PURIFICATION AND PROPERTIES OF N-ACETYL-D-GLUCOSAMINE KINASE FROM STREPTOCOCCUS PYOGENES

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Abstract

Zelevnik, L. D. (CIBA Pharmaceutical Co., Summit, N.J.), H. Hänk, J. J. Boltralk, H. Heymann, and S. S. Barkulis. Purification and properties of N-acetyl-D-glucosamine kinase from Streptococcus pyogenes. J. Bacteriol. 88:1288-1295. 1964.—A kinase from Streptococcus pyogenes which catalyzes adenosine triphosphate-dependent phosphorylation of D-glucose and N-acetyl-D-glucosamine has been purified 1,500-fold. The ratio of the enzymatic activity on both substrates remained constant throughout the fractionation. Similarity in heat stability, p-hydroxymercuribenzoate inhibition, protection by either carbohydrate, and lack of repression of enzymatic activity when the bacteria were grown exclusively on one of the two substrates supports the hypothesis that kinase activity is associated with one enzyme.

N-acetyl-D-glucosamine is a major component of the group-specific “C” carbohydrate and the glycopeptide portions of the cell wall of Streptococcus pyogenes (Heidelberger and Kendall, 1936; Zittle and Harris, 1942; Schmidt, 1952). Roseman et al. (1954) and Davidson, Blumenthal, and Roseman (1957) have shown that N-acetyl-D-glucosamine-6-phosphate can be formed in this organism by the acetylation of glucosamine-6-phosphate. Specific kinases for N-acetyl-D-glucosamine have been found in other organisms and in mammalian tissue (Soodak, 1955; Faulkner and Quastel, 1956; Leloir, Cardini, and Olavarria, 1958; O’Brien, Glick, and Zilliken, 1960). In Escherichia coli, an N-acetyl-D-glucosamine kinase was separated from a glucokinase (Asensio, 1960).

We previously reported the ability of extracts of S. pyogenes to catalyze the adenosine triphosphate (ATP)-dependent phosphorylation of N-acetyl-D-glucosamine and of D-glucose (Barkulis et al., 1962). For each 1 mole of N-acetyl-D-glucosamine and ATP that disappeared, 1 mole of acid-stable phosphate and 1 mole of adenosine diphosphate (ADP) were formed; the product of the phosphorylation was N-acetyl-D-glucosamine-6-phosphate.

In this paper, we report the partial purification and several properties of the enzyme. Glucokinase was not separated from N-acetyl-D-glucosamine kinase, although an overall purification of 1,500-fold of each was achieved.

Materials and Methods

Growth of microorganisms. S. pyogenes strain S22, serotype 14, was grown according to Barkulis and Jones (1957). Inocula of 100 ml of log-phase cells were used per 1 liter of medium contained in a 4-liter Erlenmeyer flask. The cells were harvested by centrifugation after 16 hr of stationary growth at 37°C, and were washed twice with distilled water. The yield of cells was 4 to 5 g (wet weight) per liter of medium. Similar kinase activity was found in cells grown on medium supplemented with N-acetyl-D-glucosamine instead of D-glucose.

Materials. The following were obtained from commercial sources: crystalline ATP, glucose, nicotinamide adenine dinucleotide phosphate (NADP), and glucose-6-phosphate dehydrogenase. N-acetyl-D-glucosamine was a gift of Chas. Pfizer & Co., Inc.

Procedures for studying stoichiometry of the kinase reaction. The stoichiometry of the phosphorylation of N-acetyl-D-glucosamine kinase was studied in reaction mixtures incubated for 1 hr at 37°C in a volume of 4 ml and containing 0.6 ml of crude enzyme (12 mg of protein), 0.01 M ATP, 0.05 M MgCl₂, 0.12 M tris(hydroxymethyl)aminomethane (tris)-HCl buffer (pH 8.3), 0.025 M NaF, and 0.0075 M substrate. The reaction was stopped by addition of cold 5% trichloroacetic acid to a sample, and the protein precipitated was...
sedimented by centrifugation. Phosphate was determined by the method of Fiske and Subbarow (1925) on the supernatant liquid before and after hydrolysis for 7 min at 100 C in 1 N H2SO4. Free substrate was determined in another sample after precipitation of sugar phosphates with Ba(OH)2-ZnSO4 (Brown, 1951; Somogyi, 1945), by the method of Reissig, Strominger, and Leloir (1955). N-acetyl-d-glucosamine-6-phosphate was isolated by the method of Distler, Merrick, and Roseman (1958), whose color correction was applied in analytical determinations of the product from the Reissig procedure. ADP was determined by the method of Kornberg and Pricer (1951). Disappearance of ATP was calculated by difference from the phosphate and ADP determinations.

Procedures in identification of N-acetyl-d-glucosamine-6-phosphate. The final step in the isolation of the product by the method of Distler et al. (1958), referred to in the preceding section, is elution from a column of Dowex 1-acetate by a linear gradient (Bock and Ling, 1954) between 0.10 and 0.25 N H2SO4. The product was eluted between 0.120 and 0.125 N H2SO4. This eluate was further purified by paper chromatography on Whatman no. 3MM filter paper in ethanol-ammonium acetate (1 M, pH 5)-ethylenediaminetetraacetic acid (EDTA, 1 M, pH 5; 70:30:0.1; Wavskiewics, 1961). A single spot was obtained with an Rf of 0.42.

A sample of the compound was hydrolyzed in 1 N HCl for 1 hr at 100 C. The inorganic phosphate and N-acetyl-d-glucosamine content of the hydrolysate were determined. Another sample was treated with intestinal phosphatase (Pentex, Inc., Kankakee, Ill.). The products were chromatographed on Whatman no. 1 filter paper with the solvent system indicated above.

The position of the phosphate group of the product was determined after borohydride reduction (Kabat and Mayer, 1961) of a sample, followed by periodate oxidation in acetate buffer (pH 4.6) by the method of Jeanloz and Forchielli (1951). The products were chromatographed on Whatman no. 1 paper with the solvent system employed in isolating the reaction product. To facilitate product identification, the same procedures were carried out on α-glycerol-1-phosphate and glucose-6-phosphate, and the chromatographed products were compared. Phosphate esters were located on chromatograms by the method of Hanes and Isherwood (1949), and reducing sugars by the method of Trevelyan, Proctor, and Harrison (1950).

Enzymatic assays. Kinase activity was measured by the rate of hydrogen ion liberation during the phosphorylation of the sugar and formation of ADP from ATP in a constant-pH microtitrimetric procedure (Schwartz and Myers, 1958). Reactions were carried out in 4-ml volumes at 30 C and pH 7.4 with 0.002 M ATP, 0.02 M MgCl2, 0.002 M EDTA, 0.02 M NaF, enzyme, and 0.0015 M sugar. The pH was maintained at 7.4 by addition of 0.1 N NaOH from a microtitrator (Micro-Metric Instrument Co., Cleveland, Ohio). One unit of activity was defined as the amount of enzyme required to form 1 μmole of hydrogen ion per min at 30 C, and specific activity (SA) as the number of units per mg of protein. Assays were conducted for 3-min periods, with recordings of NaOH utilization made at 15-sec intervals. The reaction rate (Fig. 1) was a linear function of the amount of enzyme between 0.25 and 0.75 unit of enzymatic activity, and assays were conducted on samples or dilutions of enzyme which were within these limits.

Protein was determined by a modification of the method of Lowry et al. (1951). Control reactions (lacking substrate) were used to determine adenosine triphosphatase activity present

FIG. 1. Constant-pH microtitrimetric assay of enzymatic activity. The same results are obtained with either N-acetyl-d-glucosamine or D-glucose as substrate.
in the extracts. Glucokinase was also measured by spectrophotometric assay at 340 m\(\text{A}\) of glucose-6-phosphate formed, in cuvettes with a 10-mm light path; the test solution (1 ml) contained 0.03 M tris buffer, 0.006 M MgCl\(_2\), 0.0006 M EDTA (pH 8.0), 0.0002 M NADP, and 1 \(\mu\)lter of glucose-6-phosphate dehydrogenase (1.40 K units/ml; Zeleznick, 1955).

**RESULTS**

Table 1 summarizes the results obtained by incubating a crude preparation of enzyme with an excess of N-acetyl-d-glucosamine. The disappearance of the substrate was accompanied by the appearance of an almost equimolar amount of ADP. However, the disappearance of ATP was larger than the consumption of substrate or the formation of ADP. This was due to the activity of phosphatases in the enzyme preparation which catalyze the formation of adenosine monophosphate and adenosine.

**Table 1. Stoichiometry of the kinase reaction with N-acetyl-d-glucosamine as substrate**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Increase or decrease ((\mu)moles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Acetyl-d-glucosamine</td>
<td>-17.6</td>
</tr>
<tr>
<td>N-Acetyl-d-glucosamine-6-phosphate</td>
<td>18.6</td>
</tr>
<tr>
<td>ATP</td>
<td>-21.1</td>
</tr>
<tr>
<td>ADP</td>
<td>18.1</td>
</tr>
</tbody>
</table>

The reaction resulted in the formation of a phosphorylated sugar in an amount approximately equivalent to the substrate consumed. Acid hydrolysis of the product liberated equimolar amounts of N-acetyl-d-glucosamine and inorganic phosphate. The same cleavage products were identified by paper chromatography after treatment of the sugar phosphate with intestinal phosphatase. The position of the phosphate moiety was identified by reducing a sample of the phosphate ester with borohydride, followed by oxidation with periodate. The same procedures were performed on \(\alpha\)-glycerol-1-phosphate and glucose-6-phosphate. The oxidation products of the three compounds were chromatographed on paper, and glycolaldehyde phosphate was identified to be present as the only phosphate ester in each sample. It was concluded that N-acetyl-d-glucosamine-6-phosphate was the product obtained by incubating the enzyme with ATP and N-acetyl-d-glucosamine.

**Purification of enzyme.** Streptococci were suspended [20% (wet weight)/volume] in 0.05 M tris buffer (pH 8.0) containing 0.01 M MgCl\(_2\), 0.001 M EDTA, and 0.2 M glucose; 70 ml of the suspension were mixed with 15 ml of Ballotini #12 beads, and were added to the large head of a Raytheon sonic oscillator. The instrument was cooled with water and operated at 10 kc for 20 min. The resulting crude extract (step 1, Table 2) was centrifuged at 29,000 \(\times\) g at 4 C for 30 min. The supernatant liquid was removed and cen-

**Table 2. Purification of kinase**

<table>
<thead>
<tr>
<th>Step</th>
<th>Fraction</th>
<th>Total protein (mg)</th>
<th>Glucokinase (units/mg)</th>
<th>N-Acetylglucosamine kinase (units/mg)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Crude extract</td>
<td>40.380</td>
<td>10,818</td>
<td>0.26</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>Supernatant after streptomycin treatment</td>
<td>19,872</td>
<td>9,000</td>
<td>0.45</td>
<td>83</td>
</tr>
<tr>
<td>3</td>
<td>First (NH(_4))(_2)SO(_4) fractionation (0 to 65%)</td>
<td>10,300</td>
<td>7,416</td>
<td>0.72</td>
<td>69</td>
</tr>
<tr>
<td>4</td>
<td>Supernatant after heat treatment</td>
<td>5,460</td>
<td>5,405</td>
<td>0.99</td>
<td>50</td>
</tr>
<tr>
<td>5</td>
<td>Second (NH(_4))(_2)SO(_4) fractionation (0 to 35%)</td>
<td>1,686</td>
<td>5,040</td>
<td>2.90</td>
<td>46</td>
</tr>
<tr>
<td>6</td>
<td>Alcohol-acetone fractionation</td>
<td>177.6</td>
<td>2,500</td>
<td>14.0</td>
<td>23</td>
</tr>
<tr>
<td>7a</td>
<td>Third (NH(_4))(_2)SO(_4) fractionation (18 to 35%)</td>
<td>62.0</td>
<td>2,180</td>
<td>35.0</td>
<td>20</td>
</tr>
<tr>
<td>7b</td>
<td>Fourth (NH(_4))(_2)SO(_4) fractionation (18 to 35%)</td>
<td>17.1</td>
<td>1,162</td>
<td>68.0</td>
<td>11</td>
</tr>
<tr>
<td>7c</td>
<td>Fifth (NH(_4))(_2)SO(_4) fractionation (18 to 35%)</td>
<td>4.6</td>
<td>1,160</td>
<td>250.0</td>
<td>11</td>
</tr>
</tbody>
</table>
trifuged again in a Spinco model L centrifuge at 105,000 × g for 1 hr at 4 C. The residue was discarded, and the clear yellow supernatant fluid was used as the starting material for fractionation. All subsequent operations, except as otherwise stated, were carried out at 0 to 4 C.

Streptomycin sulfate (1 ml of a 20% solution) was added with stirring to each 100 ml of the supernatant fraction. The fine precipitate of nucleic acids was sedimented and discarded (step 2, Table 2).

Solid ammonium sulfate was added gradually to the supernatant liquid until the salt concentration was 65% of saturation. The resulting precipitate, containing most of the enzyme, was collected by centrifugation and was dissolved in the minimal volume of 0.005 M tris buffer (pH 7.6) containing 0.001 M MgCl₂, 0.0001 M EDTA, and 0.2 M glucose or N-acetyl-D-glucosamine (step 3, Table 2). The same buffer mixture was used for redissolving the enzyme fractions obtained in the subsequent steps. The presence of either sugar substrate was essential for preventing the inactivation of the enzyme during purification.

A considerable amount of inert protein was removed at this point by heating, performed in the presence of ammonium sulfate to avoid inactivation. Even with this precaution, approximately 25% of the enzymatic activity was lost. The dissolved enzyme from step 3 was brought to 13% (NH₄)₂SO₄ saturation by addition of the solid salt. The solution was placed into a bath (60 C) with constant agitation and was kept there for 1 min after it had attained the bath temperature. It was cooled rapidly in an ice bath and centrifuged at 29,000 × g for 30 min to remove an inert precipitate (step 4, Table 2). The supernatant fluid was brought to 35% saturation by further addition of solid (NH₄)₂SO₄. The resulting precipitate, containing the major portion of the enzymatic activity, was collected by centrifugation and dissolved in buffer (step 5, Table 2).

Fractionation with alcohol and acetone was used to bring about substantial purification, but considerable inactivation of enzyme also took place. The maintenance of 0.2 M glucose or N-acetyl-D-glucosamine based on total volume was essential in this procedure. Sufficient sugar was added prior to each addition of alcohol and acetone to insure a final concentration of 0.2 M. Absolute ethanol chilled to -40 C was added drop by drop to the solution from step 5 (Table 2) until its concentration attained 50% (v/v). The enzyme solution was maintained at 0 to -10 C during this procedure. An inactive precipitate was removed by centrifugation. Acetone cooled to -40 C was added to the ethanol supernatant fraction, which was maintained at -10 to -15 C. When 10% acetone (v/v) was attained, the major portion of enzyme activity was precipitated (step 6, Table 2).

The protein was dissolved in supplemented tris buffer. Solid (NH₄)₂SO₄ was added to 18% saturation, and an inert precipitate was collected by centrifugation. The supernatant liquid was brought to 35% (NH₄)₂SO₄ saturation and allowed to stand for 3 hr at 0 to 4 C. The resulting precipitate contained most of the enzymatic activity. It was centrifuged and redissolved in supplemented tris buffer (step 7a, Table 2). This procedure was repeated twice (steps 7b and c), yielding a preparation purified 1,000-fold in kinase activity. A critical feature for obtaining purification in steps 7a, b, and c was the dilution of the protein to concentrations below 5 mg/ml in supplemented tris buffer before beginning the addition of (NH₄)₂SO₄.

The particular fractionation shown in Table 2 resulted in 1,000-fold purification. Other fractionations, in which small variations of the procedure were employed, yielded preparations with a specific activity up to 400 units/mg or approximately 1,500-fold purification. The ratio of glucokinase to N-acetyl-d-glucosamine kinase activity remained the same throughout.

A variety of chromatographic procedures were tried, and either failed to increase the specific activity of the enzyme or were inactivating. These included diethylaminoethyl cellulose, carboxymethyl cellulose, Amberlite CG 45, and starch columns. A three- to sixfold purification could be achieved at step 7a by use of Sephadex G-200. However, purification was not obtained from Sephadex gels of lower cross-linkage.

**Substrate specificity.** The enzyme catalyzed the phosphorylation of N-acetyl-D-glucosamine and D-glucose at equal rates. N-Glucosamine was phosphorylated at a rate 0.4 times that observed for glucose or N-acetyl-D-glucosamine by relatively crude preparations (SA 4). Purified preparations were inactive on D-glucosamine and the substituted D-glucosamines in Table 3. Maltose, D-galactose, N-acetyl-D-galactosamine, D-fructose,
ammonium sulfate, the enzyme is stable at 60 °C for 1 hr; at 70 °C, it is completely inactivated in 3 min. The ratio of kinase activities did not change during denaturation at various temperatures and for varying periods of time.

Effect of sulfhydryl reagents. p-Hydroxymercuribenzoate (PHMB) inhibited equally the enzymatic activity on N-acetyl-α-D-glucosamine and d-glucose; 1.25 × 10⁻⁶ M PHMB was the minimal concentration for complete inhibition of the enzymatic activities (0.58 mg of protein, SA 2.3). The substrates did not protect against this inhibition. Thioethanol protected the enzyme against PHMB inhibition only to the extent of neutralizing the unreacted PHMB, but was unable to reactivate the enzyme. Thioethanol (1.25 × 10⁻⁴ M) protected the enzyme against an equimolar amount of PHMB added subsequently. However, complete inhibition of the enzyme with 1.25 × 10⁻⁶ M PHMB was not reversed by 2.5 × 10⁻⁴ M thioethanol.

In contrast, the PHMB inhibition of the kinase was reversed by cysteine. Enzyme (2.2 mg of protein, SA 2.3) was incubated for 18 hr at 0 °C with 10⁻⁴ M PHMB, which resulted in complete inhibition of activity. Various quantities of Dl-cysteine (neutralized to pH 7.4 with NaOH) were added, and the enzymatic activity was determined after 30 min. Figure 2 shows the cysteine reversal of the PHMB-inhibited kinase, with both N-acetyl-p-glucosamine and d-glucose as substrates.

Kinetic studies. The Michaelis constants (Kₘ)
FIG. 2. Reactivation of PHMB-reactivated kinase with DL-cysteine.

FIG. 3. Lineweaver-Burk plots of the rates of reaction with each substrate alone and in the presence of each other. The velocity, V, is expressed as total moles of glucose-6-phosphate or N-acetylglucosamine-6-phosphate formed per minute. Substrate concentrations, S, ranged from $0.125 \times 10^{-2}$ to $10^{-2}$ moles/ml, inhibitor concentration $= 0.75 \times 10^{-3}$ moles/ml. Letters identify substrate and inhibitor: A, N-acetyl-D-glucosamine as substrate; B, N-acetyl-D-glucosamine as substrate with D-glucose as inhibitor; C, D-glucose as substrate; D, D-glucose as substrate with N-acetyl-D-glucosamine as inhibitor.

TABLE 5. Kinetic constants for the action of streptococcal hexokinase on D-glucose and N-acetyl-D-glucosamine

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$</th>
<th>$K_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Glucose</td>
<td>$1.09 \times 10^{-2}$</td>
<td>$1.8 \times 10^{-2}$</td>
</tr>
<tr>
<td>N-Acetyl-D-glucosamine</td>
<td>$0.77 \times 10^{-3}$</td>
<td>$0.9 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

for the two substrates were calculated from Lineweaver-Burk (1934) plots by the method of Dixon (1933).

When both substrates were present in the incubation mixture, the rate of phosphorylation was lower than the sum of the rates observed with each substrate alone, indicating an inhibition. Total phosphorylation occurring during incubation for 3 min was measured titrimetrically. The amount of glucose-6-phosphate formed at the end of 3 min was measured, in a sample heated to 100 C, by means of glucose-6-phosphate dehydrogenase. The quantity of N-acetyl-D-glucosamine phosphorylated during the interval was determined by difference. Each substrate acted as competitive inhibitor of the other (Fig. 3). The inhibitor constants ($K_i$) were calculated from the plots of Fig. 3; the values for $K_m$ and $K_i$ are listed in Table 5.

**DISCUSSION**

N-Acetyl-D-hexosamine does not serve as a substrate for the purified hexokinases or glucokinases heretofore reported. A crude extract from *E. coli*, which phosphorylated both D-glucose and N-acetyl-D-glucosamine, was separated into two enzyme fractions, one phosphorylating glucose, the other phosphorylating N-acetyl-D-glucosamine (Asensio, 1960). O'Brien et al. (1960) found a kinase for N-acetyl-D-glucosamine which did not phosphorylate glucose in a strain of *Lactobacillus bifidus* requiring the former for growth. Several reports of enzyme extracts active in phosphorylating N-acetyl-D-glucosamine (Soodak, 1955; Faulkner and Quastel, 1956; Leloir et al., 1958) failed to indicate whether glucose could serve as a substrate. No kinase described catalyzes the ATP-dependent phosphorylation of both D-glucose and N-acetyl-D-glucosamine. Our studies demonstrating the parallel purification of N-acetyl-D-glucosamine kinase and D-glucokinase...
without change of the ratio of their specific activities appear to argue for the presence in group A streptococci of a single enzyme utilizing both substrates.

The kinetic studies show that the preparation at hand gives typical Lineweaver-Burk plots, and that the substrates are competitive inhibitors of one another. Since the values for $K_m$ and $K_I$ may not be taken as simply and similarly related to the true dissociation constants of the enzyme substrate complexes, their further interpretation at this time appears fruitless. Further observations pointing to the presence of a single enzyme species in our preparation are: no repression of either activity when the bacteria are grown exclusively on one or the other of the two substrates, the similarity in heat stability and PHMB inhibition, and the protection by either substrate of both activities. The observations reported are compatible with the hypothesis that the kinase activities are associated with a single species of molecule, but they do not permit of dismissal of the supposition that two enzymes, not separable by any of the expedients tried, are present.

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