Biophysical Characterization of the Interaction between Hepatic Glucokinase and Its Regulatory Protein

IMPACT OF PHYSIOLOGICAL AND PHARMACOLOGICAL EFFECTORS*

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Glucokinase (GK) is a key enzyme of glucose metabolism in liver and pancreatic β-cells, and small molecule activators of GK (GKAs) are under evaluation for the treatment of type 2 diabetes. In liver, GK activity is controlled by the GK regulatory protein (GKRP), which forms an inhibitory complex with the enzyme. Here, we performed isothermal titration calorimetry and surface plasmon resonance experiments to characterize GK-GKRP binding and to study the influence that physiological and pharmacological effectors of GK have on the protein-protein interaction. In the presence of fructose-6-phosphate, GK-GKRP complex formation displayed a strong entropic driving force opposed by a large positive enthalpy; a negative change in heat capacity was observed (KD = 45 nM, ΔH = 15.6 kcal/mol, TΔS = 25.7 kcal/mol, ΔCp = −354 cal mol−1 K−1). With koff = 1.3 × 10−2 s−1, the complex dissociated quickly. The thermodynamic profile suggested a largely hydrophobic interaction. In addition, effects of pH and buffer demonstrated the coupled uptake of one proton and indicated an ionic contribution to binding. Glucose decreased the binding affinity between GK and GKRP. This decrease was potentiated by an ATP analogue. Prototypical GKAs of the amino-heteroaryl-amide type bound to GK in a glucose-dependent manner and impaired the association of GK with GKRP. This mechanism might contribute to the antidiabetic effects of GKAs.

Glucokinase (GK)2 is the predominant glucose-phosphorylating enzyme in liver and pancreatic β-cells and plays a central role in blood glucose homeostasis (1, 2). Enhancing GK activity by small molecule GK activators (GKAs) is currently under evaluation as an approach for the treatment of diabetes (3). Hepatic GK activity is controlled by an endogenous inhibitor, a 68-kDa GK regulatory protein (GKRP) (4). During starvation, the enzyme is bound to GKRP, leading to its inactivation and sequestration in the nucleus. After refeeding, the GK-GKRP complex dissociates and GK translocates into the cytoplasm (5, 6). In a rodent model of type 2 diabetes, this translocation is impaired, which could contribute to the defective blood glucose homeostasis (7). Further evidence for the metabolic impact of GKRP comes from recent human genome-wide association studies that show a strong linkage between a GKRP gene polymorphism and serum triglyceride levels (8, 9). The formation of the GK-GKRP complex is favored by fructose-6-phosphate (F6P) and inhibited by fructose-1-phosphate (F1P) (10). Both sugar phosphates bind to the same site on the regulatory protein (11). An increase of the intracellular F1P concentration leading to the release of GK from its inhibitory complex with GKRP is most likely the reason for the stimulation of hepatic glucose metabolism by catalytic amounts of fructose or sorbitol (6, 12, 13). Site-directed mutagenesis experiments indicate that the binding interface for GKRP lies close to the binding site of allosteric GKAs of the amino-heteroaryl-amide type (14). However, it is controversial if these GKAs have an effect on the formation of the GK-GKRP complex (15, 16). Furthermore, there are conflicting reports concerning whether glucose has a direct effect on the GK-GKRP complex (15, 17).

Current knowledge of the molecular details of the GK-GKRP interaction and its regulation is limited and originates mainly from indirect or qualitative methods such as enzymatic assays or co-immunoprecipitation (16, 18). However, despite the importance of biophysical methods for the validation and characterization of protein-protein interactions (19), such studies are lacking for GK and GKRP. In the present study we performed isothermal titration calorimetry (ITC) and surface plasmon resonance (SPR) experiments for a detailed quantitative characterization of the GK-GKRP interaction and its modulation by small molecules.

EXPERIMENTAL PROCEDURES

Chemical Synthesis—The prototypical small-molecule glucokinase activators of the amino-heteroaryl-amide type, GKA1 (15) and RO-28–1675 (termed GKA2 in the present study) (3) were synthesized in-house.

Expression and Purification of Glucokinase—The cDNA of human liver GK was cloned into pQE30 (Qiagen, Hilden, Germany), which adds an N-terminal hexahistidyl tag to the open reading frame. The plasmid was transformed into Escherichia coli strain M15(pRep4) (Qiagen). After induction with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside at ΔD600 = 0.7, cells were grown for 18 h at 18 °C. Harvested cells were resuspended...
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and lysed by lysozyme treatment and ultrasonication. GK was enriched from the filtered lysate using nickel ion affinity chromatography (0–0.5 M imidazole gradient). After desalting, the sample was applied to a Q-Sepharose anion exchange column and eluted with a 0.05–1 M NaCl gradient. GK was concentrated by ultrafiltration and applied to a Superdex 200 gel filtration column (GE Healthcare, Chalfont St. Giles, UK). After elution in 25 mM Bis-Tris propane, 150 mM NaCl, 50 mM glucose, 5 mM dithiothreitol, 5% glycerol, pH 7, samples were concentrated, and aliquots were stored at −80 °C.

Expression and Purification of Glucokinase Regulatory Protein—The cDNA of rat GKR (kindly provided by Emilie Van Schaftingen, Université Catholique de Louvain, Brussels) was ligated into pET21a (Merck Chemicals, Darmstadt, Germany); this appends a C-terminal hexahistidyl tag to GKR. The GKR mutant S183A/V187A was generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and custom primers. Expression of GKR was performed in E. coli strain BL21-CodonPlus(DE3)-RIL (Stratagene). After induction with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside at $D_{600} = 0.7$, cells were grown at 18 °C for 60 h. Harvested cells were resuspended and lysed by lysozyme treatment and ultrasonication. The cleared lysate was applied to an immobilized nickel ion affinity column and eluted with a 20–500 mM imidazole gradient. After dialysis in 20 mM Tris, 20 mM NaCl, 1 mM dithiothreitol, pH 7.5, the sample was applied to a Q-Sepharose anion exchange column and eluted with a 0.02–1 M NaCl gradient. 20% glycerol was added to the concentrated samples, and aliquots were stored at −80 °C.

Enzymatic Assay of Glucokinase Regulatory Protein—The effect of GKR on GK activity was determined spectrophotometrically using the glucose-6-phosphate dehydrogenase coupled assay as described elsewhere (20). One unit of GKR corresponds to the amount of protein that causes 50% inhibition of GK activity assayed in the presence of 5 mM glucose and 200 μM F6P (10).

Isothermal Titration Calorimetry—ITC measurements were carried out on a VP-ITC ultrasensitive titration calorimeter (MicroCal Inc.). Protein samples were dialysed twice for several hours at 4 °C against the measuring buffer and centrifuged (20,000 × g, 15 min, 4 °C) to remove insoluble matter. Unless otherwise stated, the standard buffer for the GK-GKR titrations was 50 mM Hepes, 25 mM KCl, 4 mM MgCl$_2$, 1 mM β-mercaptoethanol, 200 μM F6P, pH 7.1. Protein concentration was determined from UV absorbance measurements using a molar extinction coefficient at 280 nm of 32,890 M$^{-1}$ cm$^{-1}$ (GK) and 47,900 M$^{-1}$ cm$^{-1}$ (GKR). In a typical experiment, the stirred measuring cell contained 10 μM GKR and the syringe contained 80 μM GK as titrant. In experiments, where the influence of effectors on the GK-GKR interaction was studied, these compounds were added at equal concentrations to both cell and syringe solutions. ITC titrations and data analyses were performed as described (21). Unless otherwise stated, the titrations were performed at 25 °C. Proton transfer linked to GK-GKR binding was determined by measuring the pH dependence of binding affinity (22). Furthermore, proton linkage was determined by measuring $\Delta H_i$ of binding in buffers with different enthalpies of ionization $\Delta H_{ion}$ (23): Values for $\Delta H_{ion}$ were obtained from the literature (24, 25). To determine $\Delta C_p$ (= $d\Delta H/dT$) for the GK-GKR interaction, ITC titrations were performed over a range of temperatures. To predict the change in accessible surface area ($\Delta ASA$) upon binding from the binding heat capacity ($\Delta C_p$) and enthalpy ($\Delta H$) changes, the following empirical relationships were applied: $\Delta C_p = 0.45\Delta ASA_{np} - 0.26\Delta ASA_p$; $\Delta H_{60} = -8.44\Delta ASA_{np} + 31.4\Delta ASA_p$, where $\Delta H_{60}$ is the extrapolated binding enthalpy change at 60 °C in kcal/mol (26, 27). In these calculations, $\Delta H$ was corrected for the contribution of buffer ionization enthalpy.

Surface Plasmon Resonance Experiments—Experiments were performed on a Biacore S51 instrument (GE Healthcare, Chalfont St. Giles, UK). For the immobilization of rat GKR to CM5 sensor chips, standard amine coupling was used. GKR was diluted in 10 mM sodium acetate buffer pH 5.5 including 20 mM KCl and 200 μM F6P to a final concentration of 12 μg/ml. The immobilization level was aimed at 2500 RU. Series of various concentrations of GK that was previously dialysed in the running buffer were injected over the flow cell (flow rate 30 μl/min, contact time 120 s, dissociation time 180s). For steady-state equilibrium analysis, the sensorgrams were analyzed using Biacore S51 evaluation software version 1.2. As the global fitting routine of the evaluation software did not produce satisfactory results, the association and dissociation time courses were separately fitted to a single-exponential model, as described in Ref. 28, using Prism 4 (GraphPad Software, San Diego, CA). The influence of glucose and GKA on the dissociation of the preformed GK-GKR complex was tested using the regeneration scout tool provided by Biacore S51 control software. Here, after complex formation under running buffer conditions, the “regeneration buffer” was used for the dissociation phase, which contained the desired additives.

Statistical Analysis—Unpaired Student’s t-tests (two-tailed $p$, confidence interval 95%) were performed using Prism 4.

RESULTS

Characterization of Recombinant GK and GKR—Because recombinant human GK can be obtained only at very low yields (20, 29), we studied the interaction between GK and GKR derived from different species; human and rat, respectively. Human and rat liver GK share 97% sequence identity and are inhibited to the same extent by rat GKR (20, 30). The heterologous interaction between rat GKR and human GK is a well established model system (11, 30–33). However, human GKR has a higher affinity for F6P compared with rat GKR and is a more potent inhibitor of GK in the absence of F6P (20).

The preparations of GK and GKR for the present study were ~95% pure and homogenous, as judged by Coomassie Blue staining (exemplarily shown for GKR, Fig. 1A) and analytical gel filtration. GK was inhibited by GKR in a dose-dependent manner (Fig. 1B). From these data we determined a specific GK-GKR activity of ~700 units/mg in the presence of 200 μM F6P, which compares well to previously reported activities (33). Consistent with published data (34), F6P enhanced the potency of GKR, while F1P reduced the inhibitory effect (Fig. 1C). Using ITC, we determined a $K_i = 17 ± 4$ μM for F6P binding to GKR (Fig. 1D). This directly determined affinity is in good agreement with the reported $K_i$ of 20 μM that was derived from

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is in remarkable contrast to thermodynamic profiles determined for a number of protein-protein interactions; these very often show similar contributions of both enthalpy and entropy to the binding free energy (35) (Fig. 2B). The observed large binding entropy suggests a distinctively hydrophobic binding interface (36–38). Recently, a hydrophobic sequence motif within GKRP identified by phage display was postulated to form a binding site for GK (39). We therefore expressed a GKRP variant where the consensus residues within the identified sequence were mutated to alanine (GKRP S183A/V187A) and determined its binding parameters. In the presence of F6P the affinity of GKRP for GK was decreased only slightly (31335, with no significant changes of interaction increased with rising glucose concentrations (Fig. 2C). Despite its clear effect in the enzymatic assay, we could not calorimetrically detect binding of F1P to GKRP under a number of different experimental conditions. Possibly the binding of F1P is exclusively of entropic nature and shows a negligible heat change.

Thermodynamics of the GK-GKRP Interaction and the Influence of Physiological Effectors—In the presence of 200 μM F6P, ITC titrations of GK and GKRP showed a binding signal with strongly endothermic peaks (Fig. 2A). A $K_d$ value of 45 nM was obtained as an average result from multiple titrations (Table 1). As expected, in the absence of F6P binding became considerably weaker, with a $K_d$ value of 984 nM (Table 1). In the presence of saturating concentrations of F1P, the affinity of GKRP for GK was decreased, with $K_d = 7$ μM. To our knowledge, these are the first direct determinations of affinity for the GK-GKRP interaction. Under all measurement conditions the GK-GKRP interaction was exclusively entropy driven ($\Delta H > 0$) with a large enthalpic “penalty” ($\Delta S > 0$) (Fig. 2B and Table 1). This next, we studied the effect of glucose on the GK-GKRP interaction. The $K_d$ values determined for the protein-protein interaction increased with rising glucose concentrations (Fig. 2C; Table 1, thermodynamic parameters determined in the presence of 100 mM glucose). The presence of neither glycerol nor sorbitol at 100 mM influenced the GK-GKRP interaction, indicating that the effect of glucose is specific and not caused by the high osmolarity of the buffer (not shown). These data clearly establish that glucose directly weakens the GK-GKRP interaction. Interestingly, the addition of the non-hydrolyzable ATP analogue AMP-PNP at 1 mM enhanced the effect of glucose (Fig. 2C; Table 1, thermodynamic parameters determined in
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**TABLE 1**

| Effector | $N^e$ | $K_d^{ITC}$ | $\Delta H$ | $T\Delta S$ |
|----------|-------|-------------|-------------|-------------|
| None     | 1.0 ± 0.03 | 984 ± 190 | 23.3 ± 1.4 | 31.4 ± 1.3 |
| 100 μM F6P | 0.9 ± 0.00 | 6789 ± 253 | 21.7 ± 2.5 | 28.7 ± 2.4 |
| 200 μM F6P | 1.0 ± 0.02 | 45 ± 10 | 15.6 ± 0.7 | 25.7 ± 0.6 |
| 200 μM F6P + 100 mM glucose | 1.0 ± 0.00 | 173 ± 17 | 6.0 ± 0.8 | 15.2 ± 0.7 |
| 200 μM F6P + 25 mM glucose + 1 mM AMP-PNP | 1.0 ± 0.01 | 164 ± 18 | 5.9 ± 0.9 | 15.2 ± 0.9 |

*a* Stoichiometry of the complex.

**FIGURE 3. Effects of pH, buffer and temperature on the GK-GKRP binding thermodynamics.** Experiments under (A) and (B) were performed at 25 °C in the presence of 200 μM F6P. A, ITC titrations in HEPES buffer at different pH values. Values of −log $K_d$ are plotted versus pH; B, ITC titrations at pH 7.1 (squares) and pH 8 (triangles) in HEPES or Tris buffer. The observed binding enthalpy determined from the fit of the ITC isotherms is plotted versus the buffer ionization enthalpy. Lines, linear regressions of the data. Each data point represents the average from two independent ITC titrations. C, heat capacity changes ($\Delta C_p$) for GK-GKRP binding. ITC titrations were performed at different temperatures, in the absence (open squares) or presence of 200 μM F6P (solid squares), or 200 μM F6P + 100 mM glucose (triangles). The obtained $\Delta H$ values are plotted versus temperature, and the slope of the linear regressions gives $\Delta C_p$. The presence of 25 mM glucose + 1 mM AMP-PNP) AMP-PNP alone did not influence the interaction of the proteins (data not shown).

**Effects of pH, Buffer, and Temperature on the Thermodynamic Parameters**—To assess a potential role of protonable residues for GKRP-GK binding, we performed ITC measurements in the presence of F6P at different pH values. The pH dependence of the binding affinity (Fig. 3A) with a slope (−log $K_d$ versus pH) = 1.1 pointed to a single protonation event that is important for binding. This was confirmed by the buffer dependence of the observed binding enthalpy (Fig. 3B): With the buffer dependence measured at pH 8, the slope ($\Delta H_{\text{observed}}$ versus $\Delta H_{\text{buffer ionization}}$) = 0.9 again reflected the net uptake of one proton. On the other hand, at pH 7.1, only a minor buffer dependence of $\Delta H$ was apparent (slope = 0.2), meaning that at this pH no coupled protonation took place. These data point to a pK value of the protonated group near neutrality, which makes histidine a potential candidate residue that becomes positively charged in the course of GK-GKRP binding.

The change in heat capacity upon binding ($\Delta C_p = \Delta H/dT$) is sensitive for changes in the binding interface of interacting proteins (40). In the presence of 200 μM F6P, a negative $\Delta C_p$ value of $-354 \pm 36$ cal mol$^{-1}$ K$^{-1}$ was obtained (Fig. 3C). When glucose was added to the titration buffer, $\Delta C_p$ amounted to $-362 \pm 33$ cal mol$^{-1}$ K$^{-1}$. Thus, the presence of glucose had no significant effect on the heat capacity change upon binding. On the other hand, the positive $\Delta C_p = +1389 \pm 145$ cal mol$^{-1}$ K$^{-1}$ determined in the absence of F6P was strikingly different. These data therefore indicate that the binding interface is unaffected by glucose but strongly influenced by F6P. The heat capacity change can be tentatively used to estimate the change in accessible surface area ($\DeltaASA$) upon binding (see “Experimental Procedures”) (26, 27). This approach predicts that in the presence of F6P about 900 Å$^2$ of total surface area becomes buried during GK-GKRP complex formation.

**Binding of Small Molecule GK Activators and Their Effect on the GK-GKRP Interaction**—There is controversy whether the small molecule GKAs of the amino-heteroarylamide type also affect the GK-GKRP complex (2, 3, 15). To address this question, we first applied ITC to characterize the binding of prototypical GKAs of this class to GK. The binding of GKA1 (15) to GK absolutely depended on the presence of glucose (Fig. 4A). Following binding parameters were obtained for GKA1: stoichiometry $n = 1.0 \pm 0.03$, $K_d = 0.9 \pm 0.1$ μM, $\Delta H = -3.8 \pm 0.1$ kcal/mol, $T\Delta S = 4.5 \pm 0.2$ kcal/mol in HEPES buffer at 25 °C. Binding of another activator of this type, termed GKA2 (3), was also glucose-dependent. Here, $n = 1.0 \pm 0.1$, $K_d = 0.5 \pm 0.2$ μM, $\Delta H = -6.2 \pm 0.5$ kcal/mol, $T\Delta S = 2.4 \pm 0.4$ kcal/mol. When GKA1 was titrated to GK, which was already in a complex with GKRP, we did not observe binding of the compound (Fig. 4A). Next, GKRP was titrated to the GK-GKA1 complex formed in the presence of a saturating concentration of glucose (100 mM). Here, an interaction of GK and its regulatory protein could not be detected with the applied ITC settings (detection limit: $K_d \sim 10$ μM) (Fig. 4B). Titrations at different temperatures were performed to exclude that the lack of a binding signal was due to an accidental zero-crossing of $\Delta H$ (not shown). We found this impairment of the GK-GKRP complex also for GKA2. These findings suggest that the binding of this class of GKAs and GKRP to GK is mutually exclusive.

**Kinetic Analysis of the GK-GKRP Interaction**—Next we performed surface plasmon resonance experiments to further characterize the binding of GK to GKRP. GKRP was immobilized on the chip, while GK was used as analyte in solution. The presence of 100 mM glucose in the running buffer strongly lowered the SPR signal (Fig. 5A). 100 mM sorbitol had no effect on the resonance signal (not shown), excluding the possibility that the effect of glucose is caused by a high solute concentration. In agreement with the ITC results the SPR experiments showed that glucose impairs the interaction between GK and GKRP. The addition of GKA1 further lowered the SPR signal relative to glucose-containing buffer conditions (Fig. 5B). A similar obser-
molar $K_d^{\text{kin}}$ values obtained in the presence of GKAs were surprisingly low, given the apparent lack of binding in the ITC experiment. We therefore additionally determined the GKRP affinity in the presence of GKA1 or GKA2 using steady-state equilibrium analysis of the SPR data. Here, in both cases, higher $K_d^{\text{eq}}$ values of $4 \, \mu\text{M}$ were obtained. It appears that the actual affinity in the presence of GKAs is somewhat underestimated from $K_d^{\text{kin}}$. Finally we examined if glucose or GKAs also influenced the dissociation of the preformed GK-GKRP complex. To this end, we performed a SPR “chase” experiment in which GK was first bound to GKRP and the compound under investigation was added immediately before the dissociation phase. 100 mM glucose enhanced complex dissociation, while the two prototypical GKAs had no or only minor effects on the dissociation rate (Fig. 5D). The chase experiment indicates that glucose, but not GKAs, can bind to the preformed GK-GKRP complex. Taken together, the SPR experiments confirmed that both glucose and GKAs interfere with binding of the regulatory protein to GK.

**DISCUSSION**

Despite the significance of the regulation of GK by GKRP for blood glucose homeostasis, the current knowledge of its molecular details is limited. This is in part caused by difficulties in obtaining sufficient amounts of purified protein, especially of GKRP (20). In the present study, we established protocols for the expression of substantial amounts of both recombinant GK and GKRP. This allowed us to apply biophysical techniques to characterize the interaction between these proteins. One of our interests was the influence that physiological and pharmacological effectors of GK activity have on this protein-protein interaction. In our experiments F1P and F6P modulated GK-GKRP affinity in an opposing manner, with F1P attenuating and F6P strengthening the protein interaction. These results are in line with the data obtained by enzymatic analysis, and thereby validated our biophysical approach.

The binding process between GK and GKRP was highly endothermic and solely driven by the entropy change. From this, we suggest a distinctly hydrophobic binding interface and a rigid-body interaction of the two proteins (36–38). A hydrophobic binding site is in line with the observation that long-chain acyl-CoAs and GKRP compete for binding to GK (41). Our site-directed mutations of GKRP (S183A/V187A) indicate that further contact sites in addition to the recently reported motif (39) must be involved in this interaction. Furthermore, the pH and buffer dependence of the thermodynamic binding parameters suggested that the protonation of presumably a histidine residue is critical during the binding process. This points to an additional involvement of ionic attractive forces beside the hydrophobic interaction. An ionic contribution would agree with the observed thermodynamic profile, as charge interactions are often entropy-driven (42). A loop of GK that is rich in basic residues is critical for GKRP binding (43). Tentatively, the observed protonation could occur within this basic loop.

The heat capacity changes ($\Delta C_p$) that accompany biomolecular interactions are sensitive for the nature of the binding interface (40). A negative $\Delta C_p$ value, as determined for the GK-GKRP interaction in the presence of F6P, is typical for many
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![Graphs and diagrams]

FIGURE 5. Analysis of GK-GKRP binding by surface plasmon resonance. GK was used as analyte and GKRP as immobilized ligand; all running buffers contained 200 μM F6P. A, sensorgram with 220 nM GK as analyte. The running buffer contained either no glucose (dashed line) or 100 mM glucose (solid line). B, sensorgram with 250 nM GK as analyte. The running buffer contained 100 mM glucose, with the addition of either 0.1% DMSO (dashed line) or 10 μM GKA1 (solid line). C, sensorgram with different GK concentrations (13–425 nM). Solid line, experimental time courses; dashed line, separate fit of the association and dissociation phase to a one-exponential model. D, chase experiment with 425 nM GK; the indicated substances were added after formation of the GK-GKRP complex immediately before the dissociation phase. Inset, exemplary dissociation time courses in the absence (dashed line) or presence (solid line) of 100 mM glucose; gray lines, fit to one-exponential model. Column bar graph, effect of the indicated additive on the dissociation constant; the results are given as the quotient of $k_{off}$ obtained with regeneration buffer versus running buffer within the same experiment. Chase experiments where GKA1 were added contained 100 mM glucose in the running buffer.

TABLE 2

Kinetic data for the GK-GKRP interaction obtained by surface plasmon resonance experiments

| Effector          | $k_{on}$ | $k_{off}$ | $K_{d,SPR,kin}$ |
|-------------------|----------|-----------|------------------|
| 200 μM F6P        | $10^9$ M$^{-1}$ s$^{-1}$ | $10^{-2}$ s$^{-1}$ | ns               |
| 200 μM F6P + 100 mM glucose | 2.5 ± 0.32 | 1.3 ± 0.09 | 50              |
| 200 μM F6P + 100 mM glucose + 0.5 μM GKA1 | 1.2 ± 0.14 | 1.6 ± 0.04 | 131             |
| 200 μM F6P + 100 mM glucose + 10 μM GKA1 | 0.5 ± 0.08 | 2.0 ± 0.05 | 375             |
| 200 μM F6P + 100 mM glucose + 10 μM GKA2 | 0.3 ± 0.05 | 2.0 ± 0.07 | 661             |

$K_{d,SPR,kin} = k_{off}/k_{on}$

protein-protein interactions; it originates mainly from the release of water from protein surfaces (44). When we determined the heat capacity change upon GK-GKRP binding in the absence of F6P, we obtained a positive $\Delta C_p$ value. This could indicate additional hydration processes. Reported examples for positive $\Delta C_p$ values of biomolecular interactions are relatively rare and have been related to ionic interactions (45–47). Overall, from the comparison of the $\Delta C_p$ values obtained in the presence and absence of F6P, we propose that F6P induces substantial conformational changes within the GK-binding interface of GK and GKRP. The estimation from $\Delta C_p$ values of the change in accessible surface area upon binding ($\Delta A_{\text{ASA}}$) is discussed controversially in the literature (40, 42). When we applied the proposed empirical relationships, we determined 900 Å$^2$ as the sum of GK and GKRP buried surface in the presence of F6P. This value suggests a small binding interface. Reported values for total $\Delta A_{\text{ASA}}$ of heterodimeric protein-protein interactions range between 1300 and 6500 Å$^2$, with an average value of 2000 Å$^2$ (48). Furthermore, the dissociation rates of the GK-GKRP complex of $\sim 10^{-2}$ s$^{-1}$ as determined by SPR analysis were at the upper limit of the typical range of $10^{-2}$ to $10^{-6}$ s$^{-1}$ observed for biomolecular interactions (49). Dissociation rates primarily depend on the strength and number of direct contacts between the proteins (50), and a rapid dissociation as observed here could therefore also point to a small contact interface.

In the liver, an elevation of the cellular glucose concentration induces a translocation of active GK from the nucleus to the cytoplasm, which requires a dissociation from GKRP (5, 6). However, it is controversial if glucose by itself dissociates GK and GKRP (15, 17). Both our ITC and SPR results showed that glucose directly weakens GK-GKRP binding. This effect was potentiated by the ATP analogue AMP-PNP, in agreement with data obtained recently by co-IP experiments (16). Our quantitative analysis demonstrated that in the presence of AMP-PNP, the GK-GKRP interaction became substantially impaired at glucose concentrations that occur in the liver in the postprandial state. Therefore, the direct effect of glucose on the GK-GKRP affinity could be of physiological significance. This hypothesis might be supported by the SPR chase experiment that showed that glucose did not only decrease the binding affinity between GK and GKRP, but was also able to dissociate the preformed GK-GKRP complex. In structural terms this indicates that the active site of GK in a complex with the inhibitory protein is still accessible for the substrate glucose.

Based on x-ray structures it was proposed that the GK enzyme shuttles between at least three conformations: the “resting” super-open conformation and the active open and closed forms (51). The attenuating effect of glucose on the GK-GKRP interaction suggests that GKRP binds to the super-open conformation of GK, which prevails in the absence of glucose (51, 52). This model is further supported by the heat capacity change measurements. Here, the addition of glucose did not change the $\Delta C_p$ of the GK-GKRP interaction, relative to the
value determined in the presence of F6P alone. This indicates that although glucose lowers the binding affinity, it does not affect the binding interface between GK and GKRP. In the light of the global conformational change of GK during the inactive-to-active transition (51), this finding suggests that GKRP does not recognize the active, open conformation of GK. Rather, binding in the presence of glucose can only occur after isomerization of GK to the inactive super-open form. Overall, these results provide thermodynamic evidence that GK occurs in a pre-existing equilibrium as has been proposed recently (51, 52). The kinetic SPR data further support this concept. Here, glucose primarily affected the on-rate of the GK-GKRP interaction. This could indicate that the energetic barrier imposed by glucose precedes the actual binding step.

Small molecule GKAs of the amino-heteroaryl-amide type bind to a hydrophobic pocket at the interface between the two GK domains and allosterically activate the enzyme (51). Our ITC and SPR data demonstrate that prototypical GKAs of this class prevent binding of GKRP to GK. This observation is in line with recently published biochemical data on the effect of related GKAs (53). We found this impairment of the GK-GKRP association also for a compound (GKA1) that was previously suggested not to interfere with this protein-protein interaction (15). A likely explanation for this discrepancy is that in Ref. 15, the interaction study was performed in the absence of glucose, a condition under which GKAs cannot bind ((53), present study). The requirement of glucose for the binding of the studied GKAs indicates that these compounds cannot bind to the resting, super-open conformation of GK. On the other hand, our data strongly support the idea that GKRP binds only to the super-open conformation, as discussed above. In addition, the ITC titrations of the GKA to the preformed GK-GKRP complex as well as the SPR “chase” experiment indicated that the amino-heteroaryl-amide compounds do not bind to GK that is already in complex with its inhibitor protein. Overall, our results demonstrate that binding of GKA and GKRP to GK is mutually exclusive. We propose a dual mode of action of this class of GKA in the liver: These compounds directly activate the enzyme and additionally prevent binding of the inhibitory GKRP to hepatic GK. Potentially, both contribute to the antidiabetic effect of these molecules.

In conclusion, this study provides the first biophysical characterization of the interaction between GK and GKRP and its modulation by physiological and pharmacological effectors. In the absence of structural information, our findings put important constraints on models of the GK-GKRP interaction. The regulation of hepatic GK is central for glucose homeostasis, and GK activation is a promising approach to decrease hyperglycemia in type 2 diabetes. Against this background, it is of importance to precisely understand the complex allosteric regulation of GK.

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REFERENCES

1. Matschinsky, F. M., Magnuson, M. A., Zelent, D., Jetton, T. L., Doliba, N., Han, Y., Taub, R., and Grimsby, J. (2006) Diabetes 55, 1–12
2. Baltrusch, S., and Tiedge, M. (2006) Diabetes 55, Suppl. 2, 552–564
3. Grimsby, J., Saraba, R., Corbett, W. L., Haynes, N. E., Bizzarro, F. T., Coffey, J. W., Guertin, K. R., Hilliard, D. W., Kester, R. F., Mahaney, P. E., Marcus, L., Qi, L., Spence, C. L., Tengi, J., Magnuson, M. A., Chu, C. A., Dvoroznak, M. T., Matschinsky, F. M., and Grippio, J. F. (2003) Science 301, 370–373
4. Van Schaftingen, E., Veiga-da-Cunha, M., and Niculescu, L. (1997) Biochim. Soc. Trans. 25, 136–140
5. Agius, L. (1998) Adv. Enzyme Regul. 303–331
6. Chu, C. A., Fujimoto, Y., Iwaga, K., Grimsby, J., Grippio, J. F., Magnuson, M. A., and Cherrington, A. D., and Shiota, M. (2004) Am. J. Physiol. Gastrointest. Liver Physiol. 286, G627–G634
7. Shin, J. S., Torres, T. P., Catlin, R. L., Donahue, E. P., and Shiota, M. (2007) Am. J. Physiol. Regul. Comp Physiol. 292, R1381–R1390
8. Saxena, R., Voight, B. F., Lyssenko, V., Burtt, N. P., de Bakker, P. I., Chen, H., Roix, J. I., Kathiresan, S., Hirschhorn, J. N., Daly, M. J., Hughes, T. E., Groop, L., Altshuler, D., Almgren, P., Florez, J. C., Meyer, J., Ardlie, K., Bengtsson, B. K., Isomaa, B., Lettre, G., Lindblad, U., Lyon, H. N., Melander, O., Newton-Cheah, C., Nilsson, P., Orho-Melander, M., Rastam, L., Speiotes, E. K., Taskinen, M. R., Tuomi, T., Guiducci, C., Berglund, A., Carlsson, J., Gianniny, L., Hackett, R., Hall, L., Holmknist, J., Laurila, E., Sjogren, M., Sterner, M., Surti, A., Svensson, M., Svensson, M., Tewhey, R., Blumenstiel, B., Parkin, M., Defelice, M., Barry, R., Brodeur, W., Camarata, J., Chia, N., Fava, M., Gibbons, J., Handsaker, B., Healy, C., Nguyen, K., Gates, C., Sougnez, C., Gage, D., Nizzi, M., Gabriel, S. B., Chirn, G. W., Ma, Q., Parikh, H., Richardson, D., Ricker, D., and Purcell, S. (2007) Science 316, 1331–1336
9. Willer, C. J., Sanna, S. J., Jackson, A. U., Scuteri, A., Bonnycastle, L. L., Clarke, R., Heath, S. C., Timpson, N. J., Najjar, S. S., Stengham, H. M., Strait, J., Duren, W. L., Maschio, A., Busonero, F., Mullas, A., Albai, G., Swift, A. J., Morken, M. A., Narisu, N., Bennett, D., Parish, S., Shen, H., Galan, P., Meneton, P., Herberg, S., Zielenka, D., Chen, W. M., Li, Y., Scott, L. J., Scheet, P. A., Sundvall, J., Watanabe, R. M., Nagara, R., Ebrahim, S., Lawlor, D. A., Ben-Shlomo, Y., Davey-Smith, G., Shuldiner, A. R., Collins, R., Bergman, R. N., Uda, M., Tuomilehto, J., Cao, A., Collins, F. S., Lakatta, E., Lathrop, G. M., Boehnke, M., Schlessinger, D., Mohlke, K. L., and Abecasis, G. R. (2008) Nat. Genet. 40, 161–169
10. Van Schaftingen, E. (1989) Eur. J. Biochem. 179, 179–184
11. Veiga-da-Cunha, M., and Van Schaftingen, E. (2002) J. Biol. Chem. 277, 8466–8473
12. Agius, L., and Peak, M. (1993) Biochem. J. 296, 785–796
13. Shiota, M., Galassetti, P., Monohan, M., Neal, D. W., and Cherrington, A. D. (1998) Diabetes 47, 867–873
14. Sagen, J. V., Odlid, S., Bjorkhaug, L., Zelent, D., Buettiger, C., Kwag, J., Stanley, C., Dahl-Jorgensen, K., de Beaufort, C., Bell, G. I., Han, Y., Grimsby, J., Taub, R., Molven, A., Sovik, O., Nelson, P. R., and Matschinsky, F. M. (2003) Diabetes 52, 1713–1722
15. Brocklehurst, K. J., Payne, V. A., Carroll, D., Vertigan, H. L., Wightman, H. J., Aiston, S., Waddell, I. D., Leighton, B., Coghlan, M., and Agius, L. (2004) Diabetes 53, 535–541
16. Futamura, M., Hosaia, H., Kodotani, A., Shimazaki, H., Sasaki, K., Ohyama, S., Nishimura, T., Eiki, J., and Nagata, Y. (2006) J. Biol. Chem. 281, 37668–37674
17. Agius, L., and Peak, M. (1997) Biochem. Soc. Trans. 25, 145–150
18. Van Schaftingen, E., Vandercammen, A., Detheux, M., and Davies, D. R. (1992) Adv. Enzyme Regul. 32, 133–148
19. Mackay, I. P., Sunde, M., Lowry, J. A., Crossley, M., and Matthews, J. M. (2007) Trends Biochem. Sci. 32, 530–531
20. Brocklehurst, K. J., Davies, R. A., and Agius, L. (2004) Biochem. J. 378, 693–697
21. Anderka, O., Loenen, P., Klubante, T., Dreyer, M. K., Defossa, E., Wendt, K. U., and Schmoll, D. (2008) Biochemistry 47, 4683–4691
22. Doyle, M. L., Louie, G., Dal Monte, P. R., and Sokoloski, T. D. (1995) Methods Enzymol. 259, 183–194
Interaction between Glucokinase and Its Regulatory Protein

23. Murphy, K. P., Xie, D., Garcia, K. C., Amzel, L. M., and Freire, E. (1993) *Proteins* 15, 113–120
24. Fukada, H., and Takahashi, K. (1998) *Proteins* 33, 159–166
25. Thomson, J. A., and Ladbury, J. E. (2004) in *Biocalorimetry* 2 (Ladbury, J. E., and Doyle, M. L., eds) pp 37–58, John Wiley & Sons Ltd, Chichester, England
26. Murphy, K. P., and Freire, E. (1992) *Adv. Protein Chem.* 43, 313–361
27. Xie, D., and Freire, E. (1994) *Proteins* 19, 291–301
28. Gakamsky, D. M., Luescher, I. F., and Pecht, I. (2004) *Proc. Natl. Acad. Sci. U. S. A.* 101, 9063–9066
29. Veiga-da-Cunha, M., Delplanque, J., Gillain, A., Bonthron, D. T., Boutin, P., Van Schaftingen, E., and Froguel, P. (2003) *Diabetologia* 46, 704–711
30. Veiga-da-Cunha, M., Xu, L. Z., Lee, Y. H., Marotta, D., Pilks, S. J., and Van Schaftingen, E. (1996) *Diabetologia* 39, 1173–1179
31. Gloyn, A. L., Odili, S., Zelent, D., Buettger, C., Castleden, H. A., Steele, A. M., Stride, A., Shiota, C., Magnuson, M. A., Lorini, R., d’Anunzio, G., Stanley, C. A., Kwagh, J., Van Schaftingen, E., Veiga-da-Cunha, M., Barbetti, F., Dunten, P., Han, Y., Grimsby, J., Tash, R., Ellard, S., Hattersley, A. T., and Matschinsky, F. M. (2005) *J. Biol. Chem.* 280, 14105–14113
32. Moukil, M. A., Veiga-da-Cunha, M., and Van Schaftingen, E. (2000) *Diabetes* 49, 195–201
33. Mookhtiar, K. A., Kalinowski, S. S., Brown, K. S., Tsay, Y. H., Smith-Monroy, C., and Robinson, G. W. (1996) *Diabetes* 45, 1670–1677
34. Detheux, M., Vandercammen, A., and Van Schaftingen, E. (1991) *Eur. J. Biochem.* 200, 553–561
35. Stites, W. E. (1997) *Chem. Rev.* 97, 1233–1250
36. Leavitt, S., and Freire, E. (2001) *Curr. Opin. Struct. Biol.* 11, 560–566
37. Perozzo, R., Folker, G., and Scapozza, L. (2004) *J. Recept. Signal. Transduct. Res.* 24, 1–52
38. O’Brien, R., and Haq, I. (2004) in *Biocalorimetry* 2 (Ladbury, J. E., and Doyle, M. L., eds) pp 3–34, John Wiley & Sons, Chichester
39. Baltrusch, S., Lenzen, S., Okar, D. A., Lange, A. J., and Tiedge, M. (2001) *J. Biol. Chem.* 276, 43915–43923
40. Ladbury, J. E., and Williams, M. A. (2004) *Curr. Opin. Struct. Biol.* 14, 562–569
41. Vandercammen, A., and Van Schaftingen, E. (1991) *Eur. J. Biochem.* 200, 545–551
42. Holdgate, G. A. (2001) *BioTechniques* 31, 164–166
43. Veiga-da-Cunha, M., Courtois, S., Michel, A., Gosselain, E., and Van Schaftingen, E. (1996) *J. Biol. Chem.* 271, 6292–6297
44. Sturtevant, J. M. (1977) *Proc. Natl. Acad. Sci. U. S. A.* 74, 2236–2240
45. Luther, M. A., Cai, G. Z., and Lee, J. C. (1986) *Biochemistry* 25, 7931–7937
46. Hileman, R. E., Jennings, R. N., and Linhardt, R. J. (1998) *Biochemistry* 37, 15231–15237
47. Matulis, D., Rouzina, I., and Bloomfield, V. A. (2000) *J. Mol. Biol.* 296, 1053–1063
48. Jones, S., and Thornton, J. M. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 13–20
49. *Kinetic and Affinity Analysis using BIA* (1997) Biacore AB, Uppsala, Sweden
50. Selzer, T., Albeck, S., and Schreiber, G. (2000) *Nat. Struct. Biol.* 7, 537–541
51. Kamata, K., Mitsuya, M., Nishimura, T., Eiki, J., and Nagata, Y. (2004) *Structure* 12, 429–438
52. Kim, Y. B., Kalinowski, S. S., and Marcinkeviciene, J. (2007) *Biochemistry* 46, 1423–1431
53. Ralph, E. C., Thomson, J., Almaden, J., and Sun, S. (2008) *Biochemistry* 47, 5028–5036