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Thermal Stability of Glucokinase (GK) as Influenced by the Substrate Glucose, an Allosteric Glucokinase Activator Drug (GKA) and the Osmolytes Glycerol and Urea

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Abstract

We investigated how glycerol, urea, glucose and a GKA influence kinetics and stability of wildtype and mutant GK. Glycerol and glucose stabilized GK additively. Glycerol barely affected the TF spectra of all GKs but decreased k_{cat} , glucose $S_{0.5}$ and K_D values and ATP K_M while leaving cooperativity unchanged. Glycerol sensitized all GKs to GKA as shown by TF. Glucose increased TF of GKs without influence of glycerol on the effect. Glycerol and GKA affected kinetics and binding additively. The activation energies for thermal denaturation of GK were a function of glucose with K_D s of 3 and 1mM without and with glycerol, respectively. High urea denatured wild type GK reversibly at 20 and 60 °C and urea treatment of irreversibly heat denatured GK allowed refolding as demonstrated by TF including glucose response. We concluded: Glycerol stabilizes GK indirectly without changing the folding structure of the apoenzyme, by restructuring the surface water of the protein, whereas glucose stabilizes GK directly by binding to its substrate site and inducing a compact conformation. Glucose or glycerol (alone or combined) are unable to prevent irreversible heat denaturation above 40 °C. However, urea denatures GK reversibly even at 60 °C by binding to the protein backbone and directly interacting with hydrophobic side chains. It prevents irreversible aggregation allowing complete refolding when urea is removed. This study establishes the foundation for exploring numerous instability mutants among the more than 600 variant GKs causing diabetes in animals and humans.

Keywords

Diabetes; Glucokinase; Tryptophan fluorescence; Protein stability; Denaturation; Calorimetry

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1. INTRODUCTION

The kinetics and biophysics of glucokinase (GK; EC 2.7.11) are being intensively studied to advance the understanding of the key roles that this enzyme plays in glucose homeostasis serving as glucose sensor in insulin producing pancreatic beta-cells and as regulator of hepatic glycolysis, glycogen metabolism and gluconeogenesis [1–4]. Interest in basic studies is further heightened by the enzyme's medical relevance as illustrated by intensive biochemical genetic studies of GK linked diabetes mellitus and hyperinsulinism and the discovery of GK activators (GKAs) with potential for new antidiabetic pharmacotherapy [4–16]. The unique molecular features of GK requiring further in depth exploration include: 1) the mechanism for the enzyme's cooperative kinetics with glucose, 2) the molecular nature of its large conformational change induced by glucose binding and 3) biophysical aspects of its functional and structural stability, chosen as topic of the present study. Functional and structural stability of GK are important factors that need to be considered in basic physiological chemical explorations of glucose homeostasis and, equally significant, in biochemical genetic investigations of diabetes and hyperinsulinism linked to GK. For example, several instability mutants of GK have been discovered in mouse or man (e.g. V62M, G72R, H137R, K140E, L165F, V203A, S263P, G264S, E265K, M298K, E300K, V367M, K414E and P417R) where functional or structural stability is the sole or a contributory molecular cause of monogenic diabetes mellitus but the biophysical basis of their instability remain incompletely understood [8, 9, 12, 13, 16 and 17]. It also seems that activating GK mutants causing hyperinsulinemic hypoglycemia may be more stable than normal GK as demonstrated for GK-Y214C [18]. As an essential first step in the longterm project addressing this issue we employ in the present study the stabilizing and destabilizing osmolytes glycerol and urea, respectively, to explore functional and structural stability of GK using enzyme kinetics, tryptophan fluorescence (TF) and differential scanning calorimetry (DSC) of recombinant human wild type pancreatic islet GK and of three distinct mutant GKs which retain only one of its three tryptophans (either W99, W167 or W257) as fluorescence probes for local intramolecular domains [19,20]. The osmolyte glycerol is used as a biochemical tool to alter the structure of the macro molecule's surface water and thereby influence indirectly signature parameters of the protein including functional and structural stability and to retard or prevent the thermal denaturation of the protein [20]. Glycerol has already been employed as effective reagent in previous enzymological studies which demonstrated that GK exhibits “burst” and “lag” kinetics of reaction progress curves, a characteristic of many cooperative enzymes, also termed “hysteretic” enzymes [12, 22, 23]. Hysteresis (from the Greek hysterein which means straggling behind or being late) results from the slow conversion of one conformation of an enzyme to another with different kinetic characteristics following the rapid formation of the enzyme/substrate complex [24, 25]. This hysteretic behavior explains why in the presence of high glycerol GK's cooperativity with high glucose is lost when based on initial velocity measurements [22, 26] as compared to steady state analysis which showed that the sigmoidal glucose dependency of the GK reaction is maintained [12,22]. Therefore all studies performed here are based on steady state measurements to facilitate the interpretation of results from different approaches. Urea was employed to denature GK chemically to explore a mechanistic alternative to thermal protein denaturation and also because it had been reported previously

that GK readily refolded to a kinetically normal enzyme after having been inactivated by treatment with 4 or 8 M urea [27]. Recent evidence suggests that this effect of urea is direct and attributed to interaction of the CO group with the peptide backbone amides and to van der Waal bonding to hydrophobic side chains. The experiments with molar concentration of glycerol or urea which were expected to affect the kinetics and biophysics of GK by general, unspecific mechanisms similarly as they would other proteins were contrasted with corresponding studies using millimolar concentrations of the substrate glucose which operates through a specific mechanism based on binding to the monomeric enzyme's catalytic site and were finally complemented by some focused experiments employing microM concentrations of a GKA which affects GK structure and function by activating it via the allosteric modifier region of the enzyme [4, 28].

The present studies demonstrate that glycerol causes large functional changes and increases stability probably as a result of generalized restructuring of surface water of GK by an indirect mechanism and that these changes can occur with minimal perturbation of the protein folding structure and with preservation of the unique cooperative kinetics of the enzyme whereas glucose stabilizes the enzyme through a specific, ligand induced intramolecular transition from an open to a closed conformation. The stabilization by glucose and glycerol is however limited since thermal GK denaturation above 40 °C is irreversible even in their presence. In contrast, unfolding induced by urea at temperatures between 20 and 60 °C is fully reversible as demonstrated by TF and explained by direct effects of the denaturing osmolyte on the protein backbone and hydrophobic side chains.

2. METHODS

2.1. Materials

Sigma Chemical Co. (St. Louis, MO) supplied D-glucose, glycerol, urea and acrylamide. A non fluorescent GKA was used in these studies: RO0274375-000 [4, 28].

2.2 Generation of recombinant wild-type and mutant glucokinase

Recombinant wild type and mutant human β -cell glucokinases were generated and expressed as GST fusion proteins in *E. coli* as previously described [12, 13, 19]. GST-GK fusion proteins were cleaved with factor Xa and submitted to a second round of purification by removing GST with glutathione agarose and Factor Xa with Benzamidine Sepharose 6B following protocols provided by the manufacturer. Point mutations were introduced into the pGEX-3X vector using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, California, USA). All mutants were transformed into *E. coli* cells and verified by DNA sequencing. The enzyme variants made for this project were: GK-W99 (W167F/W257F), GK-W167 (W99R/W257F) and GK- W257 (W99R/W167F).

All mutant glucokinase proteins were expressed in significant amounts and were found to be essentially pure as indicated by the presence of a single band at 50 kD using gel chromatography.

2.3. Kinetic measurement

Assays to determine catalysis parameters were carried out as previously described using an NADP⁺-coupled spectrophotometric assay [12,13]. The Hill equation was used to estimate the Hill coefficient (n_H) of GK towards glucose. This equation is: $v/V_{\max} = [\text{glucose}]^H / (K' + [\text{glucose}]^H)$ where $K' = [\text{glucose}]_{0.5}$.

2.4. Absorption and fluorescence measurements

2.4.1. UV/Vis absorption spectra—were measured using a Hitachi Perkin-Elmer U-3000 spectrophotometer (Newtown, Pennsylvania).

2.4.2. Fluorescence intensity and spectra—were measured with a Fluorolog-3-21 Jobin-Yvon Spex Instrument SA (Edison, NJ) equipped with a 450 W Xenon lamp for excitation and a cooled R2658P Hamamatsu photomultiplier tube for detection. Ninety-degree geometry was employed for all measurements and 280 and 295 nm excitation wavelengths were used to observe fluorescence emission in the 300–500 nm range. For tryptophan fluorescence spectra slit width was set to provide a band-path of 4 nm for excitation and 3 nm for emission. Spectra were corrected for instrumental response using the Spex instrument software. A thermostatically controlled cell holder maintained the sample temperature. A thermocouple was used to measure the temperature of the sample.

2.4.3. Fluorescence quantum yields—of tryptophan (Φ) were determined for GK wild type and mutants using the following equation [29]:

$$\Phi_S = \Phi_R (A_R / A_S) (F_S / F_R) (n_S^2 / n_R^2)$$

The subscripts S and R refer to sample and reference standard, respectively; A_R and A_S denotes the absorbances at the excitation wavelength, F_S and F_R denote the integral intensities of the recorded fluorescence spectra measured under identical instrument settings. n_S and n_R are the refractive indices. Fluorescence quantum yields were determined relative to N-acetyl-L-tryptophanamide (NATA, $\Phi = 0.14$) [30].

2.4.4. Temperature dependent fluorescence spectra—of GK wild type and mutants were measured over the temperature range from 10 °C to 65 °C. Temperature dependent denaturation of the proteins were presented as the ratio of fluorescence intensities at 320 nm and at 360 nm (I_{320}/I_{360}).

2.4.5. Acrylamide quenching of TF fluorescence—The effect of acrylamide on the fluorescence of single tryptophan enzymes in the presence and absence of glucose was studied as described [31–33].

2.4.6. Effects of urea on unfolding/refolding of GK—The ΔG of GK unfolding as influenced by glycerol and glucose alone or combined was determined by urea titration using TF recording [34, 35]. Studies of reversibility of thermal or urea induced denaturation (unfolding and/or oligomeric aggregation) were carried out using TF by observing characteristic spectral shifts (from blue to red and back) and the concentration dependent fluorescence increase resulting from glucose binding [19].

2.4.7. Titration curves for glucose binding—were obtained by stepwise addition of the appropriate amount of glucose to the fluorescence cuvette. The fluorescence increase of GK proteins during glucose titration were measured with time dependent scans. A band-path for excitation was 0.5 nm at 280 nm wavelength and 12 nm for emission at 340 nm wavelength. Data were fit to the Langmuir saturation function $F = F_0 + (F_{\text{sat}} - F_0)([\text{gluc}]/([\text{gluc}] + K_D))$. The temperature dependence of binding was described using the Van't Hoff equation: $d \ln K / dt = -H / RT^2$.

2.5. Calorimetry

Samples were degassed using MicroCal Thermovac degassing apparatus. The heat capacity was determined using MicroCal VP-DSC calorimeter. In this instrument, the heat capacity difference between reference and sample cells is monitored; the reference solution included all substances except the protein. The thermograms were analyzed using MicroCal Origin software. The T_m and calorimetric enthalpy, H , was calculated as an integration of the DSC thermogram (C_p (kcal/mole/°C) vs temperature (°C)).

Experimentally observed T_m and C_p 's are sometimes a function of the scan-rate [36]. To check for this, the value T_m indicated by DSC was measured as a function of scan-rate and glucose concentration. The values are given in the supplemental Table S1. The values for T_m using fast (1.5 deg/min) and slow (0.25 deg/min) scan rates were within about 1 degree of each other, indicating that the fast scan rate produces a transition that is not quite at equilibrium. In all subsequent experiments the slow scan rate was accordingly used. Since during the course of heat denaturation at the slow rate of 0.25 degree/min equilibria are not established between the native (GK^N) and the denatured (GK^D) states of the protein before conversion to a fully unfolded (GK^{fd}) state kinetic information can be obtained on the process of protein unfolding. As pointed out by Sanches-Ruiz et al. [36,37] for a two state irreversible denaturation process $GK^{\text{native}} \xrightarrow{k} GK^{\text{denatured}} \rightarrow \rightarrow GK^{\text{fully denatured and/or aggregated}}$ the rate constant of unfolding relates to the heating rate v (in degree K/min) by $k = vC_p / (Q_t - Q)$ where C_p is the excess heat capacity, Q_t is the total heat change of the denaturation process and Q is the heat evolved at a given temperature. C_p^M is the apparent heat capacity at the maximum. The energy of activation, E_a , is obtained with a 0.25deg/min heating rate using the Arrhenius relationship $k = A \exp(-E_a / RT)$.

3. RESULTS

3.1. Crystal Structures of GK and Solvent Accessibility of the Enzyme's Three Tryptophans

The conduct of basic studies of GK are greatly influenced by current knowledge about its structure, perhaps most strongly by the crystal structures reported by Kamata and colleagues demonstrating two conformations of the enzyme, a ligand free "super-open" form and a closed form of the enzyme in ternary complex with glucose and an allosteric activator [38]. The ternary complex is fully described while that of the ligand free protein is missing a stretch of residues (amino acids 157–179) which is part of the glucose binding site and contains W167 which is important as an internal fluorescence probe (Figure 1). The active site is therefore only partly defined in the open conformation (Figure 1A and C) but clearly outlined in the closed conformation (Figure 1B and D). Figure 1 also depicts the allosteric

activator site in the open and closed configuration to illustrate its marked glucose induced reconfiguration and relocations of the contact amino acids and also of W99 which seems to be involved in affecting access and affinity of the enzyme for GKAs. Recent reports have further expanded the crystallographic data base by including the binary GK/glucose structure (PDB code 3IDH) which is apparently not different from Kamata's ternary GK/glucose/GKA structure and also providing the structure of the ternary GK/glucose/ATP complex (PDB code 3ID8). Figure 1 is consistent with this newer information. Some knowledge about the microenvironment of the three tryptophans at positions 99, 167 and 257 is critical for interpreting the fluorescence data of the present studies. It has been reported that the solvent accessibility for these tryptophans is differentially affected by glucose [20], i.e. that solvation of W99 is greatly increased and that of W257 is apparently unchanged (with no information on W167 available because the structure of the open form is insufficient). Using acrylamide quenching of TF of GK mutants which contain only a single tryptophan we have confirmed this observation and have demonstrated in addition that the solvation of W167 is greatly decreased by glucose explaining the glucose induced spectral blue shift and fluorescence increase (Tables 1, also Supplemental Figures S1, S2 and Table S2).

3.2. Effect of Glycerol on the Kinetics of GK and Additivity of GKA and Glycerol Actions

Tryptophan has been widely used as a molecular probe and the usefulness of this strategy for GK has been explored previously. Most relevant for the present studies are investigations with mutant GK retaining only one of its three tryptophans or having no tryptophan left [18]. The results showed that tryptophans at positions 99, 167 and 257 are not essential for function and structure of GK. Most strikingly, even the tryptophan free enzyme remained catalytically highly competent and retained the cooperativity with regard to glucose, its responsiveness to allosteric activators and was inhibited by the endogenous regulatory protein, characteristics unique to GK. This interpretation is not undermined by the fact that two or even three amino acid substitutions caused some quantitative changes in substrate affinities and the k_{cat} of the mutants. In another relevant study (20) the three tryptophans were mutated one at a time and the effects of these single substitutions explored with results that closely reflected the outcome with single tryptophan enzymes cited above [19]. Here we show that 20–30% (v/v or 2–3M) glycerol affects wild type and single tryptophan enzymes similarly (Table 2) demonstrating again that these amino acids influence the function of the enzyme relatively little and are therefore highly suited as molecular probes for investigating circumscribed domains of GK. Using steady state measurements we found that high glycerol lowers the glucose $S_{0.5}$, the ATP K_M and the k_{cat} of all GK species studied here markedly and nearly to the same extent but affects cooperativity with regard to glucose little or not at all. It is remarkable that in most cases glycerol does not affect the catalytic capacity of the enzymes as expressed in the $k_{cat}/S_{0.5}$ ratio. It was found that the efficacy of a GKA was little influenced by the presence of glycerol as indicated by the fact that the glucose K_D and $S_{0.5}$ were lowered even further and the k_{cat} reduction due to glycerol was reversed. The effect of high glycerol differs thus significantly from that of allosteric GKAs which similarly increase GK affinity for glucose, but lower its affinity for ATP, usually increase the k_{cat} and in many instances lower the Hill coefficient. The near constancy of the Hill coefficient of the four enzymes in the presence of 20% (v/v) glycerol even when activated by a GKA corroborates

results of Neet et al. (22) but is contrary to those of Pollard-Knight et al. [26] who found that high glycerol decreases cooperativity markedly. The difference is explainable by different methods of kinetic analysis: steady state vs. initial velocity measurements and the use of the full range of glucose concentrations including saturation of the enzyme between 0.1 and 100mM vs. reliance on glucose levels <5 mM (in [22] vs. [26]).

Glucose binding to hexokinases including GK is a spontaneous process increased by high temperature. Previous studies of the thermodynamics of glucose binding to human wild type GST-GK have shown that it is predominantly entropy driven [20]. We reinvestigated here the effect of glycerol on the thermodynamics of glucose binding using pure wild type GK (Figure 2 and supplemental Table S3). Our results with pure GK confirmed the published thermodynamic constants: $G = -13.1$ kJ/mol; $H = 34 \pm 2$ kJ/mol and $S = 157 \pm 4$ J/mol*K. The glycerol enhancement of GK glucose binding manifested itself in corresponding changes of the thermodynamic constants: $G = -16.0$ kJ/mol; $H = 20 \pm 3$ kJ/mol and $S = 120 \pm 9$ J/mol*K. These values from Vant Hoff plots are based on the marked effects that temperature has on the glucose K_D in the absence and presence of glycerol ranging from 13.7 mM at 11 °C in the absence of glycerol to 1.15 mM at 45 °C in the presence of it. Temperature has, however, only a minor effect on the glucose $S_{0.5}$ and the Hill coefficient of GK (Supplemental Table S4). The Q_{10} of the GK reaction was 1.29 in the absence of glycerol and 1.47 in its presence.

3.3. Effects of Glycerol on TF of Wild Type and Single Tryptophan Containing Mutants in the Presence and Absence of Glucose

The effect of 20% (v/v) glycerol on the spectral characteristics of tryptophan fluorescence of GK is comparatively small in view of the large effect that the osmolyte has on glucose binding and enzyme kinetics (and on the thermal stability to be documented below). This holds true for the wild type and all three single tryptophan enzymes (Figure 3, Tables 3). With glycerol present there is a tendency for an increase of the TF quantum yield in the basal and in the glucose activated state with a slight spectral blue shift (Figure 3, Table 4). The phenomenon is most pronounced for W167 and least apparent for W257. The basal fluorescence of W167 increased by 26% and the relative glucose enhancement fell by 21%. This localized structural effect on W167 by glycerol is associated with an effect on the glucose K_D which drops to 0.09 from 0.38 mM, more than the 2–3 fold change in the other cases (Table 2). W167 is an activated enzyme to begin with, but its catalytic capacity ($k_{cat}/S_{0.5}$) is not altered by glycerol. In the presence of glycerol it resembles high affinity hexokinase. Substitution of W167 by phenylalanine increases the glucose K_D and $S_{0.5}$ and lowers the k_{cat} of the W99 and W257 enzymes markedly. GKAs do usually not bind to GK in the absence of glucose as indicated by a lack of the TF spectrum changes (Figure 4A and reference [4]). However, in the presence of high glycerol there was evidence of GKA binding to all four enzymes studied here as exemplified by Figure 4B in the case of wild type GK, and most pronounced in the case of W167 which exhibited a 1.5 fold fluorescence increase at 20 μ M of the drug (Figure 5; note that the activator increases TF of W167 even in the absence of glycerol). The emission spectrum of N-acetyl-L-tryptophanamide (the reference cpd) measured in buffer and in buffer plus 20% glycerol retains the same spectral

characteristics since there is no change in the quantum efficiency ($\Phi = 0.14$) and the emission max at 350 nm (data not shown).

3.4. Effect of Non-Denaturing Temperature Changes on TF of Wild Type and Single Tryptophan GKs

Studying the general physical effect of moderate, non denaturing temperature changes (from 10 to 37 °C, a range where thermal effects on GK have been reported [38] and are shown below to be reversible) on TF of GKs containing only one tryptophan promised to provide useful information on the site specific characteristics of these intramolecular probes which can be compared and contrasted with the effects of glucose, urea and glycerol arising from very different mechanisms. Rather than intensity changes used to measure binding, here we are focusing primarily on another fluorescence parameter – the spectrum. The electron density of the excited state of tryptophan is significantly different from the ground state molecule, resulting in a larger dipole moment in the ground state [40, 41]. A consequence of the photophysics of tryptophan is that its fluorescence spectrum is sensitive to the solvent polarity, being blue shifted in a hydrophobic solvent (with max ~ 310 nm) and red shifted in aqueous liquid solution (with max ~ 340–350 nm) [42]. The amount of red shift is a function of the presence of water around the indole moiety and the ability of these water molecules to rearrange during the excited state lifetime. The necessity of solvent molecules to rearrange during the excited lifetime is demonstrated in water solutions containing glycerol or propylene glycol: as temperature decreases and water relaxation time increases, the spectrum of indole becomes increasingly blue shifted [31] and at very low temperatures the blue spectral shift is comparable to spectra obtained in hydrophobic solvents [43, 44].

With this background, fluorescence spectra of GK were used to monitor the local environment of the tryptophans in the protein as a function of temperature (Figures 6 and 7). In figure 6, the intensities of the glucose bound and glucose-free enzyme were normalized to 1 at 10 °C. The figures show the decrease in intensity and spectral shift with temperature for GK at a given condition. For wild type GK without D-glucose, the fluorescence maximum is at 326 nm at 10 °C and 331 nm at 37.5 °C. The ratio of fluorescence at 320 and 360 nm is 1.01 without glucose at 10 °C and 0.65 at 37.5 °C. The intensity ratio of W167 is 1.1 with glucose and 0.82 without glucose at 10 °C. The ratio of intensity at two wavelengths thus is indicative of the overall shift of the emission spectrum. However, even when the protein is fully denatured, tryptophan remains only partially accessible to water (see supplemental Figure S3).

The fluorescence emission spectrum of W99 is the most red shifted and high glucose augments this shift rather than switching it back to the blue region as true for all other enzymes tested here (Table 4). W99 is solvated most highly compared to the other tryptophans [20] and results shown earlier. Maxima of 334/336 nm (at 10 °C) and 335/338 nm (37.5 °C) are consistent with highly solvated tryptophan. The fluorescence spectrum of W167 is shifted towards blue upon glucose binding and the intensity increases. The observed blue shift upon glucose binding is in agreement with results showing low solvation of the indole ring of W167 (see above and [20]). The fluorescence of W257 shows a shift in spectrum with temperature change for both glucose-free and glucose-bound enzyme which

is similar to that of wild type and W167. W167 and W257 are thus the predominant qualitative determinants of the wild type fluorescence spectrum. It should be recalled here that the magnitude of the glucose induced fluorescence increase is primarily determined by W99 and W167 but little affected by W257 (see supplemental Table S5 and Figure S4).

3.5. Thermal stability of Wild Type and Single Tryptophan GKs in the Absence and Presence of High Glucose

TF spectroscopy and DSC provide an opportunity for exploring different aspects of GK thermo stability because one relies on the sensitive local recording of temperature effects whereas the other assesses these effects by integrating their global impact. The characteristic red shift of the spectrum in fluorescence spectroscopy of thermally induced unfolding of proteins associated with the gradual reduction of fluorescence intensity is recorded in terms of the fluorescence ratio of I_{320}/I_{360} and of its derivative $d(I_{320}/I_{360})/dT$ allowing the determination of T_{mF} , the temperature at which the local environment of the tryptophans in half of the population is sufficiently changed to cause this red shift of fluorescence. In DSC of thermal denaturation T_{mC} , the calorimetric melting temperature, is defined as the temperature at which half of the protein population is denatured implying a global structural change. It follows from this that T_{mF} and T_{mC} may differ considerably as actually observed in the present investigation (Figure 7, and Table 5). It should be realized in this context that T_m values determined by activity measurements may also differ from T_{mC} and T_{mF} [7–9].

High concentrations of glucose stabilize all GKs as indicated by a significant increase of the heat capacities and the T_m values (Figure 7 and Table 5). The T_{mF} and T_{mC} values for wild type GK in the absence of glucose differ by about 5 °C, i.e. they are 42 and 47 °C, respectively. They are the same, however, when the enzyme is saturated with glucose, i.e. 52 °C. This difference in the absence of glucose may be explained on the basis of results with the W167 and W257 enzymes. The T_{mF} of W257 is practically the same whether glucose is present or not (about 45 °C) and differs greatly from the protein's T_{mC} both in the glucose free and bound state. It seems W257 is uniquely susceptible to the rise in temperature for reasons that are not clear from the crystal structures. The emission spectrum of W167 changes little with temperature in the absence of glucose but is significantly red shifted in the presence of glucose. In contrast the temperature dependent fluorescence changes of W99 are very small, both in the absence and presence of glucose. The temperature dependent spectral changes of the wild type appear thus to be primarily explained by the behavior of the W167 and W257 spectra.

The observation of others needs to be considered here that GK aggregates at temperatures above 40 °C [20] which may impact on the I_{320}/I_{360} ratio and its derivative. Under the conditions used here we have seen no evidence for aggregation in the TF spectra of WT and single tryptophan enzymes alike between 320–400nm at temperatures ranging from 10 to 60 °C (not shown). The temperature dependent TF ratio recordings and their derivatives are thus valid indicators of the domain specific denaturation process. Most strikingly, the microenvironment of W99 seems to change little during temperature denaturation no matter whether glucose is present or not. This is also true for the open form of W167, whereas W257 is affected markedly and in a parallel manner in both conformations. The melting

curves of GK-WT in the absence and presence of glucose are comparable to those reported previously by others (Ralph et al. [45, 46]).

3.6. D-glucose and Glycerol Stabilize GK additively

Since substrate and osmolytes affect thermal stability of enzymes by different mechanisms their interaction in influencing GK was studied by TF and by DSC. An inspection of the temperature (Figure 8) and the glucose (Figure 9 and Table 6) dependency curves of the thermo stability tests clearly show for the wild type enzyme that the effects of the substrate glucose and the osmolyte glycerol are additive. When glycerol is present the temperature induced intensity decrease and red shift of the TF spectrum occur at higher temperatures both with and without saturation levels of glucose (Figure 8A and 8B). The respective T_{mF} values are 42 and 48 °C in the absence of glucose and they are 52 and 56 °C at 300 mM glucose. Studies with DSC had a very similar outcome (Figure 8C and 8D). As observed earlier in these studies with glycerol there is also a discrepancy of several degrees between the fluorometric and the calorimetric results in the absence of glucose explained by the greater sensitivity of the local tryptophan probes as compared to the global heat capacity determinations. The glucose dependencies of the calorimetric T_m measurements in the presence and absence of 20% (v/v) glycerol disclosed comparable half maximal values (EC_{50} values) of 36 and 44 mM, respectively (Figure 9A). Heat capacity values at the T_m showed glucose dependencies with similarly high half maximal effective glucose concentrations (Figure 9B). EC_{50} values are operationally defined and simply refer to the concentrations that exert the half maximal effect of glucose as GK stabilizer in the DSC thermolability test. The data are insufficient to allow any conclusions about the relative distribution of ligand free and bound forms of native and denatured GK at the T_m . The 35 year old observation of Grossman that glucose acts also as an osmolyte at concentrations above 100 mM further complicates the interpretation [47]. Computations of the activation energy provide further insight into the thermodynamics of the thermal denaturation process as a function of the glucose concentration both with and without glycerol. The Arrhenius plots show linearity at temperatures below the T_m which suggests that at these temperatures the assumption of an equilibrium between the native and the denatured states may apply (Figures 10B and 10C, supplemental Figure S5; see also section 3.7). In view of the marked temperature effect on the glucose binding by GK (Figure 2 and supplemental Table S3) the glucose dependency of the E_a for thermal denaturation of the protein was fit to a hyperbolic function using K_D s at the T_m s of the melting transitions in the absence and presence of glycerol. Using apparent glucose K_D values of 1 and 3 mM resulted in concentration dependency curves which imply that the tightness of the GK/glucose complex determines the magnitude of the energy barrier during the thermal melting transition and that glycerol increases the barrier by increasing glucose binding (Figure 10A).

3.7. Urea preserves reversibility of thermal denaturation of GK

Protein denaturation is in principle a reversible process [34]. For the case of GK it has been difficult to demonstrate this principle. Although one early report indicates that chemical inactivation of GK by high urea is reversible recent studies found that the inactivation by Guanidine HCl and thermal denaturation above 40 °C are not [27, 39]. This important issue was therefore reinvestigated. The irreversibility of thermal denaturation above 40 °C was

confirmed both for the apoenzyme with and without glycerol and also for GK in the presence of high glucose with or without glycerol (Figure 11). The moderate rise of the I_{320}/I_{360} ratios that is observed upon cooling from different temperature starting points above 40 °C requires comment. The TF spectra (not shown) provide evidence for significant blue shifts of the fluorescence maxima which suggests partial refolding. However, since the loss of glucose responsiveness of the denatured enzyme was not reversed it is likely that cooling results in misfolded nonfunctional protein. Exploring chemical denaturation by urea it was found that the ΔG of denaturation of the GK apoenzyme was small with 1.63 Kcal/mol and that glucose and glycerol alone or combined increased this value to maximally to 2.87 Kcal/mol (Figure 12). The process of GK unfolding with 4 or 8 M urea was fully reversible based on the results of TF studies (Figure 13). In the presence of urea TF of GK is increased, red shifted and its response to glucose is totally lost. Following removal of urea by dialysis the TF spectrum is normalized and the glucose response restored. Reversibility of the process is complete following a 30 minute denaturation in the presence of 4 M urea at 20 °C (Fig. 13, panel A) and at 8M urea both at 20 and 56 °C (the 20 °C data are shown in Fig. 13, panel B). A 30 min urea treatment of GK that had been thermally denatured at 60 °C followed by dialysis to dilute urea (to < 0.13 M) was sufficient to fully renature the enzyme based on TF data (indistinguishable from results in Fig. 13, panel B, but not shown). Similar unfolding and refolding process of GK was also obtained in 20% glycerol (Figure 13, panel C).

4. DISCUSSION

The present results are discussed under two headings, one addressing basic biophysical aspects of protein stability and the other focusing on the biomedical implications of functional and structural instability of GK

4.1. Basic Biophysical Considerations

To rationalize the basic biophysical results, we begin with the mechanisms underlying thermal protein denaturation for glucose binding by GK (equations 1 and 2).

Thermal denaturation of GK is conceptualized as occurring in two steps (equation (1)), an initial equilibrium process followed by an irreversible step. The assumption is made that denaturation is in principle reversible at least for a significant part of the TF and DSC melting transitions allowing the application of equilibrium thermodynamic analysis [36, 37]. Much evidence has accrued that suggest that the functional activation of GK by glucose follows the sequence given in equation 2 (equation 2) where GK_{open} has a low affinity for glucose and GK_{closed} has a high affinity [38]. The binding of glucose triggers a conformational change to a more compact form of the enzyme [20, 22, 23, 26, 38]. Our data show that glucose binding to GK increases GK's T_m , C_p and ΔH (Table 5). There are many examples of an increase in T_m and C_p with the binding of substrate, since, when ligand binds to the folded form, the effect is to increase thermodynamic stability [48]. The increase in heat capacity upon glucose binding is related to increase in internal H-bonds and increased van der Waals forces. As seen in Figure 1, the glucose-bound enzyme has increased secondary structure, which is especially apparent in the region around W167. The

beta strands and alpha-helix seen in the glucose-bound enzyme disappear when glucose is removed (compare Figure 1C and D). The heat capacity changes at T_m are due to the loss of internal H-bonds. The polypeptide of GK without glucose is less bonding than GK with glucose.

Glucose OH groups H-bond to the carboxyl of E290, E256 and D205 [19, 20, 38]. In considering glucose binding to GK one must think of both water and D-glucose interaction with the protein. The co-solvents can affect bulk water and the solvation of protein and D-glucose. Glycerol is fully miscible in water, but glycerol disrupts the H-bond percolation network of water [49]. The hydration around glucose has also been studied [50], and both glycerol and sugars present hydroxy groups which H-bond to water, and aliphatic groups which do not, or to a much lesser extent.

Proteins are long known to be stabilized by glycerol [43,44,47]. In support of general stabilization of the protein, is the observation that the cosolvent increases T_m and C_p in the absence of D-glucose (Table 6). Non-aqueous solvents are known to stabilize proteins and to shift the melting temperature. The effect can be very large, when water is very much depleted. For example, RNase A has a $T_m = 61^\circ\text{C}$ in water and 124°C in nonane [54]. In comparison, the T_m of GK shifts from 46.9 to 51.3°C in 20 % glycerol (Table 6). If we consider that GK is in equilibrium between open forms with low glucose binding and closed forms with high glucose binding affinities, then the increase in C_p and binding constants ($1/K_D$) can both be explained. A likely effect is that co-solvents stabilize proteins by reducing water interactions at the surface. As recently shown by Vagenende and coworkers, glycerol binds to hydrophobic sites on the surface through van der Waals force with the aliphatic groups, allowing glycerol's OH groups to interact with water, and this favorable interaction stabilizes the protein [21]. Glycerol may also directly aid in glucose binding by "removing" water molecules at the binding site. In this view, water is an inhibitor of glucose binding. This opinion has been also put forward by Reid and Rand, who observed that high concentrations of osmolytes increase the binding affinity of glucose to the related protein, yeast hexokinase [55]. Finally, one can also consider the possibility that glycerol increases viscosity and this could dampen large fluctuations of the protein. However, glycerol at 20% does not change viscosity greatly. The viscosity of water is 1.3 at 10°C , 1 at 20°C , 0.6 at 30°C and 0.65 at 40°C . The respective values for 20% glycerol are 2.41, 1.76, 1.35, and 1.07 [56].

Although the inflection point of the thermogram obtained by TF can be the same as that determined by DSC (e.g. in the presence of high glucose), the fluorescence spectra also indicate changes in the protein at temperatures lower than the DSC T_m . These fluorescence changes indicate dynamical change before over-all denaturing unfolding. W257 shows the largest dynamical changes before the denaturing unfolding transition. The amino acid W257 is in the "structured" core region of the large lobe. The similarities of fluorescence response for with and without glycerol suggest that internal motions are responsible for fluorescence changes. W257 is located near the glucose binding site. We note also that glycerol does not change the cooperativity of kinetics. The cooperativity is attributed to the slow relaxation of the closed form to the open form [22, 23, 25, 56–58]. Our data show that the cooperativity is

retained in the presence of glycerol. The reformation of the many internal H-bonds in GK when glucose binds is the likely rate limiting process.

Sugar binding to enzymes is exothermic [59]. Previous [20] and present results show that the process is predominantly entropy driven. Glycerol increased the free energy change of glucose binding to the enzyme. The OH's of glucose are equatorial, and glucose structures water to a larger degree than other sugars [50]. Water molecules above and below the plane would be ordered or "ice-like". Binding of glucose to the enzyme would release these molecules, resulting in increased entropy. The latent heat of fusion for water is 334 kJ/kg or 6.007 kJ/mol (1.433 kcal/mol). The heat capacity of the protein for unfolding changes from 13 to 33 (extrapolated) kcal/mol upon glucose binding, kcal ~ 20. In terms of energy a kcal of 20 is equivalent to ~14 moles of water melting. The removal of this water would result in increased entropy and would be the driving force for the conformational change of GK. In addition to H-bonding effects, van der Waals forces can aid in the binding. The polarizable groups of F152 and Y297 are near the active site and Y289 is also near by perpendicular to W257. Glucose binding to GK involves both H-bonding and van der Waals forces. Substitution at carbon 1 or 2 allowed for sugar binding (i.e. D-mannose and D-mannoheptulose bind), but substitution at carbons 3 to 5 produced sugars that bind no longer or only very weakly [19]. The orientation of the OH bond is critical. H-bonding is very angle dependent [60], and changing the OH-O angle from 0 to 90 completely abolishes H-bonding [61].

The present investigation illustrates with the GK case that the range of potential conformational and functional states of proteins is much wider than the crystallographic analysis might suggest. This potential for multiformity of GK manifests itself in many ways: in characteristic TF changes when glucose or GKA are bound; in the substrate binding and kinetic studies, which demonstrate the possibility for a hundredfold range of glucose K_D values; in the different characteristics of the protein melting curves that are induced thermally or chemically by urea, one monophasic but the other one biphasic suggesting the existence of well defined intermediates; and in the propensity for aggregation at temperatures above 40 degrees C explaining the apparent irreversibility of thermal denaturation [39]. It is remarkable that the destabilizing osmolyte urea preserves reversibility of the thermal denaturation/unfolding process whereas the substrate glucose, the stabilizing osmolyte glycerol or the denaturant guanidineHCl do not [39]. This unique ("chaperone-like") capability of urea is explained by its ability to bind directly to the peptide amides and the hydrophobic side chains of proteins. The insights on multiple conformational and structural states obtained here with wild type and selected tryptophan mutants of GK promises to guide the analysis of the large number of disease causing instability mutants of the enzyme.

These deliberations suggest a working model that could conceptualize the currently available information (Figure 14). The model hypothesizes that at least ten distinct GK conformations are demonstrable experimentally: Conformations 1–3 (designated GK^{ol} , GK^{cl} and GK^{acl}) illustrate the established effects of the ligands glucose and GKAs on GK structure. Conformations 4–6 illustrate the effect of thermal or chemical denaturation and its reversal demonstrated in this study (designated GK^{pd} , GK^{fd} and GK^*). And finally

conformation 7–10 illustrate the effect of the stabilizing osmolyte glycerol on glucose and GKA binding also demonstrated in this investigation (designated GK^{ot} , GK^{ct} , GK^{act} and $GK^{ac(**)t}$). Six of these proposed conformations (1–6) constitute the biological pathways of GK stabilization and thermal denaturation modifiable by urea including the pharmacological effects of GKAs whereas the other 4 (7–10) are postulated to explain the effects of the osmolyte glycerol. The model is supported by crystallographic, kinetic, TF and DSC results. This empirical minimal model based on crystallography (38) and the present study needs to be seen in light of recent molecular dynamics [62] and NMR studies [63,64] which strongly suggest that the conformational transition from an open to the closed forms (depicted as 1–3 in Figure 14) may include as many as six intermediates of a dynamic equilibrium of structures governed by ligand occupancy most importantly glucose.

4.2. Biomedical Considerations

An understanding of functional and structural stability of GK is required to develop a full grasp of the role of this enzyme in normal glucose homeostasis, of genetically based hyper- and hypoglycemia syndromes and in the action of GKAs. The high biological significance of GK stability characteristics for glucose homeostasis is most compellingly demonstrated by the dominant impact of certain diabetes causing mutant enzymes with minimal deviation of the catalytic capacity but thermally more labile than the normal protein as discussed in the introduction for 14 such MODY-2 mutants. Of particular theoretical and practical significance are K140E and P417R which were discovered in the course of nitroso-urea mutagenesis studies in the mouse [16] causing gene dosage dependent diabetes mellitus (models for both MODY-2 and Permanent Neonatal Diabetes Mellitus, PNDM). A readily available mouse model for MODY due to GK instability mutants affords an opportunity to explore in detail the impact of protein instability of GK on intermediary metabolism and function of the wide variety of GK expressing cells including insulin and glucagon producing cells in the pancreas, endocrine cells of the gut, liver tissue, anterior pituitary cells and certain nuclei of the brain. The relevance of thermo lability as molecular basis of diabetes was questioned for some of the human MODY-2 mutants (V62M and G72R) [46]. The controversy about V62M and G72R remains unsettled particularly since it has been demonstrated in cell based studies with the MIN-6 beta-cell line that these mutants do cause a cellular loss of GK protein and not just of catalytic activity [17, 18]. In the present study with wild type and single tryptophan GKs an attempt is made to lay the biophysical groundwork for a definitive evaluation which of the group of 14 GK mutants considered thermally unstable (see introduction) should indeed be classified as such and how significant thermal instability is pathogenetically. It is anticipated that many more of the currently known GK mutants counting in excess of 600 will show thermal instability [5]. As demonstrated here, TF is an extremely sensitive indicator for thermally induced local changes of structure, too subtle to be detected by DSC, but probably manifest in altered $k_{cat}/S_{0.5}$ values as suggested by pilot studies which show good agreement of results from catalytic and TF measurements for K140E, S263P, M298K and P417R [16].

The discussion of biomedical relevance of the present study would be incomplete if it did not consider the possible involvement of the allosteric modifier region of GK in the processes by which glucose and glycerol, alone or combined, stabilize GK [1, 4, 14–16, 38,

65]. This region is clearly distinct from the catalytic site but its folding structure is greatly influenced by glucose binding which causes a transition from a ligand free open to a closed binary complex. In the wild type enzyme the large glucose induced conformational change which opens a receptor site for GKAs and does probably result in the dissociation of the catalytically inhibited GK/GKRP complex is associated with a large, near twofold intensity increase of TF. It should be recalled here that GKRP (i.e. GK regulatory protein) is a hepato-specific nuclear protein not found in pancreatic islet cells [8, 62]. Also highly relevant for this discussion is the fact that occupancy of the modifier region by GKA and GKRP is mutually exclusive and that glucose facilitates GKA but inhibits GKRP binding and action [4, 65, 67]. The restructuring of the allosteric modifier region and the closure of the substrate site by glucose result in a more compact, more stable enzyme as demonstrated by TF and DSC. In striking contrast, GK stabilization by glycerol occurs without significant alteration of the folding structure of GK as demonstrated by the minimal spectral change suggesting, at first glance, that the allosteric modifier region is changed only little. Small, glycerol induced spectral changes can not be overlooked, however, particularly in the basal state of W167 (the quantum yield increasing from 0.125 to 0.157). Present data do indeed demonstrate that glycerol enhances GKA binding markedly in the absence of glucose as illustrated with the W167 enzyme (Figure 5). Similar though less pronounced enhancements of GKA binding by glycerol are seen with the other enzymes studied here. It is noteworthy that the glucose independent binding of the GKA is facilitated by the substitution of W99 by R99 suggesting that the presence of tryptophan at that location interferes with access of the activator to its binding site in the substrate free form. Results with the GKA probe show that glycerol not only facilitates substrate binding (i.e. a lowering of the glucose $S_{0.5}$ and $ATPK_M$ but also opens the allosteric activator site. Fluorescent GKAs are available [68] which offers an opportunity to study in detail this glycerol/GKA interplay in the process of GK activation and stabilization. Nothing is known about glycerol's effect on GKRP binding and action which are also mediated by binding of this natural hepatic inhibitor to the allosteric modifier region. Since GKAs have considerable potential as new antidiabetic drugs a further exploration of a potentiating drug action on glycerol and glucose mediated GK stabilization is desirable. It has indeed been shown that GKAs increase the GK content of pancreatic islet tissue in a manner that depends on the presence of glucose or the competitive not phosphorylatable inhibitor mannoheptulose, a result most likely explained by stabilization and mass increase of the enzyme protein not depending on insulin signalling [4, 28].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

1. Structural and Functional Instability of Glucokinase (GK) Cause Diabetes Mellitus
2. Stability and Structure of GK are studied by Tryptophan Fluorescence (TF) and DSC
3. Glucose Influences Solvent Accessibility of GK's 3 Tryptophans Differentially
4. Glucose and Glycerol Affect GK Kinetics and Stability Additively
5. Thermal Inactivation of GK Above 40 Degree Celsius and reactivation (8 M Urea)

Figure 1.

Comparison of tryptophan containing domains with glucose and activator binding sites of the GK crystal structures. A and B: Open and closed crystal structures with locations of the three tryptophans and glucose and activator as indicated. C: and D: active site residues involved in the binding of D-glucose. E and F show the crystallographic allosteric activator site (GKA binding site) with residues involved in the binding of activator in the open and the closed conformations (based on [37]).

Figure 2.

Thermodynamics of glucose binding to GK in the absence and presence of glycerol. The graph shows the vanHoff plots for glucose binding to pure wild type GK in phosphate buffer at pH 7.3 with and without 20% glycerol. A temperature range of 11 to 45 °C was covered. Glucose binding was studied by tryptophan fluorescence. Glucose K_D values and thermodynamic constants at 25 °C are given in supplemental Table 3 and also in the text.

Figure 3.

Effect of glycerol on TF and glucose binding of wild type and single tryptophan GKs. The left panels show the basal and glucose enhanced fluorescence spectra of control and single tryptophan containing enzymes in the presence and absence of 20% glycerol. Thin lines in the absence and solid lines in the presence of glycerol. The right panels show the glycerol effect on glucose binding curves of these enzymes as indicated. For related quantitative information see Tables 2 and 3. Samples contained about 3 μ M protein in 5 mM K-

phosphate buffer, pH 7.3, and 100 mM KCl. Measurements were made in the absence of and with 300 mM D-glucose as indicated. Excitation: 295 nm, bandpass 4 nm; emission: 3 nm.

Figure 4.

Fluorimetric evidence for glucose independent GKA binding by GK wild type enzyme in the presence of glycerol. A and B: Effects of GKA on basal fluorescence and the slow fluorescence transitions resulting from ligand binding to GK are shown. C and D: The TF spectra of GK-WT in the absence and presence of 20% glycerol are shown.

Figure 5.

Fluorimetric evidence for glucose independent GKA binding by the W167 enzyme with and without glycerol present. A: Effect of GKA on fluorescence increase in the absence and presence of glycerol and after addition of 10 mM Glucose, B: The TF spectra of W167 in the absence and presence of 20% glycerol and after addition of GKA and glucose are shown, C and D: TF spectra of W167 from B with normalized intensity.

Figure 6.

Emission spectra of GK-WT and indicated single Trp mutants at 10 and 37.5 °C. Sample contained ~3 μ M protein in 5 mM K-phosphate buffer, pH, 7.3 and 100 mM KCl in the absence of glycerol and in the presence of 20% glycerol. Dotted lines: no glucose; solid line: 300 mM D-glucose. Excitation: 295 nm bandpass 4 nm; emission bandpass: 3 nm. The spectra are normalized to the 10 °C results.

Figure 7.

Comparison of the fluorescence ratio, derivative of the ratio and heat capacity for GK-WT and single Trp mutants as a function of temperature. Sample contained 3–6 μ M GK in 5 mM phosphate, 100 mM KCl. Open symbols: no glucose; closed symbols: 300 mM glucose. A. Fluorescence spectral red shifts are recorded by the $I_{320\text{ nm}}/I_{360\text{ nm}}$ ratios. Protein concentration: 3 μ M. B. Derivative of fluorescence change. C. C_p without D-glucose (thin line) and with 300 mM D-glucose. Protein concentration: 6 μ M.

Figure 8.

Comparison of fluorescence ratio, derivative of ratio and DSC for GK-WT in 20% glycerol. Open symbols: no glucose; closed symbols: 300 mM glucose. A. Fluorescence spectral shifts are indicated by the I_{320}/I_{360} ratios. Samples contained 3 μ M GK in 5 mM phosphate, 100 mM KCl. B. Derivative of fluorescence change. C and D. C_p with out D-glucose (thin line) and with 300 mM D-glucose. Samples contained 0.3 mg/ml (6 μ M) GK. Dotted lines: no glycerol.

Figure 9.

A. T_m versus glucose concentration. Open circles: 5 mM phosphate, 100 mM KCl, pH 7.3; Closed circles: buffer and 20% glycerol. Lines are fit for a hyperbolic function with an EC_{50} of 44 mM in buffer and 36 mM in 20% glycerol. B. Heat capacity (C_p) for thermal denaturation as a function of glucose without (open circles) and with glycerol (closed circles). Lines are fit to hyperbolic functions with EC_{50} values of 43 and 35 mM in the absence and presence of 20% glycerol, respectively.

Figure 10.

Effect of glycerol on the activation energy (E_a) for thermal denaturation as a function of glucose concentration. A. Concentration dependency curves in the absence (open circles) and presence of glycerol (closed circles). The curves were fit to K_D s at 45 °C in the absence of glycerol and at 52 °C in its presence of glycerol (the temperatures at the foot of the thermal melting transitions of Figure 8) according to results of supplemental Table S3. C and D show corresponding Arrhenius plots to illustrate that linearity exists for the ascending portion of the DSC transition.

Figure 11.

Virtual irreversibility during thermal unfolding of GK-WT in 20% Glycerol. Thermal lability of GK-WT in 20% Glycerol in the open glucose-free form (A) and in the closed glucose-bound form (B) shown as the ratio of blue to red portion of the fluorescence spectra as a function of up ($\rightarrow$) and down ($\leftarrow$) temperature changes. Panel C shows the time dependent changes of the blue to red ratio of the fluorescence spectra at 40 $^{\circ}\text{C}$, 45 $^{\circ}\text{C}$, 51 $^{\circ}\text{C}$ and 56 $^{\circ}\text{C}$.

Figure 12.

A. The urea denaturation curves of GK-WT in the buffer (open circles), in 20% glycerol (open triangles), in the buffer with 300 mM D-glucose (closed circles) and in 20% glycerol with 300 mM D-glucose (close triangles) (20mM PO₄, 50mM KCl, 1mM EDTA, 1mM DTT, pH 7.3, 20°C) assuming a two-state mechanism. The fluorescence intensity ratio I_{320}/I_{360} was measured after excitation at 295 nm. B. The variation of ΔG_u with urea concentration for GK-WT. $\Delta G_u(\text{H}_2\text{O}) = 1.63, 1.84, 2.48$ and 2.87 Kcal/mol for GK-WT in the buffer (open circles), in 20% glycerol (open triangles), in the buffer with 300mM D-

glucose (closed circles) and in 20% glycerol with 300 mM D-glucose (close triangles), respectively.

Figure 13.

Fluorescence spectra of GK-WT in the absence and in the presence of 4 M urea (panel A) and 8 M urea (panels B and C) at 20 °C. Studies recorded in panel C were performed with 20% glycerol. Horizontal arrow shows the red and blue shifts of the Trp fluorescence spectra during the denaturation and refolding processes, respectively. Vertical arrows show the Trp fluorescence increase upon glucose binding of the refolded GK. The unfolded enzyme does not bind glucose as indicated by the absence of a TF response to 300 mM glucose.

Figure 14.

Stabilization and denaturation pathways of GK as influenced by glucose, osmolytes and GKA. The model is described in the discussion section under the heading “Basic Biophysical Considerations”. The GK icon illustrates the enzyme’s two lobar structure, the intramolecular location of the substrate and allosteric activator sites and the positions of the three tryptophans (green in the basal state, blue in the stabilized and red in the denatured conformations). Glycerol causes a generalized compacting or tightening (t) of a relaxed or loose (l) native structure. The superscripts (o) and (c) refer to the open and closed conformations. The rough or loose surface of the native protein is contrasted with the smooth or tight surface of the enzyme compacted by glycerol. Note that in the presence of glycerol GKAs can bind independently of glucose, a case not represented by a separate icon but indicated by the opening of the allosteric binding site.

Figure 15.

Equations 1 and 2

Equation 1 represents the process of heat denaturation [35, equation 2 the process of glucose binding by GK including the slow conformational transitions responsible for the enzymes cooperativity with glucose [21–25].

Table 1

The static solvent accessibility of Trp in GK-WT in the Open (apoenzyme) and Closed (glucose saturated GK) states as calculated from acrylamide quenching of Trp fluorescence studied here or molecular dynamics [20].

Trp of GK-WT	Open/Closed Relative Accessibility (%) (from quenching [*])	Open/Closed Static Solvent Accessibility (%) (from [20])
W-99	11.9 / 13.1	27 / 45
W-167	14.4 / 5.8	X / 4.6
W-257	6.2 / 5.2	0.8 / 0.0%

^{*}
Data from Table S2

Table 2

Effect of glycerol on D-glucose binding (−/+20%) and kinetics (−/+30%) of wild-type and single tryptophan GK's in the presence and absence of near saturation levels (20 microM) of a GKA (Data with 20–30% glycerol present are recorded *in italics*).

Enzyme	K _D (mM)	Glucose S _{0.5} (mM)	ATP K _M (mM)	nH ^{***}	k _{cat} (sec ^{−1})	k _{cat} /S _{0.5} (sec ^{−1} mM ^{−1})
GK-WT (with GKA)	7.40 / 1.80 0.83 / 0.27	6.26 / 3.15 1.48 / 0.73	0.46 [*] / 0.23 0.57 / 0.35	1.69 / 1.78 1.46 / 1.27	83.7 / 37.7 166 / 60.5	11.8 / 12.0 112 / 82.9
W-99 (with GKA)	18.8 / 4.06 3.05 / 0.36	21.0 / 6.53 3.04 / 0.95	1.63 [*] / 0.71 1.43 [*] / 1.09	1.24 / 1.52 1.25 / 1.42	8.47 / 6.10 12.7 / 9.43	0.40 / 0.93 4.16 / 9.91
W-167 (with GKA)	0.38 / 0.09 0.09 / 0.08	2.28 / 0.82 0.56 / 0.35	0.78 [*] / 0.30 0.49 [*] / 0.55	1.53 / 1.32 1.23 / 1.37	69.9 / 26.2 91.3 / 38.9	32.0 / 32.0 163 / 112
W-257 (with GKA)	11.3 / 2.09 1.77 / 0.49	9.82 / 4.70 1.20 / 0.48	3.35 [*] / 0.71 2.94 [*] / 1.43	1.23 / 1.43 1.10 / 1.54	12.4 / 6.37 11.1 / 5.99	1.27 / 1.36 9.17 / 12.5

* Kinetic data for the W99 enzyme for the absence of glycerol are from Zelent et al. [19].

** The average nH value for all four enzymes in the absence of glycerol is 1.42+/-0.12 compared to 1.51+/-0.12 in its presence. With GKA added to the medium the corresponding values are: 1.26+/-0.09 and 1.40+/-0.07. This demonstrates that glycerol does not decrease cooperativity under the present assay conditions. The GKA does however lower the nH on average.

Table 3

GK-WT Trp fluorescence quantum yield, Φ , at 20°C in the absence and presence of 300 mM D-glucose and 20% Glycerol

Enzyme	Φ Buffer no D-glucose	Φ Buffer D-glucose	Φ 20% glycerol no D-glucose	Φ 20% glycerol D-glucose
GK-WT	0.094	0.187	0.099	0.212
W-99	0.070	0.109	0.074	0.115
W-167	0.125	0.223	0.152	0.225
W-257	0.086	0.103	0.087	0.105

Table 4

GK-WT Trp fluorescence emission maximum at 10 and 37.5 C

Enzyme	Emission max, 10 C no glucose	Emission max, 37.5 C no glucose	Emission max, 10 C 300 mM D-glucose	Emission max, 37.5 C 300 mM D-glucose
GK-WT	326	331	326	327
W-99	334	335	336	338
W-167	329	330	326	327
W-257	322	324	320	322
GK-WT in 20% glycerol	326	329	325	326

T_m, C_p^m and H for thermal denaturation of GK-WT and single Trp enzymes in buffer and for GK-WT with glycerol

Table 5

Medium condition	T _m (°C)	C _p ^m (kJ/mol/°C)	H (kJ/mol)	300 mM D-glucose		
				T _m (°C)	C _p ^m (kJ/mol/°C)	H (kJ/mol)
No D-glucose						
GK-WT in buffer	46.9	55.2	289.5	52.2	130.5	564.8
W-99 in buffer	46.0	58.6	382.0	49.6	100.8	426.8
W-167 in buffer	47.1	57.7	338.1	53.4	90.4	410.0
W-257 in buffer	48.2	46.0	246.9	51.2	122.6	661.1
GK-WT in 20% glycerol	51.3	122.6	527.2	56.9	222.2	707.1

Table 6
Glucose dependent T_m , C_p^m and E_a for thermal denaturation of GK-WT in the absence and presence of 20% glycerol

D-Glucose (mM)	T_m (°C) Buffer	T_m (°C) 20% Glycerol	C_p^m (kJ/mol/°C) Buffer	C_p^m (kJ/mol/°C) 20% Glycerol	E_a (kJ/mol) Buffer	E_a (kJ/mol) 20% glycerol
0	46.9	51.3	55.2	121.8	420	550.2
7.5	48.0	52.6	63.2	148.1	502	733.6
25	49.5	-	90.8	-	562.2	-
50	50.0	54.8	101.7	186.2	575.2	795.5
100	50.9	55.5	109.6	196.2	571	809.9
200	52.2	56.3	128.4	212.5	586.9	795.2
300	52.2	56.9	130.5	222.2	572	791.9