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Biosynthesis of Bacterial Glycogen: Purification and Properties of *Salmonella typhimurium* LT-2 Adenosine Diphosphate Glucose Pyrophosphorylase

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The adenosine diphosphate glucose pyrophosphorylase from a *Salmonella typhimurium* LT-2 mutant, JP102, derepressed in the glycogen biosynthetic enzymes was purified to homogeneity. The enzyme was found to be identical with the parent wild-type enzyme with respect to regulatory properties, immunological reactivity, and kinetic constants for the allosteric effectors and for the substrate, adenosine triphosphate. The JP102 enzyme was composed of four identical subunits, each with a molecular weight of about 48,000. This was supported by the findings that (i) gel electrophoresis under denaturing conditions showed only one component; (ii) digestion with carboxypeptidase B released stoichiometric amounts of arginine; and (iii) amino-terminal sequencing showed a single sequence for the first 27 residues. The properties of the purified *S. typhimurium* enzyme were compared with the properties of the previously purified *Escherichia coli* B enzyme.

The biosynthesis of the α-1,4-glucosidic linkages of bacterial glycogen is believed to occur via the following reactions (for reviews on the subject, see references 21 and 31):

$$\text{ATP} + \alpha\text{-glucose 1-phosphate} \rightleftharpoons \text{ADP-glucose} + \text{PP}_i \quad (1)$$

$$\text{ADP-glucose} + \alpha\text{-glucan} \rightarrow \alpha\text{-1,4-glucosyl-glucan} + \text{ADP} \quad (2)$$

ADPglucose pyrophosphorylase (EC 2.7.7.27; glucose 1-phosphate adenylyltransferase) catalyzes reaction 1, and in most cases has been shown to be activated by glycolytic intermediates and inhibited by either AMP, P_i, or ADP (21, 31). The particular glycolytic intermediate that may activate is dependent on the source of the ADPglucose pyrophosphorylase. For example, many enteric bacteria contain an ADP-glucose pyrophosphorylase that is activated by fructose 1,6-diphosphate, NADPH, and pyridoxal phosphate (31, 34, 35). Evidence obtained in the study of mutants (5, 13, 32, 33, 35) and in correlating in vivo concentrations of fructose 1,6-diphosphate with rates of glycogen accumulation (7, 8) strongly suggest that fructose 1,6-diphosphate is a physiologically important activator of ADPglucose pyrophosphorylase and therefore of glycogen synthesis in *Escherichia coli* and *Salmonella typhimurium*.

Recent studies have identified the activator binding site of the *E. coli* ADPglucose pyrophosphorylase (28, 29). It was therefore of interest to compare the structural and chemical properties of the *E. coli* B enzyme with other enteric ADPglucose pyrophosphorylases. This report is concerned with the purification and characterization of the *S. typhimurium* LT-2 ADPglucose pyrophosphorylase and comparison of its properties with the ADPglucose pyrophosphorylase of *E. coli*.

MATERIALS AND METHODS

Reagents. Carboxypeptidase A (DFP treated; 21 mg/ml, 55 U/mg), yeast inorganic pyrophosphatase (600 U/mg), and carboxypeptidase B (DFP treated; 5 mg/ml, 90 U/mg) were obtained from Sigma Chemical Co. Iodoacetic acid was purchased from Sigma and recrystallized from CCl_4. Iodo-[2-^{14}C]acetic acid (12.6 mCi/mmol) and α-D-[*U*-^{14}C]glucose 1-phosphate (150 mCi/mmol) were obtained from Amersham Corp., and $^{32}P_i$ was from New England Nuclear Corp. All other reagents were obtained from commercial sources at the highest possible purity.

Bacteria. The following microorganisms were used: *S. typhimurium* LT-2 (obtained from S. Kustu, University of California, Davis) and *S. typhimurium* JP102, a glycogen-excess mutant containing three- to fourfold higher levels of ADPglucose pyrophosphorylase activity than the parent wild type, *S. typhimurium* LT-2. The isolation and characterization of this derepressed mutant have been described (35).

Growth conditions. *S. typhimurium* JP102 was grown aerobically in 100-liter batches in a New Brunswick Fermacell in minimal medium containing 0.6% glucose, 0.68% KH_2PO_4, 1.42% Na_2HPO_4, 0.12% $(NH_4)_2SO_4$, 0.0246% $MgSO_4 \cdot 7H_2O$, 0.0011% $CaCl_2$, and 1 ml of a trace element solution per liter (33). Temperature was maintained at 37°C; after 16 to 18 h of

growth, cells were harvested with a Sharples continuous ultracentrifuge and stored as a cell paste at −20°C. About 700 g (wet weight) of cells was obtained per 100-liter culture. *S. typhimurium* LT-2 was also grown aerobically on the same minimal medium, containing 0.6% glucose in 1 liter of culture.

Enzyme assays. ADPglucose pyrophosphorylase was assayed in pyrophosphorolysis and synthesis directions as described previously (35). The reaction mixtures contained: for assay A (activated pyrophosphorolysis), 20 μmol of Tris-chloride buffer (pH 8.0), 2 μmol of $MgCl_2$, 100 μg of bovine plasma albumin, 0.2 μmol of ADP-glucose, 0.5 μmol of $^{32}P_i$ (1×10^6 to 3×10^6 cpm/μmol), activator in the indicated concentrations, and enzyme in a final volume of 0.25 ml; for assay B (activated synthesis), 20 μmol of Tris-chloride buffer (pH 8.0), 0.3 μmol of ATP, 0.1 μmol of [*U*-^{14}C]-glucose 1-phosphate (1.05×10^6 cpm/μmol), 1 μmol of $MgCl_2$, 100 μg of bovine plasma albumin, 0.24 μg of yeast inorganic pyrophosphatase, activator, and enzyme in a final volume of 0.2 ml. For assay C (unactivated synthesis), in the absence of activator, the amounts of ATP, glucose 1-phosphate, and $MgCl_2$ were increased to 1.5, 0.2, and 5.0 μmol, respectively. One unit of enzyme activity equals 1 μmol of ATP formed per min under the conditions of assay A in the presence of 1.5 mM fructose diphosphate at 37°C.

Determination of kinetic constants. Kinetic data were plotted as velocity versus substrate or effector concentration and were replotted as Hill plots (3, 17). V_{max} is determined from double-reciprocal plots. $S_{0.5}$, $A_{0.5}$, $I_{0.5}$ (corresponding to the concentration of substrate, activator, and inhibitor, respectively, required for half-maximal velocity, activation, and inhibition) and $\bar{n}$ (interaction coefficient) are determined from the Hill plots (3, 17).

Preparation of enzyme. ADPglucose pyrophosphorylase was purified from *S. typhimurium* JP102 by the following procedure. (i) Cells (700 g) were suspended in 3 volumes of 50 mM ice-cold glycylglycine buffer (pH 7.2) containing 5 mM dithioerythritol, filtered through two layers of cheesecloth, and passed three times through a Manton-Gaulin homogenizer at 7,000 lb/in^2. The temperature was kept below 15°C. The homogenate was disrupted ultrasonically in 500-ml batches for 2 min with a Bronwill Biosonik III probe to reduce the viscosity.

(ii) In the presence of 30 mM potassium phosphate buffer (pH 7.0), 800-ml batches of the crude extract were heated to 55°C for 5 min, cooled to 5°C, and centrifuged (45 min, 16,500 × *g*). The pellet was extracted with 250 ml of the above glycylglycine buffer, and to the combined washes and 16,500 × *g* supernatant, solid ammonium sulfate was added to 55% saturation at 4°C. The precipitate obtained after centrifugation of the resultant suspension (20 min, 16,500 × *g*) was suspended in sufficient 50 mM Tris-chloride buffer (pH 7.2) containing 1 mM dithioerythritol and 15 mM potassium phosphate to give a workable slurry, and was dialyzed for 12 h against 10 liters of the same buffer containing 5% glycerol. The slurry was then centrifuged (1 h, 100,000 × *g*), and the pellets were extracted with 400 ml of the dialysis buffer and recentrifuged. The combined supernatants were dialyzed again for 20 h against three changes of 10 liters of dialysis buffer.

(iii) The dialyzed ammonium sulfate fraction was adsorbed on a DEAE-cellulose column (36 by 5 cm; 706-ml resin bed volume) equilibrated with 15 mM potassium phosphate buffer (pH 7.5) containing 1 mM dithioerythritol. The column was washed with 800 ml of equilibration buffer, and the protein was eluted (250 ml/h, fractions) with a linear gradient of 7 liters containing the equilibration buffer in the mixing chamber and 0.1 M potassium phosphate (pH 7.0), 0.3 M KCl, and 1 mM dithioerythritol in the reservoir. Fractions containing the enzyme were pooled and concentrated by precipitation with 60% ammonium sulfate. The resultant precipitate was dissolved and dialyzed for 12 h against two changes of 5 liters of the same buffer as in step (ii).

(iv) The dialyzed DEAE-cellulose fraction was diluted to about 3 mg of protein per ml with 20 mM Tris-chloride buffer (pH 7.2) containing 0.2 M KCl, 1 mM dithioerythritol, 1 mM EDTA, and 10% glycerol and absorbed on a P^1-(6-amino,1-hexyl)-P^2-(6-phospho,1-hexyl)pyrophosphate Sepharose column (14) (30 ml) equilibrated with the same buffer used to dilute the DEAE-cellulose fraction. The column was washed with 100 ml of equilibration buffer, and the enzyme was eluted (50 ml/h, 12-ml fractions) with 200 ml of the equilibration buffer containing 1 mM AMP. The affinity column was washed with equilibration buffer containing 1 M NaCl and then equilibrated with the equilibration buffer. The enzyme was then readsorbed onto an affinity column (20-ml resin bed volume) and eluted (50 ml/h, 6-ml fractions) with a linear gradient (200 ml) containing the equilibration buffer in the mixing chamber and equilibration buffer plus 0.1 M potassium phosphate (pH 7.0) in the reservoir. Active fractions were pooled, made 20% in glycerol, and concentrated in an Amicon cell, using a PM30 membrane and pressure below 20 lb/in^2 to about 5 ml. The protein solution was then dialyzed against 50 mM Tris-chloride buffer (pH 7.2) containing 10 mM potassium phosphate, 0.5 mM dithioerythritol, and 20% glycerol and stored at −70°C.

Enzyme prepared in this way had a specific activity of about 100 U/mg when measured in pyrophosphorolysis direction in the presence of 1.5 mM fructose diphosphate.

Partial purification of *S. typhimurium* LT-2 ADPglucose pyrophosphorylase for immunological studies. Bacterial cells (1 to 5 g) were suspended in 10 ml of 50 mM glycylglycine buffer (pH 7.2) containing 5 mM dithioerythritol and sonicated for 2 min at 0°C. After addition of potassium phosphate to a final concentration of 30 mM, the homogenate was heated for 5 min at 55°C, cooled, and centrifuged (10 min, 30,000 × *g*). The enzyme was concentrated by ammonium sulfate precipitation (30 to 60% saturation) to about 10 U/ml if possible and dialyzed against 50 mM Tris-chloride buffer (pH 7.2) containing 1 mM dithioerythritol and 15 mM phosphate for 12 h and stored at 0°C.

Polyacrylamide gel electrophoresis. The system of O'Farrell was used for gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) (25). The

running gel was composed of 10% acrylamide, 0.1% SDS, and 0.375 M Tris-chloride buffer (pH 8.8). The stacking gel contained 2.75% acrylamide, 0.1% SDS, and 0.125 M Tris-chloride buffer (pH 6.8). Protein (5 to 50 μg) was applied, and electrophoresis was conducted at room temperature. Protein was stained by the procedure of Fairbanks et al. (11). Standard proteins used were bovine serum albumin (molecular weight, 68,000), hog pancreas α-amylase (molecular weight, 51,000), myoglobin (molecular weight, 17,000), and rabbit skeletal muscle phosphorylase (molecular weight, 100,000). Electrophoresis under nondenaturating conditions was performed as described by Davis (6), using acrylamide concentrations between 6 and 10%. The running gel contained 50 mM Tris-chloride buffer (pH 8.9), and the stacking gel contained 10 mM Tris-phosphate buffer (pH 7.2). Protein was stained with Coomassie blue (4), and ADPglucose pyrophosphorylase activity was determined by an activity stain (27). Estimation of the molecular weight of the native protein was carried out by the method of Hedrick and Smith (16), using bovine plasma albumin (molecular weight, 68,000), aldolase (Boheringer Mannheim Corp., rabbit muscle; molecular weight, 160,000), and glucose 6-phosphate dehydrogenase (Sigma, baker's yeast type V; molecular weight, 240,000) as standards.

Carboxymethylation of proteins. The carboxymethylation procedure was that of Hirs (18) as modified for use in guanidine-hydrochloride (14). Protein (5 mg) was dissolved in 7 M guanidine-hydrochloride containing 0.2% EDTA to a final volume of 5 ml and reduced with 0.01 ml of mercaptoethanol with nitrogen as the gas phase (25°C, 3 h). Then 5 mg of [^{14}C]-iodoacetic acid (specific radioactivity, 6×10^6 cpm/μmol) was added, and the pH of the mixture kept at 8.5 with 10% ethanolamine. The reaction was judged complete as determined by no further decrease in pH. Then 25 mg of unlabeled iodoacetic acid, dissolved in 0.1 N NaOH, was added. The pH was adjusted to 3.0 with glacial acetic acid, and the mixture was dialyzed extensively against 5% acetic acid at 4°C for 60 h. The protein was concentrated by lyophilization.

Amino acid composition. Amino acid analyses were performed on carboxymethylated proteins hydrolyzed in 5.7 N HCl at 110°C for 24, 48, and 72 h. Before hydrolysis, samples were carefully degassed and sealed under vacuum. Norleucine was added as an internal standard. Quantitation of amino acids was carried out with a Durrum D-500 amino acid analyzer. Appropriate extrapolations were made to correct for losses of serine and threonine. All other values were uncorrected. Cysteine was determined as carboxymethylcysteine, and tryptophan was spectrophotometrically determined by the method of Edelhoch (9).

NH_2-terminal sequence determination. For determination of the amino-terminal sequence, automated Edman degradation (10) was performed, using a Beckman Sequenator model 890 C. The program used in sequence determination was Beckman program 030176, using diluted Quadrol as a buffer. The protein (4 mg) was prepared for sequence analysis by reduction and carboxymethylation with [^{14}C]iodoacetic acid as described above. The lyophilized protein was dissolved in 0.5 ml of 70% formic acid and delivered to the spinning cup. The formic acid was removed by the vacuum steps and N_2 flush in the sample application subroutine program. Before automatic sequencing was begun, the protein was subjected to one heptafluorobutyric acid and butylchloride extraction step. Reagents used were Beckman Sequanal grade. Amino acid phenylthiohydantoins and their trimethylsilyl derivatives were identified by gas-liquid chromatography (19, 30), using a Packard model 419 gas chromatography system. All residues were also identified by thin-layer chromatography (22) and counted for radioactivity. Arginine was identified as free amino acid after hydrolysis of the phenylthiohydantoin derivative (24). Residues were obtained at a repetitive yield of 96% with an extrapolated yield of 75%.

Digestion with carboxypeptidases. ADPglucose pyrophosphorylase preparations (1.5 mg/ml) were dialyzed for 12 h against 10 mM potassium phosphate buffer (pH 7.5). *N*-Ethylmorpholine-acetate buffer (pH 8.5) was added to a final concentration of 0.2 M. Samples (0.2 mg) were digested with carboxypeptidase A or B for 0.5 to 5 h, using a carboxypeptidase/protein ratio of 1:50 or 1:150 (wt/wt), respectively. Before digestion, carboxypeptidase A was washed with water and solubilized by the addition of 2 M NH_4HCO_3. The reaction was terminated by heating for 1 min at 100°C, and the samples were dried under nitrogen. Released amino acids were identified by thin-layer chromatography, using 1-butanol–acetone–1 N HCl (60:15:25, by volume) as a solvent. The plates were developed with either fluorescamine (12) or phenanthrenequinone (36). Quantitation of amino acids was carried out with the Durrum D-500 amino acid analyzer.

Immunological methods. Two rabbits were injected subcutaneously in the neck region, at intervals of 1 week (four times) and then 1 month (two times), with 0.35 mg of purified ADPglucose pyrophosphorylase. For the injections, the protein (1.4 mg/ml) was mixed with equal amounts of Freund complete adjuvant. Serum (8 to 10 ml) was collected between 7 and 9 days after the third and all following injections and stored either at 0°C in the presence of 0.02% NaN_3 or at −70°C.

Ouchterlony double immunodiffusion (26) was carried out on 0.8% agarose gels containing 0.9% NaCl, 0.01 M potassium phosphate (pH 7.2), and 0.02% NaN_3. Inhibition of enzyme by serum was performed by mixing enzyme (0.04 U/ml) and antiserum in 0.1 M Tris-chloride buffer (pH 8.0). After incubation for 20 min at room temperature, 0.01 ml of the mixture was assayed for enzymatic activity in the pyrophosphorylsis direction in the presence of 1.5 mM fructose diphosphate. The antiserum generally used was that collected 12 weeks after the first injection. Preimmuneserum was used as a control.

Sucrose density centrifugation. The determination of the molecular weight of ADPglucose pyrophosphorylase was carried out as described by Martin and Ames (23). Purified enzyme (0.3 U) was mixed with 100 U of lactic dehydrogenase (Sigma, rabbit muscle) and 40 U of pyruvate kinase (Sigma, rabbit muscle type III) in a final volume of 0.1 ml of 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid) buffer (pH 7.0). The mixture was layered on top of a linear sucrose gradient (4.15 ml, 5 to 25% sucrose) and centrifuged for 16 h (0°C, 34,500 × *g*),

using a Beckman L5-50 ultracentrifuge equipped with an SW60 rotor. Fractions of about 0.17 ml were taken from the gradient and assayed for activity of pyruvate kinase (2), lactic dehydrogenase (20), and ADPglucose pyrophosphorylase in pyrophosphorolysis direction in the presence of 1.5 mM fructose diphosphate.

RESULTS

Purification of enzyme. As indicated previously, strain JP102 has about a fourfold-greater amount of the glycogen biosynthetic enzymes than does the parent strain, *S. typhimurium* LT-2 (35), and therefore was used as the source of ADPglucose pyrophosphorylase.

Table 1 summarizes the purification of ADPglucose pyrophosphorylase from mutant JP102. Although the cell suspension was passed three times through the homogenizer, viscosity of the extract had to be reduced by ultrasonication. Heat treatment of the crude extract increased the total activity threefold, probably due to the inactivation of interfering reactions or of an inhibitor. A similar phenomenon had been previously observed with extracts from *S. typhimurium* LT-2 (34). The ammonium sulfate step, as well as increasing the specific activity fourfold, is time-saving, since it decreased the volume of the heat-treated fractions for ultracentrifugation.

After the first affinity column (AMP eluate), gel electrophoresis showed a main and a faint band, both of which were enzymatically active when assayed by an activity stain (27). By readsorption of the protein and elution with a phosphate gradient, it was possible to remove the faint band. To avoid losses, concentration of the enzyme after the second affinity column step was carried out by using an Amicon cell concentrator. The enzyme was found to be stable in the presence of 10 mM potassium phosphate (pH 7.0) and 20% glycerol.

Gel electrophoresis. Purified ADPglucose pyrophosphorylase (20 μg) run in the Tris-glycine system of Davis (6) with gel concentrations of 6 to 10% acrylamide showed one protein band, which coincided with the enzyme activity seen by using an activity stain (27). Determination of the molecular weight of the native protein by the method of Hedrick and Smith (16) gave a value of 195,000 ± 10,000. Sucrose density gradient centrifugation (16) of the pure ADPglucose pyrophosphorylase indicated a molecular weight of 190,000. When the protein was denatured and 5 to 50 μg was subject to slab gel electrophoresis according to O'Farrell (25), it migrated as a single, homogeneous band. The molecular weight of the subunit was determined to be 48,000. The amino acid composition of the enzyme is shown in Table 2.

Kinetic properties of ADPglucose pyrophosphorylase from *S. typhimurium* JP102. Fructose diphosphate has been found to be the physiological modulator of the ADPglucose pyrophosphorylase of *S. typhimurium* (34). Fructose-diphosphate as well as NADPH and pyridoxal phosphate have been found to be effective activators of other enteric ADPglucose pyrophosphorylases (34). Table 3 shows the activation of the purified ADPglucose pyrophosphorylase from JP102 by various effectors. The derepressed mutant showed activator specificity similar to that of the parent strain enzyme. Comparison of kinetic constants of activators, ATP, and inhibitors of ADPglucose pyrophosphorylase from *S. typhimurium* LT-2 and JP102 indicated that the enzymes from the mutant and parent organism were quite similar (Table 4).

NH_2-terminal sequence studies. The results of amino acid sequence analysis of the first 27 residues of ADPglucose pyrophosphorylase from JP102 are shown in Fig. 1. Residue 16 could not be positively identified, because identification of the corresponding phenylthiohydantoin derivative by gas chromatography did not result in a significant appearance of an amino acid, whereas thin-layer chromatography indicated glutamic acid. However, back-hydrolysis showed that residue 16 may be either glutamic acid or proline. Figure 1 also shows the corresponding sequence of the NH_2 terminal of ADPglucose

TABLE 1. *Purification of ADPglucose pyrophosphorylase from S. typhimurium JP102*[a]

Purification step	Vol (ml)	Total units (μmol/min)	Protein (mg/ml)	Sp act (U/mg)
Crude homogenate	2,530	605	18.5	0.0013
Heat treatment fraction	2,550	1,762	16.3	0.042
$(NH_4)_2SO_4$ precipitation	1,790	1,307	4.5	0.16
Chromatography on:				
DEAE-cellulose	260	1,211	3.9	1.2
HDP[b]-Sepharose (AMP eluate)	40	1,080	0.32	84.4
HDP-Sepharose (phosphate eluate)	6	761	1.2	106.0

[a] Enzyme was isolated from 700 g of cell paste as described in the text. Enzyme activity was measured in the pyrophosphorolysis direction in the presence of 1.5 mM fructose 1,6-diphosphate.

[b] Hexanediol 1,6-diphosphate.

TABLE 2. *Composition of ADPglucose pyrophosphorylase from S. typhimurium JP102*[a]

Amino acid	Concn (mol/48,000 g)
Aspartic acid	45.2 ± 3.3
Threonine	15.5 ± 0.4
Serine	39.4 ± 1.1
Glutamic acid	43.6 ± 2.6
Proline	19.6 ± 0.6
Glycine	31.0 ± 1.6
Alanine	37.1 ± 2.7
Valine	34.0 ± 1.5
Methionine	12.3 ± 0.7
Isoleucine	26.3 ± 0.4
Leucine	35.9 ± 1.6
Tyrosine	15.1 ± 0.92
Phenylalanine	13.2 ± 0.3
Histidine	8.2 ± 0.1
Lysine	21.3 ± 1.7
Arginine	31.6 ± 1.7
Tryptophan[b]	5.6 ± 0.3
CM-cysteine[c]	10.4

[a] Protein was reduced, carboxymethylated, and hydrolyzed for 24, 48, and 72 h as described in the text. The results are based on a 94% yield and are the average of two determinations on two different preparations of pure enzyme.

[b] Determination was carried out spectrophotometrically (9).

[c] Determined as *S*-carboxymethyl (CM)-cysteine.

TABLE 3. *Activation of ADPglucose pyrophosphorylase from S. typhimurium JP102 by various metabolites*[a]

Effector	ADPglucose formed (nmol/10 min)
None	0.3
3-Phosphoglycerate	0.5
2-Phosphoglycerate	1.8
PLP[b], 0.05 mM	9.4
NADP, 1 mM	0.9
NADPH, 1 mM	8.6
Fructose-diphosphate	9.2
Ribose 5-phosphate	0.5
Glucose 1,6-diphosphate	1.0
3-Phosphoglyceraldehyde	0.95
Glycerol 1,3-diphosphate	4.2
6-Phosphogluconate	0.6

[a] Purified enzyme was assayed in the synthesis direction in the presence of 1.5 mM effector, unless otherwise indicated.

[b] Pyridoxal phosphate.

pyrophosphorylase from *E. coli* B (14, 29). Compared with the sequence of mutant JP102, there were at least two, and possibly three (residue 16), amino acid changes in the first 27 residues starting from the NH_2-terminal amino acid.

Carboxyl-terminal studies. Incubation of carboxypeptidase A with *S. typhimurium* ADPglucose pyrophosphorylase did not release amino acids as seen by thin-layer chromatography. Digestion with carboxypeptidase B and subsequent quantitation by amino acid analysis resulted in the release of 1.1 mol of arginine per

TABLE 4. *Kinetic parameters of ADPglucose pyrophosphorylase from S. typhimurium LT-2 and JP102*

Effector	JP102			LT-2	
	V_{max} (μmol min^{-1} mg^{-1})	$S_{0.5}$ ATP[a] (mM)	$\bar{n}$	$S_{0.5}$ ATP (mM)	$\bar{n}$
None	22.9	2.0	1.6	2.4	1.5
Fructose 1,6-diphosphate, 1 mM	91.6	0.50	2.0	0.48	2.2
NADPH, 0.5 mM	76.6	0.36	1.6	0.37	1.5
Effector	V_{max} (μmol min^{-1} mg^{-1})	$A_{0.5}$ (mM)	$\bar{n}$	$A_{0.5}$ (mM)	$\bar{n}$
Fructose 1,6-diphosphate	91.6	0.12	2.4	0.10	2.3
NADPH	76.6	0.13	2.4	0.11	2.3
Glycerol 1,3-diphosphate	41.2	0.30	2.3	0.31	2.2
Inhibitor		$I_{0.5}$ (mM)	$\bar{n}$	$I_{0.5}$ (mM)	$\bar{n}$
AMP (0.25)[b]		0.032	1.5	0.028	1.7
AMP (1.0)		0.096	1.9	0.11	2.0
ADP (0.25)		0.42	1.4	0.42	1.8
ADP (1.0)		1.0	2.0	1.1	2.4
PO_4^{3-} (0.25)		1.3	1.5	0.93	1.7
PO_4^{3-} (1.0)		2.0	2.0	1.7	2.5

[a] Concentration of ATP at which 50% maximal velocity is attained.

[b] Numbers in parentheses indicate concentration (millimolar) of fructose 1,6-diphosphate.

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     1                5                  10
     Val-Ser-Leu-Glu-Lys-Asn-Asp-Arg-Val-Met
A    Leu-Ala-Arg-Gln-Leu-(*)-Leu-Lys-Ser-Val
     Ala-Leu-Ile-Leu-Ala-Gly-Gly-

     1                5                  10
     Val-Ser-Leu-Glu-Lys-Asn-Asp-His-Leu-Met
B    Leu-Ala-Arg-Gln-Leu-Pro-Leu-Lys-Ser-Val
     Ala Leu Ile-Leu-Ala-Gly-Gly-
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FIG. 1. *NH_2-terminal sequence of ADPglucose pyrophosphorylases from S. typhimurium JP102 (A) and Escherichia coli B (B). The S. typhimurium amino acid sequence was determined as described in the text. Residue 16 could not be positively identified by gas chromatography; thin-layer chromatography indicated glutamic acid, and back-hydrolysis of the phenylthiohydantoin derivative showed glutamic acid or proline. The E. coli B amino acid sequence was previously determined (14, 29).*

48,000 g. All other amino acids were found in amounts less than 0.1 mol/48,000 g.

Immunology. When anti-*S. typhimurium* JP102 ADPglucose pyrophosphorylase serum was reacted against the purified enzyme from JP102 in double-immunodiffusion gels (26), only one precipitin band was visible. Partly purified ADPglucose pyrophosphorylases from *S. typhimurium* LT-2 was prepared as previously described (34) and also reacted with the antiserum in double-immunodiffusion gels. When at least 0.15 U of enzyme activity was placed on the gel, a single precipitin band formed and was cross-reactive with the band produced with the purified enzyme from JP102. In contrast, the purified ADPglucose pyrophosphorylase from *E. coli* B (14) formed a cross-reactive precipitin with the *S. typhimurium* enzyme antiserum which contained a spur. Preimmune serum did not form precipitin bands with either enzyme. Figure 2 shows the effect of antibody on the activity of the ADP-glucose pyrophosphorylases from *S. typhimurium* JP102, *S. typhimurium* LT-2, and *E. coli* B. The amounts of antibody required to inhibit 50% of the enzyme activity of purified ADP-glucose pyrophosphorylases from JP102 and *E. coli* B, of enzyme in a crude extract of JP102, and of a partially purified ADPglucose pyrophosphorylase fraction from *S. typhimurium* LT-2 were about 5.0, 13, 5.2, and 5.5 μl of serum per U of enzyme, respectively. Preimmune serum, used as a control, had no effect on any of these enzymes. These results indicate that the two *S. typhimurium* enzymes are antigenically similar, whereas the *E. coli* B enzyme has some antigenic dissimilarities when compared with the *S. typhimurium* enzymes.

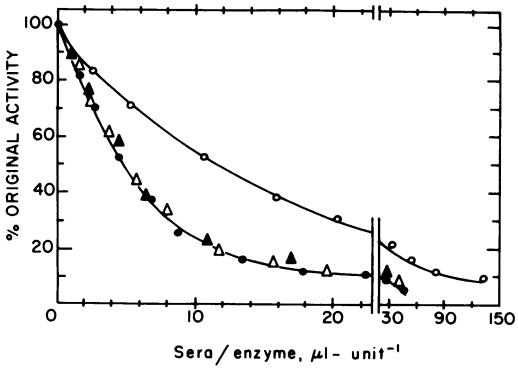

FIG. 2. *Inhibition of ADPglucose pyrophosphorylase activity purified from E. coli* (○), *from S. typhimurium LT-2* (△), *from S. typhimurium LT-2 mutant JP102* (●), *and from a crude extract from S. typhimurium LT-2 mutant JP102* (▲). *Conditions are described in the text.*

DISCUSSION

These results indicate that the ADPglucose pyrophosphorylase from *S. typhimurium* LT-2 mutant JP102 is very similar if not identical in all tested respects with the enzyme from the parent strain, *S. typhimurium* LT-2. Therefore, the mutant JP102 appears to synthesize the wild-type parent enzyme but in greater amounts. Kinetic studies showed that the two enzymes are similar with respect to kinetic constants for the allosteric effectors, fructose diphosphate, NADPH, AMP, ADP, P_i, and the substrate, ATP. Furthermore, the immunological reactions of both enzymes toward antibody produced from the mutant JP102 enzyme were identical in double-immunodiffusion gels and in neutralization of activity experiments.

The purified enzyme from mutant JP102 has been shown to have a subunit molecular weight of about 48,000. Both gel electrophoresis and sucrose gradient density ultracentrifugation experiments suggest that the native molecular weight is 190,000 to 195,000, indicating that the native ADPglucose pyrophosphorylase is a tetramer. Amino-terminal sequence studies showed only one sequence and suggest that the subunits are similar if not identical. Moreover, the release of no amino acids by carboxypeptidase A and the release of 1 mol of arginine per 48,000 g of enzyme by carboxypeptidase B are also consistent with similar subunits of 48,000 molecular weight.

Although some antigenic dissimilarities are noted between the *E. coli* B and *S. typhimurium* enzymes, there is a cross-reaction between the antibodies produced against each enzyme (this report; 14). Both enzymes have the same activator specificity, with the major activators being fructose diphosphate, pyridoxal phosphate, and NADPH, and are very sensitive to AMP inhibition (13, 34). Both enzymes have similar subunit and native molecular weights, and the amino acid compositions are similar (14). The similarity extends also to the amino-terminal sequence. Of the first 27 amino acids in the sequence, there are only two positive differences (residues 8 and 9) and perhaps a third (residue 16). The changes at residues 8 and 9 are conservative, since there is a change of arginine for histidine and valine for leucine. The conservation of the amino terminal of these two enzymes takes on added significance since it has been shown that a lysine residue, 37 amino acids from the amino-terminal valine, is involved in the binding of the allosteric activator (15, 28, 29).

The similarity of the ADPglucose pyrophosphorylase of *E. coli* and *S. typhimurium* corre-

lates also with the close evolutionary relatedness of these two organisms (1). The availability of antiserum prepared against the *E. coli* and *S. typhimurium* enzymes will enable more extensive immunological comparisons of the ADPglucose pyrophosphorylases of other organisms of the *Enterobacteriaceae* having the same activator specificity as the enzyme from the above two organisms as well as from other related bacteria. This will be the subject of another report.

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