Purification and Properties of Bacillus subtilis Nucleoside Diphosphokinase*

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SUMMARY

Bacillus subtilis nucleoside diphosphokinase was purified 1,100-fold to apparent homogeneity (a single band upon disc gel electrophoresis). Gel filtration through Sephadex G-200 indicated an approximate molecular weight of 100,000. The corrected Michaelis constants for the various nucleoside triphosphate substrates ranged from 0.10 mM for GTP to 0.42 mM for CTP. Deoxyribonucleoside triphosphates as well as ribonucleoside triphosphates served as substrates for the enzyme.

Mn$^{2+}$ and Mg$^{2+}$ both were able to fulfill the divalent cation requirements for enzyme activity. Mn$^{2+}$ was maximally activating at 1 mM while Mg$^{2+}$ was maximally activating at 10 mM.

The purified enzyme reacts with [$\gamma$-$^{32}$P]ATP yielding a phosphorylated enzyme in which the phosphate group is bound via a 1-phosphoheistine bond. The equilibrium between the free and phosphorylated enzyme forms favors the free form, indicating that the 1-phosphoheistine bond has a free energy of hydrolysis more negative than the terminal ATP bond.

Initial velocity and product inhibition kinetic studies suggest a ping pong reaction mechanism (the first product dissociates before the second substrate combines with the enzyme). This is consistent with the postulation that the phosphorylated protein associated with enzyme activity is an intermediate in the enzymatic reaction.

Nucleoside diphosphokinase (ATP : nucleoside diphosphate phosphotransferase, EC 2.7.4.6) catalyzes the phosphorylation of nucleoside diphosphates by nucleoside triphosphates; e.g. XTP + YDP $\Rightarrow$ XDP + YTP.

In the course of investigation of succinyl-CoA synthetase in Bacillus subtilis, an additional protein capable of being phosphorylated by [$\gamma$-$^{32}$P]ATP was found. This protein was identified as nucleoside diphosphokinase based on the coelution of the phosphorylated protein and enzyme activity on Sephadex G-200 and DEAE-Sephadex column chromatography and by comigration of the phosphorylated protein and enzyme activity in polyacrylamide gel electrophoresis.

Although nucleoside diphosphokinase has been well documented in eukaryotic organisms, little information is available on this enzyme from prokaryotic organisms. Therefore, an investigation of the properties of B. subtilis nucleoside diphosphokinase was undertaken.

This investigation entailed purification of the B. subtilis enzyme to apparent homogeneity and studies of some kinetic and physical properties of the purified enzyme and its phosphorylated form.

MATERIALS

The sodium salts of all nucleoside di- and triphosphates used were obtained from Sigma Chemical Company. [$\beta$-$^{32}$P]Pi was obtained from New England Nuclear Corporation. [$\gamma$-$^{32}$P]ATP was prepared by the Pi e ATP exchange reaction catalyzed by the succinyl-CoA synthetase from Escherichia coli. This enzyme was purified as described by Ramaley et al. (2). TPN, hexokinase, and glucose 6-phosphate dehydrogenase were obtained from Sigma. Sephadex G-200 and DEAE-Sephadex A-50 were purchased from Pharmacia Fine Chemicals. Lyphogel was obtained from the Gelman Company. Protein molecular weight markers and ammonium sulfate were purchased from Mann Laboratories. Hydroxylapatite was prepared by the method of Siegelman, Wieczorek, and Turner (3) and fine particles were removed by settling. Succinyl-CoA synthetase (specific activity 55 pmoles per min per mg of protein) was purified from succinate grown E. coli Crookes strain (a gift from Dr. E. Wooding, Commercial Solvent Corporation, Terre Haute, Indiana) by the method of Ramaley et al. (2). Whatman DEAE-cellulose (DE-23) was obtained from the Reeve Angel Company. All other materials used were of the highest grade of purity commercially available.

METHODS

Enzymatic Assays

Two different types of enzymatic assay for nucleoside diphosphokinase were employed: the coupled hexokinase-glucose 6-phosphate dehydrogenase assay and a direct assay measuring transphosphorylation from [$\gamma$-$^{32}$P]ATP to CDP.

Coupled Hexokinase-Glucose 6-Phosphate Dehydrogenase Assay

This assay is similar to the one used by Mourad and Parks (4). The reaction mixture contained, in 1 ml: glucose, 1 mM;
reaction mixture containing 10 mM MgCl₂, 0.015 mM ATP (1.2 μM), 0.25 mM Tris-acetate (pH 7.5), 0.15 mM ADP, 0.15 mM (unless otherwise specified); and 0.1 mM (unless otherwise specified). The reaction was started by the addition of nucleoside diphosphokinase preparations and measurements were made by following the increase in absorbance at 340 nm using a Beckman DU spectrophotometer equipped with a Gilford 2000 multiple sample recorder. One unit of activity is defined as that amount of enzyme which catalyzes the production of 1 μmole of ATP per min under the conditions of the standard assay. Since some of the nucleoside triphosphates could serve as substrates for yeast hexokinase, a control without nucleoside phosphokinase was run and subtracted from the value obtained in the presence of nucleoside phosphokinase in the nucleoside triphosphate specificity studies.

**Direct Assay**—The reaction mixture (final volume of 0.2 ml) contained MgCl₂, 10 mM; Tris-acetate (pH 7.5), 0.015 mM; [γ-³²P]-ATP and CDP in the concentrations specified; and nucleoside phosphokinase. The reaction mixture, minus nucleoside phosphokinase, was kept in an ice bath and then preheated for 5 min at the desired temperature before the reaction was initiated by the addition of enzyme. At 30 sec, 1 min, 2 min, 3 min, and 4 min, 5-μl samples were withdrawn, spotted on a piece of Whatman No. 1 filter paper (26 × 18 cm) 2.5 cm from the bottom and the reaction terminated by the immediate addition of an equal volume of absolute alcohol to the same spot. After the addition of 0.1 μmole of unlabeled CTP and 0.1 μmole of unlabeled ATP (so the spot could be located under an ultraviolet lamp) ascending chromatography was carried out in fresh 0.1 M sodium phosphate, ammonium sulfate, and 1-propanol, 100:80:2, v/v/v. Under these conditions CTP had an Rₚ value of 0.80 and ATP had an Rₚ value of 0.48. The chromatograms were allowed to dry in air and then the CTP and ATP spots were cut out and placed in vials containing scintillation fluid (7.0 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene per liter of toluene). Radioactivity was determined with a Packard liquid scintillation counter (window open and 2% gain). Radioactivity appearing in the products was plotted as a function of time and the initial velocity was calculated from the slope. In most cases the reaction was linear throughout the entire time of sampling. When it was not, the early linear portion of the curve was used to determine the initial velocity.

**Determination of Protein**

Protein was estimated by the procedure of Lowry et al. (5) with bovine serum albumin from Sigma as the standard.

**Determination of Protein-bound ³²P**

Protein-bound radioactivity was measured by phenol extraction of ATP-treated samples as previously described (2).

**Determination of ATPase Activity**

ATPase activity was measured as previously described (2) by the release of inorganic phosphate from [γ-³²P]ATP in a reaction mixture containing 10 mM MgCl₂, 0.015 mM Tris-acetate (pH 7.5), 0.25 mM ATP (1.2 × 10⁶ cpm per μmole).

**Purification of B. subtilis Nucleoside Diphosphokinase**

**Step 1: Growth of Cells**—B. subtilis 168 was obtained from Frank Young and John Spizizen of the Scripps Clinic, La Jolla, California. The organism was grown in a 180-liter fermentor (Stainless and Steel Division, Butler Manufacturing, St. Paul, Minnesota) in a medium containing, per liter of distilled-deionized water: 85% H₂PO₄, 0.3 ml; MgSO₄·7H₂O, 1.0 g; MnSO₄·H₂O, 25 μg; FeSO₄(NH₄)₂·6H₂O, 25 μg; NaCl, 0.4 g; KCl, 0.4 g; and CaCl₂, 10 mg. The salts medium for 1 liter was made up to 800 ml, the pH was adjusted to 7.9 with concentrated KOH, and the mixture was sterilized. The final medium was prepared by the addition of 100 ml of 0.2 M glucose and of 100 ml of 0.5 M glutamate (sterilized separately by autoclaving) for each 800 ml of salts medium.

**Step 2: Preparation of Cell-free Extract**—Cells were harvested in the late logarithmic phase of growth, washed twice in 0.5 M potassium phosphate (pH 7.2), and stored at 20°C until needed. The cells were thawed and resuspended in 0.02 M potassium phosphate-0.001 M MgCl₂ (pH 8.0) in a 1:1 ratio of volume of buffer to wet weight of cells. The resuspended cells, in 80- to 100-mI batches, were then disrupted sonically for 10 2-min intervals with a Bronson Heat System Company sonicator using tap 6. The suspension was stirred throughout with a magnetic stirrer and maintained at 2-6°C by immersion in an ice bath. The sonicated treated cells were brought to twice the original volume with phosphate buffer and cellular debris was removed by centrifugation for 150 min at 30,000 rpm and 2°C (Spinco L2-65 centrifuge with type 30 rotor).

**Step 3: First Ammonium Sulfate Fractionation**—The supernatant fraction was brought to 50% (NH₄)₂SO₄ saturation by the gradual addition of solid (NH₄)₂SO₄ while maintaining the pH between 7 to 7.5 by the addition of concentrated ammonium hydroxide. During the addition of (NH₄)₂SO₄ the suspension was stirred with a magnetic stirrer and maintained between 0-4°C. After the addition of (NH₄)₂SO₄ the suspension was allowed to stir for 2 hours and then centrifuged for 30 min at 16,500 rpm with SS 34 rotor in a Sorvall centrifuge. The residue was discarded and the supernatant fluid brought to 100% (NH₄)₂SO₄ concentration as described. The residue collected by centrifugation was resuspended in 0.025 M potassium phosphate (pH 7.2) and the ammonium sulfate removed by dialysis against the same buffer.

**Step 4: Chromatography on DEAE-cellulose**—The diazol solution was poured onto a column (8 × 83 cm) of DEAE-cellulose (DE-23) equilibrated with 0.025 M potassium phosphate (pH 7.2), 0.001 M MgCl₂, and 0.001 M 2-mercaptoethanol. The column was washed with 500 ml of the same buffer and the proteins were eluted with a linear gradient of potassium chloride (6 liters of the wash buffer and 6 liters of the wash buffer containing 1.0 M KCl). Fractions of 22 ml each were collected. The fractions containing the major portion of the enzyme activity (Fractions 150 to 200) were pooled.

**Step 5: Second (NH₄)₂SO₄ Fractionation**—The pooled fractions from the DEAE-cellulose column were brought to 70% (NH₄)₂SO₄ saturation by the addition of solid (NH₄)₂SO₄ as described in Step 3 and centrifuged at 16,500 rpm with a SS 34 rotor in a Sorvall centrifuge. The supernatant fraction was discarded and the residue resuspended in a minimal volume of 0.05 M KCl, 0.05 M potassium phosphate (pH 7.2). The resuspended residue was then dialyzed against three changes of the phosphate buffer to remove (NH₄)₂SO₄.

**Step 6: Chromatography on Sephadex G-200**—The diazol solution from Step 5 was applied to a Sephadex G-200 column.
(5 × 82 cm) equilibrated at 4° with 0.05 M KCl-0.05 M potassium phosphate (pH 7.2) and eluted with the same buffer. The eluent was collected in 14.2-ml fractions. Fractions containing the major portion of the enzyme activity (Fractions 70 to 90) were pooled.

**Step 7: First DEAE-Sephadex Chromatography**—The pooled fractions from the Sephadex G-200 column of Step 6 were applied to a column (1.75 × 90 cm) of DEAE-Sephadex A-50 equilibrated with 0.05 M potassium phosphate (pH 7.2)-0.05 M KCl and eluted with a 1500-ml linear gradient from 0.05 to 0.5 M KCl in 0.05 M potassium phosphate (pH 7.2) at 4°. Fractions of 13 ml each were collected. Fractions containing most of the enzyme activity (Fractions 70 to 90) were pooled and dialyzed against 0.006 M potassium phosphate-0.05 M Tris-0.1 M KCl (pH 7.2).

**Step 8: Chromatography on Hydroxylapatite**—The dialyzed enzyme from the DEAE-Sephadex column of Step 7 was placed on a column (68 × 1.75 cm) of hydroxylapatite previously equilibrated with 0.005 M potassium phosphate-0.05 M Tris-0.1 M KCl (pH 7.3) and eluted with a 1200-ml linear gradient from 0.005-0.1 M potassium phosphate in 0.05 M Tris-0.1 M KCl (pH 7.2) at 4°.

**Step 9: Second DEAE-Sephadex Chromatography**—Pooled fractions from the hydroxylapatite column were dialyzed against 0.05 M potassium phosphate-0.05 M KCl (pH 7.2) and then applied to a column (32 × 2 cm) of DEAE-Sephadex A-50. Nucleoside diphosphokinase was eluted with a 600-ml linear gradient from 0.05-0.5 M KCl in 0.05 M potassium phosphate (pH 7.2) at 4°.

**Step 10: Lyphogel Concentration**—Pooled fractions (Fractions 50 to 58) from the second DEAE-Sephadex column were concentrated by the addition of 11 g of Lyphogel. Lyphogel was left in the enzyme solution overnight at 4° and then the gel was removed by centrifugation using 40-ml perforated centrifuge tube inserts (Ocecony Associates, Chicago, Illinois) which retained the gel and permitted the concentrated enzyme solution to pass through. Centrifugation was performed at 3000 rpm for 6 min using a S3 34 rotor in a refrigerated Sorvall centrifuge. The concentrated enzyme solution was stored at −20°.

**Disc Gel Electrophoresis of "Purified" Nucleoside Diphosphokinase**

Disc gel electrophoresis was performed with a Canalo gel model 26 apparatus using a Buchler International model 3-1014A power supply. A 50-mm 7% acrylamide lower gel (Canalo) and a 20-mm upper sample gel (Canalo) were used. The lower gel was adjusted to pH 8.3 and polymerized in front of a fluorescent lamp and then the upper gel, consisting of an equal volume of Canalo upper gel (pH 6.7) and purified nucleoside diphosphokinase, was applied and polymerized. The electrophoresis buffer contained, per liter, Tris (6 g) and glycerine, (28.5 g) (pH was adjusted to 8.3 with HCl). Electrophoresis was carried out at room temperature using 2.5 ma of current per tube until a bromphenol blue indicator had moved 40 mm. Gels were stained for 1 hour in a solution of 1% Amido black 10B and 7% acetic acid. Destaining was effected with 7% acetic acid at 37°. The 7% acetic acid used for destaining was changed every 4 to 5 hours until complete destaining was achieved. A duplicated series of gels were sliced into 1.7-mm fractions and placed in 0.1 ml of 0.15 M Tris-acetate (pH 7.5) to clute the enzyme. After standing overnight at 4°, the buffer was then assayed for enzyme activity and the ability to form protein-bound 32P from [γ-32P]ATP as described earlier (1).

**Determination of Estimated Molecular Weight by Sephadex G-200 Gel Filtration**

Purified nucleoside diphosphokinase (9 units of specific activity, 217 units per mg of protein) was applied to a column (2.5 × 35 cm) of Sephadex G-200 in 1 ml of 0.05 M potassium phosphate (pH 7.2)-0.05 M KCl and eluted with the same buffer. The Sephadex G-200 was prepared in the phosphate-potassium chloride buffer, the fine particles were removed, and the column was precalibrated with 5 mg of the indicated proteins obtained from Pharmacia Fine Chemicals, Inc. (prepared by Worthington Biochemical Corporation). Fractions of 25 drops each were collected and the apparent molecular weight was estimated from plotting the molecular weight of the standards against the peak tube number on a semilogarithmic plot (6).

**Determinant of Apparent Equilibrium Constant for Phosphorylation of Enzyme by [γ-32P]ATP**

Approximately 0.08 nmole of enzyme (specific activity, 217) was incubated for 10 min at pH 7.5, 30° in a 0.1-ml total volume containing 10 mM MgCl₂, 0.015 M Tris-acetate, 0.21 mM [γ-32P]ATP (specific activity 1.67 × 10⁸ cpm per μmole), and ADP to give the ratios as indicated. Enzyme-bound 32P was determined by phenol extraction (2). No significant change was observed with an additional 20-min incubation, showing that equilibrium had been reached.

**RESULTS**

**Purification of Nucleoside Diphosphokinase**—As seen in Table I, the purification scheme resulted in a 1070-fold purification of the enzyme from glucose-glutamate grown cells of B. subtilis. Disc gel electrophoresis (Fig. 1) indicated that the major protein component in the purified nucleoside diphosphokinase migrated with the nucleoside diphosphokinase activity. Fractions containing nucleoside diphosphokinase activity also had the ability to form a phosphorylated protein from [γ-32P]ATP (not shown). The purified enzyme is relatively stable to storage in 0.05 M potassium phosphate (pH 7.2)-0.05 M KCl at −20° at a protein concentration of 0.05 mg per ml. Repeated freezing and thawing over a period of 5 months resulted in a 25% loss of enzyme activity. Attempts to crystallize the purified enzyme have thus far not been successful.

**Substrate Specificity**—The nucleoside trisphosphate specificity of B. subtilis nucleoside diphosphokinase was examined with the hexokinase-glucose 6-phosphate dehydrogenase-linked assay using 0.15 mM ADP. The reaction was linearly dependent on enzyme concentration up to 0.01 enzyme units.

In common with other nucleoside diphosphokinases isolated from other sources (4, 7, 8), B. subtilis nucleoside diphosphokinase is nonspecific with regard to the nucleoside triphosphate substrate (Table II). Although there are differences in the apparent kinetic constants, the enzyme reacts with either ribo- or deoxyribonucleoside triphosphates.
TABLE I
Summary of purification of B. subtilis nucleoside diphosphokinase

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Volume</th>
<th>Units</th>
<th>Protein</th>
<th>Purity (%)</th>
<th>Yield</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial extract</td>
<td>915</td>
<td>4110</td>
<td>21.4</td>
<td>0.21</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ (50 to 100%)</td>
<td>300</td>
<td>1330</td>
<td>21.8</td>
<td>0.29</td>
<td>47</td>
<td>1.4</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>600</td>
<td>1170</td>
<td>0.75</td>
<td>2.01</td>
<td>28.3</td>
<td>12.4</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ (70%)</td>
<td>30</td>
<td>335</td>
<td>12.6</td>
<td>2.19</td>
<td>19.6</td>
<td>10.3</td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>300</td>
<td>530</td>
<td>0.16</td>
<td>11.10</td>
<td>12.9</td>
<td>53.0</td>
</tr>
<tr>
<td>First DEAE-Sephadex purification</td>
<td>92</td>
<td>432</td>
<td>0.18</td>
<td>20.0</td>
<td>10.0</td>
<td>124</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>80</td>
<td>348</td>
<td>0.10</td>
<td>43.5</td>
<td>8.45</td>
<td>207</td>
</tr>
<tr>
<td>Second DEAE-Sephadex purification</td>
<td>65</td>
<td>230</td>
<td>0.015</td>
<td>235</td>
<td>5.6</td>
<td>1100</td>
</tr>
<tr>
<td>Lyphogel concentration</td>
<td>10</td>
<td>168</td>
<td>0.080</td>
<td>218</td>
<td>4.1</td>
<td>1070</td>
</tr>
</tbody>
</table>

**TABLE II**
Kinetic constants of nucleoside diphosphokinase with various nucleotides

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Apparent $K_m$ (mM)</th>
<th>Corrected $K_m$ (mM)</th>
<th>Apparent $V_m$ (nmoles/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTP</td>
<td>0.059 ± 0.008</td>
<td>0.10 ± 0.01</td>
<td>13.2 ± 0.8</td>
</tr>
<tr>
<td>dGTP</td>
<td>0.065 ± 0.015</td>
<td>0.16 ± 0.03</td>
<td>14.6 ± 1.3</td>
</tr>
<tr>
<td>CTP</td>
<td>0.28 ± 0.10</td>
<td>0.42 ± 0.04</td>
<td>10.8 ± 3.3</td>
</tr>
<tr>
<td>dCTP</td>
<td>0.30 ± 0.08</td>
<td>0.55 ± 0.07</td>
<td>17.1 ± 3.8</td>
</tr>
<tr>
<td>TTP</td>
<td>0.11 ± 0.01</td>
<td>0.20 ± 0.03</td>
<td>15.7 ± 1.3</td>
</tr>
<tr>
<td>dTTP</td>
<td>0.12 ± 0.03</td>
<td>0.30 ± 0.06</td>
<td>9.8 ± 1.5</td>
</tr>
<tr>
<td>ATP</td>
<td>0.15 ± 0.06</td>
<td>0.22 ± 0.06</td>
<td>2.2 ± 1.8</td>
</tr>
<tr>
<td>ADP</td>
<td>0.006 ± 0.03</td>
<td>0.01 ± 0.02</td>
<td>8.4 ± 1.5</td>
</tr>
<tr>
<td>CDP</td>
<td>0.008 ± 0.02</td>
<td>0.01 ± 0.02</td>
<td>10.4 ± 1.5</td>
</tr>
</tbody>
</table>

* The actual active substrates are presumed to be magnesium-nucleotide complexes at the concentrations of magnesium employed (10 and 25 mM).
* Corrected $K_m$ (apparent $K_m$ value increases as second substrate approaches saturation concentration). Second substrate used shown under each corrected $K_m$.
* Coupled assay using 0.14 mM ADP.
* Data from Fig. 6 (increasing concentrations of CDP).
* Data from Fig. 5 (increasing concentration of GTP).
* Data not given (increasing concentration of CTP).

**FIG. 2.** Determination of estimated molecular weight by Sephadex G-200 gel filtration. The Sephadex G-200 column was calibrated with known proteins (○) as described under “Methods.” Nucleoside diphosphokinase (NDP kinase) activity (△) was determined with the coupled assay following gel filtration of 41 μg of purified nucleoside diphosphokinase.

The purified enzyme preparation had no nucleoside di- or triphosphatase activity (e.g. ATPase).

**Estimation of Molecular Weight by Sephadex G-200 Column Chromatography**—The data in Fig. 2 show an apparent molecular weight (6) of 100,000 for B. subtilis nucleoside diphosphokinase.

**Divalent Cation Requirements for Transphosphorylation**—The
effect of Mg\(^{2+}\) and Mn\(^{2+}\) concentration upon the reaction velocity (as measured by the direct assay) at constant ATP (0.357 mM) and CDP (2.5 mM) concentrations is seen in Fig. 3. Both Mg\(^{2+}\) and Mn\(^{2+}\) can fulfill the divalent cation requirement for the reaction but Mn\(^{2+}\) is maximally effective at a 10-fold lower concentration and over a narrower range of concentration than is Mg\(^{2+}\). Mn\(^{2+}\) has an inhibitory effect at concentrations above 1.0 mM and Mg\(^{2+}\) has an inhibitory effect at concentrations above 10.0 mM.

Equilibrium for Phosphorylation of Enzyme with ATP—The purified enzyme required only the addition of Mg\(^{2+}\) and ATP for the formation of a phosphorylated protein. Measurement of the apparent equilibrium for formation of enzyme-bound phosphate (E-P) from ATP was accomplished by incubating the enzyme with various ratios of ADP:[$\gamma$-32P]ATP in the presence of 10 mM Mg\(^{2+}\) and then determining the protein-bound radioactivity by phenol extraction. Results of such an experiment are shown in Fig. 4. They suffice for an estimate of an apparent partial equilibrium constant $K_{eq}$ defined as

$$K_{eq} = \frac{(E-P)(ADP)}{(E)(ATP)}$$

Plots of 1/(E-P) against (ADP:ATP) have an intercept of 1/E \(_t\) (where \(E_t\) is the maximum amount of enzyme-bound P) and a slope of 1/$K_{eq}E_t$. Determination of \(E_t\) from the intercept then permits determination of $K_{eq}$ from the slope. In this case the value of $K_{eq}$ is 0.01. From this an approximate $\Delta F$ for the phosphorylation of the protein can be calculated as 300 cal. However, these results are obtained by phenol denaturation of the enzyme followed by an extensive extraction procedure. This method could be introducing a shift in equilibrium before the enzyme is completely inactive and thus some caution is indicated in comparison of $\Delta F$ obtained by other means.

The maximum phosphorylation obtained in the presence of

![Fig. 3. Effect of divalent cation concentration upon the rate of transphosphorylation between ATP and CDP as catalyzed by B. subtilis nucleoside diphosphokinase. The velocity (micro-
moles per min per ml) of transphosphorylation was determined by the direct assay as described under “Methods.” Nucleoside diphosphokinase (specific activity 218) concentration was 0.05 units per ml.](image)

![Fig. 4. Apparent equilibrium for the phosphorylation of B. subtilis nucleoside diphosphokinase by [$\gamma$-32P]ATP. Conditions for the phosphorylation are described under “Methods.” Enzyme (0.08 nmoles) was used in a final volume of 0.1 ml (0.80 nmoles per ml). E-P was determined by phenol extraction and is given as nanomoles per ml (picomoles per liter) of enzymebound P. $E_{total}$ is the extrapolated enzyme-bound 32P obtained at a constant ATP concentration of 0.21 mM and is 0.83 pmoles per liter. $K_{eq}$ is the calculated partial equilibrium constant for the phosphorylation of the enzyme by ATP (i.e. $K_{eq} = \frac{(E-P)}{(E_{total})(ATP)} = 0.61$).](image)

![Fig. 5. Plot of the reciprocal of the initial velocity (micro-
moles per min per ml) against the reciprocal of concentrations of ADP (millimolar). The reaction components are those described for the hexokinase-glucose-6-P dehydrogenase coupled assay with 25 mM Mg\(^{2+}\). Nucleoside diphosphokinase (specific activity, 218) concentration was 0.008 units per ml.](image)
ATP and Mg\(^{2+}\), as calculated from the data presented in Fig. 4, corresponds to about 1 phosphoryl group per molecule of enzyme.

**Kinetic Analysis of B. subtilis Nucleoside Diphosphokinase**

The reciprocal plots of the data obtained in initial velocity studies using the hexokinase-glucose 6-phosphate dehydrogenase assay system with ADP as the variable substrate and GTP (Fig. 5) as the changing fixed substrate yielded a family of parallel lines. Essentially the same results were obtained when ADP replaced GTP as the changing fixed substrate and GTP was the variable substrate. Families of parallel lines were also obtained with CDP, UTP, ITP, and dGTP as the changing fixed substrate.

Similar results were also obtained utilizing the direct assay. Fig. 6 shows that the plots of initial velocity data with CDP as the variable substrate and ATP as the changing fixed substrate yielded a family of parallel lines thus substantiating the results of the linked assay system.

These results are consistent with a ping pong reaction mechanism (9). A sequence for such a reaction using ATP and CDP as examples is given in Scheme 1.

\[
\begin{array}{c}
\text{ATP} \quad \text{ADP} \quad \text{CDP} \quad \text{CTP} \\
\text{E} \quad \text{E}^*\text{ADP} \quad \text{E}^*\text{CTP} \\
\end{array}
\]

**Scheme 1**

The ping pong mechanism depicted also predicts competitive inhibition between nucleoside diphosphates and competitive inhibition between nucleoside triphosphates. The direct assay was employed to investigate these possible inhibitions since it was observed that high substrate concentration inhibited the activity of hexokinase in the coupled assay.

Fig. 7 shows that ADP acts as a competitive inhibitor of CDP (\(K_i = 0.05\) mM) and Fig. 8 shows that CTP acts as a competitive inhibitor of ATP (\(K_i = 0.30\) mM).
DISCUSSION

This paper marks the first report of an investigation of the properties of nucleoside diphosphokinase from a prokaryotic organism, the bacterium *B. subtilis*, and indicates that its properties are similar to nucleoside diphosphokinase isolated from other sources. Nucleoside diphosphokinase has been purified from glucose-glutamate grown cells of *B. subtilis* to an apparent homogeneous state free from myokinase and ATPase.

The purified *B. subtilis* enzyme had a broad substrate specificity typical of other nucleoside diphosphokinases studied thus far 

The nature of this enzyme phosphoryl bond has been tentatively in which the phosphoryl group is bound to the enzyme has a equilibrium favors the free enzyme, and suggests that the form phokinase by 4TP at 10 mM Mg2+ was 0.61 (Fig. 4). This constant for the phosphorylation of *B. subtilis* nucleoside diphosphate, when subjected to the same hydrolytic procedure, exhibited ble in the alkaline hydrolysates. E. coli succinyl-CoA synthetase, no phosphohistidine or 3-phosphohistidine were detectable by Garces and Cleland (12) for yeast nucleoside diphosphokinase.

A comparison with the data of Garces and Cleland (12) for yeast nucleoside diphosphokinase supports the suggestion of Garces and Cleland (12) that the physiological function of nucleoside diphosphokinase in the cell is to rephosphorylate other nucleoside diphosphates at the expense of ATP.

The control and regulation of such an enzyme would be of some importance in view of the emerging energy charge 

Garces and Cleland (12) have reported a comprehensive kinetic study with crystalline yeast nucleoside diphosphokinase in which they found a kinetic partial equilibrium of 0.188 for the phosphorylation of the enzyme by ATP and suggested that the yeast enzyme may contain four catalytic sites based on the 32P incorporation from [γ-32P]ATP.

The present available kinetic data with the *B. subtilis* nucleoside diphosphokinase indicates that the kinetic constants thus far obtained are not sufficiently accurate to be verified by the Haldane relationships. Further study is required to resolve the apparent discrepancy between the partial equilibrium of phosphorylation determined by direct analysis (King = 0.61) and our tentative kinetic phosphorylation equilibrium of (King = 0.29).

It is clear that, in contrast to succinyl-CoA synthetase, the free rather than the phosphorylated form of nucleoside diphosphokinase is favored at equal concentrations of ATP and ADP. This plus the fact that the King values observed for ATP and the other nucleoside triphosphates are higher than those reported for ADP support the suggestion of Garces and Cleland (12) that the physiological function of nucleoside diphosphokinase in the cell is to rephosphorylate other nucleoside diphosphates at the expense of ATP.

The control and regulation of such an enzyme would be of some importance in view of the emerging concepts of energy charge (19). If the nucleoside diphosphokinase is not under inhibitory control, then the concept of energy charge which to date has been restricted to adenylate nucleotides might have to be extended to include the pools of total nucleotides even though the regulatory energy charge may be mediated through the adenylate nucleotide effectors.

We have investigated the effect of various growth conditions and metabolic inhibitors on the level of nucleoside diphosphokinase in *B. subtilis* and have found that there is a correlation between growth rate and nucleoside diphosphokinase level. However, we have no present evidence concerning the basis of the control of the level of nucleoside diphosphokinase in *B. subtilis*.

The present report of the occurrence, purification, and properties of nucleoside diphosphokinase from a prokaryotic organism indicates that this enzyme is similar in all of its major respects to nucleoside diphosphokinase isolated from other organisms. This suggests that nucleoside diphosphokinase conditional mutants obtained in microbial systems would be extremely useful in studies on the effects of cellular metabolism via energy charge regulation (19) and that the result might be applicable to the study of regulation of metabolism in other more complex organisms.

Note Added in Proof—The *B. subtilis* nucleoside diphosphokinase, like the pI 7.3 isoenzyme of nucleoside diphosphokinase from human erythrocytes (20), yields a diphasic Arrhenius plot. The transition point for the *B. subtilis* enzyme is 25°. Energy of activation above and below 25° are 4.2 and 8.5 kcal m-1, respectively.

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Purification and Properties of *Bacillus subtilis* Nucleoside Diphosphokinase
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