen for *S. griseus* on the nondenaturing polyacrylamide activity gels. This band migrates at a rate intermediate to that of the *E. coli* and *P. aeruginosa* ATCase holoenzyme standards which were run on the same gel. This gives *S. griseus* ATCase an estimated molecular weight of near 400 kDa.

**Sephacryl S-400 column chromatography**

The maximal ATCase and DHOase activities were found in the same column fraction (Fig. 11). This suggests that both activities could reside in the same polypeptide, such as is found for CPSase and ATCase in *Saccharomyces cerevisiae* and for CPSase, ATCase, and DHOase in mammals. This unusual phenomenon for a bacterium is being pursued with purified proteins and will include an assay for carbamoylphosphate synthetase activity as well.

**Salvage enzyme assays**

The presence of specific enzymes for the salvage of pyrimidine bases and nucleosides was examined using HPLC reversed-phase. By incubating cell extract with the substrates cytosine, cytidine or uridine, it was possible to
Figure 11. Percent maximal activity of ATCase and DHOase from Sephacryl S-400 column fractions.
observe the activities of the enzymes by the disappearance of substrate and the formation of products.

With cytosine as substrate, there was no change after incubation, indicating that *S. griseus* did not utilize this base and lacks the enzyme cytosine deaminase (Fig. 12). When incubated in cytidine for one hour, there was a visible increase in uridine and a concomitant decrease in cytidine (Fig. 13). By the end of two hours, cytidine had almost disappeared from the solution, to be been replaced by uridine and uracil (Fig. 14). This indicated the presence of cytidine deaminase to catalyze the conversion of cytidine to uridine.

Since there was no conversion of cytidine to cytosine but uridine was utilized to produce uracil, this indicated the likely presence of a uridine-specific phosphorylase. If *S. griseus* had contained a nucleoside hydrolase, both the reaction from cytidine to cytosine and from uridine to uracil should have been present due to the traditional non-specific nature of that enzyme. To test this assumption, the extract was dialyzed to remove phosphates. The dialyzed extract was used to repeat the incubation in uridine. After dialysis, there was no longer any uracil produced. This supports the presence of the uridine-specific phosphorylase for catalysis of uridine to uracil in *S. griseus*. The phosphorylase requires inorganic phosphate for the reaction, while the nucleoside hydrolase has no such requirement.
Figure 12. HPLC chromatogram of products from a two-hour incubation of \textit{S. griseus} cell extract with cytosine substrate.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Retention Time (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosine (C)</td>
<td>2.40</td>
</tr>
<tr>
<td>Uracil (U)</td>
<td>3.55</td>
</tr>
<tr>
<td>Cytidine (CR)</td>
<td>4.24</td>
</tr>
<tr>
<td>Uridine (UR)</td>
<td>7.49</td>
</tr>
</tbody>
</table>

*actual peak area less than background*
Figure 13. HPLC chromatogram of products from a one-hour incubation of *S. griseus* cell extract with cytidine substrate.
Figure 14. HPLC chromatogram of products from a two-hour incubation of *S. griseus* cell extract with cytidine substrate.
Chromatogram: 254 nm

Retention Time (mins)

- Cytosine (C) 2.40
- Uracil (U) 3.55
- Cytidine (CR) 4.24
- Uridine (UR) 7.49

AU

Time (mins)

+0.1100

0.00

-0.0100
With phosphates removed by dialysis, the cessation of activity shows that the phosphorylase is present.

Based on the information obtained by the HPLC studies of the utilization of nucleosides and bases in *S. griseus*, it was possible to construct the salvage pathway for this organism (Fig. 15).

Conclusions

The study of pyrimidine metabolism in *S. griseus* has revealed yet more interesting facets to the physiology of members of this remarkable genus. *Streptomyces* and other actinomycetes have long eluded simple classification in relation to other organisms, for a time even being placed with the fungi. The aspects of pyrimidine metabolism examined in this research and the findings may be useful in the classification of the genus.

The kinetics of ATCase in *S. griseus* would seem to place it with the Class A bacterial ATCases. Like *P. aeruginosa*, a member of that class, *S. griseus* ATCase has velocity-substrate curves for both aspartate and carbamoylphosphate which are hyperbolic in nature. Additionally, the ATCase of both organisms is inhibited by the effector molecules ATP, CTP, and UTP.

Determination of the approximate mass of ATCase in *S. griseus* by a nondenaturing polyacrylamide activity gel does
Figure 15. Pyrimidine salvage pathway utilized by *S. griseus*. \( cdd \) = cytidine deaminase, \( udk \) = uridine kinase, \( udp \) = uridine phosphorylase, \( upp \) = uracil phosphoribosyl transferase.
not yet present such a clear picture. The enzyme motility is intermediate between that of \textit{P. aeruginosa}, a member of Class A, and \textit{E. coli}, representative of Class B. Further study to determine the exact mass of the enzyme as well as to define its subunit composition will be necessary before this relationship can be resolved.

The most intriguing finding is the association between ATCase and DHOase of \textit{S. griseus} as seen in the preliminary column separation studies. This is similar to the multienzymic proteins found in fungi and mammals. The association between these two enzyme activities in \textit{Streptomyces} will be the subject of further research. Once again, a streptomycete appears to have blurred the classic distinctions among organisms by possessing a trait heretofore seen only in eukaryotes. Indeed, \textit{Streptomyces} ATCase may define the genus as on the interface between prokaryotes and eukaryotes. A related finding is the suggestion that these organisms contain a linear chromosome (D.A. Hopwood, Chiron Corporation Lecture, American Society for Microbiology General Meeting, 1993).

The salvage pathway does little to clarify the overall placement of \textit{S. griseus}. Among those organisms with a similar salvage scheme are \textit{Bacillus}, another Gram positive bacterium, and \textit{Homo sapiens}, a eukaryote with a multienzymic polypeptide containing the first three pyrimidine activities (Jones, 1980).
It is apparent that streptomycetes will continue to defy simple explanation and resist placement in a neat "pigeonhole". Their uniqueness makes a study of Streptomyces all the more rewarding. With the general aspects of pyrimidine metabolism defined, further research will be initiated. In order to unravel the mysteries of pyrimidine metabolism in Streptomyces, there must be a focus on the genetic aspects of the pathway. Examination of the genes involved will best be accomplished by using mutants of in another species, S. coelicolor A3(2), which has become a model organism for studies of genetics in Streptomyces (Chater & Hopwood, 1983).


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