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Determinants of Human Glucokinase Activation and Implications for Small Molecule Allosteric Control

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Abstract

Glucokinase (GK) is an enzyme that catalyzes the ATP-dependent phosphorylation of glucose to form glucose-6-phosphate, and it is a tightly regulated checkpoint in glucose homeostasis. GK is known to undergo substantial conformational changes upon glucose binding. The monomeric enzyme possesses a highly exotic kinetic activity profile with an unusual sigmoidal dependence on glucose concentration. In this interdisciplinary study, which draws on small angle X-ray scattering (SAXS) integrated with 250 nanoseconds of atomistic molecular dynamics (MD) simulations and experimental glucose binding thermodynamics, we reveal that the critical regulation of this glucose sensor is due to a solvent controlled “switch”. We demonstrate that the “solvent switch” is driven by specific protein structural dynamics, which leads to an enzyme structure that has a much more favorable solvation energy than most of the protein ensemble. These findings uncover the physical workings of an agile and flexible protein scaffold, which derives its long-range allosteric control through specific regions with favorable solvation energy. The physiological framework presented herein provides insights that have direct implications for the design of small molecule GK activators as anti-diabetes therapeutics as well as for understanding how proteins can be designed to have built-in regulatory functions via solvation energy dynamics.

Keywords

allostery; glucokinase; SAXS/MD; diabetes; protein-solvent interactions

1. Introduction

The classic view of allosteric regulation, centering on ligand - mediated shifts in interaction between multiple protein subunits (e.g. positive cooperativity in hemoglobin), was formulated around proteins that were multimeric with the consideration of conformational transitions due to equilibrium ligand binding [1]. However, based on a growing number of monomeric enzyme systems that display various forms of cooperativity, one must consider a broader definition of allostery, which includes how binding events at one location in a

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protein (or ligand-protein complex) affect dynamics, catalysis and the distribution of conformations at remote locations [2].

In contrast to the classic allosteric theories, which considered that the ligand induced conformational transitions are faster than the catalytic steps, the “mnemonical” model and the “slow transition” model have been proposed to explain the apparent positive cooperativity of monomeric proteins [3]. Remarkably, monomeric allostery arises from conformational transitions that occur at a pace that is no longer fast in comparison to the rate of the catalytic reaction, k_{cat} . It is exceedingly challenging to elucidate the source of catalytic enhancement (or inhibition) due to remote binding of allosteric effectors, where there is a lack of atomistic structural information; one of the paradigms for revealing the novel and broader principles of allostery is characterized by the monomeric human glucokinase (GK).

Glucokinase (GK, also known as hexokinase IV) catalyzes the phosphorylation of glucose to glucose-6-phosphate (G6P) in the liver and the pancreas [4]. In hepatocytes, GK catalyzes the rate-determining step in glycolysis; while in pancreatic β -cells, it is involved in controlling insulin release [5]. GK has a relatively high K_M for glucose and exhibits an apparent positive cooperativity as indicated by a hill coefficient of 1.7, which identifies it as a unique member of the hexokinase family [6]. It is believed that the distinct kinetic characteristics of GK forms account for its role as a sensor of glucose metabolism and homeostasis. Human GK has attracted much research interest as a novel drug target for type 2 diabetes (T2D) in recent years [7, 8]. Small-molecule allosteric activators of GK (GKA) were effective in lowering plasma glucose levels, increasing insulin secretion and decreasing hepatic glucose yield in T2D patients [9, 10], but recent clinical studies suggested that GKAs administration corresponded with an increase of hypoglycemia occurrence [10].

GK allosteric activators validate the importance of GK from a therapeutic perspective, but the nature of allosteric activation of GK remains unclear. The GK allosteric activator site, the pocket where GKAs bind, is ~ 20 Å remote from the active site (Figure 1A). The allosteric site is the locus of many heterozygous GK-activating mutations that are clinically hyperinsulinemic and hypoglycemic [11]. Numerous published structural studies of GK have demonstrated that it undergoes a large conformational change from open to closed upon glucose binding [3, 12]. However, there is a paucity of structural information on the activating mutants of GK. GKAs and clinically activating mutants were suggested to enhance GK activity via shifting the enzyme structure toward a glucose-bound conformation [3, 12, 13]. In 2008, Christesen et al., found S64Y mutant among four GK activating mutants, in patients who were diagnosed with congenital hyperinsulinemia (CHI). Located in the GK allosteric activator site, the S64Y mutant was biochemically characterized to increase activity in comparison to GK wild-type (GK-wt) [14].

In the current study, focusing on the clinically significant mutant GK-S64Y, we determine that the GK hyperactive mutant has distinct structural and thermodynamic characteristics during substrate recognition using small angle X-ray scattering (SAXS) and fluorescent based glucose binding assays. Atomistic models from a series of experimentally correlated molecular dynamics (MD) simulations in the 100s of nanoseconds timescale showed that

solvent interactions, in association with enzyme dynamics, contribute to the favorable binding energies observed in the activated GK catalysis of glucose. This integrated experimental and computational approach reveals that GK's adaptable affinity for glucose is driven by specific changes in the protein solvation energy. We refer to these dynamics as a "solvent switch", which we show is the source of GK's remarkable intrinsic regulatory properties. These studies provide an atomistic framework for the discovery and design of more effective potential therapeutics for treating diabetes.

2. Materials and Methods

2.1 Small Angle X-ray Scattering Titration.

GK-wt and GK-S64Y were purified fresh before SAXS experiments. Purified proteins were dialyzed overnight at 4°C against appropriate dialysis buffers (50 mM Tris, 100 mM NaCl, 5 mM DTT, pH 8.0 with 0 mM, 5 mM, 25 mM and 100 mM glucose). Each dialyzed sample were filtered through a 0.02 µm syringe filter and homogeneity was assessed via Dynamic Light Scattering on a DynaPro NanoStar (Wyatt Technology Corp., Santa Barbara, CA) at 25°C.

The same SAXS titration experimental study was collected twice independently at the Advanced Light Source (ALS, Lawrence Berkeley National Laboratories) and once at the Advanced Photon Source (Argonne National Laboratory, Chicago, IL). The trend of the changes in R_g between GK-wt and GK-S64Y was consistent, here we show the data set collected at the Advanced Light Source (12.3.1 SIBYLS beamline, wavelength 1.2398 Å). Three different concentrations of each protein sample (1.7, 3.3 and 5 mg/mL) were exposed to the synchrotron radiation at the Advanced Light Source (12.3.1 SIBYLS beamline, wavelength 1.2398 Å) for 0.5, 1, 2 and 4 seconds at 10°C. Buffer scatterings (buffer matched by corresponding overnight dialysis solution) were subtracted, and the scattering profiles were analyzed with ATSAS Program package[15]. No concentration dependence was observed when comparing the initial exposures for each protein concentration, but radiation damage was seen with increased exposure time. Data from the lowest concentration and 0.5 exposure time were used for data analysis.

2.2 Intrinsic Tryptophan Fluorescence Measurements of Binding Affinities.

Fluorescence intensity and spectra were measured in a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, Santa Clara, CA). Measurements were performed at a 90° angle with excitation and emission wavelengths of 295 nm and 310–400 nm. For time based intensity glucose titrations, the change in intrinsic tryptophan fluorescence of 5 µM proteins at 335 nm, using a 10-nm slit width, upon addition of varying glucose concentration (0 – 50 mM) in a buffer containing 5 mM phosphate, 25 mM KCl, 2 mM DTT, with or without 20% glycerol pH 7.4 was monitored. Dilution effect of glucose addition was offset by control titrations of buffer alone. Experiments were performed in triplicate, averaged and fitted to the following equation using PRISM 7(GraphPad Software, La Jolla, CA):

$$\Delta F = (\Delta F_{max} \times [glucose]) / (K_D + [glucose])$$

2.3 Molecular Dynamics (MD) Simulations.

In order to correspond to the temperature range from 11°C to 49°C used in our experimental intrinsic fluorescent glucose binding studies, the MD simulations of apo-GK-wt and apo-GK-S64Y were performed at three temperatures of 283 K, 298K and 313 K. At each temperature, the simulations of apo-GK-wt and apo-GK-S64Y were performed with YASARA version 15.3.8 (YASARA Biosciences GmbH, Austria). The AMBER03 force field [16, 17] was used with long-range electrostatic potentials calculated with the Particle Mesh Ewald (PME) method [18], with a cutoff of 7.86 Å. A periodic simulation cell with boundaries extend 10 Å from the surface of the protein of interest was employed. The hydrogen bonding network was optimized to address the potential issues due to incorrect oriented or protonated side-chains [19] that are not resolved by the electron density. YASARA's pK_a utility was used to assign pK_a values at pH 7.4 [20]. The simulation cell was filled with water to reach a solvent density of 0.997 g/mL, and the cell was neutralized with NaCl (0.9% by mass). Energy minimization of the entire system using a steepest descent step to remove conformational stress was completed before initiation of the simulations using the NVT ensemble at 283 K, 298K and 313 K respectively. Integration time steps for intramolecular and intermolecular forces were calculated every 2.5 fs. A MD snapshot was generated every 250 ps during the ~250 ns MD simulations, and equilibrated ensembles were reached after ~50 ns of simulations time. From each MD simulation, 866 snapshots after enzyme equilibration were retained for further analysis. The Supplementary Information provides contains a detailed description of the MD Clustering and Analysis [21].

2.4 Stopped-Flow Spectrophotometry.

Pre-steady-state kinetics was detected by measuring the increase of fluorescent signals by the rapid mixing of enzyme and substrate at 25°C using an Applied Photophysics SX20 stopped-flow spectrophotometer (dead time of 0.45ms). Apo-GK-wt or apo-GK-S64Y (final concentration 3 µM) and glucose (final concentration 0–10mM) in aqueous buffer (5mM K₂SO₄, 25mM KCl, 5mM DTT, pH 7.4) were rapidly mixed in the stopped-flow apparatus by injections from separate syringes. The increase of intrinsic fluorescent signal was monitored using a 320nm cutoff filter. For each glucose concentration, 15 data acquisitions were collected and averaged for data fitting. GraphPad Prism 7(GraphPad Software, La Jolla, CA) was used to fit a one-phase association model which is described by the equation

$$\text{here: } F = F_{max} * \left(1 - e^{(-k_{obs} * X)} \right).$$

3. Results and Discussion

3.1 The Construction of GK-S64Y Mutant.

The congenital hyperinsulinemia (CHI), persistent hyperinsulinaemic hypoglycemia of infancy (PHHI)-associated, hyperactive mutant GK-S64Y, results in placement of a tyrosine

residue in the region that composes part of the allosteric binding pocket of GKA (Figure 1A). Furthermore, Ser 64 residue is not conserved across the hexokinase family (consists of hexokinase I, II, III and GK(IV)) (Figure 1B). Based on analytical circular dichroism [8] spectra of purified enzymes, both apo-GK-wt and apo-GK-S64Y isoforms appear to be similarly well folded (Figure S1). Previously published X-ray crystallographic studies suggest that GKAs enhance GK activity by shifting GK ensembles toward a more compact conformation that is favorable for both glucose binding and phosphorylation [3]. The presence of the activating mutant in the GKA binding pocket suggests that apo-GK-S64Y may possess allosteric properties in common with (GK-wt)/Glu/GKA and/or (GK-wt)/Glu complexes [3, 12]; alternatively, it may possess very distinct dynamics from apo-GK-wt.

3.2 Small Angle X-ray Scattering (SAXS) Titration Experiment on GK-wt vs. GK-S64Y.

Since crystallographic structures of (GK-wt)/Glu/GKA and (GK-wt)/Glu complexes are nearly identical [12], we hypothesize that the salient structural and dynamic differences between GK-wt and GK-S64Y are in the apo-state. To examine the global conformational change of GK-S64Y in comparison to GK-wt in solution, a series of glucose binding titration experiments using SAXS in four glucose concentrations at 0 mM, 5 mM, 25 mM and 100 mM were performed. The radius of gyration (R_g) calculated from SAXS, which reflects the average size of the protein, were used to probe conformational distributions of GK-wt and GK-S64Y at various glucose concentrations (Figure 2A).

Based on previously published X-ray crystallographic studies, GK is in its relaxed, or “open” state while not bound to glucose; it undergoes a relatively large closure upon binding its substrate [3]. The negative correlation between the R_g values and glucose concentrations in our SAXS data indicated that both GK-wt ($R_g = -3.5 \text{ \AA}$) and GK-S64Y ($R_g = -1.7 \text{ \AA}$) undergo substantial conformational changes in solution upon glucose binding (Figure 2A). It is noteworthy that R_g values of GK-wt ($R_g = 31.9 \text{ \AA}$) and GK-S64Y ($R_g = 31.6 \text{ \AA}$) converged to be statistically insignificant when glucose concentration was high at 100 mM (Figure 2A), and the most distinct difference between the R_g of GK-wt ($R_g = 35.4 \text{ \AA}$) and GK-S64Y ($R_g = 33.3 \text{ \AA}$) occurred when glucose concentration was 0 mM (Figure 2B and S2). The strong structural similarity between GK-wt and GK-S64Y in 100 mM glucose was reflected in the Volatility of Ratio metric [22] from SAXS, while the GK-wt and GK-S64Y showed relatively low structural correspondence in the absence of glucose (Figure S3).

R_g values of GK-wt and GK-S64Y converge at 100 mM glucose (in which GK is fully saturated), which may be like the case of (GK-wt)/Glu/GKA and (GK-wt)/Glu complexes; in each case, the ligand bound complexes do not show significant structural differences. More specifically, it is interesting that the changes of R_g (R_g) in GK-wt and GK-S64Y are quite similar between 5mM and 25mM glucose ($\sim 2 \text{ \AA}$), but the change of R_g (R_g) in between 0mM and 5mM glucose is much more pronounced in GK-wt ($\sim 1.4 \text{ \AA}$) than that of GK-S64Y ($\sim 0.3 \text{ \AA}$). In addition, this is not reflective of glucose affinities (see below), but rather reflects the large conformational changes that occur when GK-wt transitions between the “super-open” form of the apo and the “closed” state. This finding underscores the fact that the activating GK mutant is, in its apo form, in a pre-organized configuration for binding glucose, which is addressed further below. In the absence of glucose, apo-GK-

S64Y's R_g is 2.1 Å less than apo-GK-wt, suggesting that apo-GK-S64Y is more closely packed than apo-GK-wt, a global conformation that resembles the (GK-wt)/Glu “closed” state. Thus, we provide direct evidence that, the apo-state of the clinically hyperactive mutant GK-S64Y is likely to exhibit a more compact structure than apo-GK-wt, which is associated with characteristics that favor glucose phosphorylation.

3.3 Thermodynamic Signatures of Glucose Binding for GK-wt and GK-S64Y.

We assessed any functional connection between the very distinct apo-GK-wt and apo-GK-S64Y SAXS parameters via the thermodynamic signatures of glucose binding using the van't Hoff analysis. The van't Hoff equation relates the equilibrium constant for glucose binding to the change in temperature, and it was used to extract thermodynamic parameters for ligand binding. A modified version of a well-established fluorescent binding assay was followed [23], which monitored the increase in intrinsic fluorescence that occurs upon glucose binding. The equilibrium constant, K_D , of GK-wt and GK-S64Y in aqueous buffer were determined at temperatures ranging from 11°C to 39°C at pH 7.4. The resulting thermodynamic parameters indicate that glucose binding of GK is a spontaneous (i.e. exergonic), endothermic reaction driven by entropy (Figure 3C). Consistent with previous studies [23–25], the glucose binding reaction yields a favorable entropy of 175.0 ± 13.5 J*K⁻¹ *mol⁻¹ to overcome an unfavorable enthalpy of 37.6 ± 4.0 kJ*mol⁻¹ in GK-wt (Figure 3C and Table 1). In contrast, glucose binding in the hyperactive mutant, GK-S64Y, produces a less favorable entropy of 144.0 ± 6.7 J*K⁻¹ *mol⁻¹, in association with a less unfavorable enthalpy of 27.3 ± 1.9 kJ*mol⁻¹ (Table 1). As the temperature decreases, glucose binding in the GK-wt is impeded greatly, displaying a steeper slope in the van't Hoff plot (Figure 3A). The thermodynamic signatures show glucose binding in GK-S64Y results in an altered and flattened slope, indicating that GK-S64Y is less responsive to the environmental temperatures than GK-wt (Figure 3A).

3.4 The Presence of Glycerol Alters Thermodynamic Signatures in Both GK-Wt and GK-S64Y.

The contribution of entropy as a driving force in GK glucose binding was consistent with studies performed by others [23]. Interestingly, early studies published by Rand et al., on the binding of glucose to yeast hexokinase under osmotic stress demonstrated a significant role played by solvation during substrate binding and catalysis [26, 27]. Furthermore, additional studies published by Filfil et al., showed that glucose binding in yeast hexokinase induced changes in hydration, and the glucose-induced changes in GK hydration were associated with enzyme conformational compaction [28]. A structural alignment of apo-yeast hexokinase with human apo-GK-wt was performed, and it showed that human apo-GK-wt displayed a more extended and open conformation in comparison to the yeast isoform (Figure S4). Due to the existence of a more “opened” conformation of human apo-GK-wt to other apo-hexokinases, it is reasonable to speculate that GK would be more dependent on solvation when binding glucose. Our SAXS experiments demonstrated that apo-GK-wt experienced a substantially larger degree of conformational packing in the presence of glucose than apo-GK-S64Y (Figure 2), which may be a function of protein solvation. Thus, the large changes in protein-water structure may be a major contributor to the unusually large glucose-induced conformational changes seen in the human GK isoform.

If solvation plays a significant role in mediating the distribution of the GK ensemble, then this may help to explain the large R_g discrepancy between the apo-GK-wt and apo-GK-S64Y in SAXS. Although the details of the GK-Glucose binding trajectory were not fully assessed, our SAXS glucose titration study provides strong evidence for a continuum of various open structures of apo-GK-wt and apo-GK-S64Y, but not of GK/Glu. Nevertheless, the data suggests that differences in protein solvation do shift the distribution in the respective apo-ensembles. Remarkably, the degree of solvation exposure of apo-GK isoforms was reflected in the enhanced compaction upon substrate binding. The implications for these solvation dynamics to our SAXS studies are detailed below.

Glycerol is known to shift native protein ensembles to more compact and less flexible conformations [29]. Moreover, the protein compaction induced by glycerol is caused by the excluded volume effects originating from the electrostatic orientation of glycerol at protein surfaces [29]. Previously published work on GK-wt suggested that apo-GK-wt exists in multiple forms that are interconvertible and the cooperativity is a consequence of changing the proportions of the different enzyme forms [6, 30]. GK-wt in the presence of glycerol causes the loss of cooperativity with respect to glucose, suggesting glycerol favors enzyme conformations with higher affinities for glucose [6, 30]. To examine the role of preferential water interaction on thermodynamic parameters of both GK-wt and GK-S64Y, glucose binding in 20% glycerol was performed at temperatures ranging from 16°C to 44°C. In contrast to GK-wt in aqueous buffer, van't Hoff analysis of glucose binding with GK-wt in 20% glycerol produces a less favorable entropy of $128.5 \pm 8.1 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ in association with a less unfavorable enthalpy of $22.5 \pm 2.5 \text{ kJ} \cdot \text{mol}^{-1}$ (Figure 3A and Table 1), and this is consistent with thermodynamic values of GK-wt produced by others [25].

Moreover, our GK-S64Y glucose-binding experiment in 20% glycerol yielded an even less favorable entropy, $S = 96.7 \pm 3.4 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ accompanied by the least unfavorable enthalpy of $11.7 \pm 1.0 \text{ kJ} \cdot \text{mol}^{-1}$ in comparison to buffer without glycerol (Figure 3C and Table 1). We have found that, a common signature for enhanced glucose binding, which is shared by GK-S64Y and buffer conditions containing 20% glycerol, was a less favorable entropy and a corresponding unfavorable enthalpy. It should be noted that, the enthalpy-entropy changes of GK-wt in 20% glycerol were like the enthalpy-entropy values of GK-S64Y in aqueous solution alone; thus, the enzyme distribution of GK-S64Y in aqueous solution can be mirrored by GK-wt in 20% glycerol through both compaction of macromolecular structures and reduction of interactions from water molecules around protein surfaces. In addition, other factors may contribute to the overall observed enthalpy and entropy changes in GK-wt and GK-S64Y, such as amino acids side chain pKa values, and thus changes in heat of ionizations. The reduced entropy and more favorable free energy changes produced by 20% glycerol in both GK-wt and GK-S64Y suggests that, the presence of glycerol favors glucose binding in both GK-wt and GK-S64Y through the excluded volume effect and the tangential consequence of various structural compressions in both GK isoforms. In other words, GK's capability to function with adaptable affinities depends on its ability to access the open forms, and this is compromised by desolvating agents. In conclusion, the glucose binding studies, together with the SAXS-glucose titration experiments, strongly suggests that ligand binding in GK is a continuous and gradual

process which involves enzyme desolvation in association with progressive shifts in GK conformational distributions toward more compact structures that favor glucose binding.

3.5 Temperature Based Atomistic Molecular Dynamics (MD) Simulation Studies.

As discussed above, structural evidence gathered from our experimental SAXS data (Figure 2) and published crystallographic structures of GK strongly suggested that apo-GK-wt and apo-GK-S64Y dynamics play a crucial role in catalysis and likely, monomeric allosteric control. Obtaining definitive evidence for GK allostery is problematic due to the inherent complexity of apo-GK-wt [31], and it can be challenging to represent the less populated conformations of apo-GK-wt which favors glucose binding using X-ray crystallographic structures [3, 12]. In combination with the experimental SAXS structural information, our MD-based atomistic GK model was produced using knowledge based structural modeling [32–34]. A detailed description of our atomistic model generation can be found in the Supplementary Information (SI). Our SAXS data was used as a statistically independent experimental authentication for constructing a realistic all-atom model of apo-GK-wt. Thus, our complete atomistic model of apo-GK-wt was a significantly improved representation of the enzyme in solution ($\chi^2 = 2.63$), relative to the known X-ray crystal structure ($\chi^2 = 12.58$ and $\chi^2 = 31.67$) (Figure 4 and S5).

To develop an atomistic framework for understanding how apo-GK conformational dynamics may be linked to its catalytic and allosteric properties, we have performed a series of atomistic molecular dynamics (MD) simulations. A temperature range that maps to our experimental binding studies with van't Hoff analysis was used for the MD simulations to examine how apo-GK dynamics change as a function of temperature. A ~250 ns MD simulation of apo-GK-wt and apo-GK-S64Y in explicit solvent was performed at each temperature of 10°C, 25°C and 40°C. Our complete atomistic homology model of apo-GK-wt was used in conducting MD simulations of apo-GK-wt with YASARA Structure package version 15.3.8 (YASARA Biosciences GmbH, Austria)[20, 34, 35], and a single site mutation was made when performing MD simulations of apo-GK-S64Y using the same methodology. The area under the curve integration [36] from root mean square fluctuation (RMSF; a measurement of the average atomic mobility) of MD simulations showed that apo-GK-wt experienced more structural fluctuations than apo-GK-S64Y after achieving equilibrium in ~250 ns of MD across the three examined temperatures (Figure 5B), which suggested that apo-GK-wt may be exploring more distinct forms than the mutant enzyme. These apo-GK-wt and apo-GK-S64Y ensembles from MD simulations were highly distinct from one another in terms of protein flexibility and solvation energy, as discussed below.

The macromolecular conformational entropies (S) of apo-GK-wt and apo-GK-S64Y ensembles were calculated to reflect enzyme structural diversity and flexibility by using Boltzmann sampling [37] from clustering 866 MD snapshots for every MD simulation with a 3 Å cutoff (Figure 5). Apo-GK-wt exhibited a more favorable macromolecular conformational entropy (S) than apo-GK-S64Y at all three temperatures examined (Figure 5A), which indicated that apo-GK-wt was generally more structurally flexible. The variance of apo-GK-wt macromolecular conformational entropies (S) was more temperature sensitive than the clinically hyperactive mutant, GK-S64Y, as observed by a steeper slope (Figure

5A). Remarkably, the thermal dependency of macromolecular entropy (S) of apo-GK-wt correlated with the thermal dependency of glucose affinity measured for apo-GK-wt from the van't Hoff plots based on the experimental binding studies across the same temperature range (Figure 3A). An important implication of the flexibility differences between apo-GK-wt and apo-GK-S64Y ensembles is how physical properties may be altered due to these changes in the inherent flexibility.

Considering the known water excluding effect of glycerol, as seen in the van't Hoff plots based on the temperature-dependent intrinsic fluorescent glucose binding measurements (Figure 3A), we were interested in the connection between MD ensembles and the diversity of their solvation properties. Solvent-accessible surface areas (SASA) of each MD clustered structural centroid of apo-GK-wt and apo-GK-S64Y were evaluated to estimate the solvent exposure of the two enzymes at 10°C, 25°C and 40°C. Poisson-Boltzmann solvation (PBS) energies [38] of apo-GK-wt and apo-GK-S64Y were calculated to yield the Coulombic energy of solvation under physiological salt condition at 10°C, 25°C and 40°C. With macromolecular conformational entropies (S) mentioned above, SASA calculations of apo-GK-wt and apo-GK-S64Y provided signs that the two isoforms hold very distinct conformational diversities associated with discrete solvent interaction patterns, and the calculation of the PBS solvation energy further elucidated the relationship between protein solvation and enzyme ensemble. From the SASA plots, apo-GK-wt had greater SASA than apo-GK-S64Y across all three examined temperatures, and the largest SASA of apo-GK-wt was observed at the lowest temperature of 10°C (Figure 5C). SASA values were proportional to the extent to which amino acids interact with the solvent environment, and it indicated that apo-GK-wt possessed more interactions with solvent. Furthermore, temperature elevation to 40°C resulted in a sharper decrease in the SASA of apo-GK-wt in comparison to apo-GK-S64Y. Interestingly, this thermally sensitive trend seen in apo-GK-wt SASA values, as illustrated by a steeper slope (Figure 5C), correlated with our van't Hoff plots from the experimental binding studies across the same temperature range (Figure 3A).

The $G_{\text{solvation}}$ energies of apo-GK-wt and apo-GK-S64Y were calculated using the Adaptive Poisson-Boltzmann solvation (APBS) method. As shown in Figure 5D, apo-GK-wt yields a similar $G_{\text{solvation}}$ as apo-GK-S64Y at the highest temperature of 40°C, but apo-GK-wt manifests more favorable $G_{\text{solvation}}$ energies than apo-GK-S64Y at lower temperatures of 25°C and 10°C. This suggests that, lowering the temperature greatly increased the solubility of apo-GK-wt ensembles. In addition, compared to apo-GK-wt, apo-GK-S64Y's $G_{\text{solvation}}$ energies varied less across the examined temperature range, as reflected by a more horizontal slope (Figure 5D). Remarkably, the temperature-dependent patterns exhibited in the van't Hoff analysis from our experimental studies describing G of glucose binding in GK-wt and GK-S64Y (Figure 3B) are recapitulated by our plots illustrating $G_{\text{solvation}}$ of apo-GK-wt and apo-GK-S64Y across the same temperature range.

Overall, the clinically hyperactive mutant, apo-GK-S64Y, exhibited a less structurally diverse ensemble, which had less favorable solvent interactions than apo-GK-wt. The temperature sensitive character of apo-GK-wt $G_{\text{solvation}}$ energies agreed with the temperature sensitive patterns observed in our calculated macromolecular conformational entropy (S), as well as the distribution of SASA in the ensemble of the same temperature

range. Although the exact nature of the loss of ΔS in the experimental system itself is difficult to ascribe, our thermal based simulation studies show that at least a portion of effect is derived from protein dynamics. However, the differential desolvation between GK-S64Y and GK-wt may also significantly contribute to the observed ΔS as well. More importantly, the most salient differences in the macromolecular entropy (S), SASA, and $G_{\text{solvation}}$ energy between apo-GK-wt and apo-GK-S64Y occurred at the lowest analyzed temperature of 10°C (Figure 5). Clustered centroids of apo-GK-wt and apo-GK-S64Y from MD simulations at 10°C were investigated further to scrutinize the degree and the nature of the differences between the protein ensembles of apo-GK-wt and apo-GK-S64Y.

3.6 GK Allosteric Control Occurs Via a Conformationally Linked “Solvent Switch”.

Our calculated macromolecular conformational entropies (S) highlighted the diverse population of GK-wt ensembles in comparison to apo-GK-S64Y; apo-GK-wt was more flexible, especially at lower temperatures (Figure 5A and S6). In general, apo-GK-S64Y experienced less fluctuation per residue as demonstrated by RMSF calculations (Figure 5B and Figure 6A), and the most significant fluctuation of the entire protein occurred in a region (residues 151–180) corresponding to a disordered loop (Figure 6A).

As demonstrated above, a disordered loop region (residues 151–180) near the active site appeared to exhibit the salient difference between apo-GK-wt and apo-GK-S64Y. In the X-ray crystal structure of apo-GK-wt (PDB: 1V4T) [3], residues 157–179, which accounted for the majority part of the dynamic loop region, were not defined by electron density; but this disordered loop formed a β – hairpin in the X-ray crystal structure of (GK-wt)/Glu (PDBID: 1V4S) [3]. Additionally, previously reported NMR and proteolysis studies revealed that this disordered loop experienced fast dynamics and it was the primary locus of apo-GK structural disorder [24]. Our explicit MD analysis show that the disordered loop was the focus of structural fluctuation (Figure 6A), and this structural fluctuation is tightly linked to the magnitude of solvent exposure as demonstrated by our SASA evaluation of the disordered loop (Figure 6B). Thus, our assessment determined the extent that the preferential interactions between the disordered loop and solvent molecules contribute to the global conformational distribution and structural dynamics of apo-GK-wt and apo-GK-S64Y ensembles.

Superposition of clustered centroids of apo-GK-wt and apo-GK-S64Y allowed a precise view of the nuanced relationship between solvent interactions, global conformational distribution and regional enzyme dynamics. We found that the dynamic loop of apo-GK-wt clustered centroids showed a wide range of conformational orientations in association with different protein $G_{\text{solvation}}$ values (Figure 7B); configurations of the disordered loop can be illustrated by the three most representative forms shown in Figure 7A. When the disordered loop was “compact” (GK-wt_form1), the active site was protected from the solvent environment (Figure 7A), and it was associated with the least favorable $G_{\text{solvation}}$ among the three out of nine examined clustered forms shown (Figure 7B). Furthermore, the “compact” orientation of the disordered loop occurred infrequently, constituting only 0.80% of all analyzed MD snapshots of GK-wt. However, when the disordered loop was in an extended, “super-open” configuration (GK-wt_form3), fully exposing the active site to

solvent (Figure 7A), the structure was associated with the most favorable $G_{\text{solvation}}$ among all analyzed clustered forms (Figure 7B). This “super - open” configuration of the disordered loop seldom occurred; it made up as little as 0.11% of all investigated apo-GK-wt ensembles. In comparison to the two uncommon forms of apo-GK-wt clustered centroids discussed, the dominant clustered centroid (GK-wt_form2), which represented as much as 80% of the entire apo-GK-wt ensemble population, organized its dynamic loop in a relatively extended, “open” conformation. This dominant, “open” conformation of the disordered loop was positioned in-between the “compact” and the “super-open” loop orientations (Figure 7A). Although not extended fully, this “open” conformation of the disordered loop suggested that the active site of apo-GK-wt was largely exposed to the solvent environment. Frequently, this orientation of the disordered loop allowed GK-wt active site to form preferential solvent interactions as suggested by a rather favorable

$G_{\text{solvation}}$ (Figure 7B). The disordered loop in apo-GK-wt sampled several orientations in between “compact” and “super-open” states that facilitated favorable solvent interactions to various extents, which strengthened the idea that apo-GK-wt ensembles were flexible with the capacity to explore more conformations during catalysis. The apo-GK-S64Y ensembles were less structurally diverse, associated with limited enzyme flexibilities and less favorable

$G_{\text{solvation}}$ as suggested by our earlier property calculations from the temperature-based MD simulations (Figure 5).

Unlike apo-GK-wt, apo-GK-S64Y displayed a strong preference for the “compact” conformation of its disordered loop region. This conformational preference of apo-GK-S64Y can also be described by the three representative clustered forms as illustrated in Figure 7C. GK-S64Y_form1 and GK-S64Y_form3, associated with the least favorable and the most favorable $G_{\text{solvation}}$ out of three clustered centroids respectively (Figure 7D), exhibited a disordered loop that either sealed off the active site or exposed the active site to solvent interactions (Figure 7C). Moreover, compositions of the disordered loop of GK-S64Y_form 1 and GK-S64Y_form3 were infrequent, constituting only 2.54% and 1.27% respectively, of the analyzed population of GK-S64Y ensemble. The dominant, GK-S64Y_form2 possessed a rather “compact” dynamic loop that represented as much as 96% of all apo-GK-S64Y ensembles (Figure 7C), and this relatively “compact” configuration of apo-GK-S64Y (GK-S64Y_form2) prevented favorable solvent interactions and resulted in a rather unfavorable $G_{\text{solvation}}$ (Figure 7D). Previous proteolysis experiments with thermolysin on other GK activating mutants at residue S64 in the apo-states supports our experimental and computational findings of the limited structural flexibility of apo-GK-S64Y [24] regarding the unconserved dynamic loop (Figure 7E). In summary, these MD simulations on apo-GK-wt vs. apo-GK-S64Y, indicated a conformational link between protein structures and solvation. This conformational link pointed to an enhanced protein solvation energy in apo-GK-wt which was tied to its structural flexibility and conformational distribution.

3.7 How the Solvent Switch Affects Glucose Binding and GK Turnover: The Allosteric Control of a Glucose Sensor.

Our experimental SAXS results, along with previously published studies [39], have identified a linkage between the enhanced activity of GK mutant’s structural compactness of

the apo-form with enzyme activity. From our subsequent MD based calculations, apo-GK-wt ensemble manifested favorable macromolecular conformational entropy (S), enhanced flexibility and greater SASA than apo-GK-S64Y. Moreover, the temperature sensitive behavior of apo-GK-wt, and the relatively temperature insensitive character of apo-GK-S64Y observed in properties calculated from MD simulation studies mirrored the trends seen in apo-GK-wt and apo-GK-S64Y glucose binding studies, as shown by the van't Hoff analyses, respectively (Figure 5 and Figure 3A).

Our series of experimental and computational studies on GK-wt and the clinically hyperactive mutant, GK-S64Y, showed that the substrate recognition of glucose entailed an elegant and continuous conformational transition of the apo-enzyme. Specifically, a partially disordered loop (residues 151–180) near the active site acted as a “solvent switch” (i.e., a specific structural change in GK vastly changed its solvation energy). The conformational distribution, and protein flexibility were clearly salient aspects of glucose binding, yet these dynamics were also associated with a wide range of changes in protein solvation energy, which was an integral part of the reaction coordinate (i.e., protein desolvation energy played an important role in the energetics of catalysis). Previously published GK stopped-flow fluorescence spectrophotometry measurements of glucose binding kinetics suggested that glucose binding was the slow step (85-fold slower than the k_{cat}) [40]. Thus, the physical step of GK substrate binding was the dominant kinetic barrier in GK catalysis. This slow glucose-binding step was obviously affected by the apo- ground state energies of the GK ensembles. One can surmise that the enhanced rate of the overall GK catalyzed reaction is driven by ground state differences in the respective apo-systems (Figure 8), giving the more favorable ΔG° binding of glucose in GK-S64Y relative to GK-wt (Figure 3B), and the large additional desolvation penalty of the GK-wt relative to the mutant (Figure 8).

In order to test the possibility outlined in Figure 8, in which the differences in apo-GK ground state energies are driving rate differences in glucose binding, we performed stopped-flow studies on apo-GK-wt and apo-GK-S64Y binding to glucose (Figure 9). As expected, the GK-S64Y exhibits faster substrate binding, in agreement with the scheme outlined in Figure 8. More specifically, our stopped-flow pre-steady glucose binding kinetics assays with GK-wt and GK-S64Y demonstrated that, the rate of fast-step bimolecular binding of GK-S64Y and glucose is indeed threefold more rapid than that of GK-wt and glucose (Figure 9). In other words, the glucose binding step is a major contributor to the overall GK catalytic turnover. These findings, together with our SAXS, computational and thermodynamic studies agree to the enhanced solvation energy of the “solvent switch” as the driving regulator of GK activity and adaptability.

4. Conclusions

Since enzyme desolvation is a necessary step during glucose binding, apo-GK-S64Y is more poised than apo-GK-wt to follow the same continuous trajectory of substrate binding. Because apo-GK-S64Y ensemble is more structurally organized, exhibiting limited protein flexibility, and less solvent interactions, apo-GK-S64Y is essentially pre-activated for glucose binding. Lastly, both our glucose binding experiments in the presence of 20% glycerol, as well as in D_2O performed by others [40], showed enhancements of glucose

binding to GK-wt, indicating that restricted water interactions can facilitate the activation of GK-wt in a manner similar to the de-solvated/pre-activated ground state apo-GK-S64Y. Certainly, the de-solvated/pre-activated ground state of apo-GK-S64Y may provide insights in the design of tunable regulatory elements in protein-based adaptability, and in the development of allosteric activators to treat type 2 diabetes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. X-ray crystal structure and protein sequence of GK.

(A) X-ray crystal structure of GK-wt (cyan) in complex with substrate glucose (blue) and GKA (magenta) (PDB:1V4S). GKA and Ser 64 residue (red) are in the allosteric site. The glucose binding site is ~20 Å away from the allosteric site. (B) Sequence alignment of the four members of the human hexokinase family. Glucokinase is represented as hexokinase 4 (HXK4_HUMAN). Residues are highlighted from light to dark in response to the degree of conservation. Ser 64 residue is shown in red (Residues 1–13, 462–465 were missing from PDB:1V4S).

Figure 2. Conformational changes in GK-wt and GK-S64Y captured by substrate binding titration using SAXS.

(A) Radius of gyration (R_g) of GK-wt (black) and GK-S64Y (red) at glucose concentration of 0 mM, 5 mM, 25 mM and 100 mM. For the scattering data of GK-wt and GK-S64Y at 0 mM glucose, the inset displays the linear segment of the Guinier regions and the residuals after fitting to the experimental data in the low- q region, such that $q \cdot R_g < 1.3$, yielding R_g values of 35.4 Å and 33.3 Å for GK-wt and GK-S64Y, respectively. (B) R_g values of GK-wt and GK-S64Y at glucose concentration of 0 mM, 5 mM, 25 mM and 100 mM. Error bars, SD. Two-way ANOVA test was used with p values shown ($n=3$).

Figure 3. Thermodynamic parameters of GK-wt and GK-S64Y glucose binding in aqueous buffer vs. glycerol.

(A) Van't Hoff plots of GK-wt (black) and GK-S64Y (red) glucose binding in buffer pH of 7.4 in aqueous buffer (solid lines) or in 20% glycerol (dotted lines) over a temperature range of 11°C to 44°C. (B) G of glucose binding across the examined temperature range using van't Hoff derived enthalpy and entropy. (C) Thermodynamic signatures of GK-wt and GK-S64Y glucose binding at 11°C and 39°C in buffers with and without 20% glycerol.

Figure 4. Goodness of fit (χ^2) of MD-based atomistic homology models.

Representative MD-based atomistic homology models generated for apo-GK-wt (red), X-ray crystal structure of GK/Glu/GKA complex (PDB:1V4S; green) and X-ray crystal structure of apo-GK-wt (PDB: IV4T; purple) with their calculated R_g values. The MD-based atomistic homology model used in the subsequent MD simulation studies is shown with a large red dot, which displays a relative low discrepancy score(χ^2) and represents ~ 80% of generated atomistic homology models.

Figure 5. Analysis of Molecular Dynamic trajectories of apo-GK-wt (black) and apo-GK-S64Y (red) from simulations performed at 10°C, 25°C and 40°C.

(A) Weighted averages of conformational macromolecular entropies (S) of apo-GK-wt and apo-GK-S64Y ensembles over examined temperature range. Conformational entropies (S) were calculated using Boltzmann sampling [37] over structural clustering of 866 snapshots for every MD simulation (see Methods for more details). Weighted averages were calculated considering the relative importance of each clustered centroid (i.e., a more populated clustered structural centroid was given more weight based on its population size). (B) Areas under the curve [7] of the RMSF plots of apo-GK-wt (black) and apo-GK-S64Y (red) at examined temperatures of 10°C, 25°C and 40°C. AUC values were calculated by integrating the RMSF curves from MD simulations of apo-GK-wt and apo-GK-S64Y at examined temperatures. (C) Weighted average values of solvent surface accessible areas (SASA) of apo-GK-wt and apo-GK-S64Y over examined temperature range. (D) Weighted averages of Poisson-Boltzmann solvation (PBS) energy of GK-wt and GK-S64Y [38].

Figure 6. MD calculations associated to the disordered loop at 10°C.

(A) Calculated RMSF of each amino acid residues apo-GK-wt(black) and apo-GK-S64Y (red). The arrowed region matches the disordered loop (residues 151–180) region. (B) Solvent Accessible Surface Areas (SASA) of the dynamic loop (residues 151–180) in apo-GK-wt (black) and apo-GK-S64Y (red) during MD simulations after reaching equilibration. The average SASA values are shown as a straight line for clarity.

Figure 7. Structural comparison of apo-GK-wt and apo-GK-S64Y clustered centroids from MD at 10°C.

The glucose bind site is represented as a black dot. (A) Superposition of three representative clustered forms of apo-GK-wt, the loop regions are differentiated with colors. GK-wt_form1 (gray), GK-wt_form2 (cyan) and GK-wt_form3 (blue). (B) $G_{\text{solvation}}$ of GK-wt_form1 (gray), GK-wt_form2 (cyan) and GK-wt_form3 (blue). (C) Same as A, but showing superposition of three representative clustered forms of apo-GK-S64Y with differentiated colors, GK-S64Y_form1 (gray), GK-S64Y_form2 (magenta) and GK-S64Y_form3 (red). (D) $G_{\text{solvation}}$ of GK-S64Y_form1 (gray), GK-S64Y_form2 (magenta) and GK-S64Y_form3 (red). (E) Protein sequence alignment from residue 121 to residue 180 across all four members of the hexokinase family. Residues are highlighted from light to dark based on the degree of conservation. The disordered loop region (residues 151–180) is shown in red.

Figure 8.
Energy diagram of the allosteric activation of GK via differences in ensemble solvation.

Figure 9. Fitted curves of observed rate constants of glucose binding to GK-wt and GK-S64Y measured by stopped-flow fluorescence spectrophotometry.

(A) Linear plot of bimolecular binding rate, k_{obs} , of GK-wt (black) and GK-S64Y (red) with respect to the concentration of glucose. (B) Stopped-flow fluorescence spectra of GK-wt (black) and GK-S64Y (red) binding with 10mM glucose fitted to the one-phase association model.

Table 1

Change of enthalpy (ΔH) and change of entropy (ΔS) of GK-wt and GK-S64Y in the absence or presence of 20% glycerol using van't Hoff analysis. Error, SD.

Protein Sample	ΔH , kJ * mol ⁻¹	ΔS , J * K ⁻¹ * mol ⁻¹
wt in buffer	38 ± 4	180 ± 14
S64Y in buffer	28 ± 2	145 ± 7
wt in 20% glycerol	23 ± 3	130 ± 8
S64Y in 20% glycerol	12 ± 1	97 ± 3