The Preparation and Characterization of Pure Rat Liver Glucokinase*  

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SUMMARY

Glucokinase from rat liver has been purified over 10,000-fold and appears homogeneous by the criterion of sodium dodecyl sulfate gel electrophoresis. The enzymatically active protein represents 0.007% of the total liver protein and possesses a specific activity of 80 units per mg for purified enzyme. The use of potassium phosphate buffer gradients greatly enhances enzyme resolution during ion-exchange chromatographic procedures. In addition, use of substrate and sulfhydryl protecting reagents in all buffers prevents significant losses of activity. To our knowledge this is the highest degree of purity ever achieved for a mammalian hepatic ATP-d-hexose-6-phosphotransferase.

Rat liver glucokinase has been characterized with respect to stability, $K_m$, $V_{max}$, and phosphorylation coefficient toward several substrates as well as behavior in the presence of several inhibitors. Spectral analysis fails to reveal the presence of a prosthetic group. The molecular weight determined by sodium dodecyl sulfate acrylamide gel electrophoresis and Sephadex G-100 gel filtration is 53,000 and 57,000, respectively. The turnover number is calculated to be 4,346 moles of $D$-glucose phosphorylated by ATP per min per mole of enzyme at 25°C.

Several attempts have been made to obtain purified rat liver glucokinase (EC 2.7.1.2) (1–3). This enzyme is of particular interest not only because of its key role in metabolism but also because of its response to hormones and diet (4) as well as its deficiency in certain hepatomas (5). Of the reported purifications, none gives a protocol which results in a homogeneous preparation, although the recent report by Pilkis (3) gives a procedure for obtaining enzyme enriched by a factor of almost 3,000. However, as judged by the criterion of disc gel electrophoresis, this preparation still contains several other proteins. The difficulties encountered in purification are derived from the instability of the crude preparation and the small amount of glucokinase in rat liver.

Our initial interest in glucokinase was stimulated by the possibility of obtaining fluorescent antibodies against the enzyme in order to study its occurrence in several types of hepatic cells. Accordingly, we have developed a procedure for the purification of glucokinase to apparent homogeneity. In this paper we report the purification procedure and partial characterization. To our knowledge this is the highest degree of purity ever achieved for a mammalian liver ATP-d-hexose-6-phosphotransferase.

EXPERIMENTAL PROCEDURE

Chemicals—ATP, ADP, NADP, NADH, P-enolpyruvate, fructose-1-P, fructose-1,6-P$_2$, glucose-6-P dehydrogenase, 6-P-glucuronate dehydrogenase, lactate dehydrogenase, and pyruvate kinase were obtained from Boehringer Mannheim Corp. DEAE-Sephadex, CM-Sephadex, and Sephadex G-100 were obtained from Pharmacia Fine Chemicals. Hydroxypatite, alumina gel Cy, and phosphocellulose were purchased from Bio-Rad Laboratories. Glycylglycine, Tris, SDS, N-mercaptoethanol, dithiothreitol, N-acetylsalicylamide, deoxyribonuclease, ribonuclease, pepsin, trypsin, bovine serum albumin, and ovalbumin were products of Sigma Chemical Corp. DEAE-cellulose was obtained from Whatman Biochemicals, Ltd. Galactose, turanose, d-sorbitol, d-arabinose, and D-mannose were purchased from Nutritional Biochemicals Corp. Fluorescamine was obtained from Fisher Scientific. Actin, purified from the slime mold Physarum polycephalum, was donated by Dr. Wallace LeStourgeon (McArdle Laboratory) and myosin was a gift from Miss Fay Wang (McArdle Laboratory). m-Nitrobenzoylglucosamine was a generous gift from Professor H. Lardy (University of Wisconsin).

Assay of Glucokinase—Glucokinase activity was assayed at 25°C by recording the increase in absorbance at 340 nm of a reaction mixture containing: 50 mM glycylglycine (pH 7.5), 100 mM KCl, 7.5 mM MgSO$_4$, 0.5 mM NADP, 5 mM ATP (pH 7.0), 0.9 unit of glucose-6-P dehydrogenase, 0.03 unit of 6-phosphogluconate dehydrogenase, 100 mM glucose, and enzyme in a total volume of 3.0 ml. When hexokinase having a low $K_m$ (EC 2.7.1.1) was assayed, the glucose was omitted in the reaction mixture since the enzyme preparation contained sufficient glucose (approximately 0.50 mM) to saturate the hexokinases but well below that necessary to demonstrate glucokinase activity. Hexokinase activity and glucose dehydrogenase activity were subtracted from the total activity observed with 100 mM glucose as substrate to give the glucokinase activity. All reaction mixtures were incubated at 25°C for 5 min prior to assay. One unit of glucokinase activity represents 1 amole of glucose-6-P formed from glucose and ATP per min at 25°C (1).

When substrate specificity was studied, the activity was measured by recording at 25°C the decrease in absorbance at 340 nm of a mixture containing 100 mM triethanolamine-HCl (pH 7.5), 100 mM KCl, 7.5 mM MgSO$_4$, 0.75 mM P-enolpyruvate, 5 mM ATP, 0.25 mM NADH, 40 units of lactate dehydrogenase, 3 units of pyruvate kinase, and substrate. The mixture was incubated for 10 min at 25°C.
25° before initiating the reaction by the addition of enzyme to a final volume of 2.5 ml (1).

**Protein Determination**—When protein concentrations were greater than 100 μg per ml and phosphate concentration below 0.1 M, protein was determined by measurement of the absorbance at 280 nm assuming 1 mg of protein corresponded to an optical density of 1. At low protein concentrations, or when column fractions containing increasing amounts of potassium phosphate were analyzed, the fluorescamine procedure (6) was used, with bovine serum albumin as the standard.

**SDS Gel Acrylamide Electrophoresis**—Analytical SDS gel electrophoresis was performed according to the procedures of Laemmli (7).

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**RESULTS**

**Enzyme Purification**—Sprague-Dawley rats weighing between 250 and 300 g were maintained on a 60% carbohydrate diet ad libitum for at least 1 week prior to sacrifice. Livers from 20 to 30 rats were combined and homogenized in 2 volumes of Buffer P (0.02 M potassium phosphate, 100 mM KCl, 1 mM EDTA, 1 mM MgSO4, 50 mM glucose, and 10 mM mercaptoethanol, pH 7.0) for 30 to 40 s at high speed in a Polytron homogenizer (Kinematica GmbH, Lucerne, Switzerland). All procedures were carried out at 4°. The crude homogenate was immediately centrifuged for 1 hour at 165,000 × g. The resulting high speed super-

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**FIG. 1** (upper left). First Sephadex G-100 gel filtration of glucokinase. Enzyme was applied to a column (7 × 80 cm) previously washed with 0.1 M EDTA, then equilibrated and developed with Buffer P. Eighteen-milliliter fractions were collected.

**FIG. 2** (upper right). DEAE-cellulose ion exchange chromatography of glucokinase. Enzyme was adsorbed onto a column (3 × 60 cm) equilibrated with buffer consisting of 0.1 M potassium phosphate, 1 mM MgSO4, 1 mM EDTA, 50 mM glucose, and 10 mM mercaptoethanol, pH 7.0. Enzyme was eluted by a linear gradient of potassium phosphate prepared from 1 liter of 0.1 M potassium phosphate, pH 7.0, in the mixing chamber and 1 liter of 0.5 M potassium phosphate, pH 7.0, in the reservoir chamber each with 1 mM MgSO4, 1 mM EDTA, 50 mM glucose, and 10 mM mercaptoethanol. Eighteen-milliliter fractions were collected. The inset shows a SDS acrylamide gel of Fraction 92 stained for protein. Approximately 15 μg of protein were run. Glucokinase, as determined from the \( R_f \) value of the purified enzyme (Fig. 4), is indicated by the arrow.

**FIG. 3** (lower left). Second Sephadex G-100 gel filtration of glucokinase. The column (3.5 × 100 cm) was equilibrated and developed with buffer consisting of 0.1 M potassium phosphate, 0.1 M KCl, 5 mM EDTA, 5 mM MgSO4, 2 mM dithiothreitol, and 0.5 M glucose, pH 7.0. Eighteen-milliliter fractions were collected. The inset shows a SDS acrylamide gel of Fraction 34 stained for protein. Approximately 40 μg of protein were run. Glucokinase, as determined from the \( R_f \) value of the purified enzyme (Fig. 4), is indicated by the arrow.

**FIG. 4** (lower right). DEAE-Sephadex ion exchange chromatography of glucokinase. The column (1 × 100 cm) was equilibrated and developed with the same buffer as used in Fig. 2 except that 2 liters of each gradient buffer were used. Six-milliliter fractions were collected. The inset shows a SDS acrylamide gel of purified glucokinase from Fraction 15 stained for protein. Approximately 6 μg of protein were run.
Table 1

Purification of glucokinase

| Procedure                              | Volume | Total units | Total protein | Specific activity | Yield | Purification |
|----------------------------------------|--------|-------------|---------------|------------------|-------|--------------|
| Crude homogenate                       | 6174   | 1742        | 229,210       | 0.0076           |       |              |
| 105,000 × g supernatant                | 4147   | 1548        | 48,375        | 0.032            | 89    | 4.2          |
| 0.42 to 0.68 ammonium sulfate fractionation | 1290   | 875         | 5,633         | 0.12             | 50    | 15.7         |
| First Sephadex G-100                   | 5469   | 447         | 300           | 1.49             | 25    | 196          |
| DEAE-Sephadex batchwise               | 1830   | 144         | 15            | 9.0              | 8.2   | 1,184        |
| DEAE-cellulose                        | 200    | 115         | 6             | 19.2             | 6.6   | 2,526        |
| Second Sephadex G-100                 | 90     | 43.2        | 0.54          | 80.0             | 2.4   |              |
| DEAE-Sephadex column                  | 30     |             |               |                  |       |              |
combined active fractions from the first gel filtration step must be batchwise treated immediately with DEAE-Sephadex because at this stage of purification a delay of one day results in a 75% or more loss of activity.

Enzyme obtained from the batchwise DEAE-Sephadex step and all subsequent steps is very stable. We have had such preparation stored at 4°C for several weeks with only a 10% loss of activity. It is important that the purified enzyme be stored in 50 mM glucose, 100 mM potassium ion, and 10 mM mercaptoethanol since omission of any of these results in rapid losses of activity.

**Thermal Stability**—At any stage of the purification of glucokinase, the activity decreases rapidly when the enzyme is heated for 10 min at temperatures above 42°C. In the absence of glucose all activity is lost when enzyme is held at 52°C for 10 min. However, 0.5 M glucose has a significant effect in protecting glucokinase against thermal inactivation at 48.5°C, and the effect increases with increased enzyme purity (Fig. 6). In fact, purified glucokinase can be heated for 60 min at 48.5°C in the presence of 0.5 M glucose without any detectable loss of activity.

**Kₘ and Vₘₐₓ**—The Michaelis constants for glucokinase activity towards several substrates are given in Table II. Included are the values for the phosphorylation coefficient defined as:

\[
\frac{Kₘ(\text{glucose}) \times Vₘₐₓ(\text{substrate})}{Kₘ(\text{substrate}) \times Vₘₐₓ(\text{glucose})}
\]

The phosphorylation coefficient is a useful indicator of the relative susceptibility of a substrate to phosphorylation (8). The values for Kₘ that we report are somewhat lower than those of Parry and Walker (1) although our Kₘ value for glucose is in agreement with that reported by Pilks (3).

The following compounds did not act as substrate: d-sorbose, d-sorbitol, d-arabinose, fructose-1-P, N-acetyleneuraminic acid, L-mannose, and α-methyl glucoside.

With yeast hexokinase only the 2-hydroxyl group of the carbohydrate substrate can be modified and still permit binding to the enzyme (9). Therefore, d-mannose and d-glucose act as substrates, while glucosamine and N-acetylglucosamine function as competitive inhibitors. A recent report (10) in which deoxyfluoro-d-glucopyranoses were used confirms this specificity. Results of Parry and Walker (1) and those reported in Table II suggest a similar binding specificity is exhibited by glucokinase. d-Glucosonic acid, N-acetyleneuraminic acid, sucrose, d-sorbose, d-sorbitol, β-galactose, turanose, d-arabinose, L-mannose, fructose-1,6-P₂, and fructose-1-P are not inhibitors. However, m-nitrobenzoylglocosamine inhibits glucokinase with a Kᵢ of 0.75 mM (Fig. 7).

**Spectral Analysis**—Purified glucokinase exhibits the absorption spectrum expected from a protein devoid of the usual coenzymes. Absorption maxima were observed at 278 and 231 nm. The sample of enzyme used in this experiment had a protein concentration of 200 mg per ml when determined by the fluorescamine assay for protein and an optical density reading of approximately 0.20 units at 278 nm.

**Molecular Weight and Turnover Number**—Glucokinase has a molecular weight of 57,000 when determined by Sephadex G-100 gel filtration (Fig. 8). As seen in Fig. 9, glucokinase and glutamate dehydrogenase (subunit mol wt 53,000) are migrated as single protein bands when subjected to SDS acrylamide gel electrophoresis. The difference in molecular weight obtained by
FIG. 7. Lineweaver-Burk plot exhibiting the competitive inhibition of glucokinase by m-nitrobenzoylglucosamine (I).

FIG. 8. Estimation of the molecular weight of glucokinase by Sephadex G-100 gel filtration. A column (2.5 X 28 cm, $V_e = 137$ ml) of Sephadex G-100 was equilibrated with 0.1 M potassium phosphate, pH 7.0, plus 1 mM EDTA, 1 mM MgSO$_4$, 10 mM mercaptoethanol, and 50 mM glucose. The void volume ($V_0$) was 38 ml. One milligram of each protein standard in 0.5 ml of buffer plus 0.05 ml of 3 M glucose was layered on the column. When glucokinase was examined, approximately 1 unit of activity was added and the elution volume ($V_e$) determined by activity assay. $K_{av} = (V_e - V_0)/(V_e - V_t)$.

FIG. 9. Estimation of the molecular weight of glucokinase by SDS gel electrophoresis. Gels and samples were prepared as indicated by Laemmli (7). The gel on the left contained 5 µg of glutamate dehydrogenase ($D$, subunit mol wt 53,000). The center gel contained a mixture of 2 µg of myosin ($G$, mol wt 220,000), 5 µg of bovine serum albumin ($F$, mol wt 68,000), 5 µg of pyruvate kinase ($E$, subunit mol wt 57,000), 5 µg of glutamate dehydrogenase ($D$), 3 µg of glucokinase ($C$), 5 µg of actin ($B$, mol wt 46,000), and 5 µg of deoxyribonuclease ($A$, mol wt 31,000). The gel on the right contained 3 µg of glucokinase ($C$).

The two methods is probably due to the hydrodynamic properties of the native enzyme, which become important during gel filtration. Nevertheless, comparison of the values obtained by the two procedures, suggests that glucokinase is a monomer. The turnover number, assuming one catalytically active site, a molecular weight of 55,000, and a $V_{max}$ of 82 units per mg for pure enzyme, is 4,346 moles of $\beta$-glucose phosphorylated by ATP per min per mole of glucokinase.

**DISCUSSION**

Glucokinase is difficult to purify because of its instability during initial fractionation procedures and the small amount in the liver. We have calculated that protein with glucokinase activity comprises approximately 0.007% of the total liver protein and 0.018% of the soluble liver protein. It is clear from the value for the over-all yield presented in this paper that sufficient material for characterization and immunological studies can be obtained only after the purification procedure has been repeated several times. However, owing to the stability of the purified enzyme this task is not beyond the scope of possibility. In our laboratory, we have repeated this procedure three times to date and are in the process of accumulating milligram quantities of pure glucokinase.

Our purification protocol differs from the three previously published methods in several respects. In the purification reported by Gonzalez et al. (2), CM-Sephadex and hydroxyapatite were employed. We have been unable to achieve significant purification or improvement of stability by using either of these materials. In addition, these workers reported precipitation of glucokinase activity between 0.55 and 0.80 saturation with ammonium sulfate, whereas our results show maximum increases in specific activity are obtained between 0.42 and 0.68 saturation. Inclusion of glucose in our procedure during the fractionation or differences in protein concentration may account for the effectiveness of a lower ammonium sulfate concentration.

The purification reported by Parry and Walker (1) results in material purified approximately 1,100-fold, with a specific activity of 11 units per mg. According to our calculation, this enzyme preparation is only 14% pure. At no point in their chromatographic separations did they obtain a protein peak which corresponded to the activity profile. In contrast to their use of Tris-HCl buffer throughout the purification, we found potassium phosphate buffer to be superior because it enhanced enzyme stability. Phosphate buffer gradients gave sharp separations during ion exchange chromatography. In addition the KCl requirement for stability could be met by potassium phosphate.

The purification protocol presented by Pilkis (3) is a modification of that of Parry and Walker (1). A significant purification and yield were obtained using preparative starch gel electrophoresis, a procedure, which in the hands of many investigators, including ours, has proven less than satisfactory. Use of gel
filtration with Sephadex G-100 is, however, a major reason for the success of his purification (2940-fold; specific activity, 29.4 units per mg) and ours. We have found that use of large gel filtration columns early in the purification achieves significant purification and removes the ammonium sulfate from the previous step, making dialysis unnecessary before batchwise ion-exchange chromatography.

As seen in Fig. 3 after the second filtration of glucokinase through Sephadex G-100, a considerable amount of contaminating protein is still present, even though the specific activity represents a purification of 2,520-fold. Special reference is made to the two closely migrating, heavily staining protein bands at the bottom of the gel. Disc gel electrophoresis of this preparation in the absence of sodium dodecyl sulfate allows the detection of only one protein band and it is our conclusion that this band represents the undenatured protein resulting in the double band in the sodium dodecyl sulfate gel. It therefore appears to us, that prior to the last step of our purification, a dimeric protein of molecular weight 52,000 is being co-purified with glucokinase. Use of a long, slowly running DEAE-Sephadex column with a shallow potassium phosphate gradient has been used in our procedure to effect separation of this impurity from the glucokinase.

We have confirmed the need for inclusion of 50 mM NaCl, 100 mM potassium ion, and 10 mM mercaptoethanol in all buffers during purification and storage of glucokinase. As indicated, potassium phosphate buffer at pH 7.0 was found most suitable for the initial liver homogenizing medium and subsequent steps. The enzyme is most labile in the high speed rat liver supernatant and after the first gel filtration chromatography. However, it is quite stable during all subsequent steps of the purification and during storage at 4°C with the stabilizers present. In addition, the ability of glucokinase to resist thermal inactivation in the presence of 0.5 mM glucose increases with increasing enzyme purity.

Determination of the phosphorylation coefficient reveal the following order of capacity to be phosphorylated by ATP: glucose > mannose > 2-deoxyglucose > fructose. Of the several other compounds tested only those with an altered C-2 hydroxyl group would act as inhibitors confirming the observation that hexokinases require unaltered C-3, C-4, C-5, and C-6 hydroxyls in the pyranose configuration of the compound for binding (10). Like brain hexokinase, glucokinase is also inhibited by nitrobenzoylglucosamine (11). From spectral analysis, glucokinase appears to be devoid of prosthetic groups, exhibiting absorption maxima at 278 and 231 nm.

The molecular weight of glucokinase, when determined by Sephadex G-100 gel filtration is 57,000 and when determined by sodium dodecyl sulfate acrylamide gel electrophoresis is 53,000. These results are in disagreement with previous findings (3) in which the molecular weight was reported to be 48,000 in the presence of 0.15 M KCl and 65,000 in the absence of salt when examined by the method of gel filtration with Sephadex G-100 (3). At the same time, it was reported that glucokinase behaves like a protein with a molecular weight of 68,000 by gel filtration on Bio-Gel P-100 and by sucrose density gradient centrifugation. The cause for these discrepancies is uncertain but it has been suggested (3) that glucokinase undergoes a conformational change during gel filtration in buffers of lower ionic strength. We have carefully examined the behavior of pure glucokinase in sodium dodecyl sulfate acrylamide gel electrophoresis with reference proteins having molecular weights near that suspected for glucokinase. Since glucokinase and glutamate dehydrogenase (subunit mol wt 53,000) exhibit identical $K_r$ values when examined by sodium dodecyl sulfate acrylamide gel electrophoresis, we report a molecular weight of 63,000 for rat liver glucokinase with some certainty. It is difficult to compare our result obtained on gel filtration with those reported by others since our buffer system and ionic strength differed from theirs, even though our result, molecular weight 57,000 with this technique, falls between the reported values of 48,000 and 68,000. Comparison of the molecular weight values obtained by gel filtration and gel electrophoresis here suggest that glucokinase is a monomer in its native state.

At the outset of this work it was our intention to obtain sufficient pure glucokinase to produce fluorescent antibodies which could be used to ascertain whether individual liver cells contain widely differing amounts of glucokinase depending on their state of development. At present it is unclear whether the low or questionable levels of glucokinase found in fetal liver and most hepatomas (5) means that all cells produce a reduced amount of enzyme or that some cells are producing normal amounts while others fail to do so. It is possible that slowly growing hepatomas contain a high proportion of cells that have differentiated function, therefore exhibiting glucokinase and pyruvate kinase type I (or L) while rapidly growing hepatomas, almost devoid of glucokinase, are essentially all enzymatically undifferentiated (12). The availability of pure glucokinase for the preparation of fluorescent antibodies makes possible a direct approach to the problem.

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REFERENCES
1. Pakry, M. J., and Walker, D. G. (1966) Biochem. J. 99, 266-274
2. Gonzalez, C., Ureta, T., Babul, J., Rabajille, E., and Niemeyer, H. (1967) Biochemistry 6, 400-408
3. Pilks, S. J. (1972) Arch. Biochem. Biophys. 149, 349-350
4. Sharma, C., Manjeshwar, R., and Weinhouse, S. (1964) Advan. Enzyme Regul. 2, 189-200
5. Shatton, J. B., Morris, H. P., and Weinhouse, S. (1969) Cancer Res. 29, 1161-1172
6. Bohlen, P., Stein, S., and Udenfriend, S. (1973) Arch. Biochem. Biophys. 155, 213-220
7. Lajem, U. K. (1970) Nature 227, 650-651
8. Solis, A., and Crane, R. K. (1954) J. Biol. Chem. 210, 581-595
9. Walker, D. G. (1966) Essays in Biochem. 2, 33-67
10. Bessell, E. M., Foster, A. B., Westwood, J. H. (1972) Biochem. J. 128, 199-204
11. Boles, F., and Lardy, H. A. (1956) J. Biol. Chem. 214, 765-773
12. Pilks, S. J., Hansen, R. J., and Krahl, M. E. (1968) Biochim. Biophys. Acta 154, 250-252
13. Walker, P. R., and Potter, V. R. (1972) Advan. Enzyme Regul. 10, 339-364
