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Purification and Characterization of Dog Liver Glucokinase*

Purificación y Caracterización de la Glucoquinasa de Hígado de Perro

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Glucokinase from dog liver has been purified to homogeneity by a procedure involving DEAE-cellulose chromatography, ammonium sulfate fractionation, gel filtration chromatography, and affinity chromatography on glucosamine-Sepharose. The purified enzyme was characterized with respect to stability, molecular weight, amino acid composition, SH groups, and physicochemical and kinetic properties. A molecular weight of 49,000 and 47,000 was estimated by sodium dodecylsulfate gel electrophoresis and gel filtration in non-denaturing conditions, respectively, indicating a monomeric structure for the enzyme. Glucokinase exhibits a sigmoidal saturation function for glucose with a Hill coefficient of 1.5 and a half-saturation value of 4 mM at pH 7.5.

During the last 15 years this laboratory has been dedicated to the study of the glucose phosphorylating isozymes with respect to their number, purification, kinetic properties, cellular distribution, response to changes in the amount and quality of the diet, role in metabolism, their distribution along phylogenetic paths and time of appearance during development (1-5). Attention has been focused on glucokinase because of its relative high amount, adaptive character, and its sigmoidal saturation function with glucose. All these features had led to conclude that glucokinase plays a key role in the utilization of glucose by the liver (2,6).

Several goups have attempted to purify this enzyme in order to study its properties and to understand its role at the molecular level (7-13). Difficulties in obtaining a pure preparation are the small amounts present in the liver and its instability. This work describes the purification and properties of glucokinase using dog liver as a convenient starting material to obtain large quantities of the enzyme.

MATERIALS AND METHODS

Materials

ATP, NADP, NAD, EDTA, dithiothreitol, Trizma base, glucose, glucose 6-phosphate, fructose, arabinose, 2-deoxyglucose,

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glucosamine, mannose, N-acetylglucosamine, galactose, xylose, α-methylglucoside, ninhydrine, riboflavine, blue dextran 2000, glycine, Coomassie brilliant blue R, amido black, nitrobluetetrazolium, phenazine metosulfate, 1 ethyl-3 (3 dimethylaminopropyl) carbodiimide, sodium lauryl sulfate, mannose 6-phosphate isomerase, glucose 6-phosphate isomerase, TPCK-trypsine, ribonuclease, and pepsine were from Sigma Chemical Co. Urea and p-chloromercuribenzoate were products of Calbiochem; Ampholines R were from LKB; glucose-^{14}C and leucine-^{14}C were from New England Nuclear; acrylamide, bisacrylamide, tetramethylethylenediamine, and ammonium persulfate were from Polysciences; Sephadex G-100, Sephadex G-25 DEAE-Sephadex A-25, CH-Sepharose-4B were from Pharmacia Fine Chemicals; DEAE-cellulose, hydroxyapatite, and Dowex-1 were from Bio Rad; cytochrome *c*, chymotrypsinogen, ovalbumin, bovine serum albumin were from Schwarz Man; 6-phosphogluconic dehydrogenase was obtained by a modification of the procedure of Glock and McLean (13).

Animals

Male mongrel dogs of varying ages between 10 and 15 Kg were fed during 4 to 7 days with a daily diet containing 175 g of a mixture of proteins, lipids, mineral salts, and vitamins, and 250 g of noodles as carbohydrate source. Forty eight hours before scrifice this diet was doubled. Dogs were sacrificed by an intravenous injection of saturated KCl.

Activity measurements

Glucokinase and hexokinase were determined spectrophotometrically (15). The rate of glucose 6-phosphate formation was measured by coupling the reaction with $NADP^+$ and an excess of glucose 6-phosphate dehydrogenase. NADPH formation was followed at 340 nm in a Gilford 2000 spectrophotometer at 30°C. The reaction mixture contained (final concentrations): 80 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 1 mM EDTA, 10 mM KCl, 0.5 mM NADP, 5 mM ATP, 0.1 units of glucose 6-phosphate dehydrogenase, substrate and enzyme in a final volume of 0.5 ml. A similar reaction mixture with ATP omitted acted as a blank. When glucokinase was assayed in crude extracts, two glucose concentrations, 100 and 0.5 mM, were used. The total phosphorylating activity was measured using 100 mM glucose and represented the glucokinase and hexokinase activities. The hexokinases were measured using 0.5 mM glucose. The difference between the activity values measured at 100 mM and 0.5 mM corresponded to glucokinase activity. When the enzyme was assayed in fractions at the initial stages of purification before the first DEAE-cellulose chromatography, an excess of 6-phosphogluconic dehydrogenase was added to the reaction mixture. Under these conditions two molecules of NADPH are formed per molecule of glucose phosphorylated (16) and thus, the absorbance changes were divided by two to obtain glucokinase activity. After the DEAE-cellulose step, 6-phosphogluconic dehydrogenase no longer accompanies glucokinase so its addition as an auxiliary enzyme is not necessary.

For substrate specificity studies glucokinase activity was measured through ADP production by coupling the reaction with pyruvate kinase and lactate dehydrogenase (17). The reaction mixture contained (final concentrations): 80 mM Tris-HCl (pH 7.5), 5 mM ATP, 0.25 mM NADH, 12 mM $MgCl_2$, 2.5 mM phosphoenolpyruvate, 1.5 mM EDTA, 1 unit of each of the auxiliary enzymes, substrate and enzyme in a final volume of 0.5 ml.

Hexokinase activity was also measured by the radioactive method of Radojkovic *et al.* (18). The reaction mixture was similar to the one described for the glucose 6-phosphate formation except for the omission of NADP and the dehydrogenases, and the inclusion of 0.5 mM glucose containing about 500,000 cpm. The labelled glucose 6-phosphate formed was separated from the unreacted glucose by ion exchange in small Dowex-1 columns. Elution was performed with 0.4 M ammonium formate and aliquots of these eluates were counted in a Unilux

spectrometer with a standard scintillation mixture.

One unit of ATP: D-hexosephosphotransferase activity is the amount of enzyme that catalyzes the phosphorylation of 1 μmol of glucose in 1 min at 30°C under the specified conditions.

Protein concentration

In column effluents proteins were detected by measuring the absorbance at 280 nm. In crude extracts or impure preparations the protein concentration was measured by the method of Lowry *et al.* (19). In pure preparations the protein concentration was measured using an $A_{280}^{1\%}$ of 12.5. This value was calculated by measuring the absorbance at 280 nm of a solution of pure glucokinase whose concentration was calculated using an $A_{210\ nm}^{1\%}$ of 27 (20).

Spectral measurements

Absorption spectra were obtained using a Cary model 18 spectrophometer. The tryptophan content of glucokinase was determined according to Bencze and Schmidt (21). Tyrosine contents were calculated from spectrophotometric titrations at 243 nm as in Stellwagen (22). Sulfhydryl groups were determined with p-chloromercuribenzoate as described by Boyer (23). The enzyme was dialyzed extensively against distilled water, and equilibrated in a mixture containing 50 mM Tris-HCl pH 7.2 and 8 M urea. The titration was also performed in the absence of urea using 0.1 M glucose and 50 mM KCl to stabilize the enzyme.

Molecular weight

The elution volume of several well characterized proteins on a Sephadex G-100 column was compared with the elution volume of purified glucokinase in order to estimate its molecular weight (24). A 1×100 cm column was equilibrated with 10 mM potassium phosphate, 1 mM EDTA, 5 mM 2-mercaptoethanol, and 0.3 M KCl, pH 7.0. The reference proteins, 2 mg each in 0.5 ml, and purified glucokinase (2 units) were eluted with the same buffer. The reference proteins and their respective molecular weights are indicated in Figure 7. The molecular weight of pure glucokinase was also measured by thin layer gel filtration with Sephadex G-100 superfine according to the procedure of Andrews (24). The gel was equilibrated with 10 mM potassium phosphate, 0.1 M glucose, 5 mM 2-mercaptoethanol and 0.3 M KCl. The reference proteins used are indicated in Figure 8. The proteins and glucokinase, 40 μg each, were applied to the plates in a volume of 20 μl. After 44 hours they were detected as blue spots by placing a dry sheet of Whatman M 1 paper over the gel until wet, soaking the paper with a mixture of methanol: acetic acid (90:10 v/v) with 0.25% Coomassie blue for 1 min and drying. Destaining was achieved in methanol: acetic acid: water (50:10:50 v/v) for 30 min with several changes. The molecular weight of glucokinase was estimated by plotting the molecular weight of the reference proteins versus the distance between the point of application and the centre of each spot.

Gel electrophoresis

Polyacrylamide gel electrophoresis in non-denaturing solvents was performed as described by Davis (25). Electrophoresis in 6.25 M urea, 0.3 M Tris-HCl pH 8.6, was performed in 10% acrylamide gels for 2 hr at 3 mA/gel at room temperature. The gels were stained with 1% Amido black-7% acetic acid and destained with 7% acetic acid. Electrophoresis in the presence of sodium dodecyl sulfate was carried out in 5% acrylamide gels following the method of Shapiro *et al.* (26). The gels were stained as in Fairbanks *et al.* (27).

Glucokinase activity was detected after electrophoresis with the method of Grossman and Potter (28) with slight modifications. Electrophoresis was carried out in 7% acrylamide gels at 3 mA/gel using Tris-HCl pH 8.0 2 mM dithiothreitol, 4 mM EDTA, 5 mM $MgSO_4$, and 50 mM glucose as electrophoresis buffer.

The isoelectric point of purified glucokinase was determined by gel electrofocusing (29) using 7% polyacrylamide gels and 1% ampholine pH range 3-10 for 2 hr at 3 mA/gel. The anode solution was H_3PO_4

0.2% and the cathode solution was NaOH 0.28%. The sample, 50 to 80 μg in 0.5 ml, was dialyzed extensively in 2 mM potassium phosphate pH 7.0 before polymerization. The gels were stained with bromophenol blue 0.2% in a mixture of ethanol: acetic acid: water (50:5:45 v/v) and destained in a mixture of the same compounds (30:5:65 v/v). In order to determine the pH gradient, an unstained gel was cut in 2 mm fractions and incubated with 2 ml of water for 1 hr at 30°. Afterwards the pH of each fraction was measured.

Aminoacid analysis

For aminoacid analysis glucokinase solutions were dialyzed exhaustively in 2 mM potassium phosphate pH 7.0 and were diluted with an equal volume of 11.6 N HCl and hydrolyzed for 22-24 hr at 110° in an evacuated sealed tube. The hydrolyzates were analyzed in a Beckman 120 C aminoacid analyzer according to Spackman *et al.* (30). Norleucine was used as an internal reference standard.

Peptide maps

For peptide mapping a solution of 0.8 mg of glucokinase was dialyzed in 2 mM potassium phosphate pH 7.0 and heated for 15 min in boiling water. Afterwards the protein was digested by the addition of trypsin (tosyl-amido-2-phenylethyl chloromethyl ketone treated) dissolved in 10 μl of 1 mM HCl (substrate to trypsin 40:1, w/w). The mixture was incubated at 25° and the pH was kept at 8.2 by the addition of diluted NH_4OH. The reaction was stopped after 16 hr by lowering the pH to 4.0. A small amount of precipitate was centrifuged and the supernatant liquid was lyophilized. The dried digest was dissolved in 50 μl of distilled water and was applied to a 46×56 cm Whatman 3MM paper. Peptide mapping was as described by Katz *et al.* (31). Separation was achieved by descending chromatography using butanol-acetic acid-pyridine-water (15:3:10:12, v/v) for 16 hr and by electrophoresis (Savant) in acetic acid pH 3.5 at 2000 volts for 90 min at 15°. Phenol red was used as a marker during chromatography. The peptides were visualized after spraying with 0.25% ninhydrin in absolute ethanol and heating at 70°.

Affinity chromatography

Coupling of glucosamine to aminohexyl Sepharose 4B was performed through reaction with 1-ethyl-3-(dimethylaminopropyl) carbodiimide. 12 ml of aminohexyl Sepharose were rinsed with 0.5 M NaCl and were reacted with glucosamine 0.1 M and carbodiimide 0.1 M in a volume of 24 ml for 24 hr at room temperature with gentle stirring. The pH of the solution was mantained at 5.0.

RESULTS

Glucose phosphorylating activities in crude liver extracts

DEAE-cellulose chromatography of crude liver extracts (see below) showed the presence of three glucose phosphorylating isozymes in dog liver (Fig. 1). The first fraction elute at 62 mM KCl and presented the same activity when

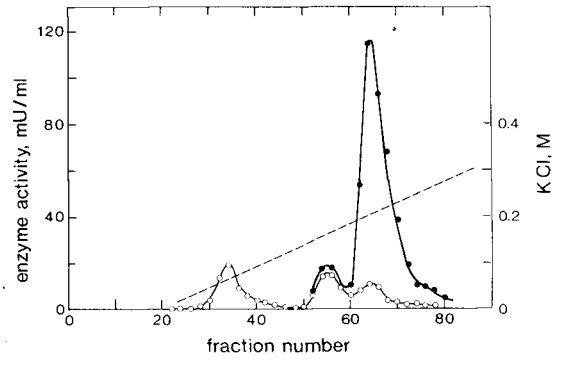

Fig. 1. Glucose phosphorylating isozymes in crude liver extracts. 5.2 g of liver were homogenized in an equal volume of 10 mM Tris-HCl (pH 7.0), 1 mM EDTA, and 1 mM dithiothreitol and centrifuged 1 hr at 105,000 g. The supernatant liquid was applied to a 20×0.9 cm DEAE-cellulose (DE-52) column and the isozymes were eluted with a 240 ml linear gradient of KCl from 0 to 0.5 M in the homogenization buffer. Fractions of 2 ml were collected and the enzyme activity determined spectrophotometrically (●) and by the radioactive assay (O) as described in Methods. The KCl gradient is also indicated (---).

assayed at 100 or 0.5 mM glucose. The second fraction eluted at a concentration of 165 mM and its activity at 100 mM was 1.2 times higher than at 0.5 mM glucose. These two fractions were identified as hexokinases A and B respectively (32) and represented approximately 20% of the total glucose phosphotransferase activity in the liver. The third peak corresponded to glucokinase whose purification and properties will be described in the present work.

PURIFICATION OF GLUCOKINASE

Crude extracts

The livers of two dogs (approximately 1 kg) were chopped and passed through a meat grinder before homogenization for 1 min in a Waring blendor. 50% homogenates (w/v) were prepared in a medium containing 10 mM potassium phosphate pH 7.0, 10 mM glucose, 1 mM EDTA, 5 mM 2-mercaptoethanol (solution 1) and 0.18 M KCl. These and all further operations were performed at 4° unless otherwise stated. After homogenization the pH was adjusted to 7.0 with dilute NH_4OH. Afterwards the homogenate was centrifuged at 16,300 g in a Sorvall RC2B centrifuge for 40 min. The supernatant liquids were then centrifuged at 78,410 g in a Spinco model L centrifuge for another 40 min. (Crude extract, Table 1).

This step is basically the same one used by Babul and Niemeyer in the purification of the rat liver enzyme (7). Approximately 700 ml of crude extract were passed through a 60×4.8 cm DEAE-cellulose column equilibrated with solution 1. The column was then washed with 1.5 *l* of solution 1 with the addition of 0.18 M KCl. This wash contained approximately 95% of the proteins originally present in the crude extract. Glucokinase was eluted using a linear gradient of KCl from 0.18 to 0.75 M KCl in solution 1. Fractions of 20 ml were collected at a flow of 8 ml/min. The enzyme appeared after approximately 700 ml elution at a KCl concentration of around 0.3 M. Fractions containing more than 0.5 units/ml were pooled (DEAE-cellulose I fraction, Table 1).

Ammonium sulfate fractionation

Solid ammonium sulfate was added to the pooled fractions of the DEAE-cellulose chromatography to bring the concentration to 55% saturation, and after stirring for 30 min the mixture was centrifuged at 16,300 g for 30 min. Afterwards the concentration of ammonium sulfate of the supernatant liquid was brought to 80% saturation and was centrifuged as before. The precipitate thus obtained could be stored at 4° for a few weeks without appreciable loss in activity.

Gel filtration chromatography

The ammonium sulfate precipitate was suspended in approximately 30 ml of 0.3 M KCl in solution 1 and was dialyzed for 2 hr against 1 *l* of the same solution but 50% in sucrose (Ammonium sulfate fraction, Table 1). The enzyme (ca 30 ml) was then applied to a 120×5 cm Sephadex G-100 column equilibrated with 0.3 M KCl in solution 1. The enzyme was eluted with equilibration buffer and 8.5 ml fractions were collected at a flow of 0.4 ml/min. Fractions containing 0.65 units/ml or more were pooled (190 ml, Sephadex G-100 fraction, Table 1).

Second DEAE-cellulose chromatography

The pooled fractions of the previous step were diluted 1:2 with solution 1 and were passed through a 35×2 cm DEAE-cellulose (DE-52, microgranular) column equilibrated with solution 1. The column was then washed with 200 ml of 0.18 M KCl in solution 1 and the enzyme was eluted using an 800 ml linear gradient of KCl from 0.18 to 0.45 M in solution 1. The enzyme appeared in approximately 130 ml at a KCl concentration of around 0.24 M. Fractions containing more than 0.4 units/ml were pooled (DEAE-cellulose II fraction, Table 1).

Glucosamine-Sepharose chromatography

The pooled fractions from the second DEAE-cellulose chromatography were diluted 1:2 with solution 1 and were concentrated using a 2 ml DEAE-cellulose

column. The enzyme was eluted with 0.5 M KCl in solution 1 and was then dialyzed against a solution containing 20 mM Tris-HCl pH 7.2, 1 mM EDTA, 5 mM 2-mercaptoethanol (solution 3) and 50 mM KCl. The enzyme, in a volume of about 150 ml, was then applied to an 8×1.5 cm glucosamine-Sepharose column (see Methods). The column was washed with four column volumes of 0.1 M KCl in solution 3. This wash removed contaminant proteins and less than 10% of the total glucokinase activity applied to the column. Finally, pure glucokinase was eluted with 0.3 M glucose and 0.1 M KCl in solution 3. Figure 2 shows the elution profile and the corresponding 280 nm absorbancy of the collected fractions (22 ml, Glucosamine-Sepharose fraction, Table 1). The enzyme could also be eluted from the column with a linear gradient of KCl from 0.5 to 1.0 M, but the enzyme obtained had a lower specific activity than when eluted with glucose.

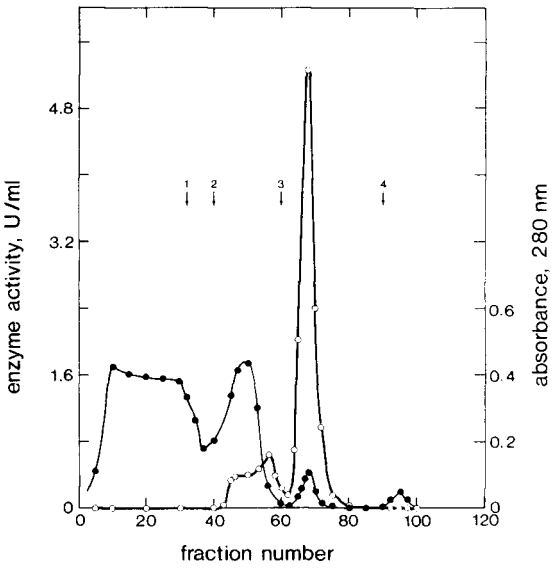

Fig. 2. Affinity chromatography of the DEAE-cellulose fraction in glucosamine-Sepharose. The pooled fractions from the second DEAE-cellulose chromatography (257 units per 12 mg in 132 ml) were equilibrated in 20 mM Tris-HCl pH 7.2, 1 mM EDTA, 5 mM 2-mercaptoethanol (solution 3) and 50 mM KCl and then applied to a 8×1.5 cm glucosamine-Sepharose column. The column was then washed with 50 mM KCl (arrow 1) and 100 mM KCl (arrow 2) in solution 3 and the enzyme eluted with 0.3 M glucose, 0.1 KCl in solution 3 (arrow 3). Elution with 1 M KCl in solution 3 is indicated by arrow 4. Fractions of 2.8 ml were collected. (○) enzyme activity; (●) absorbance at 280 nm.

Alternate procedure

Glucokinase could also be purified using an ion filtration step instead of the glucosamine-Sepharose affinity chromatography. The concentrated DEAE-cellulose II fraction was dialyzed against a solution containing 10 mM potassium phosphate, 1 mM EDTA, 10 mM glucose, 5 mM 2-mercaptoethanol, pH 7.4 (solution 2) and 0.35 M KCl. The enzyme was then applied to an 80×1.5 cm DEAE-Sephadex column equilibrated with 0.15 M KCl in solution 2. Pure glucokinase was eluted with 0.35 M KCl

TABLE I

Purification of Dog liver Glucokinase

Fraction	*Total activity units*	*Specific activity units/mg*	*Yield %*
Crude extract	1,144	0.008	100
DEAE-cellulose I	736	0.41	64
Ammonium sulfate	552	0.67	48
Sephadex G-100	421	5.1	37
DEAE-cellulose II	257	21.4	23
Glucosamine-Sepharose	134	89.3	12

in solution 2 at a flow rate of 8 ml1/hr. Glucokinase eluted as a symmetrical peak showing constant specific activity. Approximately 50% of the enzyme was recovered with a specific activity of about 80 units/mg. The glucosamine-Sepharose step was a faster one and gave better yields so it was preferred for standard preparations.

Abridged purification method

Attempts were also made to shorten the purification procedure by using the glucosamine-Sepharose step with enzyme preparations obtained after the exclusion chromatography step. The results indicate that the enzyme is pure, and that 2.1 mg were obtained with a specific activity of 90 units/mg and 15% recovery.

Purity of the preparations

Purified glucokinase migrates as a single band when subjected to zone electrophoresis either in 7% or in 10% polyacrylamide gels at pH 8.7. When electrophoresis is performed in the presence of sodium dodecyl sulfate or 6.25 M urea, the denatured protein also exhibits a single component (Figure 3). The same results were obtained when glucokinase was purified by the alternate or abridged methods (not shown). That the single band obtained in polyacrylamide gels corresponds to glucokinase was shown in an experiment where a specific stain for activity was used (28). Figure 4 shows that the band stained for activity and the one stained for protein have the same relative mobility.

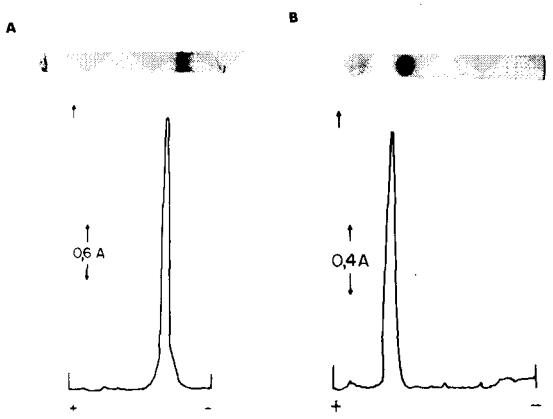

Fig. 3. Polyacrylamide gel electrophoresis of glucokinase in the presence of denaturing agents. A) 200 μg of glucokinase in 10% polyacrylamine gels in the presence of 6.25 M urea. B) 160 μg of glucokinase in 5% polyacrylamide gels in 0.1% sodium dodecyl sulfate. The densitometric tracings are shown under the gels. The experimental conditions are described in Methods.

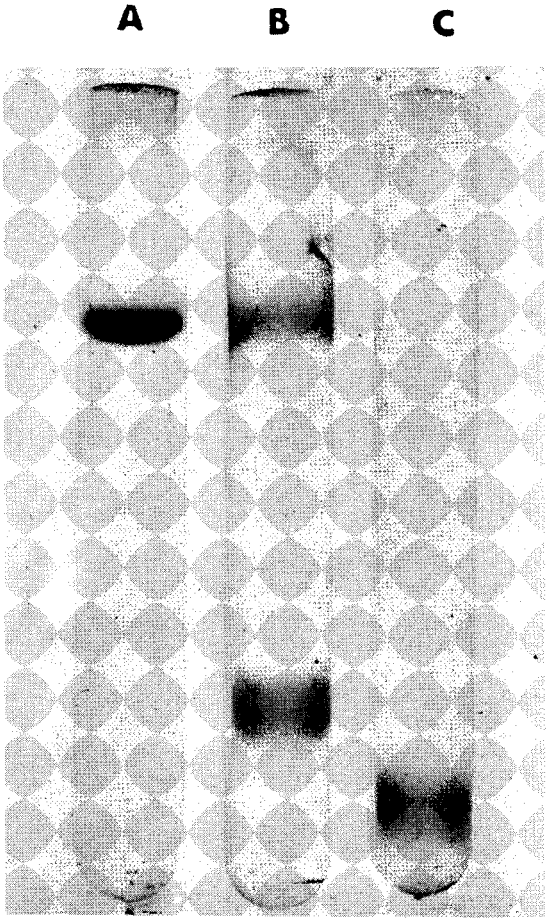

Fig. 4. Polyacrylamide gel electrophoresis of glucokinase under nondenaturing conditions. The enzyme was applied to 10% polyacrylamide gels and stained after electrophoresis with amido black (A) and for glucokinase activity (B). (C) Control gel stained as in B with ATP omitted. Experimental conditions are described in Methods.

CHARACTERIZATION

Stability

The stability of glucokinase towards thermal inactivation at different stages during the

purification procedure is shown in Fig. 5. The effect of KCl and glucose additions is also shown. In the absence of protective agents a half inactivation time of 22.5 hr could be calculated for the pure enzyme at 37° C compared to a value of 3.4 hr for the enzyme after the Sephadex G-100 step.

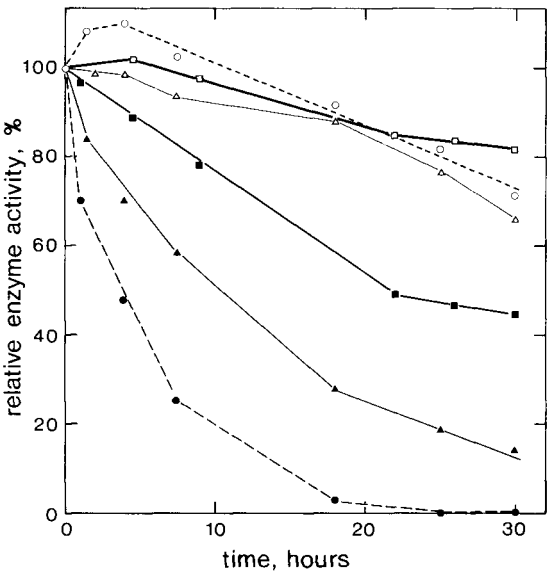

Fig. 5. Thermal inactivation of glucokinase at different stages of the purification procedure. The enzyme preparation in 10 mM potassium phosphate pH 7.0 and 1 mM EDTA was incubated at 37° in the presence (empty symbols) and in the absence (filled symbols) of 0.1 M glucose, 0.3 M KCl and 5 mM 2-mercaptoethanol. Aliquots were taken at the indicated times and assayed for enzymatic activity with the spectrophotometric assay (see Methods). Circles, Sephadex G-100 fraction; triangles, DEAE-cellulose II fraction; squares, glucosamine-Sepharose fraction (see Table I).

Figure 6 shows that glucokinase activity decreased 50% when the enzyme was incubated and its activity measured in the presence of 1.5 M urea. Almost complete inactivation is seen at 3 M urea. Pilkis reported an irreversible loss of activity for the rat liver enzyme at 2-4 M urea, and a partial protection in the presence of 1 M glucose (11).

Molecular weight

By analytical gel filtration studies on Sephadex G-100 (Fig. 7) it was possible to

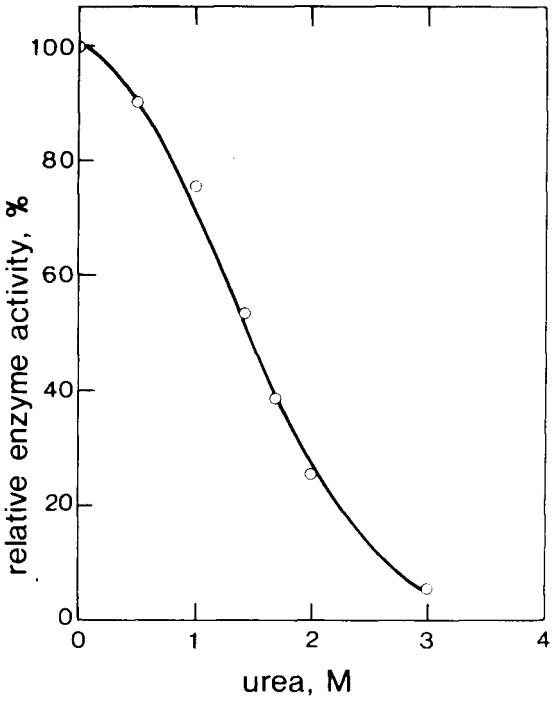

Fig. 6. Inactivation of glucokinase by urea. Purified glucokinase (0.015 units) was mixed with the assay reagents and urea at the indicated concentrations and the activity was measured as described under Methods. The auxiliary enzyme glucose 6-phosphate dehydrogenase was not affected by the urea concentrations used.

estimate a molecular weight of 47,000±2,500 for the native enzyme. A molecular weight of 55,000 was estimated by thin layer gel filtration (Fig. 8). Zone electrophoresis in the presence of sodium dodecyl sulfate resulted in a molecular weight of 49,000±2,500 (Fig. 9).

Hydrodynamic properties

A stokes radius of 29.7 Å was determined for glucokinase (Fig. 10). Analysis of the gel filtration data according to Porath (33) yields a value of 30 Å. The molecular radius of glucokinase was calculated using the following equation:

$$r_o = \left[\frac{3\ M_r}{4\pi M} (\bar{v} + d_1\ v_1^o) \right]^{1/3}$$

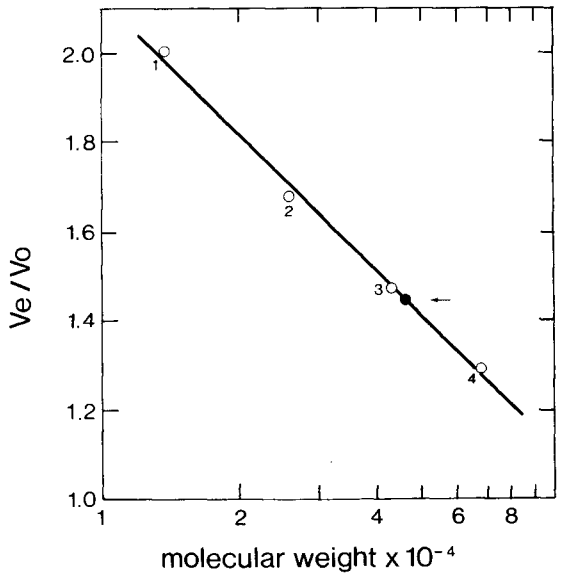

Fig. 7. Molecular weight estimation of glucokinase by gel filtration. The molecular weight is plotted against V_e/V_o (V_e, elution volume; V_o, void volume) for four standard proteins of the following molecular weights: 1) ribonuclease (13,600), 2) chymotrypsinogen (27,500), 3) ovalbumin (43,000) and 4) bovine serum albumin (68,000). The arrow indicates the position of glucokinase. Other details are given under Methods.

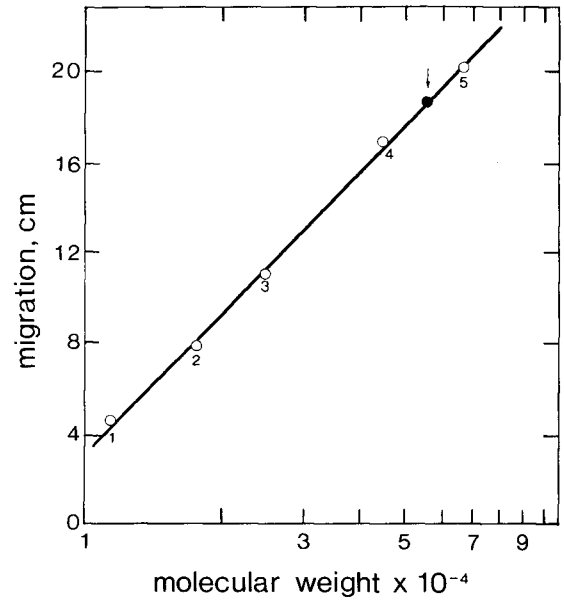

Fig. 8. Molecular weight estimation of glucokinase by thin layer gel chromatography. The molecular weight is plotted against the migration for five standard proteins of the following molecular weights: (1) cytochrome *c* (12,500), (2) myoglobin (17,200), (3) chymotrypsinogen (25,700), (4) ovalbumin (43,000) and (5) bovine serum albumin (68,000). The arrow indicates the position of glucokinase. The experimental procedure is described under Methods.

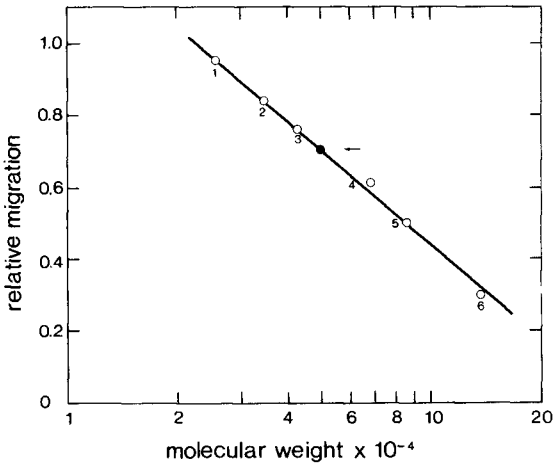

Fig. 9. Molecular weight estimation of glucokinase by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The molecular weight is plotted against the mobility relative to the indicator dye for four standard proteins of the following subunit molecular weights: 1) chymotrypsinogen (25,700), 2) pepsin (35,000), 3) ovalbumin (43,000, monomer), 4) bovine serum albumin (68,000; monomer), 5) ovalbumin (86,000; dimer), and 6) bovine serum albumin (136,000; dimer). The arrow indicates the position of glucokinase. The experimental procedure is described under Methods.

were M_r is the molecular weight, $\bar{v}$ is the partial specific volume in ml/g, N Avogadro's number, v_1° the partial specific volume of water, and d_1 is the protein hydration in g of solvent/g of protein. Assuming that glucokinase behaves in solution like an anhydrous sphere, the term $d_1 v_1^\circ$ is equal to zero. A molecular radius of 23.9 Å was obtained using a value of 0.729 ml/g for $\bar{v}$. This value was calculated from the amino acid composition (see below) according to Cohn and Edsall (34).

The value of the frictional coefficient (f/f_{min}) was calculated using the expresion:

$$f/f_{min} = f/f_o \left(\frac{\bar{v} + d_1 v_1^\circ}{v} \right)^{1/3}$$

$$f/f_o = a/ \left(\frac{3 \bar{v} M}{4 \pi N} \right)^{1/3}$$

where *a* is the stokes radius, M the molecular weight, f the frictional coefficient of the protein and f_o the frictional coefficient that

would be observed for a sphere of the same volume. Assuming that glucokinase behaves like an anhydrus particle, and using a value of 29.7 A° for the stokes radius, 48,000 for the molecular weight, and 0.729 for the partial specific volume, a value 1.24 was calculated for the frictional coefficient. This value corresponds to maximal asymmetry and gives a value of 4.7 for the axial ratio, a/b (35) when one considers the molecule as an anhydrous prolate. Assuming that glucokinase is a spherical particle one can calculate the maximal hydration. A value of 0.65 g of water/g of protein was calculated using a value of 1.24 for f/f_{min}.

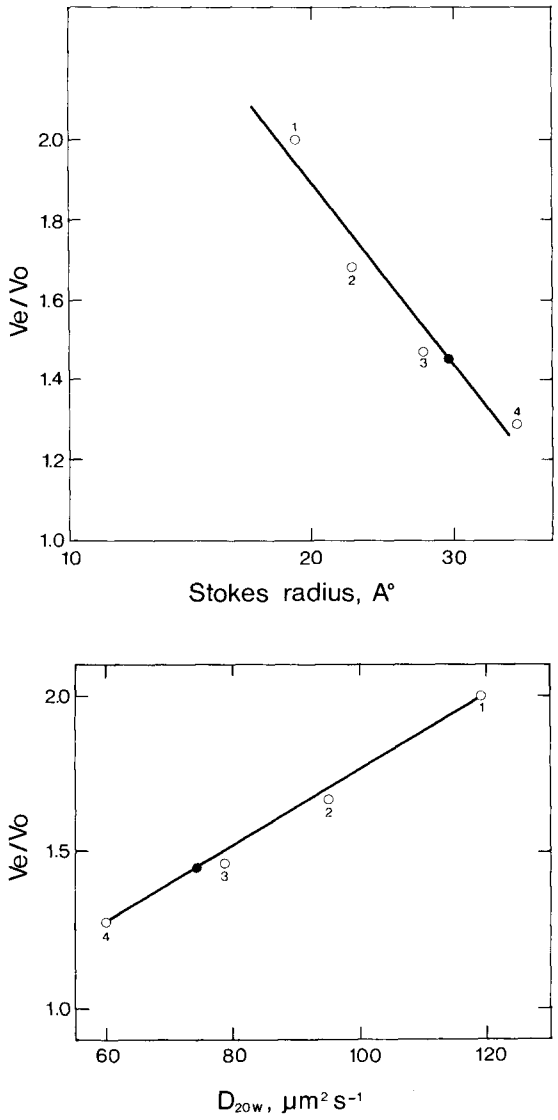

Fig. 10. Stokes radius and diffusion coefficient estimation of glucokinase by gel filtration. Experimental data from gel filtration experiment of Fig. 7. The diffusion coefficients (μm^2/sec) and stokes radii (Å) of the standard proteins used are respectively: 1) ribonuclease: 119, 19.2; 2) chymotrypsinogen: 95, 22.5; 3) ovalbumin: 77.8, 27.6; 4) bovine serum albumin: 60, 36.1. The filled symbols indicate the positions of glucokinase. Other details are given in the text.

The diffusion coefficient was calculated from the gel filtration data as shown in Fig. 10. A value of 74 μm^2/sec was obtained in this way. The diffusion coefficient was also calculated from the Stokes-Einstein equation:

$$D_{20,w} = \frac{k\,T}{6\,\pi\,\eta\,r}$$

where k is the Boltzman constant, T the absolute temperature, and η is the viscosity of water. A value of 72 μm^2/sec was obtained for $D_{20,w}$. The physical constants of dog liver glucokinase are summarized in Table 2.

Ultraviolet absortion spectra

The ultraviolet absorption spectrum of pure glucokinase is shown in Fig. 11. There is a maximum at 278 nm and a minimum at 255 nm. In the presence of 0.1 M HCl a shift in the absorbance maximum to 275 nm was observed. The spectrum in the presence of HCl was found to be the same as the one calculated from the aminoacid composition (36). Glucokinase does not absorb radiation in the 350-800 nm region in the presence or in the absence of HCl.

Aminoacid composition

The aminoacid composition of dog liver glucokinase is given in Table 3. The tyrosine content was also calculated by spectrophotometric titration in the presence and in the absence of 8 M urea (Fig. 12). The titration curves in both solvents were similar and from them a value of 15.4 moles of tyrosine/mole of enzyme was calculated, using an extinction coefficient of 11,000 M^{-1} cm^{-1} at 243 nm (22). This value compares well to 15.2 moles of tyrosine/mole of enzyme found by aminoacid analysis. A value of 6.6 moles

TABLE 2

Physicochemical Properties of Dog Liver Glucokinase

Property	*Method*	*Value*
Native molecular weight	gel filtration	47,000
	thin layer gel filtration	55,000
Denatured molecular weight	zone electrophoresis	49,000
Stokes radius	gel filtration	29.7 Å
	gel filtration (Porath)	30 Å
Diffusion coefficient, $D_{20,w}$	gel filtration	74 μm^2 sec^{-1}
	gel filtration (Stokes-Einstein)	72 μm^2 sec^{-1}
Frictional ratio, f/f_{min}		1.24
Axial ratio, a/b (maximal asymmetry)		4.7
Maximal hydration		0.65 g/g
Isoelectric point	gel electrofocusing	5.1

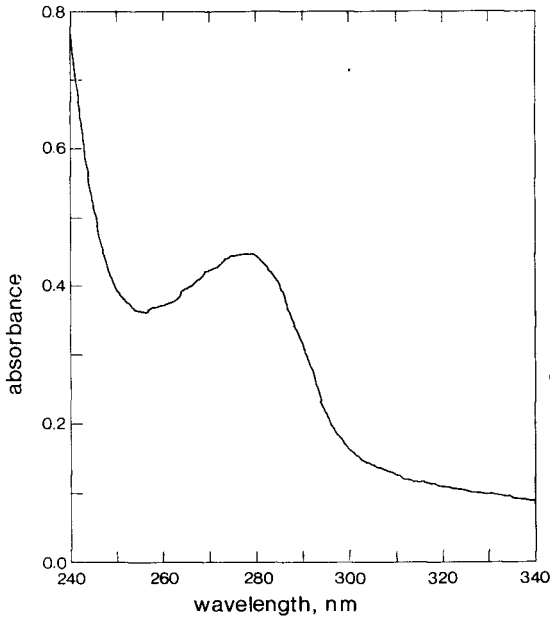

Fig. 11. Ultraviolet absorption spectrum of glucokinase. Glucokinase (0.36 mg/ml) in 2 mM potassium phosphate, pH 7.0.

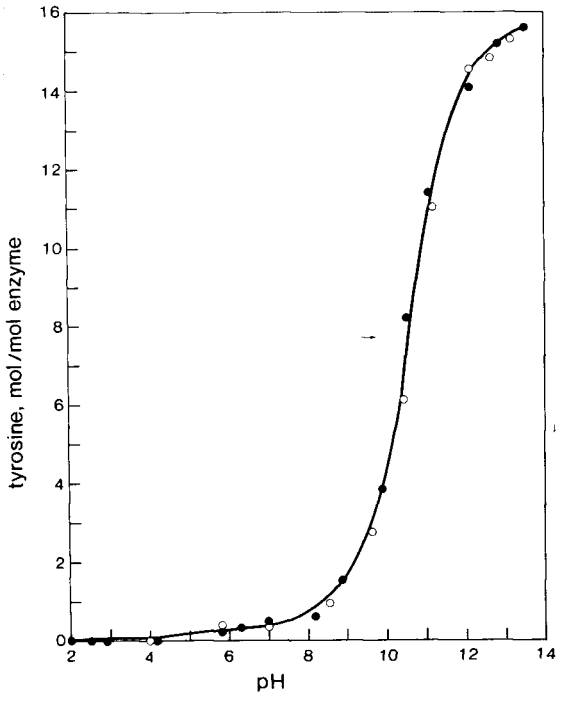

Fig. 12. Spectrophotometric titration of the phenolic groups of glucokinase at 243 nm. Glucokinase (1.42×10^{-6} M) equilibrated with 2 mM potassium phosphate pH 7.0 in the presence (●) and in the absence (○) of 8 M urea. Details are given under Methods.

TABLE 3

Aminoacid Composition of Dog Liver Glucokinase

Aminoacid	*Residues per mole*[1, 2]	*Residues per mole (round values)*
lysine	13.5±1.2	14
histidine	5.1±0.1	5
arginine	16.4±1.1	16
cysteine[3]	8.0	8
aspartic acid[4]	44.5±0.9	45
threonine	18.3±1.3	18
serine	22.5±2.1	23
glutamic acid[4]	54.3±2.0	54
proline	7.2±0.1	7
glycine	40.0±0.6	40
alanine	26.5±0.7	27
valine	27.2±0.1	27
methionine	8.8±1.6	9
isoleucine	25.8±2.0	26
leucine	32.1±1.1	32
tyrosine	15.2±0.2	15
phenyalanine	23.5±1.4	24
tryptophan[3]	6.6	7

1. Values based on a molecular weight of 48,000
2. Average of three analysis after 24 hr hydrolysis
3. Spectrophotometric determination (see Text)
4. Include the corresponding amides

of tryptophan/mole of enzyme was calculated from the spectrum of the enzyme in the presence of 0.1 M NaOH (36) using a value of 15.4 moles of tyrosine/mole of enzyme.

Figure 13 shows the titration of thiol groups with p-chloromercuribenzoate (23). The activity of the enzyme decreased with increasing concentration of the reagent, and total inactivation was observed when 8.4 thiol groups were titrated. This value is in agreement with the one found by aminoacid analysis. Addition of 10 mM dithiothreitol resulted in 100% recovery of glucokinase activity. In the presence of 8 M urea (not shown) a total of 8.6 thiol groups were titrated. From a first order plot the half time for the reaction in 50 mM Tris-HCl, pH 7.0, 100 mM KCl, was found to be 3 min. These two results suggest that the thiol groups are located in the surface of the enzyme in contact with the solvent.

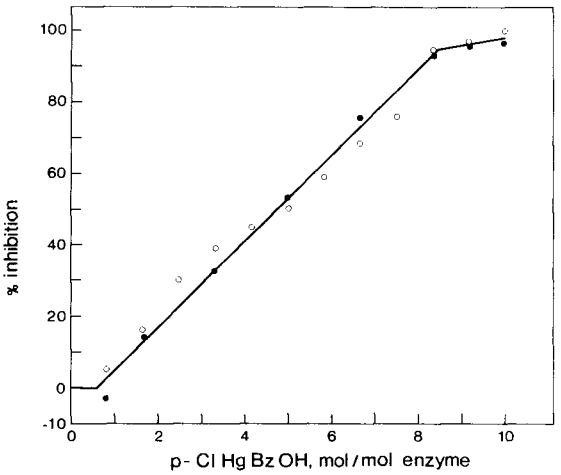

Fig. 13. Inactivation of glucokinase by p-chloromercuribenzoate. Glucokinase (2.2×10^{-6} M) in 50 mM Tris-HCl pH 7.2, 50 mM KCl, 100 mM glucose. (○) Percentage change of absorbancy at 250 nm; (●) Percentage change in glucokinase activity (inhibition). Procedure is described under Methods.

Peptide mapping

The peptide mapping of a tryptic digest of pure glucokinase is shown in Fig. 14. A total of 30 ninhydrin reactive spots were detected.

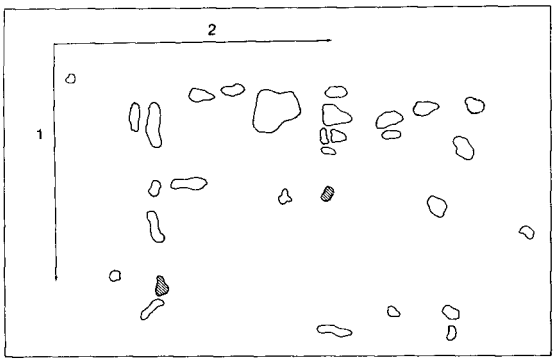

Fig. 14. Peptide map of a tryptic digest of glucokinase. Glucokinase was digested with trypsin and chromatographed in butanol : acetic acid : pyridine : water (15:3:10:12) for 16 hr. Electrophoresis was performed in pyridine : acetic acid : water (1:11:288) pH 3.4 for 90 min at 2000 volts. The hatched spots showed UV light absorption. Other details are described in Methods.

Isoelectric Point

The isoelectric point of glucokinase was determined by gel electrofocusing (29). An isoelectric point of 5.1 was determined by this technique.

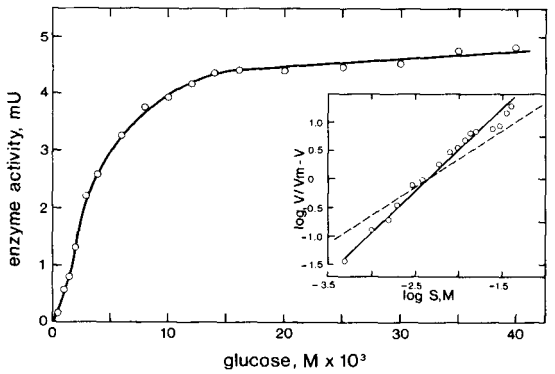

Fig. 15. Glucokinase activity as a function of glucose concentration. The enzyme was equilibrated with 10 mM sodium phosphate pH 7.0, 1 mM EDTA, 5 mM 2-mercaptoethanol, 100 mM KCl. The activity was measured with the spectrophotometric assay. The insert shows a Hill plot of the data; the dotted line corresponds to a Hill coefficient of 1.0.

Catalytic measurements

Pure glucokinase presents a sigmoidal dependence of the rate on glucose concentration (Fig. 15). A Hill coefficient of 1.5 and a $K_{0.5}$ of 4 mM was calculated from these data. Table 4 shows the relative phosphorylation rates of glucokinase with several sugars.

TABLE 4

Relative Phosphorylation Rates of Several Saccharides or Derivatives by Glucokinase[1]

Saccharide	$V_{saccharide}/V_{glucose}$
α-D-(+)-glucose	1.00
D-(+)-mannose	0.60
D-(−)-fructose	0.33
2-deoxy-D-glucose	0.31
D-(+)-glucosamine	0.00
D-(+)-galactose	0.00
α-methyl-D-glucoside	0.00
D-(−)- ribose	0.00
D-(−)-arabinose	0.00
D-(−)-xylose	0.00

[1]Concentration of substrates, 100 mM.

DISCUSSION

Purification

Several groups of investigators have attempted to purify liver glucokinase (6). Salas *et al.* (9) partially purified rabbit liver glucokinase 200 fold by fractionation with ethanol, DEAE-Sephadex chromatography, and ammonium sulfate, obtaining a final specific activity of 2 units/mg. Parry and Walker (10) described an 870 fold purification of rat liver glucokinase involving ammonium sulfate fractionation, DEAE-cellulose, DEAE-Sephadex, and polyacrylamide chromatography, achieving a specific activity of 8.7 units/mg. Babul and Niemeyer (7) reported a 400 fold purification of the rat liver enzyme by a procedure using CM-Sephadex, DEAE-cellulose, ammonium sulfate

fractionation, and hydroxylapatite chromatography, obtaining a preparation of 5.6 units/mg. Pilkis (11) improved the purification procedure of Parry and Walker (10) by including starch gel electrophoresis and obtained a preparation with 29.4 units/mg, but the yield was only 3%. Grossman *et al.* (12), with a procedure similar to the ones described above, purified the rat liver enzyme with a specific activity of 80 units/mg that represented 10,500 fold purification with a yield of 2.4%. A preliminary report from this laboratory (37) described the purification of the dog liver enzyme with a procedure similar to the one described in this work, representing aproximately 8,000 fold purification with a specific activity of 62 units/mg. Recently, Holroyde *et al.* (13) described the purification of the rat liver enzyme using affinity chromatography with N-(6-aminohexanoyl)-2 deoxyglucose attached to Sepharose (38). A specific activity of 150 units/mg was obtained with a yield of 40%. With the purification procedure used in this report, dog liver glucokinase was purified 11,000 fold with a final specific activity of 89 units/mg and a yield of 12%. This procedure could be further simplified by eliminating the second DEAE-cellullose step. The final chromatography in glucosamine-Sepharose is the key step in the procedure. The specific elution of the enzyme with glucose from this affinity column together with the fact that the enzyme was not retained by the aminohexanoyl-Sepharose without glucosamine attached to it, suggest that there is an enzyme ligand interaction.

Stability

Pure dog liver glucokinase was stabilized by the addition of 2-mercaptoethanol, glucose and K^+ ions. Similar effects were observed by Grossman *et al.* with the rat liver enzyme (12).

Molecular weight

The molecular weights of 47,000 and 49,000 found by gel filtration in native conditions and sodium dodecylsulfate gel electrophoresis, respectively, indicate that dog liver glucokinase is a monomeric enzyme. Furthermore, a tryptic digest of the pure enzyme gave a number of peptides such that, according to the number of lysines and arginines in the molecule, is also consistent with a monomeric structure. Similar molecular weights for the native and denatured rat liver glucokinase have been reported by Parry and Walker (10), Grossman *et al.* (12) and Holroyde *et al.* (13). Pilkis reported a value of 65,000 and 48,000 by gel filtration in the presence and in the absence of KCl, and suggested that glucokinase undergoes a conformational change during gel filtration in buffers of low ionic strenght. Very recently Cárdenas *et al.* (39) determined the molecular weight of rat liver glucokinase under reacting conditions and found a molecular weight of 54,000 in the absence and in the presence of substrates and products.

The fact that glucokinase, from rat and dog liver, and hexokinase, from rat brain (40), are monomeric enzymes with different molecular weights together with the fact that the antibody prepared against rat liver glucokinase does not inhibit any of the hexokinases (41), indicate that the isozymes are codified independently. Also, the existence of glucose phosphorylating isozymes cannot be explained by hybridization of different subunits like other isozymic system (42).

The aminoacid composition of dog liver glucokinase was compared to that of the rat liver enzyme using the $S\Delta_n$ index of Cornish-Bowden (43). A $S\Delta_n$ value of 292 was obtained, suggesting no identity between the two sequences. This is not surprising since a comparison between hexokinase A from rat brain and bovine brain also gives a similar $S\Delta_n$, 256 (Tito Ureta, personal communication).

Enzyme kinetics

Dog liver glucokinase shows a sigmoidal dependence of rate with varying glucose and mannose concentrations. The $K_{0.5}$ value of 4 mM is similar to the one found for several vertebrate glucokinases (5). The sigmoidal kinetics observed with glucokinase cannot be explained with the models used with oligomeric enzymes (44, 45) since glucokinase is a monomeric enzyme. Several investigators

have attempted to explain sigmoidal kinetics for monomeric enzymes (46-50). Cárdenas *et al.* (51) in our laboratory have proposed a mechanism, based on the one proposed by Ferdinand (46), which considers a conformational change of the enzyme favored by glucose. Their results are compatible with a model in which glucose binds to two different enzyme molecules, with and without ATP bound, inducing a conformational change in the enzyme. This conformational change would be faster when glucose binds to the free enzyme than when it binds to the binary complex. Further studies are in progress in our laboratory to test this proposal.

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