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Kinetic Basis of Carbohydrate-Mediated Inhibition of Human Glucokinase by the Glucokinase Regulatory Protein

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Abstract

The glucokinase regulatory protein (GKRP) is a competitive inhibitor of glucokinase (GCK), triggering its localization to the hepatocyte nucleus upon glucose deprivation. Here we establish the kinetic mechanism of GKRP action by analyzing its association with a genetically encoded, fluorescent variant of human GCK. Our results demonstrate that binding of GKRP to GCK involves two steps, formation of an initial encounter complex followed by conformational equilibration between two GKRP–GCK states. Fructose 6-phosphate, a known enhancer of GKRP action, promotes formation of the initial encounter complex via a 2.6-fold increase in k_{on} and stabilizes the complex through a 60-fold decrease in k_{off} .

Glucokinase (GCK) plays a vital role in glucose homeostatic maintenance by governing the rates of glucose catabolism in pancreatic β -cells and glycogen storage in hepatocytes.^{1–3} The misregulation or dysfunction of GCK causes a variety of diseases, including maturity onset diabetes of the young type II and persistent hyperinsulinemia of infancy.^{4–8} In hepatocytes, GCK is regulated by direct association with the glucokinase regulatory protein (GKRP), a 68 kDa polypeptide that functions as a competitive inhibitor of binding of glucose to GCK.^{9–13} Under low-glucose conditions, GKRP forms an inhibitory complex with GCK and sequesters the enzyme within the nucleus. When glucose concentrations increase, GKRP releases GCK into the cytosol where it can participate in metabolism.⁹ The importance of the GKRP–GCK complex in hepatic glucose homeostasis, combined with the observation that mutations in GKRP are associated with an increased risk of cardiovascular disease,^{14,15} suggests that the complex is a putative target for therapeutic design. Indeed, Amgen recently reported the discovery of a novel class of functionalized piperazines that disrupt the GKRP–GCK interaction.¹⁶ *In vitro* and *in vivo* studies demonstrate that these molecules stimulate GCK activity by interfering with GKRP-mediated regulation and effectively reduce blood glucose levels in diabetic model organisms.¹⁶

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.6b00349. Materials and methods, a detailed description of kinetic and transient-state assays, plots of k_{obs} and amplitude versus GKRP concentration, Table S1, and Figures S1–S4 (PDF)

The authors declare no competing financial interest.

The identity of phosphorylated carbohydrates that bind to GKRP at a site near the interface between the cap domain and the second sugar isomerase domain (SIS2) regulates the strength of the complex. Fructose 6-phosphate and sorbitol 6-phosphate stimulate formation of the inhibitory complex, whereas fructose 1-phosphate reduces the affinity of GKRP for GCK.^{10,12} The recently described crystal structure of the mammalian GCK–GKRP–fructose 6-phosphate ternary complex reveals that the binding site for carbohydrate effectors is more than 30 Å from the GCK interaction interface (Figure S1).¹³ This binding site is near but does not overlap with the binding site of piperazine-based small-molecule disruptors.¹⁶ Despite more than 25 years of biochemical investigations of GKRP, the mechanism by which the regulatory protein recognizes and inhibits GCK in a carbohydrate-dependent manner remains unclear.

We sought to establish a quantitative description of the mechanism by which the GKRP–GCK protein–protein complex is assembled. Because both GKRP and GCK contain multiple tryptophan residues, we decided against using intrinsic protein fluorescence to monitor association. Instead, we used methods developed by the Schultz group to genetically incorporate a fluorescent amino acid, L-(7-hydroxycoumarin-4-yl) ethylglycine (fUAA), into human pancreatic GCK at a site near the interaction interface.^{17,18} Insertion of fUAA in place of Leu47, a solvent-exposed residue located at the GKRP contact site, provided a sensitive and unique reporter of complex formation (Figure 1A). Equilibrium binding experiments demonstrate that association of GKRP with L47fUAA GCK causes a decrease in the magnitude of the fluorescence signal of the protein. Importantly, the kinetic parameters of L47fUAA GCK are highly similar to those of the wild-type enzyme, including its unique kinetic cooperativity (Table S1).

We used transient-state stopped-flow spectrofluorometry to monitor the time-dependent change in L47fUAA GCK fluorescence upon GKRP binding. These experiments were performed by mixing L47fUAA GCK (1 μ M) with variable concentrations of GKRP (2–45 μ M) and collecting fluorescence traces until equilibrium was reached (Figure 1B). The resulting binding traces were averaged and fit to a sum of exponentials equation, which revealed two kinetically distinguishable events (Figure 1C). To establish the mechanism of GKRP–GCK association, we globally fit our experimental data to several binding models via numerical integration using the KinTek Global Kinetic Explorer software (Figure S2A).^{19,20} Models I and II allow for a preexisting equilibrium between two conformations of GCK and GKRP, respectively, prior to complex formation. Model III posits a single, unliganded form of both proteins and describes a two-step binding process involving an initial encounter complex and a subsequent isomerization event. The goodness of fit of the data to each model is determined by the χ^2/D_oF value, where χ^2 is the average of the σ values based upon the analytical fit and D_oF represents the degrees of freedom, which is the number of data points minus the number of fitted parameters. A value of unity for χ^2/D_oF is an ideal fit. A comparison of χ^2/D_oF values for each mechanism indicated that model III provided the best fit to the experimental data, with a χ^2/D_oF value of 1.9.

Global fitting of experimental binding traces established estimates for the microscopic rate constants describing individual steps of the GKRP association mechanism (Table 1 and Figure S2B). Our analyses yielded values for k_1 and k_{-1} of $6.4 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ and 2.06 s^{-1} ,

respectively. This k_1 value is consistent with association rate constants reported for other protein–protein interactions, which generally fall in the range of 10^3 – 10^4 $\text{M}^{-1} \text{s}^{-1}$.²¹ The k_{-1}/k_1 ratio produces an intrinsic dissociation constant ($K_{\text{d,intrinsic}}$) of 32 μM for formation of the initial encounter complex. Our data also established values for k_2 and k_{-2} of 0.11 and 0.21 s^{-1} , respectively. The k_2/k_{-2} ratio of these values provides the equilibrium constant (K_2) for the conformational isomerization step. In the absence of any carbohydrate effector, the K_2 value is 0.51, indicating that at equilibrium 66% of the complex exists in the GKRP–GCK state while 34% adopts the GKRP–GCK* state. Our experimental rate constants also facilitate the calculation of the apparent dissociation constant for the binding of GKRP to L47fUAA GCK using the relationship $K_{\text{d,apparent}} = (k_{-1}/k_1)/(1 + k_2/k_{-2})$.²² The $K_{\text{d,apparent}}$ calculated in this manner is 21 μM , which is slightly higher than the apparent dissociation constant measured for the GKRP–GCK interaction using isothermal titration calorimetry.²³ We attribute this difference in dissociation constants to a modest weakening of the interaction because of the presence of the non-natural fUAA amino acid at the protein–protein interface.

Previous studies demonstrated that fructose 6-phosphate enhances the equilibrium affinity of GKRP for GCK.¹⁰ To understand the mechanistic origins of the stabilizing impact of this carbohydrate, we performed transient-state studies in the presence of saturating concentrations of fructose 6-phosphate (5 mM). Global fitting of averaged binding traces demonstrates that fructose 6-phosphate does not alter the nature of the binding mechanism but instead alters the values of individual microscopic rate constants (Table 1). In the presence of fructose 6-phosphate, the k_1 value increases 2.6-fold to $1.7 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$ and the k_{-1} value decreases 57-fold to 0.036 s^{-1} . As a result, the intrinsic dissociation constant decreases from 32 to 0.21 μM . The equilibrium constant for the subsequent conformational equilibration step, K_2 , increases slightly to 0.72 in the presence of fructose 6-phosphate. Thus, the GKRP–GCK state comprises 58% of the total complex, and the GKRP–GCK* state represents 42% of the population. The apparent K_{d} value decreases 160-fold in the presence of fructose 6-phosphate to 124 nM, which agrees well with a previously reported value obtained via isothermal titration calorimetry.²³ Together, our data indicate that fructose 6-phosphate promotes GKRP-mediated inhibition of GCK by enhancing the stability of the initial encounter complex between GCK and GKRP; this effector has little influence upon the second conformational equilibration step.

Our data demonstrate that GKRP utilizes a two-step mechanism to recognize GCK. The crystal structure of the GKRP–GCK complex determined in the presence of fructose 6-phosphate indicates that upon association with GKRP, GCK adopts a conformation similar to that observed in the crystal structure of the unliganded enzyme.¹³ This state, which has been termed the “super-open” conformation, is characterized by a wide angle of separation between the large and small domains of GCK, as well as a disordered active site loop. In the GCK–GKRP–fructose 6-phosphate ternary complex, however, the angle between the two domains is $\sim 10^\circ$ smaller than that observed in unliganded GCK (Figure S3).¹³ This slight closure of the GCK structure involves the movement of the central β -sheet and two α -helices of the small domain toward the large domain. On the basis of our data, a structural model of GKRP–GCK complex assembly can be formulated. We propose that the initial encounter complex formed in the first step of the binding mechanism involves the

association of GKRP with the “super-open” unliganded conformation of GCK reflected in Protein Data Bank entry 1V4T (Figure S3).²⁴ The subsequent conformational equilibration step is postulated to reflect subtle rearrangements of the interaction interface that correspond to the slight closure of the opening angle between the large and small domains of GCK.

Our findings demonstrate that fructose 6-phosphate modulates the inhibitory activity of GKRP by stabilizing the initial encounter complex. Structurally, it has been noted that fructose 6-phosphate causes a reorganization of the GKRP scaffold, particularly near the interface between the cap domain and the SIS2 domain.¹³ Fructose 6-phosphate binding disrupts a hydrogen bond between Arg509 from the cap domain and His351 from SIS2, leading to a large reduction in the buried surface area (Figure S4). Fructose 6-phosphate binding is also associated with repositioning of residues 462–470 in GKRP, which is located at the protein–protein interaction interface. On the basis of these structural data, we postulate that the observed impact of fructose 6-phosphate upon the values of k_1 and k_{-1} originates from subtle alterations in the position of residues 462–470, which serve to preorganize GKRP into a conformation that is more favorable for GCK binding. The validity of this model awaits future transient-state investigations of association of GCK with site-specific variants of GKRP.

In summary, we provide the first quantitative kinetic model for GKRP-mediated inhibition of GCK and establish the mechanistic basis for the modulation of GKRP’s inhibitory action by phosphorylated carbohydrates. This information provides a valuable foundation for understanding the basis of dysfunctional GKRP variants associated with hyperlipidemia and for elucidating the mechanism of action of recently described small-molecule disruptors of the GKRP–GCK complex. A mechanistic description of the GKRP–GCK complex assembly pathway is important for future efforts to target the GKRP–GCK complex with therapeutic agents to combat diseases such as diabetes and cardiovascular disease. In particular, our discovery that the protein–protein complex exists in two conformationally distinct states suggests a potential mechanism for restoring GKRP-mediated inhibition of GCK in disease variants via the development of small molecules that preferentially recognize and stabilize the rare GKRP–GCK* conformation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Interaction interface between GKR (blue) and GCK (yellow), indicating the structure, position, and spectroscopic properties of the non-natural amino acid, fUAA (red) (Protein Data Bank entry 4LC9). (B) Transient-state spectrofluorometric traces of association of L47fUAA GCK (1 μM) with GKR at 2 μM (red), 3 μM (green), 10 μM (blue), 15 μM (yellow), 30 μM (teal), and 45 μM (pink). (C) GKR binding trace (10 μM) fit to a double-

exponential equation. The experimental data are colored red with the fit in black. The residuals are shown in the inset.

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Table 1.

Microscopic Rate Constants Describing the Association of L47fUAA GCK with GKRP in the Absence and Presence of Fructose 6-Phosphate

kinetic constant	without fructose 6-phosphate	with fructose 6-phosphate
k_1 ($M^{-1} s^{-1}$)	$(6.4 \pm 0.4) \times 10^4$	$(1.7 \pm 0.1) \times 10^5$
k_{-1} (s^{-1})	2.06 ± 0.02	0.036 ± 0.005
k_2 (s^{-1})	0.11 ± 0.01	0.26 ± 0.09
k_{-2} (s^{-1})	0.21 ± 0.01	0.36 ± 0.07
K_2	0.51 ± 0.01	0.72 ± 0.2
$K_{d,intrinsic}$ (M)	$(3.2 \pm 0.6) \times 10^{-5}$	$(2.1 \pm 0.3) \times 10^{-7}$
$K_{d,apparent}$ (M)	$(2.1 \pm 0.4) \times 10^{-5}$	$(1.23 \pm 0.21) \times 10^{-7}$