Evidence for a Role of Glucose-induced Translocation of Glucokinase in the Control of Hepatic Glycogen Synthesis*

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Glucokinase reversibly partitions between a bound and a free state in the hepatocyte in response to the metabolic status of the cell. Maximum binding occurs at low [glucose] (<5 mM) and minimum binding at high [glucose] in the presence of sorbitol or fructose. In this study we determined the binding characteristics of glucokinase in the hepatocyte in situ, by adenovirus-mediated glucokinase overexpression combined with the digitonin-permeabilization technique. We also determined the sensitivity of glycogen synthesis to changes in either total glucokinase overexpression or in free glucokinase activity. Glucokinase overexpression is associated with an increase in both free and bound activity, with an overall decrease in the proportion of bound activity. In hepatocytes incubated at low [glucose] (0–5 mM), glucokinase binding involves a high-affinity binding site with a $K_d$ of $\sim 0.1$ nM and a binding capacity of $\sim 3$ pmol/mg total cell protein and low-affinity binding with a $K_d$ of $\sim 1.6$ nM. Increasing glucose concentration to 20 mM causes a dose-dependent increase in the $K_d$ of the high-affinity site to $\sim 0.6$ nM, and this effect was mimicked by 50 mM sorbitol, a precursor of fructose 1-P, confirming that this site is the regulatory protein of glucokinase.

Glycogen synthesis determined from the incorporation of [$2$H$_2$U$^{14}$C]glucose into glycogen at 5 mM or 10 mM glucose was very sensitive to small increases in total glucokinase activity and correlated more closely with the increase in free glucokinase activity. The relation between glycogenic flux and glucokinase activity is sigmoidal. Expression of the sensitivity of glycogen synthesis to glucokinase activity as the control coefficient reveals that the coefficient is greater for the incorporation of 2-tritium (which occurs exclusively by the direct pathway) than for incorporation of 14C label (which involves direct and indirect pathways) and is greater at 5 mM glucose (when glucokinase is maximally sequestered at its high-affinity site) than at 10 mM glucose. The results support the hypothesis that compartmentation of glucokinase in the hepatocyte increases the sensitivity of glycogen synthesis to small changes in total glucokinase activity and that glucose-induced translocation of glucokinase has a major role in the acute control of glycogen synthesis.

Glucokinase (hexokinase IV or D), is one of four hexokinase isoenzymes expressed in vertebrates (1). It differs from the other isoenzymes in its lower molecular mass, a low affinity for glucose and sigmoidal kinetics, and in binding to a 68-kDa regulatory protein, which decreases its affinity for glucose (2–4). Binding of glucokinase to the regulatory protein is competitive with glucose (5) and is influenced by fructose 6-P and fructose 1-P (6). Glucokinase is expressed in liver cells, insulin-secreting beta cells of the pancreas, and small populations of neuroendocrine cells (7–10). Hepatic glucokinase is conventionally assayed in the high-speed supernatant of liver homogenates and has been assumed to be present in the free state in the cytoplasm (1–3). However, studies on digitonin-permeabilized hepatocytes have shown that both glucokinase and its regulatory protein bind to the hepatocyte matrix by a Mg$^{2+}$-dependent mechanism (11, 12). The proportion of the enzyme that is bound is dependent on the substrates with which hepatocytes are incubated before permeabilization with digitonin. Maximum binding occurs at low [glucose] (<5 mM) and minimum binding occurs at high [glucose] or at low concentrations of fructose or sorbitol (13, 14), which are precursors of fructose 1-P (15). Binding of the regulatory protein to the cell matrix is not affected by these substrates (12), suggesting that the regulatory protein acts as a receptor for binding glucokinase to the cell matrix.

Adenovirus-mediated enzyme expression is potentially a very powerful tool to modulate the concentration of a single enzyme in the intact cell in situ in order to determine the consequent changes in metabolic flux (16). Marked overexpression of glucokinase in hepatocytes using adenovirus is associated with various changes in glucose metabolism including a large increase in glycogen deposition (17). The present study had two aims. First, to determine the binding characteristics of glucokinase in the hepatocyte in situ by combining adenovirus-mediated glucokinase overexpression with the digitonin-permeabilization technique. Second, to determine the sensitivity of glycogen synthesis to small increases in glucokinase overexpression. We demonstrate that there is both high-affinity and low-affinity binding for glucokinase in the hepatocyte, and that the rate of glycogen synthesis is very sensitive to small increases in total glucokinase activity and correlates more closely with the free glucokinase activity. The sensitivity of glycogen synthesis to changes in total glucokinase activity is greatest at low glucose concentration, thus demonstrating the role of glu-
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cose-induced translocation of glucokinase in the acute control of hepatic glycogen synthesis.

EXPERIMENTAL PROCEDURES

Materials—Digitonin was from British Drug House Ltd. (Poole, Dorset, United Kingdom). Glucose-6-phosphate dehydrogenase (from Leuconostoc mesenteroides) was from Sigma. [U-14C]Glucose and [2-3H]glucose were from Amersham International, PLC, Amersham, Bucks, U.K. [2-3H]Glucose was purified before use as in Ref. 11. Preparation of Recombinant Adenovirus—Recombinant adenovirus containing the cDNA encoding rat liver glucokinase (AdCMV-GKL) was prepared as in Ref. 18. Expression of the transgene is driven by the cytomegalovirus (CMV) promoter. Hepatocyte Isolation and Culture—Hepatocytes were isolated from 24-h fasted male Wistar rats by collagenase perfusion as in Ref. 19 except for the experiments in Table II where hepatocytes were isolated as in Ref. 20. The hepatocytes were suspended in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 10 mM glucose, 100 mM Hepes, 2 mM dithiothreitol, 0.0375 mg/ml digitonin, pH 7.2. Incubations for determination of either glucokinase binding or of glycogen synthesis were started after 1 h in fresh minimal essential medium containing 2 mM cycloheximide and the substrate concentrations indicated. The hepatocytes were washed once with 150 mM NaCl and permeabilized in medium containing digitonin by a modification of the method described in Ref. 11. For all experiments, other than the ones in Fig. 1, the digitonin permeabilization medium contained 2 mM cycloheximide to inhibit protein synthesis (21) and thereby further glucokinase expression. After 30–60 min the medium was replaced by fresh minimal essential medium containing 2 mM cycloheximide and the substrate concentrations indicated for subsequent determination of either glucokinase binding or of glycogen synthesis.

Determination of Glucokinase Binding—The hepatocyte monolayers were washed once with 150 mM NaCl and permeabilized in medium containing digitonin by a modification of the method described in Ref. 11. For all experiments, other than the ones in Fig. 1, the digitonin permeabilization medium contained: 300 mM sucrose, 5 mM MgCl₂, 3 mM Hepes, 2 mM dithiothreitol, 0.0375 mg/ml digitonin, pH 7.2. Incubation with this medium (300 μl/well) was for 6 min at 20°C. On termination of this incubation the culture plate was swirled gently and the digitonin medium was removed for immediate determination of glucokinase activity (designated as “free” glucokinase). The residual glucokinase activity that remained bound to the cell matrix was removed by two sequential extractions (each for about 12 min) in 150 mM KCl, 3 mM HEPES, 2 mM dithiothreitol, 0.05 mg/ml digitonin, pH 7.2. The first of these extractions recovered approximately 50% of the bound activity and the second the remaining activity. Little (<4%) or no further activity could be recovered from further extraction. Total recovery was confirmed by permeabilization in sucrose without Mg²⁺. The sum of the activity recovered in the two KCl extractions is designated the “bound” activity. The total activity represents the sum of the activity recovered in the two KCl extractions is designated the “bound” activity. The total activity represents the sum of the activity recovered in the two KCl extractions is designated the “bound” activity. The total activity represents the sum of the activity recovered in the two KCl extractions is designated the “bound” activity. The total activity represents the sum of the activity recovered in the two KCl extractions is designated the “bound” activity. The total activity represents the sum of the activity recovered in the two KCl extractions is designated the “bound” activity. The total activity represents the sum of the activity recovered in the two KCl extractions is designated the “bound” activity. The total activity represents the sum of the activity recovered in the two KCl extractions is designated the “bound” activity.

Determination of Binding Capacity and Dissociation Constant—Beginning 30 min after termination of enzyme expression with cycloheximide, hepatocytes were incubated for a minimum of 30 min in fresh medium with the concentration of substrates indicated. They were then permeabilized for determination of free (F) and bound (B) glucokinase activity as described above. Binding capacity (n) and dissociation constant (Kd) were determined from plots of F/B versus F from the equation

\[
C_k = \frac{(J_e - J_o) - C_e}{(e - e_o) - J_0}
\]

where \(J_e\) represents rates of glycogen synthesis and \(C_e\) glucokinase activity in untreated cells and \(J_o\) and \(e_o\) the values in cells overexpressing glucokinase. This equation was used for levels of glucokinase overexpression ranging from 1.1 to 1.55 relative to endogenous activity.

RESULTS

Effects of AdCMV-GKL Adenovirus on Glucokinase Activity and Regulatory Protein Expression—When hepatocytes were treated with 3, 6, or 12 plaque-forming units/cell of AdCMV-GKL, glucokinase activity after 15 h was increased to 1.6 ± 0.2, 2.4 ± 0.5, and 3.6 ± 0.9-fold relative to endogenous activity and after 19 h the activity increased to 3.3 ± 0.9, 6.1 ± 1.9, and 9.4 ± 2.5-fold, respectively (means ±S.E., n = 5). The expression of the regulatory protein determined by Western blotting was unchanged by treatment with AdCMV-GKL (7–30 plaque/cell) for up to 24 h (results not shown). Treatment of hepatocytes with recombinant adenovirus without transgene (E1-deficient) (16) had no effect on endogenous glucokinase activity, binding, translocation, or glycogen synthesis (results not shown). In all subsequent experiments untreated cells were used as controls and a range of glucokinase overexpression was achieved using varying titers of AdCMV-GKL and incubation times ranging from 15 to 21 h.

One of the objectives of this study was to correlate the incorporation of [2-3H, U-14C]glucose into glycogen with the glucokinase activity and its distribution. Since the glycogen synthesis experiments involve a 3-h incubation and glucokinase activity increases markedly during 3 h, it was necessary to inhibit protein synthesis with cycloheximide during this incubation to maintain a constant amount of glucokinase. We used 2 μM cycloheximide, which inhibits protein synthesis in hepatocytes.
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Mg\(^{2+}\)-dependent Binding of Overexpressed Glucokinase—
When hepatocytes are permeabilized with digitonin, glucokinase binding to the cell matrix is preserved in the presence of Mg\(^{2+}\) with a half-maximally effective concentration of 0.5 mM (11, 14). Fig. 1A shows the Mg\(^{2+}\) dependence of glucokinase binding in untreated cells and cells overexpressing glucokinase by about 8-fold, after preincubation for 30 min in medium without glucose. In virus-treated cells glucokinase binding has a similar Mg\(^{2+}\) dependence as the endogenous enzyme in untreated cells. However, the proportion of total activity that was bound at 5 mM MgCl\(_2\) was 40% for enzyme overexpression as compared with 80% for untreated cells (Fig. 1B). In the rest of this study the free and bound activity were determined by permeabilization with 0.0375 mg/ml digitonin and 5 mM MgCl\(_2\).

Effects of Glucokinase Overexpression on Enzyme Binding—
Fig. 2 shows the increase in free and bound glucokinase activity in cells with a range of glucokinase expression from endogenous levels to 8-fold increase. Before permeabilization with digitonin the hepatocytes were preincubated for 30 min either with 0 mM glucose to maximize binding (Fig. 2A) or with 30 mM glucose to minimize binding (Fig. 2B). There was a greater increase in free than in bound activity with increasing enzyme expression at both 0 mM and 30 mM glucose with a consequent decrease in proportion of bound activity (Fig. 2C).

The data in Fig. 2 are representative of five experiments,
Effects of glucokinase overexpression on enzyme binding

Hepatocyte monolayers were treated with AdCMV-GKL as described in the legend to Fig. 2. They were then incubated for 30 min in fresh medium containing 2 μM cycloheximide and either 0 or 30 mM glucose and free and bound glucokinase activity were determined. Data represent total or bound glucokinase activity in control (untreated) cells or cells overexpressing glucokinase by approximately 6-fold above endogenous activity. Subscripts represent the glucose concentration in the preincubation before permeabilization. Values are means ± S.E. for five experiments.

| Glucose Concentration | Total GK Activity (milliunits/mg) | Binding Capacity (nM) | Dissociation Constant (Kd) |
|------------------------|-----------------------------------|----------------------|---------------------------|
| 0 mM glucose           | 16.4 ± 2.1                        | 103 ± 11             | 0.66 ± 0.07b              |
| 5 mM glucose           | 13.7 ± 1.9                        | 36.9 ± 2.9a          | 0.04 relative to control cells. |
| 20 mM glucose          | 6.3 ± 2.4                         | 23.7 ± 4.1           | 0.07 relative to control cells. |
| 50 mM glucose          | 7.4 ± 1.3                         | 13.2 ± 1.4           | 0.04 relative to control cells. |
| 100 mM glucose         | 83 ± 4                            | 37 ± 4              | 0.04 relative to control cells. |
| 200 mM glucose         | 38 ± 4                            | 23 ± 1              | 0.04 relative to control cells. |
| 500 mM glucose         | 54 ± 5                            | 36 ± 3              | 0.04 relative to control cells. |
| 1000 mM glucose        | 45 ± 4                            | 14 ± 3              | 0.04 relative to control cells. |

* p < 0.001 relative to control cells.

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Effects of glucose and sorbitol on the Kd of the high-affinity binding site

The effects of different glucose and sorbitol concentrations on the Kd of the high-affinity binding site were determined from plots of free/bound activity (Fig. 3). The binding capacity (n) and Kd of the high-affinity binding site were determined from plots of free/bound versus free activity as in Fig. 3. Values are mean ± S.E. for the number (n) of experiments indicated.

| Substrates | n | Binding Capacity (n) | Dissociation Constant (Kd) |
|------------|---|---------------------|---------------------------|
| 0 mM glucose | 7 | 3.3 ± 0.2          | 0.14 ± 0.02               |
| 5 mM glucose | 13| 2.7 ± 0.2          | 0.27 ± 0.03a              |
| 10 mM glucose | 11| 2.5 ± 0.2         | 0.54 ± 0.09b              |
| 20 mM glucose | 7 | 2.2 ± 0.1         | 0.08 ± 0.07b              |
| 5 mM glucose + 50 μM sorbitol | 7 | 2.4 ± 0.3     | 0.92 ± 0.10b              |
| 5 mM glucose + 10 mM insulin | 3 | 3.1 ± 0.3       | 0.39 ± 0.03               |

* p < 0.05; relative to the respective controls at 0 mM glucose.

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Effects of [substrate] on the Kd of the high-affinity binding site

Hepatocytes were treated with AdCMV-GKL (2–30 pfu/cell) and incubated for 15–18 h. They were then incubated for a minimum of 30 min in DMEM with the substrate concentrations indicated and then permeabilized with digitonin (as described in the legend to Fig. 2). The binding capacity (n) and Kd of the high-affinity binding site were determined from plots of free/bound versus free activity as in Fig. 3. Values are mean ± S.E. for the number (n) of experiments indicated.

| Substrates | n | Binding Capacity (n) | Dissociation Constant (Kd) |
|------------|---|---------------------|---------------------------|
| 0 mM glucose | 7 | 3.3 ± 0.2          | 0.14 ± 0.02               |
| 5 mM glucose | 13| 2.7 ± 0.2          | 0.27 ± 0.03a              |
| 10 mM glucose | 11| 2.5 ± 0.2         | 0.54 ± 0.09b              |
| 20 mM glucose | 7 | 2.2 ± 0.1         | 0.08 ± 0.07b              |
| 5 mM glucose + 50 μM sorbitol | 7 | 2.4 ± 0.3     | 0.92 ± 0.10b              |
| 5 mM glucose + 10 mM insulin | 3 | 3.1 ± 0.3       | 0.39 ± 0.03               |

* p < 0.05; relative to the respective controls at 0 mM glucose.

Effects of Glucose and Sorbitol on the Kd of the High-affinity Binding Site—Glucokinase translocation is induced by high glucose concentrations (10–20 mM) and by micromolar concentrations of sorbitol or fructose (11, 13, 14). We investigated the effects of these substrates on the binding characteristics of glucokinase by incubating hepatocytes (with varying glucokinase overexpression) with these substrates for 30 min before permeabilization with digitonin. Increasing glucose concentration from 0 to 20 mM was associated with a 5-fold increase in the Kd of the high-affinity binding site. Sorbitol (50 μM) caused a 3-fold increase in the Kd and insulin caused a small increase (Table II). The effect of sorbitol establishes that this site is the regulatory protein.

Effects of Overexpressed Glucokinase on Incorporation of [2-3H, U-14C]Glucose into Glycogen—Fig. 4 shows the effects of increasing glucose concentration on the free (unbound) activity of glucokinase in either untreated cells expressing only the endogenous enzyme (Fig. 4A) or in relation to increasing levels of glucokinase overexpression (Fig. 4B). Although the absolute activities of free glucokinase are higher at high glucose concentration (Fig. 4B) the fold increase in free glucokinase activity (in virus-treated relative to untreated controls) is higher at low glucose concentration (Fig. 4C), because of the low free activity in control cells at low glucose. It is noteworthy that a 2-fold increase in total glucokinase activity is associated with a 2-fold increase in free glucokinase activity at 30 mM glucose but with a 6-fold increase in free activity at 0 mM glucose (Fig. 4C). Glucokinase overexpression might therefore be expected to affect glucose metabolism differently at low and high glucose depending on the relative contributions of the free or bound states of the enzyme to pathway flux. We therefore determined the effects of glucokinase overexpression on glycogen synthesis at two glucose concentrations (5 and 10 mM glucose).

Table III shows the effects of 3 levels of glucokinase overexpression (1.4-, 1.9-, and 2.8-fold relative to endogenous activity) on the incorporation of [2-3H, U-14C]glucose into glycogen, which is expressed as the fold change in labeling rate relative to untreated controls. Incorporation of [14C]glucose into glycogen approximates glycogen synthesis from glucose by direct and indirect pathways (27) whereas incorporation of [2-3H]glucose into glycogen occurs only by the direct pathway (glucose → Glu-6-P → Glu-1-P → UDP-Glu → glycogen), but is an underestimate of the direct pathway because 2-tritium is lost as 3H2O in the conversion of Glu-6-P to Fru-6-P (28, 29). The increase in 14C labeling in glycogen at 5 mM glucose (1.9-, 3.0-, and 4.1-fold) is greater than the increase in total glucokinase activity (1.4-, 1.9-, and 2.8-fold) but lower than the increase in free glucokinase activity (2.7-, 4.5-, and 7.8-fold). The increase in 2-tritium labeling (3.3-, 6.9-, and 10.2-fold) was greater than the increase in 14C labeling and similar or greater than the
Effects of glucokinase overexpression on the incorporation of [2-3H, U-14C]glucose into glycogen

Hepatocytes monolayers were treated with AdCMV-GKL (3, 6, or 12 pfu/cell) and cultured for 15 h. The incorporation of [2-3H, U-14C]glucose into glycogen was determined from 3-h incubations at either 5 or 10 mM glucose. Glucokinase binding was determined from parallel incubations at either 0, 5, 10, or 30 mM glucose. Glucokinase activity (total or free) and incorporation of [U-14C]glucose or [2-3H]glucose into glycogen are expressed as a ratio to the corresponding values in untreated cells. The basal rates (in untreated cells) of incorporation of [U-14C]glucose were 4.7 ± 1.8 and 19.4 ± 6.8 and of [2-3H]glucose, 2.1 ± 0.9 and 11.8 ± 2.2 nmol/3 h/mg at 5 and 10 mM glucose, respectively. The endogenous total glucokinase activity was 16.4 ± 2.5 and the free activity at 5 and 10 mM glucose were 4.6 ± 0.4 and 6.4 ± 0.6 milliunits/mg (assayed with 100 mM glucose as substrate). Values are means ± S.E. for five experiments except for the free glucokinase data that are means ± S.E. for three experiments.

Table III

**Effects of glucokinase overexpression on the incorporation of [2-3H, U-14C]glucose into glycogen**

| AdCMV-GKL (pfu/cell) | 3 | 6 | 12 |
|----------------------|---|---|----|
| **GK activity**      |   |   |    |
| Total                | 1.4 ± 0.1 | 1.9 ± 0.3 | 2.8 ± 0.5 |
| Free at 0 mM glucose | 2.8 ± 0.4 | 5.6 ± 0.8 | 10.3 ± 1.3 |
| Free at 5 mM glucose | 2.7 ± 0.3 | 4.5 ± 0.5 | 7.8 ± 0.9 |
| Free at 10 mM glucose| 2.1 ± 0.1 | 3.5 ± 0.3 | 6.2 ± 0.6 |
| Free at 30 mM glucose| 1.4 ± 0.2 | 2.0 ± 0.3 | 2.8 ± 0.6 |
| **[U-14C]Glucose incorporation** |   |   |    |
| at 5 mM glucose      | 1.9 ± 0.3 | 3.0 ± 0.4 | 4.1 ± 0.9 |
| at 10 mM glucose     | 1.5 ± 0.2 | 2.4 ± 0.4 | 3.1 ± 0.7 |
| **[2-3H]Glucose incorporation** |   |   |    |
| at 5 mM glucose      | 3.3 ± 0.6 | 6.9 ± 1.0 | 10.2 ± 2.4 |
| at 10 mM glucose     | 2.5 ± 0.5 | 4.5 ± 0.9 | 6.4 ± 1.6 |

increase in free glucokinase activity. The fold increase in 14C and 3H labeling at 10 mM glucose were lower than at 5 mM glucose (Table III).

The Sensitivity of Glycogen Synthesis to Glucokinase Overexpression—The sensitivity of glycogen synthesis to changes in glucokinase activity can be expressed by the control coefficient which is a measure of the increase in flux that results from small increases in enzyme activity (25, 26). The relation between flux and enzyme activity is generally non-linear and the coefficient decreases with increasing enzyme activity. The coefficient is thus defined relative to a specific enzyme activity. In order to determine the relation between glycogenic flux and glucokinase activity we performed experiments similar to the ones in Table III, but with five virus titers (1–12 pfu/cell; Fig. 5). The control coefficients were determined from the initial slope of log flux versus either log total glucokinase activity (Fig. 5, A and C) or log free glucokinase activity (Fig. 5, B and D) and describe the sensitivity of glycogen synthesis to glucokinase at the endogenous activity. The coefficients were higher for 2-tritium labeling than for 14C labeling (Fig. 5, C versus A, D versus B) and they were higher when expressed relative to total glucokinase activity than relative to free glucokinase activity (Fig. 5, A versus B and C versus D). When expressed relative to total glucokinase activity they were higher at 5 mM glucose than at 10 mM glucose (Fig. 5A, 3.6 versus 2.2 and Fig. 5C, 7.2 versus 4.8). This difference (5 > 10 mM) was no longer observed when the coefficients were expressed relative to free glucokinase activity (Fig. 5, B and D).

The control coefficients for the experiments in Table III and Fig. 5, were also estimated by the Taylor expansion (see “Experimental Procedures,” Equation 2). This approximation showed similar trends as for the coefficients determined from the slope of log flux versus log enzyme activity in Fig. 5, in that the coefficients were higher for 3H labeling than for 14C labeling; they were higher when expressed relative to total as opposed to free glucokinase activity; and when expressed relative to total glucokinase they were higher at 5 mM glucose than at 10 mM glucose (Table IV).

In estimates of control coefficients of enzymes it is often assumed that the relation between pathway flux and enzyme activity is hyperbolic (25). However, the data in Fig. 5 are not consistent with a rectangular hyperbola but they show positive cooperativity with a Hill number >2 (see data of Hill plots in Table V). It is of interest that the total glucokinase activity that caused half-maximal increase (GK50) in glycogen synthesis (~4 milliunits/mg at 5 mM glucose and 7–9 milliunits/mg at 10 mM glucose) is within the physiological range of hepatic glucokinase activity. Furthermore, the maximum rate of glycogen synthesis (GSmax) at saturating glucokinase activity is about 2.7-fold higher at 10 mM than at 5 mM glucose (Table V). Thus although the sensitivity of glycogen synthesis to total glucoki-
nase overexpression is greater at 5 mM glucose, the maximum rate of synthesis is greater at 10 mM glucose. These results are consistent with previous findings of a 3-fold higher rate of glycogen synthesis at 10 mM compared with 5 mM glucose at maximum glucokinase overexpression (17).

**DISCUSSION**

In the present study, by combining adenovirus-mediated glucokinase overexpression with digitonin permeabilization we evaluated the effect of increments in total glucokinase activity on the distribution of the enzyme between bound and free states. Because the amount of the regulatory protein as determined by Western blotting was unchanged in cells overexpressing glucokinase, it was possible to determine the capacity and affinity of glucokinase binding in situ. The data show that there is high-affinity binding for glucokinase with a binding capacity that exceeds the molar amount of endogenous glucokinase by less than 2-fold. The $K_d$ of this high-affinity binding was dependent on the substrates with which the hepatocytes were preincubated before permeabilization with digitonin. The increase in $K_d$ of this site with sorbitol (a precursor of fructose 1-P (11)) confirms that this site is the regulatory protein because binding of glucokinase to the regulatory protein is dissociated by fructose 1-P (6).

There was also low-affinity binding with a $K_d$ of $\sim 1.6 \, \mu M$ and a capacity corresponding to approximately 2–3 times that of the high-affinity binding. The low-affinity binding, unlike the high-affinity binding was very sensitive to increasing digitonin concentration suggesting that it may be associated with membranes. It is as yet unclear whether this low-affinity binding

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**TABLE IV**

| Glucose      | $C_{GK_{\text{GK}}}^{eGK}$ | $C_{GK_{\text{H}}}^{eGK}$ | $C_{GK_{\text{F}}}^{eGK}$ |
|--------------|-----------------------------|----------------------------|-----------------------------|
| 5 mM glucose | 4.1 $\pm$ 0.8               | 11.4 $\pm$ 2.2             | 0.99 $\pm$ 0.16             |
| 10 mM glucose| 2.8 $\pm$ 0.7$^a$           | 6.6 $\pm$ 1.6$^a$          | 0.94 $\pm$ 0.44             |

$^a p < 0.005$ relative to corresponding values at 5 mM glucose.

**TABLE V**

| Glucose Incorporation | [Glucose] | GK Activity | Hill No. | $K_{GK}$ | $G_{S_{\text{max}}}$ |
|-----------------------|-----------|-------------|----------|----------|--------------------|
|                       |           |             |          | (h)      | (milliunits/mg)    |
| 5A [U-14C]            | 5         | Total       | 2.6      | 3.9      | 0.19               |
| 5A [U-14C]            | 10        | Total       | 3.4      | 7.2      | 0.49               |
| 5C [2-3H]             | 5         | Total       | 2.8      | 4.3      | 0.17               |
| 5C [2-3H]             | 10        | Total       | 4.7      | 9.4      | 0.48               |
| 5B [U-14C]            | 5         | Free        | 1.8      | 1.5      | 0.20               |
| 5B [U-14C]            | 10        | Free        | 3.4      | 3.4      | 0.48               |
| 5D [2-3H]             | 5         | Free        | 2.0      | 1.7      | 0.17               |
| 5D [2-3H]             | 10        | Free        | 3.4      | 3.8      | 0.48               |
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constitutes a specific receptor for glucokinase. The possibility that this site may be a low-affinity conformation of the regulatory protein cannot be excluded. The binding characteristics of this low-affinity binding could only be determined in cells incubated at low glucose because of non-linearity of the plots (free/bound versus free activity) at high glucose. Linearity is expected if binding is non-cooperative (22). Whether the non-linearity at high glucose was due to co-operative binding could not be resolved from the data.

A key finding from the binding experiments is that a small increase in glucokinase overexpression (by less than 40% above endogenous levels) is associated with a much larger fractional increase in the free glucokinase activity because the percentage binding decreases sharply with overexpression. This effect is most marked at low glucose (0–5 mM). The physiological implication is that a small change in the total amount of hepatic glucokinase as occurs between fed and fasted states would be associated with a much larger change in free activity, if the amount of the regulatory protein does not change by a corresponding amount. The amount of regulatory protein in rat liver decreases during fasting and increases during refeeding. However, the proportional changes of the regulatory protein are smaller than the changes in the amount of glucokinase (30). Our findings therefore suggest that changes in the total amount of glucokinase during fasting or refeeding would be associated with larger changes in free glucokinase activity.

Adenovirus-mediated glucokinase overexpression is associated with a large increase in glycogen synthesis (17), consistent with a major role of changes in total amount of glucokinase in controlling glycogen synthesis. In the present study we used metabolic control analysis (determination of control coefficients) to evaluate the sensitivity of glycogen synthesis to small increases in glucokinase activity. Control coefficients express the sensitivity of pathway flux to small changes in total enzyme activity and represent the slope of plots of log flux versus log enzyme activity. Since glucokinase partitions between bound and free states and glucokinase overexpression is associated with larger increases in free than in bound activity we determined the coefficients in relation to either total glucokinase activity (which would be equivalent to the total enzyme concentration) or to the free fraction which would approximate glucokinase activity if the bound fraction is much less active than the free activity as predicted from the kinetic properties of glucokinase in association with the regulatory protein (4).

The coefficients expressed in relation to total glucokinase activity report three findings. First, that the coefficient (or sensitivity of flux) is greater for incorporation of 2-tritium than of 14C label into glycogen. Second, that the coefficients for incorporation of both 14C and 2-tritium are greater above endogenous levels as are observed during refeeding after a fast in vivo (30), result in much larger changes in glycogen synthesis as indicated by control coefficients greater than unity. Second, that glucokinase-induced translocation of glucokinase as a result of the 5-fold increase in the Kd of the high-affinity binding site is a major mechanism for the acute control of glucokinase activity as indicated by the higher control coefficient of glycogen synthesis to total glucokinase activity at 5 mM as compared with 10 mM glucose. We suggest that adenovirus-mediated glucokinase overexpression is a powerful tool to evaluate the roles of glucokinase expression and of short-term glucokinase translocation to the control of hepatic glycogen synthesis.

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