JOURNAL OF BACTERIOLOGY, June 1972, p. 895-904
Vol. 110, No. 3

Serine Biosynthesis and Its Regulation in *Bacillus subtilis*[1, 2]

MANUEL M. PONCE-DE-LEON AND LEWIS I. PIZER

Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Received for publication 22 December 1971

Cell-free extracts of *Bacillus subtilis* strains GSY and 168 convert ^{14}C-phosphoglycerate to ^{14}C-serine phosphate and ^{14}C-serine. These reactions indicate a functional phosphorylated pathway for serine biosynthesis in these cells. The addition of serine to the incubation mixture inhibited the formation of both radioactive products. Extracts of mutant strains that require serine for growth lacked the capacity to synthesize serine phosphate, confirming that the phosphorylated pathway was the only functional pathway available for serine synthesis. Serine phosphate phosphatase and phosphoglycerate dehydrogenase activity were demonstrated in cell extracts, and the phosphoglycerate dehydrogenase was shown to be inhibited specifically by L-serine. The extent of serine inhibition increased when the temperature was raised from 25 to 37 C, and the thermal stability of the enzyme was enhanced by the presence of the inhibitor serine or the coenzyme reduced nicotinamide adenine dinucleotide. At 37 C the curve representing the relationship between phosphoglycerate concentration and enzyme velocity was biphasic, and the serine inhibition which was competitive at low substrate concentrations became noncompetitive at higher concentrations.

Where it has been possible to correlate nutritional requirements for serine with enzyme deficiencies, the enzymes affected catalyzed one of the steps in the "phosphorylated pathway" (Fig. 1) (7). This correlation, which has been made with the mutant strains of *Salmonella typhimurium* (20), *Escherichia coli* (13), *Haemophilus influenzae* (16), *Pseudomonas* AM 1 (4), and *Micrococcus lysodeikticus* (11), has established that the conversion of 3-phosphoglycerate (PGA) to serine proceeds via the intermediates hydroxypyruvate phosphate (HPAP) and serine phosphate (serine-P) and is the physiological pathway for serine biosynthesis in these organisms. The first four organisms listed above are gram-negative, and, in view of the differences in the intracellular amino acid concentrations shown by gram-negative and gram-positive bacteria (6, 19), we investigated serine biosynthesis and regulation in a gram-positive organism for which information was available on the size of the serine pool.

[1] Taken in part from a thesis submitted to the University of Pennsylvania in partial fulfillment of the requirements for the M.S. degree.

[2] A preliminary report of this research was presented at the Annual Meeting of the American Society for Microbiology, Boston, 26 April-1 May 1970.

The organism chosen for this investigation, *Bacillus subtilis*, is a gram-positive aerobe which has been the subject of numerous physiological, biochemical, and genetic studies. This report describes the enzymes in the organism responsible for serine synthesis and the nature of the controls acting to regulate the biosynthetic pathway.

MATERIALS AND METHODS

Bacterial strains. *B. subtilis* GSY W23 was obtained from Neville Kallenbach of the University of Pennsylvania. N2-168M *ser*$^-$ and GSY-424 *ile*$^-$*ser*$^-$ were obtained from Eli Siegal of the Yeshiva University. N2-168R was a revertant of N2-168 M *ser*$^-$ isolated in this laboratory. The original strains were streaked on nutrient agar plates, and several single colonies were isolated and tested in minimal agar plates in the absence and presence of 200 μg of L-serine per ml. Selected colonies were grown in liquid minimal medium plus 200 μg of L-serine per ml. When the cultures reached a Klett reading of 120 they were chilled and sterile glycerol was added to give a final concentration of 15% by volume. Samples of 1 ml were frozen in an alcohol-dry ice mixture and stored at −70 C.

Media and growth conditions. The synthetic medium was a modification of Spizizen's minimal medium (17) from which the amino acids except glutamate were removed. The medium had the fol-

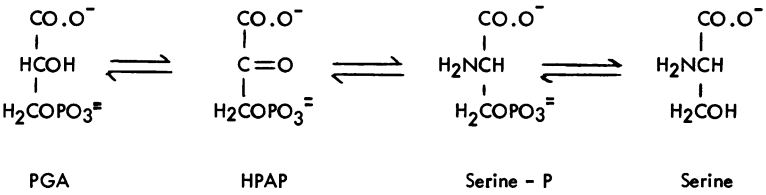

FIG. 1. *Reactions of the phosphorylated pathway.*

lowing composition, in grams per liter: K_2HPO_4, 14; KH_2PO_4, 6; $(NH_4)_2SO_4$, 2; sodium citrate, 1; and $MgSO_4$, 0.305. The medium was supplemented with glucose and glutamate, which were sterilized as 20% solutions and added to the sterile salts solutions to give final concentrations of 2 mg/ml. For the serine auxotrophs, 200 μg of L-serine per ml was also added. Cultures of GSY-424 were also supplemented with 20 μg of L-isoleucine per ml. Solid medium was prepared by addition of 1.5% agar (Difco) to the stock of minimal salts, and then the carbon sources and amino acids were added as required.

Bacteria were grown in Erlenmeyer flasks at 37 C. Aeration was achieved by shaking, and growth was measured by following turbidity in a Klett-Summerson colorimeter fitted with a blue filter no. 420.

Dose response curves. The procedure for the dose response experiments was as follows: 10 ml of minimal medium supplemented with 200 μg of L-serine per ml was inoculated with 0.5 ml of strain GSY-424 *ser*⁻ and the flask was incubated overnight at 37 C. The following morning the culture was diluted 1:10 into 20 ml of fresh minimal medium and the cells were harvested by filtration onto a membrane filter (Millipore; 0.65-μm pore diameter). The cells were washed twice with cold minimal salts and resuspended in 10 ml of minimal medium. A triplicate series of calibrated Klett tubes containing 5 ml of minimal medium, 2 mg of glucose per ml, and different concentrations of L-serine (0 to 200 μg/ml) were inoculated with 0.1 ml of the cell suspension and incubated at 37 C overnight. Aeration was achieved by rotation of the tubes. The next morning the tubes were read in a Klett colorimeter, and the averages of the triplicate determination were plotted against serine concentration.

Enzyme preparations. Overnight cultures were started by diluting the bacterial stocks 1:50 into growth medium. The following morning the cultures were diluted 1:10 into 1 liter of fresh medium and allowed to grow until the Klett reading reached 120. The cells were then centrifuged, washed once with 0.1 M phosphate buffer, *p*H 7.1, and resuspended in 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, *p*H 7.5, containing 5 mM dithiothreitol (DTT); 5 ml of buffer was used per g of wet cells. The cells were subsequently disrupted by treatment for 2 min in an MSE sonic oscillator (Measuring and Scientific Equipment, Ltd., London), and the cell debris was removed by centrifugation. The supernatant fluid was used as unfractionated extract for the investigation of serine-P formation. It could be stored at −20 C for at least a week without appreciable loss of activity. For the study of PGA dehydrogenase the supernatant fluid was fractionated by the addition of 0.3 ml of 5% (w/v) streptomycin sulfate per ml of extract to precipitate nucleic acid; after the nucleic acid precipitate was removed by centrifugation, solid ammonium sulfate was added to 70% saturation. The ammonium sulfate precipitate, which contained most of the protein, was kept in the refrigerator at 4 C. Under these conditions the PGA dehydrogenase activity was stable for several months. For individual experiments, enzyme was obtained from the ammonium sulfate suspension by centrifuging a 1-ml sample, discarding the supernatant fluid, and dissolving the precipitate in 1.0 ml of 0.1 M phosphate buffer, *p*H 7.4, containing 1 mM DTT.

Enzyme assays. The conversion of PGA to serine-P was measured by incubating ^{14}C-PGA with unfractionated extracts as described previously (13). The complete incubation mixture contained: 1 μmole of ^{14}C-PGA (20,000 counts per min per μmole); 50 μmoles of potassium phosphate buffer, *p*H 7.4; 2.5 μmoles of sodium fluoride; 5 μmoles of DTT; 10 μmoles of sodium glutamate; 10 μmoles of sodium oxalacetate; and 1 μmole of nicotinamide adenine dinucleotide (NAD). Unfractionated extracts were added to give a final volume of 0.5 ml. Incubation was at 37 C for 30 min, and the reaction was stopped with 0.5 ml of 10% trichloroacetic acid. The precipitated material was removed by centrifugation, and ^{14}C-serine-P was separated from ^{14}C-PGA by ion exchange chromatography on Dowex 50 (H^+). Samples from the fraction that contained ^{14}C-serine-P were pipetted into planchets and were then dried with an infrared lamp. The radioactivity present was counted in a Nuclear-Chicago windowless counter.

To measure serine production, the incubation mixture contained: ^{14}C-PGA (20,000 counts per min per μmole), 200 μmoles; potassium phosphate buffer, *p*H 7.5, 2 μmoles; DTT, 10 μmoles; $MgSO_4$, 10 μmoles; sodium glutamate, 20 μmoles; sodium oxalacetate, 20 μmoles; and 2 mg of enzyme; the final volume was 1.0 ml. The reaction mixture was incubated for 30 min at 37 C and after incubation, 10 μmoles of L-serine and L-alanine were added to act as carrier compounds. The protein was precipitated by the addition of 0.2 ml of 50% trichloroacetic acid, and, after the protein precipitate was removed by centrifugation, the products in the supernatant fluid were fractionated in a Dowex 50 (H^+) (1 by 50 cm; 100–200 mesh) column. ^{14}C-PGA and ^{14}C-serine-P were eluted with 0.01 N HCl, and the amino acids were eluted from the column with 5 N NH_4OH. The amino acids were separated by chromatography on a column of Dowex 50 (H^+) (1 by 50 cm, 200–400

mesh) (15). This column was developed with 1.5 N HCl. The ^{14}C-serine was located by measuring the radioactivity in 0.5 ml of each fraction of the column, and further identification of the amino acids was carried out by paper chromatography. The results are reported as total radioactivity in the samples.

To assay for serine-P phosphatase, the rate of inorganic phosphate release from serine-P was measured colorimetrically (13). The assay conditions were those previously described, and unfractionated extract was used as a source of enzyme.

PGA dehydrogenase was assayed by the HPAP-dependent oxidation of NADH (18) or by a fluorimetric assay in which the PGA-dependent reduction of acetyl-pyridine NAD was followed. For this assay reaction mixture contained per milliliter: 100 μmoles of potassium phosphate buffer, *p*H 7.5; 2.5 μmoles of acetyl-pyridine-NAD; 5 μmoles of DTT; and enzyme; the reaction was started by the addition of 10 μmoles of PGA. The appearance of acetyl-pyridine-NADH was followed in a Eppendorff fluorimeter equipped with a Lauda K-2 water bath and a Servo/Riter II recorder. Activation of the reaction was through a 313- to 366-nm Hg filter and the emitted light passed through a 400- to 3,000-nm filter. Both optical assays were run routinely at 35 C and 1 unit of activity corresponded to the production of 1 nmole of product per min. For the fluorimetric assay a standard acetyl-pyridine-NADH solution was used to calibrate the instrument since appreciable variations in fluorescence intensity were observed from day to day. The fluorimetric assay was directly dependent on added protein up to 0.5 mg/assay and the spectrophotmetric assay was directly dependent on protein up to 1.0 mg/assay.

Effect of temperature of PGA dehydrogenase. Kinetic measurements of PGA dehydrogenase were performed with the standard assay conditions described above except that the assay mixture plus enzyme was equilibrated at a variety of temperatures. The temperature was measured directly in the cuvette with a YSI model 42SC Tele-thermometer. A variation of ±0.5 C was observed during the course of the reaction.

A typical experiment was done in the following manner: the assay mixture and the enzyme were prewarmed 2 to 3 min in the cuvette and the reaction was started by addition of substrate. In experiments where the effect of ligands on the stability of the enzyme at 45 C was tested, the following procedure was used. The assay mixture was preincubated for 5 min in a water bath at 45 C; then enzyme was added and 1-ml samples were removed at different times to chilled tubes. The enzyme activity was subsequently measured at 35 C by addition of substrate as described previously. In all experiments the enzyme activity was proportional to the protein concentration.

Analytical procedures. Protein was determined in the crude and fractionated extracts by the method of Lowry et al. (9). The analysis was done after precipitation of the protein sample with trichloroacetic acid and redissolving of the precipitate with NaOH.

Chemical compounds. ^{12}C-PGA and NAD were purchased from Sigma Chemical Co., St. Louis, Mo; HPAP, amino acids, DTT, and ^{14}C-PGA were purchased from Calbiochem, Los Angeles, Calif. Acetyl-pyridine-NAD was purchased from Pabst Laboratories, Milwaukee, Wis.

RESULTS

Growth studies. The growth studies provide qualitative and quantitative information about the serine requirements of the mutants. Mutant strain GSY-424 failed to grow in the absence of serine but grew at the same rate as the wild type (GSY-W23) when the medium was supplemented with serine. Strain N2-168M also exhibited a growth requirement for serine, and a revertant (N2-168R) selected on solid media without a serine supplement had lost this requirement. The mass doubling time of the growing cultures was 90 min, and the lag that only preceded growth of the auxotrophs was variable and appeared to depend on the condition of the overnight culture used to start the growth experiment. Since both strains N2-168M and GSY-424 failed to grow in the absence of serine, they could be used to construct dose reponse curves from which quantitative information concerning the serine requirement of *B. subtilis* could be calculated. With the medium used, the extent of growth was linear with serine concentration in the range of 0 to 15 μg/ml and maximal growth was achieved with about 100 μg of L-serine per ml (Fig. 2). Both strains gave similar response curves. From the linear part of the curve it was calculated that 300 μmoles of serine was required for 1 liter of culture to grow to a Klett reading of 120 units. At this Klett reading the yield of cells was 1.25 g wet weight which corresponded to 153 mg after drying at 100 C for 16 hr.

Serine-P formation by cell-free extracts.

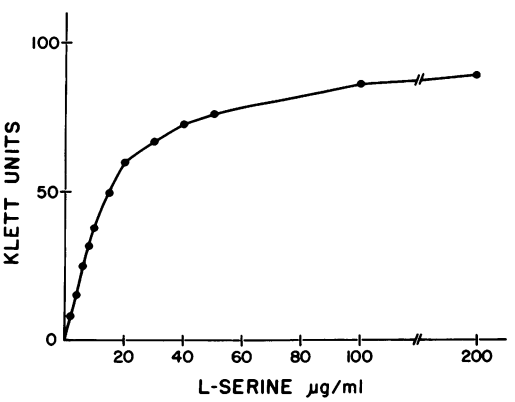

FIG. 2. *Growth response of B. subtilis strain GSY-424 ile⁻ ser⁻ to* L-*serine.*

To establish whether *B. subtilis* contains the enzymes of the phosphorylated pathway (7), the capacity of unfractionated cell-free extracts to convert ^{14}C-PGA to serine and serine-P was determined. When Mg^{2+} was added to the incubation, radioactivity was found in serine and serine-P (Fig. 3). The replacement of Mg^{2+} by 5 mM NaF reduced the amount of radioactivity in the serine and increased its quantity in serine-P. These data indicate that extracts of *B. subtilis*, like extracts of *E. coli*, convert PGA to serine via the intermediate serine-P. When fluoride replaced Mg^{2+}, the activity of serine-P phosphatase was reduced and serine-P accumulated. The addition of L-serine inhibited the synthesis of both serine and serine-P (Fig. 3), suggesting that end product inhibition acts on an early step in the pathway to reduce the conversion of PGA to serine.

To prove that the phosphorylated pathway was the only functional pathway in *B. subtilis*, the capacity of the mutant strains to make serine-P from PGA was determined. The results (Table 1) show that the strains with the nutritional requirement (GSY-414, N2-168M) fail to make serine-P, whereas a revertant (N2-168R) that can make serine has regained this capacity. By mixing mutant and wild-type extracts we have eliminated the possibility that the mutant extracts failed to synthesize serine-P because of soluble inhibitory compounds.

Requirements for serine-P synthesis. Because in early experiments the rate of serine-P formation by extracts was too low to satisfy the serine requirement (300 μmoles/g of wet cells) calculated from the dose response curves (Fig. 2), we attempted to improve the in vitro assay conditions. The requirement for an α-keto acid was tested and the specificity of the coenzyme and amino donor was determined. Oxalacetate stimulated serine-P formation (Table 2), probably by removing NADH formed by PGA oxidation. Glutamate was the preferred amino group donor and NAD could not be replaced by NADP.

Under optimal conditions serine-P synthesis

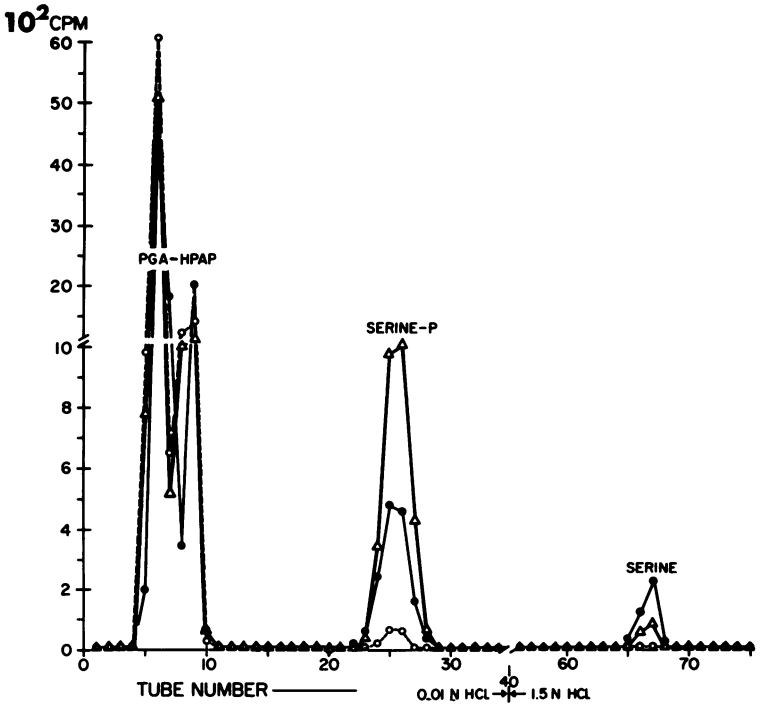

FIG. 3. *Dowex 50 (H^+) chromatography of the products formed from ^{14}C-PGA. Complete incubation mixture contained per 1 ml: ^{14}C-PGA (20,000 counts per min per μmole), 200 μmoles; potassium phosphate buffer, pH 7.5, 2 μmoles; DTT, 10 μmoles; sodium glutamate, 20 μmoles; sodium oxalacetate, 20 μmoles; $MgSO_4$, 10 μmoles; 2 mg of protein was present in the incubation. The reaction mixture was incubated for 30 min at 37 C and the reaction was stopped by addition of 0.2 ml of 50% trichloroacetic acid. Fractionation of the ^{14}C products was done as described in the text. Results are reported as total radioactivity in the samples. Symbols: ●, no addition; △, minus $MgSO_4$ plus 5 μmoles of NaF; ○, addition of 5 μmoles of L-serine. PGA and HPAP do not separate in this chromatographic system and the first split peak contains both compounds.*

TABLE 1. *Capacity for serine phosphate formation*[a]

Strain	Protein in incubation (mg)	Serine phosphate (counts/min)
GSY-W23	0.76	1,980
GSY-424	1.58	15
N2-168R	0.85	2,400
N2-168M	1.83	46

[a] Standard assay conditions were used.

TABLE 2. *Requirements for serine-P formation*[a]

Compound added (20 mM)	Serine-P produced (counts/min)
Expt I	
None	1,550
Pyruvate	760
Oxalacetate	3,240
α-Ketoglutarate	825
Expt II	
None	210
Glutamate	2,340
Aspartate	285
Alanine	210
Glutamine	255
−NAD + NADP (Glutamate)	375

[a] In Experiment I, glutamate was present in the incubation mixture with 500 μg of protein, whereas in Experiment II, oxalacetate was present in the incubation with 200 μg of protein. Standard assay conditions were used.

was linear with enzyme concentration up to 200 μg/assay and time for at least 40 min. From the measured specific activity and the total activity in an extract we calculated that a gram of packed cells would synthesize in 60 min about 50 μmoles of serine-P. Addition of serine to the growth medium did not alter the specific activity of the serine-P synthesizing system, indicating that the enzymes were not repressible.

Because the inhibition of serine-P formation by serine could have physiological significance, the specificity of the inhibition was tested. The results in Table 3, which show that L-serine is the only effective inhibitor, support the proposal that this inhibition functions in the cell.

Serine-P hydrolysis. The last step in the phosphorylated pathway is the hydrolysis of serine-P. Enzymes from mammalian sources that catalyze this reaction are strongly inhibited by serine (1, 12, 14). The unfractionated extract contained a serine-P phosphatase which was inhibited by 25% when the incubation contained 10 mM L-serine but was not affected by 10 mM glycine or L-alanine. The presence of the phosphatase completes the demonstration that the enzymes of the phosphorylated pathway exist in *B. subtilis*, and the insensitivity of this enzyme to serine inhibition makes it appear that the regulation of biosynthesis is unlike that found in animal cells.

Kinetics of PGA dehydrogenase. PGA dehydrogenase activity was found in the ammonium sulfate precipitate of the fractionated cell extract. Enzyme activity could be readily assayed by following fluorimetrically the PGA-dependent reduction of acetyl-pyridine-NAD, or spectrophotometrically the HPAP-dependent oxidation of NADH. The fluorimetric assay measures the reaction in the direction of biosynthesis, and for this reason it was studied in greater detail than the reverse reaction. However, analogous experiments were performed with HPAP as the substrate and similar results were obtained with this assay. The appearance of fluorescence was dependent on substrate (PGA) and was linear with time for at least 2 min in the range where the reaction was proportional to enzyme concentration. The specific activity in the fractionated extract was in the range of 10 units/mg at *p*H 7.5 and 35 C. Activity increased with *p*H from *p*H 7 to 10.5 and at the *p*H of the standard assay was 60% of maximum. The PGA dehydrogenase activity was inhibited by serine. This inhibition which is analogous to that found with PGA dehydrogenase from other bacteria was specific for L-serine and could account for the serine inhibition of serine-P formation described above. The inhibition curve (Fig. 4) had an abrupt break at about 2 mM, and greater than 70% inhibition was not achieved even at high serine concentrations. These characteristics suggested that the inhibition was complex and probably due to allosteric effects. Kinetic measurements

TABLE 3. *Specificity of inhibition of serine-P formation*[a]

Amino acid (20 mM)	Serine-P formed (counts/min)	Inhibition (%)
None	1,440	0
L-Serine	186	87
L-Alanine	1,365	5
Glycine	1,480	0
L-Threonine	1,365	5
D-Serine	1,300	9

[a] Standard assay conditions were used with 200 μg of protein and with the amino acids present at a concentration of 20 mM.

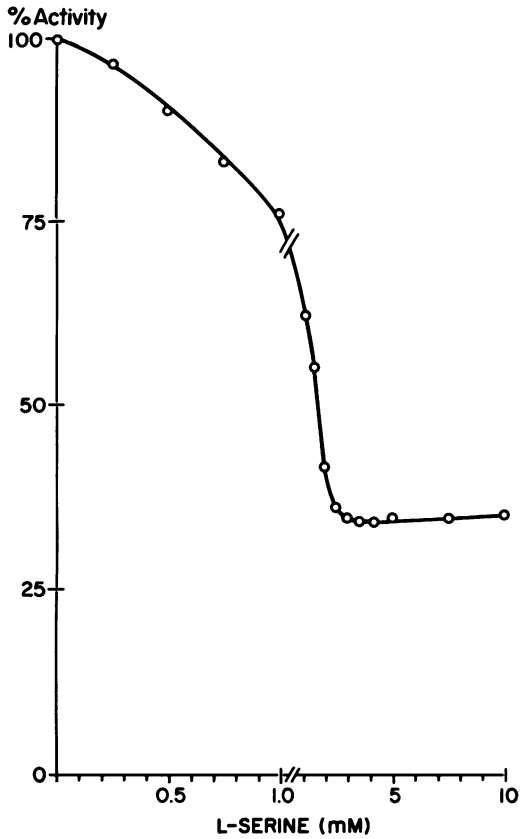

FIG. 4. *Effect of L-serine concentration of PGA dehydrogenase activity. Standard assay conditions were used with 100% activity corresponding to 1.8 units. L-Serine was added at the molar concentration indicated, and the reaction was started by the addition of PGA.*

were performed in which the substrate and coenzyme concentrations were varied. The data obtained when the coenzyme concentration was varied gave a straight line when the data were plotted according to Lineweaver and Burk. The K_m value calculated from this plot was 45 μM. Under standard assay conditions, therefore, the enzyme was saturated with coenzyme. The velocity data obtained in the presence or absence of serine, when the substrate PGA was varied, failed to fit simple Michaelis kinetics. The plot of substrate concentration (S) versus velocity (*V*) (Fig. 5A) and the double reciprocal plot (Fig. 5B) were biphasic. K_m values of 2.5 mM and 20 mM were calculated from the data obtained at low and high PGA concentrations, respectively (Fig. 5B and C). From these plots it appears that serine inhibition was competitive or mixed at low PGA concentrations and noncompetitive at high PGA concentrations. The biphasic character of the curves in Fig. 5 (A and B) diminished as the serine concentration was increased.

Effect of temperature on PGA dehydrogenase. Preliminary spectrophotometric experiments performed at room temperature showed negligible inhibition of PGA dehydrogenase by L-serine; but, in the radioactive assay for serine-P formation which was run at 37 C, serine was an effective inhibitor. It appeared that the temperature difference was influencing the inhibition of the reaction, and we investigated the effect of temperature on the reaction in the presence and absence of serine. The results show that activity increased with temperature until about 38 C, at which point activity appeared to level off (Fig. 6A). The absence of an increase in activity above 39 C could have resulted from thermal denaturation occurring at these temperatures. When these data were converted to an Arrhenius plot, a break in the curve occurred at about 40 C. When 1.5 mM serine was in the incubation mixture, the degree of inhibition increased with temperature to about 37 C and then appeared to decrease. The reduction in serine inhibition observed around 40 C (Fig. 6B) could be explained by serine stabilizing the enzyme. Consistent with this possibility, there was no break in the Arrhenius plot at 40 C in the presence of 1.5 mM serine.

That serine was stabilizing the enzyme at higher temperatures was tested by incubating the enzyme at 45 C and following the decay in enzyme activity. Preliminary experiments established that the enzyme was stable in the presence of NADH. Therefore, the enzyme was heated without ligand or with either NADH or serine. The HPAP-dependent oxidation of NADH was used to assay the activity remaining after different periods of heating as shown in Fig. 7. When the enzyme was incubated in the presence of NADH it was stable for at least 30 min whereas, in the absence of NADH and the presence of L-serine, the enzyme was inactivated 20% after 30 min. When neither NADH nor serine was present, inactivation occurred rapidly. These data confirm that at 45 C in the absence of L-serine or NADH the enzyme undergoes denaturation, but both of these ligands stabilize the structure of the enzyme.

DISCUSSION

The object of the research reported in this paper was to identify the pathway utilized by *B. subtilis* for serine synthesis and compare the regulation of the pathway with the regula-

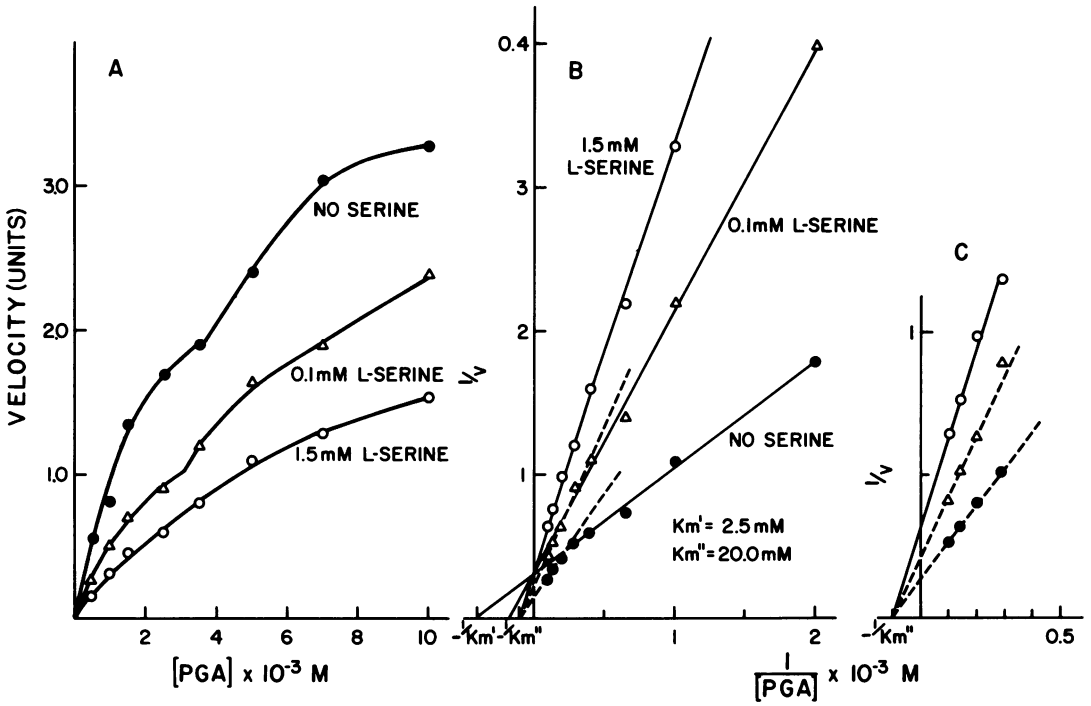

FIG. 5. *Effect of PGA concentration on the kinetics of PGA dehydrogenase. The standard assay was used with the PGA concentration varied and* L-*serine added at the concentration indicated. The three panels indicate: (A) relationship between velocity and enzyme concentration; (B) double reciprocal plot of these data; (C) an enlargement of the double reciprocal plot showing the data obtained between 3.5 to 10 mM PGA. Velocity is expressed as enzyme units, and the reciprocals of these values are plotted. PGA concentrations in panel A are millimolar, and the reciprocals of these values are plotted in panels B and C.*

tion of serine synthesis in gram-negative bacteria. Evidence for a pathway usually includes a demonstration of the postulated enzymatic steps and a correlation between the loss of one of the enzymes and the appearance of a nutritional requirement for the end product of the pathway. The results described in this paper meet the above criteria for establishment of the phosphorylated pathway as the route for serine biosynthesis in *B. subtilis*. A comparison may be made of the capacity to synthesize serine and the growth requirement displayed by auxotrophic mutants. The cell-free extract from 1 g of cells has the capacity to synthesize approximately 50 μmoles of serine-P in 60 min, and cultures with a mass doubling time of about 60 min require approximately 300 μmoles of serine per g wet weight. The latter value was calculated from the dose response curve of mutant strain GSY-424. It appears from these data that, in order to produce the amount of serine required for cell growth, the enzymes of the phosphorylated pathway would have to function six times faster in vivo than when assayed under our standard conditions.

The similarity between *B. subtilis* and gram-negative organisms extends from the reactions used to synthesize serine to the mechanisms that regulate biosynthesis in these organisms. If we compare the way in which *B. subtilis* and *E. coli* control the phosphorylated pathway, we find that neither organism regulates enzyme levels by repression of enzyme synthesis and that both organisms have PGA dehydrogenases which are subject to end product inhibition (13, 20). The details of this inhibition differ in the two organisms with the *E. coli* enzyme being more sensitive to serine inhibition. When assayed at 37 C in the direction of PGA oxidation, 75 μM serine inhibited the *E. coli* enzyme 50% and 1 mM serine close to 100% (13). As shown in Fig. 4, 1 mM serine inhibited the *B. subtilis* enzyme only 25% and complete inhibition was not achieved at any concentration tested. These inhibition data can be viewed in terms of the intracellular concentrations of serine which in *B. subtilis* range from 0.5 to 1.5 mM depending on the NaCl concentration (19) and in *E. coli* approximate 0.1 mM (Tosa and Pizer, *unpublished data*). It appears

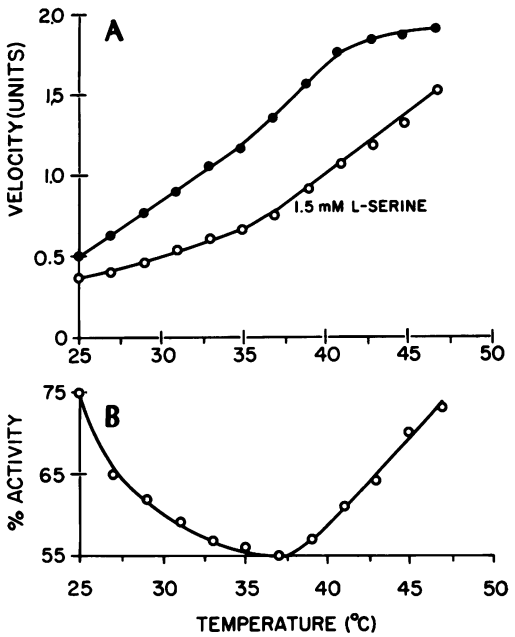

FIG. 6. *Effect of temperature on the inhibition by* L-*serine of PGA dehydrogenase. Standard assay conditions were used. Assay mixture was preincubated in the presence of the enzyme until it reached the desired temperature; then the reaction was started by addition of 10 μmoles of PGA. (A) Velocity of the reaction expressed as enzyme units in the presence and absence of* L-*serine. (B) Relative activity in the presence of 1.5 μmoles of* L-*serine.*

that in the gram-positive organism even the relatively large intracellular pool would not exert a tight control on the PGA dehydrogenase, whereas in the gram-negative organism the lower pool would strongly inhibit enzyme activity. The higher intracellular amino acid concentrations maintained in gram-positive organisms raise the question whether the amino acid biosynthesis is ever closely regulated in these organisms.

In the case of the biosynthetic threonine deaminases, a comparison of the properties of the purified enzyme from *B. subtilis* (5) can be made with the purified enzyme from the gram-negative organism *Salmonella typhimurium* (2). Both enzymes are inhibited by isoleucine. At a threonine concentration of 5 mM the *B. subtilis* enzyme is inhibited 50% by 125 μM isoleucine. The *Salmonella* enzyme is more sensitive, with 50 μM producing 50% inhibition. These differences are not as great as with the enzymes that regulate serine biosynthesis. Many more enzymes would have to be examined before a generalization could be made about the tightness of regulation in gram-positive organisms, but as additional information becomes available, it should be examined in the light of this hypothesis. The other most pronounced difference between the PGA dehydrogenases from the two sources is their behavior towards the substrate, PGA. The *E. coli* enzyme behaves in a conventional manner and the double reciprocal plot is a single straight line from which a K_m of 1 to 2 mM was calculated (18). The biphasic character of the PGA saturation curve displayed by the *B. subtilis* enzyme is distinctive and suggests that PGA affects the structure of the enzyme. It is possible that PGA produces this effect by binding to the protein at a site other than the substrate binding site,

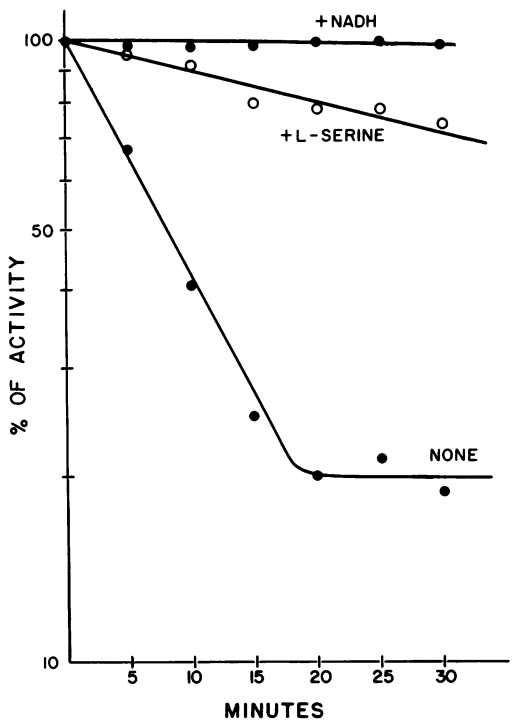

FIG. 7. *Stability of PGA dehydrogenase at 45 C. Complete incubation mixture contained: potassium phosphate buffer, pH 7.5, 1,000 μmoles; NADH, 1 μmole; DTT, 50 μmoles; and water to give a final volume of 8.4 ml. The incubation mixture was prewarmed at 45 C for 5 min and 1 ml of enzyme suspension containing 600 μg of protein was added. Samples of 1 ml were removed, at the times indicated in the figure, to chilled tubes. Activity for PGA dehydrogenase was tested at 35 C by addition of 0.18 μmole of HPAP as described in the text. Two other tubes were prepared as above except that in one NADH was substituted for by 100 μmoles of* L-*serine and in the other both NADH and* L-*serine were lacking. To the samples removed from these tubes 0.1 μmole of NADH was added before assay.*

i.e., an activation site. Alternatively, the presence of PGA on the substrate site of one subunit could alter the binding properties of the other subunits. This type of interaction involves what has been termed "negative cooperativity" (8) and requires that the enzyme consist of subunits. PGA dehydrogenase has a molecular weight greater than 150,000 daltons (Sasaki and Pizer, *unpublished data*) and is probably made of subunits.

The differences in the K_m values for PGA could have a pronounced regulatory function since, when the PGA concentration is in the mM range, the enzyme acts at a reduced rate, and considerably higher PGA concentrations are required to approach the second K_m value (20 mM). The net effect of this situation would be an extension of the range where PGA dehydrogenase activity is responsive to changes in PGA concentration.

Our cultures were grown with glucose and glutamate in the medium. Under these conditions aconitase levels are low and pyruvate produced from glycolysis is not oxidized via the tricarboxylic acid cycle (3). If glutamate is not present in the medium, aconitase levels are high and the products of glycolysis enter the tricarboxylic acid cycle. Since the growth rates are essentially unaltered by the addition of glutamate, it might be expected that the steady-state concentrations of PGA would be different depending on whether the cells were oxidizing pyruvate. A direct measurement of PGA concentrations in the cell under these conditions may provide an insight into the biological value of the substrate-velocity relationship shown by PGA dehydrogenase.

In a discussion of the comparative aspects of serine inhibition of PGA dehydrogenase, it is worthwhile to draw attention to the work of Nelson and Naylor with the enzyme from *Micrococcus lysodeikticus* (11). The enzyme from this source was relatively insensitive to serine inhibition requiring concentrations of the amino acid in the 1 to 10 mM range and after fractionation showed maximum inhibition of about 40%. The sensitivity was markedly increased by the presence of Ca^{2+}, with 50% inhibition being achieved at 0.2 mM serine and an 80% maximum inhibition being reached. Whether this characteristic will be common to enzymes from other organisms, as well as its physiological significance, will be important to determine. The PGA dehydrogenase from *M. lysodeikticus* was inhibited by adenosine monophosphate. We have observed inhibition of the *E. coli* enzyme by this nucleotide but not the enzyme from *B. subtilis* (*unpublished data*).

Certain of the questions posed by the study reported in this paper require a purified enzyme for their investigation. The purification of the PGA dehydrogenase has been undertaken in order to make a comparison of the properties of the *B. subtilis* and *E. coli* enzymes, with the expectation that these properties will be pertinent to the physiology of the cell.

ACKNOWLEDGMENT

This research was supported by Public Health Service grant AI-03957 from the National Institute of Allergy and Infectious Diseases.

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