The Topographical Location and Unique Nature of a Glucokinase Associated with the Golgi Apparatus of Rat Liver

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A particulate glucokinase was recovered in the Golgi-rich fraction of rat liver prepared by the method of Morré [*Methods Enzymol.* (1971) 22, 130–148], thus extending the demonstration by Berthillier *et al.* [*Biochim. Biophys. Acta* (1973) 293, 370–378] of particulate glucokinase activity in a microsomal subfraction that showed enrichment in Golgi characteristics. The purity of this fraction was examined and it was then subjected to several treatments, the action of Triton X-100, freezing and thawing, and sonication to establish the topographical location of the glucokinase activity thus solubilized. The evidence suggests that the glucokinase activity is either soluble in the lumen of the Golgi apparatus or loosely associated with the inside of the Golgi membranes. This Golgi glucokinase differs from the cytoplasmic enzyme in that it does not cross-react immunologically with an antiserum specific to the latter and in its binding to concanavalin A–Sephadex. The hepatic Golgi glucokinase is present in normal quantities in two situations, the livers of diabetic and neonatal rats, when the cytoplasmic enzyme activity is low or absent. A portion of the total hepatic activities of UTP–glucose 1-phosphate uridylyltransferase and phosphoglucomutase also appears to have a location in the Golgi apparatus. Together with the glucokinase these two enzymes would facilitate the biosynthesis of UDP-glucose, the donor in glucosylations, from glucose.

In addition to the well-established cytoplasmic glucokinase (EC 2.7.1.2) (Walker, 1966; Walker & Parry, 1966; Niemeyer *et al.*, 1975), some glucokinase activity possessing different kinetic characteristics is associated with microsomal fractions prepared from rat liver (Berthillier *et al.*, 1970; Berthillier & Got, 1972). Most of this activity could be recovered in a microsomal subfraction containing Golgi material prepared by zonal centrifugation (Berthillier *et al.*, 1973). The particulate glucokinase is believed to be involved in a pathway for the synthesis of UDP-glucose that is also located in the original microsomal fraction (Berthillier & Got, 1974). To confirm the existence of this glucokinase in the Golgi apparatus and to examine its topographical relationships therein we have now prepared a Golgi-rich fraction directly from livers of rats by the procedure of Morré (1971). The present paper describes experiments on this fraction that provide evidence for the location of the particulate glucokinase on the luminal side of the Golgi vesicles. Enzymic, immunological and affinity-chromatographic evidence is presented concerning the non-identity of cytoplasmic and Golgi glucokinases; the latter enzyme does not show the adaptive behaviour typical of the former. The topographical relationships of the two other enzymes implicated in

the biosynthesis of UDP-glucose have also been examined. A preliminary account of part of this work has been given (Berthillier *et al.*, 1975b).

Materials and Methods

Reagents

Glucose 6-phosphate dehydrogenase (type VII), α -glycerophosphate dehydrogenase (type I), aldolase (grade I), UDP-dehydrogenase (type III), thiamin pyrophosphate chloride, UDP-galactose (sodium salt) and most of the sugar phosphates and nucleotides were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K., and other nucleotides and lactate dehydrogenase (from rabbit muscle) from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K. UDP-D-[U-¹⁴C]galactose (ammonium salt) (sp. radioactivity 250 mCi/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K. Streptozotocin was from Upjohn Co., Kalamazoo, Mich., U.S.A. All other chemicals were AnalaR or best-quality-grade reagents.

Samples of antisera prepared in sheep against pure cytosolic glucokinase (sp. activity 150 units/mg of protein), which reacted specifically with the cytoplasmic glucokinase when tested with a purified preparation of the enzyme (M. B. Allen & D. G.

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Walker, unpublished work), were kindly supplied by Dr. M. B. Allen. These samples were capable of inactivating 13–27 units of soluble glucokinase activity/ml.

Animals

Male Wistar rats from the Departmental colony were fed *ad libitum* and weighed 250–300 g. Diabetes was induced by intraperitoneal injection of streptozotocin dissolved in 5 mM-sodium citrate buffer, pH 4.5, at the dosage of 65 mg/kg body wt. After 3 days, diabetes was confirmed by the presence of massive glucosuria associated with hyperglycaemia and weight loss. Neonatal rats were taken at random from litters in the breeding colony; the livers from many pups were pooled as indicated.

Golgi preparation

The method of Morré (1971) was used with only minor modifications. The homogenizer was a Polytron model PT 35 OD instead of a Polytron 20 ST. Briefly, this method involved homogenization of livers (10 g/20 ml) in a medium consisting of 0.5 M-sucrose in 0.05 M-Tris/HCl buffer, pH 7, for 50 s. After centrifugation at 4000 g for 20 min in a swinging-bucket rotor the upper yellow-brown phase of the pellet was resuspended in homogenization medium and layered on a two-step sucrose density gradient (Morré, 1971). After a centrifugation at 100 000 g for 30 min in an MSE 3×25 ml swing-out rotor, the Golgi-rich fraction was collected from the 1.25 M-/0.5 M-sucrose interface, resuspended in the Tris/HCl buffer, pH 7, and centrifuged for 1 h at 100 000 g. The pellet, resuspended in 0.15 M-NaCl or in an appropriate buffer, was taken as the Golgi preparation. It was normally either used as soon as possible the same day, or frozen and kept in solid CO₂ for certain experiments where undamaged membranes were not necessary.

Washing with 3 M-KCl

The pellet obtained after the last centrifugation of the Golgi preparation was resuspended in 5 mM-Tris/HCl buffer, pH 7.0, containing 3 M-KCl and left for 10 min at room temperature. The suspension was centrifuged at 100 000 g for 1 h and the pellet obtained was resuspended in 0.15 M-NaCl; enzyme activities were then measured as described below.

Freezing and thawing of Golgi preparation

The fraction, prepared as described above and resuspended in 0.15 M-NaCl, was frozen for 10 min in solid CO₂, allowed to thaw at room temperature (approx. 5 min) and then centrifuged for 1 h at

100 000 g. After determination of the enzyme activities in the supernatant and the pellet, the latter was resuspended in the same volume of 0.15 M-NaCl, frozen again in solid CO₂ and the same procedure followed again. This was repeated until no glucokinase (or other enzyme activity) remained in the pellet. When this procedure was used to prepare solubilized Golgi enzymes as a routine, the Golgi preparation was frozen and thawed four times without centrifugation between each operation. The centrifugation was performed after the fourth freezing-and-thawing step, so that the supernatant contained all of the liberated (or solubilized) activities.

Sonication

This was performed in a MSE sonicator, with a probe of 1 cm diameter operating at 60 W, 20 000 Hz, for 10 s at maximum power on the Golgi preparation suspended in 0.15 M-NaCl. After centrifugation for 1 h at 100 000 g, the same operation was repeated on the pellet resuspended in a minimum volume of 0.15 M-NaCl.

Experiments with the antisera to rat cytosolic glucokinase

A suspension of Golgi material was made 100 mM with respect to glucose and 1 mM to dithiothreitol (to help stabilize glucokinase activity) and subjected to freezing-and-thawing as described above. The incubation medium for immunoprecipitation, final volume 0.2 ml, contained either the cytosolic or the solubilized Golgi glucokinase at the same concentrations (in the range 0.02–0.05 unit/ml), and either 50 μ l or 100 μ l of an antiserum specimen that had been diluted such that the amount of antibody was a small excess of that required completely to inactivate the cytosolic glucokinase. Control incubations without added antibody or with control serum were also performed. After incubation for 1 h at 37°C, and then for 15 min at 4°C, the medium was centrifuged for 5 min in a micro-centrifuge at 14 000 g and 4°C. Enzyme activity was assayed on 100 μ l of each supernatant.

Samples of this solubilized Golgi glucokinase were concentrated about 200-fold by ultrafiltration in Minicon-A25 cells (Amicon Corp., Lexington, Mass., U.S.A.) and immunodiffused on agarose plates against the antisera; controls with pure cytosolic glucokinase were included on the plates.

Filtration on concanavalin A-Sepharose columns

Small glass columns (1 cm×8 cm) contained 2 ml of concanavalin A-Sepharose suspension (column height 2 cm). Each column was washed twice with 2 ml of 0.05 M-Tris/HCl buffer, pH 7, then twice with

2ml of either 0.25M- or 0.5M- α -methyl glucoside or 0.1M- α -methyl mannoside, each dissolved in the same Tris buffer, and finally twice with 2ml of the Tris buffer. A sample (2ml) of either the supernatant obtained after freezing-and-thawing of the Golgi preparation (i.e. the solubilized enzyme) or the 100000g supernatant prepared from the same homogenate and diluted to get the same range of activity (i.e. cytosolic glucokinase) was passed through a column. Elution was performed with two 2ml portions of Tris buffer, and two of 0.25M- or 0.5M- α -methyl glucoside or 0.1M- α -methyl mannoside. Enzyme activity was assayed in suitable samples of the eluate. Columns were not re-used in further experiments.

Enzyme assays

Glucokinase (ATP-D-glucose 6-phosphotransferase, EC 2.7.1.2) activity was measured by the method previously described (Berthillier *et al.*, 1970). Briefly, glucose 6-phosphate formed was detected by the reduction of NADP⁺ (0.2mm) in the presence of glucose 6-phosphate dehydrogenase (0.4 unit), 5 mM-ATP, 100mM-glucose, 10mM-MgCl₂ and 20mM-triethanolamine buffer, pH 8.1. The reaction volume was 1ml. Triton X-100, when required, was added at a final concentration of 0.1% (v/v). Any hexokinase (EC 2.7.1.1) activity, tested by the same method but with 1.1mM-glucose, was deducted from the above activity.

Cytochrome oxidase (ferrocytochrome c-oxygen oxidoreductase, EC 1.9.3.1) was measured by the method of Appelmans *et al.* (1955). Acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) was measured by the method of Fishman & Lerner (1953). Glucose 6-phosphatase (D-glucose 6-phosphate phosphohydrolase, EC 3.1.3.9) and 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) were measured by the methods indicated by Morré (1971); the P_i produced was measured by the method of Fiske & SubbaRow (1925). Thiamin pyrophosphatase (thiamin pyrophosphate phosphohydrolase, no EC number) was measured by the method indicated by Morré (1971), P_i being detected by the method of Ueda & Wada (1970).

Glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate-NADP⁺ oxidoreductase, EC 1.1.1.49) was measured by the method of Lohr & Waller (1965), pyruvate kinase (ATP-pyruvate phosphotransferase, EC 2.7.1.40) by the method of Bücher & Pfeleiderer (1955) and lactate dehydrogenase (L-lactate-NAD⁺ oxidoreductase, EC 1.1.1.27) by the method of Bergmeyer *et al.* (1965).

Aldolase (D-fructose 1,6-bisphosphate D-glyceraldehyde 3-phosphate-lyase, EC 4.1.2.13) was measured by the method of Bruns & Bergmeyer (1963) without adding any triose phosphate isomerase, so that 1 mol

of NAD produced corresponds to 1 mol of substrate metabolized.

Phosphofructokinase (ATP - D - fructose 6 - phosphate 1-phosphotransferase, EC 2.7.1.11) was measured by coupling with aldolase in order to detect the amount of fructose 1,6-bisphosphate formed (Ling *et al.*, 1955). The medium was the same as for the aldolase assay, except that fructose 1,6-bisphosphate was replaced by ATP and fructose 6-phosphate and 0.12 unit of aldolase activity was added.

Phosphoglucomutase (α -D-glucose 1,6-bisphosphate- α -D-glucose 1-phosphate phosphotransferase, EC 2.7.5.1) and UTP-D-glucose 1-phosphate uridylyltransferase (EC 2.7.7.9) were measured by methods previously described (Berthillier & Got, 1974).

The determination of UDP-galactose-N-acetylglucosamine galactosyltransferase (no EC number) activity was based on the incubation medium described by Morré (1971). It contained (in a final volume of 110 μ l): 10mM-piperazine/glycylglycine buffer, pH 7, 8mM-MnCl₂, 15mM-MgCl₂, 0.15mM-UDP-[¹⁴C]galactose (0.03 μ Ci) and, except for the controls, 6mM-N-acetylglucosamine. Because the control tubes generally gave high blank activities, owing to the presence of UDP-galactose pyrophosphatase activity, 5mM-ATP was added to the incubation media. This was found to inhibit this latter activity, as indicated by Palmiter (1969). The reaction was stopped by addition of 50 μ l of 0.25mM-EDTA. Separation of the substrate UDP-[¹⁴C]galactose, the product N-acetyl-[¹⁴C]lactosamine and the [¹⁴C]galactose liberated by the pyrophosphatase was performed in two steps. High-voltage electrophoresis on Whatman 3MM chromatography paper permitted the separation of UDP-[¹⁴C]galactose from the non-charged products (Berthillier *et al.*, 1973). The N-acetyl-[¹⁴C]lactosamine and [¹⁴C]galactose were subsequently separated on the same electrophoresis paper by chromatography in the same direction in butan-1-ol/ethanol/water (10:1:2, by vol.) for 65h (Helting & Erbing, 1973). After drying the sheet of paper was cut and appropriate pieces were counted for radioactivity in a Philips liquid-scintillation analyser, by using a scintillation fluid composed of 4g of 2,5-diphenyloxazole and 0.12g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/l of toluene.

The galactosyltransferase activity is expressed in d.p.m./min per mg of protein. For all the other enzymic activities one unit corresponds to the utilization of 1 μ mol of substrate/min and then normally expressed per mg of protein.

Chemical methods

Protein was determined by a biuret method, modified to avoid interference by sucrose and lipids

(Frot-Coutaz & Got, 1971). Phospholipids were extracted by the method of Bligh & Dyer (1959). After digestion with conc. HClO_4 , P_i was measured by the method of Bartlett (1959).

Results

Purity of Golgi preparation

Several marker enzymes were assayed in the whole homogenate, in the material applied to the gradient and in the Golgi preparation obtained, to assess the relative enrichment in Golgi material and the extent of contamination (Table 1). UDP-galactose-*N*-acetylglucosamine galactosyltransferase and thiamin pyrophosphatase activities were assayed as marker enzymes for the Golgi fraction. They were found to be enriched 15-fold and 13-fold respectively, over the whole homogenate, with an overall yield of about 8%. Glucose 6-phosphatase and glucose 6-phosphate dehydrogenase activities were not detectable in the Golgi fraction, thereby excluding contamination by both endoplasmic reticulum and cytosol. The specific activities of cytochrome oxidase and 5'-nucleotidase were very low and their content was in the range of 0.1–2% of the activity found in the whole homogenate. The specific activity of acid phosphatase was in the same range in the Golgi preparation as in the two previous steps of the preparation, the yield being about 0.6% of the total activity found in the whole homogenate. These results (Table 1) indicate that we obtained a Golgi fraction of relatively high purity. Electron microscopy showed that the preparation was composed largely of vesicles having the same profile as that shown by Morré (1971).

We were unable to determine precisely the amount of particulate glucokinase in the total homogenate because of the presence of the relatively large quantity

of soluble enzyme. However, glucokinase activity was enriched in the Golgi preparation fivefold over that in the material applied to the gradient, and the whole of this represented latent particulate material. UDP-galactose-*N*-acetylglucosamine galactosyltransferase and thiamin pyrophosphatase activities were enriched 11- and four-fold respectively, during the same procedure.

In order to know if the glucokinase in the Golgi-rich preparation might be involved in a glycolytic pathway or not, or arises because of cytosolic components being trapped during the homogenization, four other typical glycolytic enzymes were assayed during the preparation. Table 2 shows that, although these enzymes were still present in the material applied to the gradient, phosphofructokinase, aldolase and pyruvate kinase activities could not be detected in the Golgi preparation and only a trace of lactate dehydrogenase activity was present. The latter could easily be removed from the Golgi preparation by washing with 3M-KCl, which is known to remove protein adsorbed on membrane preparations (Reisfeld *et al.*, 1971; Robinson & Tanford, 1975). The same washing procedure left all of the glucokinase activity still associated with the Golgi preparation; this activity could only be detected, of course, by including Triton X-100 in the assay medium.

Effect of solubilizing treatments on the glucokinase activity associated with Golgi preparations

Detergent. Fig. 1 shows the results of various concentrations of Triton X-100 on the detectability of the Golgi-bound glucokinase activity. The activity appeared at a concentration close to the critical micellar concentration of Triton X-100, at 0.015%. This behaviour is suggestive of a latent enzyme, i.e. one that is located in some way within the intact

Table 1. *Distribution of particulate glucokinase and certain marker enzyme activities during preparation of the Golgi fraction from rat liver*

Enzyme activities were determined as described in the text and the results are expressed as $\mu\text{mol/min}$ per mg of protein, except for galactosyltransferase, where the results are in d.p.m./mg of protein. These results are for a single experiment but are typical of several. The particulate glucokinase activity is the latent activity measured in the presence of Triton X-100.

	Whole homogenate	Material applied to the density gradient	Golgi preparation
Total protein (mg)	3000	525	15
Enzyme activities			
Cytochrome oxidase	1.6	0.2	0.2
Acid phosphatase	1.1	1.7	1.3
Glucose 6-phosphatase	0.05	0.02	0
5'-Nucleotidase	0.0037	0.0075	0.0007
Glucose 6-phosphate dehydrogenase	0.02	0	0
UDP-Galactose- <i>N</i> -acetylglucosamine galactosyltransferase	232	320	3600
Thiamin pyrophosphatase	0.0009	0.0028	0.012
Particulate glucokinase	—	0.0012	0.006

vesicle and inaccessible to its substrate unless the membrane of the vesicle is damaged.

Freezing and thawing. Four successive freezing-and-thawing operations were necessary to liberate all the latent activity from Golgi vesicles (Fig. 2). The first three operations liberated 33, 26 and 17% of the activity bound to the pellet respectively. The last step released the whole of the remaining activity, and the total recovery was 100%. The same treatment did not liberate more than 7% of the UDP-galactose-

N-acetylglucosamine galactosyltransferase activity, or any of the phospholipids of the membranes.

Sonication. Two sonications for 10s were required for liberation of the glucokinase activity (Fig. 3). The total recovery of released glucokinase was 70%, and again not more than 8% of the total phospholipids could be detected in the supernatant.

Evidence for the non-identity of cytosolic and Golgi glucokinase

Freezing and thawing having thus been shown to be a good method to solubilize the Golgi glucokinase, without addition of any secondary reagent or the

Table 2. Distribution of cytosolic enzyme activities during preparation of the Golgi fraction from rat liver

Enzyme activities were determined as described in the text and are expressed as percentages of the total activity in the original whole homogenate. This experiment is typical of several.

	Percentage of total whole-homogenate activity found in fraction	
	Material applied to gradient	Golgi preparation
Pyruvate kinase	11	0
Aldolase	12	0
Phosphofructokinase	11	0
Lactate dehydrogenase	7	0.6 (0.002)*

* The number in parentheses is the activity in the Golgi preparation after washing with 3M-KCl.

Fig. 1. Effect of concentration of Triton X-100 on glucokinase activity associated with a Golgi preparation from rat liver

Glucokinase activity was determined as described in the text in the presence of various final concentrations of Triton X-100 ranging from 0 to 0.2% (v/v) in order to demonstrate latency.

Fig. 2. Distribution of glucokinase and galactosyltransferase activities and of phospholipids between the supernatant and remaining particulate fractions during successive freezing and thawing of a Golgi preparation

Enzyme activities and phospholipids were determined as described in the text. P represents the enzyme activity or the amount of phospholipids associated with the initial Golgi preparation and S₁, S₂ etc. and P₁, P₂ etc. represent the activities (or phospholipid content) found in the supernatant and particulate fractions respectively after one, two etc. successive freezing and thawing procedures performed as described in the text. Results are expressed as percentages of the initial activity or amount of phospholipid associated with P and are from one experiment typical of several. No phospholipids could be detected in any of the soluble (supernatant) fractions during any of these procedures.

Fig. 3. Distribution of glucokinase activity and of phospholipids during sonication of a Golgi preparation

Glucokinase activity and phospholipids were determined as described in the text. P represents the glucokinase activity or the amount of phospholipids associated with the initial Golgi preparation and S₁ and S₂, P₁ and P₂, represent the activity or phospholipid found in the supernatant and particulate fractions respectively after one or two successive sonication operations as described in the text. Results are expressed as percentages of the initial activity or amount of phospholipid associated with P and are from one experiment typical of several. No particulate glucokinase activity was detectable after the second sonication step.

need for dilution, we then compared this solubilized enzyme with cytosolic glucokinase.

Enzymic properties. The preparation of solubilized Golgi glucokinase was found to phosphorylate glucose and glucosamine but not a number of other acceptor analogues. The effect of glucose concentration on the enzyme gave a biphasic plot of v against v/s very similar to those given in Fig. 10 of Berthillier *et al.* (1970), with apparent K_m values of 6 and 100 mM. These experiments were, of necessity, performed on comparatively crude extracts of the enzyme, but gave results essentially identical with those on the partially purified microsomal enzyme (Berthillier *et al.*, 1970) and quite different from cytosolic glucokinase (Parry & Walker, 1966; A. C. Storer & A. Cornish-Bowden, unpublished work).

Antigenic behaviour. Incubation of the Golgi glucokinase, solubilized from the preparation by freezing and thawing, with an antibody specific for the cytosolic glucokinase did not cause any inhibition of the activity. Control incubations with cytosolic glucokinase under the same conditions and similar activities showed almost 100% inhibition of enzyme activity. Immunodiffusion of the solubilized Golgi glucokinase under standard conditions against the antisera showed no cross-reactivity, whereas cytosolic glucokinase gave the standard precipitin pattern.

Concanavalin A-Sepharose chromatography. After passage of the proteins, solubilized from the Golgi preparation by freezing and thawing, through a concanavalin A-Sepharose column, no glucokinase activity was detectable either in the immediate eluate or in the washings with 2 bed volumes of 0.05 M-Tris/HCl buffer, pH 7.0. About 50% of the retained glucokinase activity could be eluted with α -methyl glucoside (0.25 M). The remaining activity was recovered by increasing the concentration of α -methyl glucoside to 0.5 M, when the total yield was 100%. Elution of retained Golgi glucokinase activity could also be achieved with 0.1 M- α -methyl mannoside with a recovery of about 60%. In this case, either a higher concentration of α -methyl mannoside or 0.5 M- α -methyl glucoside did not elute the remaining activity.

Filtration of a cytosolic fraction prepared from the same homogenate as the Golgi fraction through a concanavalin A-Sepharose column under similar conditions resulted in all of the cytosolic glucokinase activity being recovered in the Tris-buffer washing alone, thus indicating that there had been no interaction of the cytosolic enzyme with the concanavalin A ligand.

Enzyme distribution in diabetic and neonatal rats

Glucokinase activity was assayed in both the cytosolic fraction and Golgi-rich preparations isolated from livers of streptozotocin-diabetic rats. Preparations from normal rats served as controls. Although only 10–18% of the normal cytosolic activity was found in three diabetic animals, the same range of Golgi glucokinase activities (cf. Table 1) was found in the diabetic and normal rats. The diabetic rats were killed from 6 to 12 days after induction of the diabetes.

Golgi-rich fractions were prepared from three pools of livers taken from 11-, 14- and 15-day-old rats. The Golgi glucokinase activity both per g of liver and specifically per mg of protein was the same as for adult rat liver in each case.

Association of phosphoglucomutase and UTP-glucose 1-phosphate uridylyltransferase with the Golgi preparation

Because these two enzymes are believed to be involved, along with the glucokinase, in a pathway for the synthesis of UDP-glucose (Berthillier & Got, 1974), we investigated whether they were present in the Golgi-rich fraction. Table 3 shows that, in a preparation checked with marker enzymes as in Table 1, UTP-glucose 1-phosphate uridylyltransferase and phosphoglucomutase activities were present. In a total of eight experiments where glucokinase was assayed, the recovery of latent glucokinase activity associated with the Golgi preparation was

Table 3. Recovery of glucokinase, phosphoglucomutase and UTP-glucose 1-phosphate uridylyltransferase activities in the Golgi preparation from rat liver

Enzyme activities were determined as described in the text and the results are from one experiment typical of several; additional individual results are given in the text.

	Material applied to gradient		Golgi preparation		
	Proportion of total activity that is latent (%)	Latent enzymic activity (nmol/min per mg of protein)	Proportion of total activity that is latent (%)	Recovery of latent enzymic activity (% of activity applied to gradient)	Latent enzymic activity (nmol/min per mg of protein)
Glucokinase	20	1.2	100	12	6
UTP-glucose 1-phosphate uridylyltransferase	30	1.8	100	14	10
Phosphoglucomutase	10	20	70	7	30

Fig. 4. Distribution of UTP-glucose 1-phosphate uridylyltransferase and phosphoglucomutase activities between the supernatant and remaining particulate fractions during successive freezing and thawing of a Golgi preparation

Procedures, the designation of fractions and the expression of results are as in Fig. 2, except that S₅ represents the combination of preparation S₄ with the supernatant obtained by washing preparation P₄ with 3M-KCl.

always between 12 and 15% of the latent activity applied to the gradient. UTP-glucose 1-phosphate uridylyltransferase and phosphoglucomutase activities were determined in three of these experiments. The transferase was 100% latent in the Golgi preparation and the recovery ranged from 14 to 20%. Phosphoglucomutase was only 70% latent in the Golgi preparations; the 30% non-latent activity was possibly due to a contamination by the very active soluble enzyme. The recovery of phosphoglucomutase in each of the three experiments was about 7%. The specific activities of the two enzymes were increased in the Golgi fraction to a similar extent to that of glucokinase.

Fig. 4 shows the effect of freezing and thawing on the release of UTP-glucose 1-phosphate uridylyltransferase and phosphoglucomutase from the Golgi preparation. Their behaviour was the same as for glucokinase, i.e. they were liberated in soluble form by four freezing-and-thawing operations. The low amount of phosphoglucomutase activity remaining

in the pellet could be removed completely by washing with 3M-KCl (Fig. 4). It was therefore probably solubilized by the freezing and thawing but re-adsorbed on to the membrane fragments.

Discussion

Isolation of a Golgi-rich fraction from rat livers by the procedure of Morré (1971) proved to be particularly useful in the present study, for it provided Golgi vesicles rapidly in good yield. Freshly prepared membranes could hence be made for most of the experiments. Our preparations were almost free of contamination by enzymes recognized as markers for mitochondria, lysosomes, endoplasmic reticulum and the cytosol.

Thiamin pyrophosphatase, an enzyme known to be a marker *in vitro* for rat liver Golgi apparatus (Morré, 1971), is enriched in our preparation to the same range as in that of Morré (1971). The specific activity of UDP-galactose-*N*-acetylglucosamine galactosyl-

transferase, another known marker enzyme for rat liver Golgi apparatus (Morré *et al.*, 1969; Fleischer & Fleischer, 1970; Leelavathi *et al.*, 1970; Schachter *et al.*, 1970), was increased 15-fold in our Golgi preparation over that in the total homogenate. The difference between this result and the apparently higher increase described by Morré (1971) can be explained by our modification of the assay procedure. Control tests indicated the presence of substantial pyrophosphatase activity in our preparation that hydrolysed the donor substrate. Rather high activities of this latter enzyme have been reported in rat mammary gland (Palmiter, 1969) and in rat liver microsomal fraction (Mookerjee & Yung, 1975). Vessey & Zakim (1975) have reported the presence of a pyrophosphatase in rat liver Golgi membranes that hydrolyses GDP-mannose. All these pyrophosphatases are known to be inhibited by ATP, which we therefore included in our assay procedure (see the Materials and Methods section). Our lower transferase activity may well be due therefore to the correction applied for pyrophosphatase activity.

Precise evaluation of particulate glucokinase activity in the homogenate was not possible, but it was enriched in the Golgi-rich preparation over the material applied to the density gradient to a similar extent as the two marker enzymes for the Golgi apparatus. Berthillier *et al.* (1973) had found 60% of the total microsomal glucokinase to be recoverable in one of six subfractions prepared by discontinuous-density-gradient centrifugation along with marker enzymes suggesting association of most of the particulate glucokinase with Golgi-enriched material. Our present results extend those observations, although we have not excluded the possibility that small sources of glucokinase-like activity may also be present in other components of the microsomal fraction.

Washing with 3M-KCl is known to remove cytoplasmic proteins adsorbed on membranes and membrane proteins bound by ionic interactions to the membrane. It can therefore remove proteins bound to the external side of vesicles (Reisfeld *et al.*, 1971; Robinson & Tanford, 1975). This washing procedure removed the traces of lactate dehydrogenase activity detected in the unwashed Golgi preparation. Glucokinase activity, however, remained particulate, indicating that this enzyme is neither adsorbed on, nor located on the outside of the Golgi vesicles.

A procedure that disrupts the entire membrane structure, such as the use of a detergent, was always required for the measurement of glucokinase activity in Golgi preparations. The typical latency curve obtained (Fig. 1) for the effect of Triton X-100 showed that, above a final concentration of 0.015%, membranes began to be disrupted, allowing the penetration of substrates to the site of the enzyme. This concentration of Triton X-100 is similar to the

critical micellar concentration values given by Makino *et al.* (1973) and Robinson & Tanford (1975). The particulate glucokinase may hence be either embedded in the phospholipid bilayer, or located inside the lumen or loosely bound to the inside of the Golgi vesicles.

The behaviour of the particulate glucokinase during freezing and thawing of the Golgi preparations was completely different from that of the galactosyl-transferase. This latter enzyme is known to be associated with the membranes of the Golgi elements (Bergeron *et al.*, 1973) and is probably embedded in the lipid bilayer. As phospholipids are constituents of the membranes, their non-liberation by freezing and thawing suggests that no membrane fragments are present in the supernatant, where all the glucokinase activity is finally recovered. Hence glucokinase is unlikely to be embedded in the lipid layer of the Golgi membranes. This correlates with the observation that the presence of Triton X-100 was not necessary during the purification of the solubilized enzyme (Berthillier *et al.*, 1970). The localization either free in the lumen or loosely bound to the inside of the Golgi vesicles is confirmed by the sonication experiments.

If Golgi vesicles were formed during the preparation procedure from a more complicated membrane structure, the fact that glucokinase activity was found inside these vesicles could imply that it represents some cytosolic glucokinase that had been entrapped during the preparation. This is unlikely, because Table 2 shows clearly that none of the cytosolic enzymes assayed, all of which are implicated in the glycolytic pathway, were entrapped in the Golgi vesicles and it is improbable that only cytosolic glucokinase would be entrapped.

All the evidence points, however, to the conclusion that the cytosolic and Golgi glucokinases are not identical. The enzyme solubilized from the Golgi preparation did not cross-react with an antiserum prepared against cytoplasmic glucokinase and showed different affinity from the cytoplasmic enzyme for concanavalin A. In the diabetic and neonatal rat livers the activities of Golgi glucokinase were similar to those found in the adult animal, which is different from the cytoplasmic enzyme, which decreases to very low activity in the diabetic rat (see Niemeyer *et al.*, 1975, for a review) and only normally first appears in the developing rat around the time of weaning from 16 days of age onwards (Walker & Holland, 1965). The latter observations suggest that the two glucokinase enzymes do not have the same metabolic role in the liver cell, even though they both have comparatively high K_m values for glucose.

The cytosolic glucokinase is considered to be involved in the phosphorylation of glucose before glycolysis and glycogen synthesis. The role of the

Golgi glucokinase warrants consideration. Berthillier & Got (1974) reported the existence of a biosynthetic pathway for the formation of UDP-glucose in the microsomal fraction of rat liver. In addition to the particulate glucokinase, two other enzymes are necessary in this pathway: phosphoglucosmutase and UTP-glucose 1-phosphate uridylyltransferase. Latent activities of both of these enzymes were enriched in the Golgi preparation to a similar extent as the glucokinase (Table 3) and their behaviour during freezing and thawing was also similar, suggesting a similar location to the glucokinase in the Golgi vesicles.

These results have been demonstrated *in vitro*. If the localization of these three enzymes in the cell is indeed as indicated, their physiological significance deserves consideration. They make up a pathway for the biosynthesis of the ultimate substrate in glucosylation reactions. Several glucosyltransferases have been located in the microsomal fraction of rat liver (Berthillier *et al.*, 1975a). The substrate, UDP-glucose, being a charged molecule, may not be able to cross the membrane (Nilsson *et al.*, 1973), so that they might require UDP-glucose to be synthesized inside the Golgi lumen directly. The submicrosomal localization of the glucosyltransferases certainly requires further examination. A related problem is the penetration of substrates through the membrane, particularly to reach the luminal glucokinase. Kuhn & White (1975) have given evidence for a glucose-transport system in mammary-gland particles, but the mechanism of UDP-galactose transport through those particles is not known. We believe the present work to be a clear demonstration of the presence of defined enzymes essentially within the lumen of the Golgi apparatus of liver cells. There is now a need for more detailed information about the nature of the Golgi apparatus and then for a consideration of the transport problems arising therefrom.

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