

Small-Molecule Allosteric Activation of Human Glucokinase in the Absence of Glucose

Joseph M. Bowler, Katherine L. Hervert, Mark L. Kearley and Brian G. Miller

Supporting Information

Protein Expression and Purification

Recombinant wild-type human pancreatic glucokinase was produced as N-terminal hexa-histidine tagged polypeptides in glucokinase-deficient *E. coli* K-12 strain BM5340(DE3). Cultures were inoculated to an initial OD₆₀₀ of 0.01 and were grown at 37°C in Luria-Bertani broth supplemented with ampicillin (150 µg/mL), kanamycin (40 µg/mL), and chloramphenicol (25 µg/mL). When the OD₆₀₀ reached 0.8, IPTG (1 mM) was added to induce gene expression and the temperature was reduced to 20° C, where growth was continued for 20 h. Cells were harvested by centrifugation at 8,000x g, and 5 g of wet cell pellet was resuspended in 17 mL of buffer A containing HEPES (50 mM, pH 7.6), KCl (50 mM), imidazole (25 mM), dithiothreitol (10 mM), and glycerol (25%, w/v). Cells were lysed using a microfluidizer and subjected to centrifugation at 25,000x g and 4° C for 45 m. The supernatant was immediately loaded onto a 5 mL HisTrap Fast Flow Affinity Column (GE Healthcare) previously equilibrated in buffer A. Following loading, the column was washed with 10 column volumes of buffer A followed by 5 columns of buffer A containing 52 mM imidazole. Glucokinase was eluted with buffer A containing 250 mM imidazole, and the enzyme was dialyzed for 1.5 h at 4° C against 1 L of buffer containing HEPES (50 mM, pH 7.6), KCl (50 mM), glycerol (5%), imidazole (40 mM), EDTA (0.25 mM), and dithiothreitol (10 mM). Dialyzed glucokinase

was concentrated to 500 μ L via centrifugation at 4000x g and 4° C using a 10,000 MWCO filter (Millipore Amicon Ultra). The concentrated sample was injected onto a Superdex 200 10/300 GL size-exclusion column (GE Healthcare) pre-equilibrated in a buffer containing HEPES (50 mM, pH 7.6), KCl (50 mM), and TCEP (4 mM). The gel filtration column was run at a flow rate of 0.02 mL/min, and fractions containing the highest A_{280} readings were pooled and retained for further analysis.

Kinetic Assays

GCK activity was assessed spectrophotometrically at 340 nm by coupling the production of glucose 6-phosphate to the reduction of NADP^+ via glucose 6-phosphate dehydrogenase. Reaction mixtures contained HEPES (250 mM, pH 7.6), NADP^+ (0.5 mM), KCl (50 mM), DTT (10 mM), ATP (10 mM), MgCl_2 (12 mM), and G6PDH (7.5 units). Stock solutions of activators (25% DMSO in HEPES 10 mM, pH 7.6) were used for assays containing the activator (20 μ M in assay) while control runs were performed using the same solution lacking GKA. Glucose concentrations were varied and reactions were initiated by the addition of ATP. The slope of the linear portion of the progress curve yielded steady-state velocities and subsequent fitting of these data to the Hill equation afforded values for $K_{0.5}$, k_{cat} , and the Hill coefficient. Viscogen dependence of catalysis was assessed using the above conditions with the inclusion of varied levels of sucrose (0-30% w/v). Relative viscosities of sucrose solutions were measured using a Cannon-Fenske viscometer. Relative maximal velocities were plotted against the corresponding relative viscosities and slopes calculated by the linear least-squares method.

For transient measurements of τ , GCK was purified as described (23) and dialyzed against HEPES (250 mM, pH 7.6), KCl (50 mM), and DTT (10 mM). After overnight dialysis at 4° C, enzyme was removed from buffer and kept on ice. To the dialysis buffer was added NADP⁺ (0.5 mM), ATP (0.25 mM), and MgCl₂ (3 mM). The buffer containing assay components was subsequently partitioned into four aliquots: three corresponding to each activator and one control solution. Activator stocks in DMSO were used to achieve a concentration of 20 μ M while the control aliquot was made using the same volume of DMSO. G6PDH (7.5 units) was added to all solutions, which were then each divided into separate “A” and “B” solutions. To each “A” solution was added GCK (10 nM final concentration), while “B” samples were prepared with the addition of 10 mM glucose (10X stock for 1:10 mixing). Transient data were collected using an Applied Photophysics SX20 stopped-flow spectrometer housed in the Protein Biophysics Laboratory at The Florida State University. Reaction progress was monitored by following the absorbance change at 340 nm using a 1 mm slit width. Absorbance traces were collected for 250 s and the resulting data were fitted to an equation for a single-exponential decay of one enzymatic state to another (Eq. 1) to yield values for transition time (τ), initial velocity (V_i), and steady-state velocity (V_{ss}) (18).

$$\text{Eq. 1} \quad A_{340} = V_{ss}t - (V_{ss} - V_i)(1 - e^{-t/\tau})\tau$$

Values obtained from twelve or more fitted progress curves were pooled for statistical analysis. Progress curves were fitted using SigmaPlot v.10 and statistical analyses were performed with Microsoft Excel 2011.

Isothermal Titration Calorimetry

For activator binding studies using ITC, purified enzyme was dialyzed against buffer containing HEPES (50 mM, pH 7.6), KCl (50 mM), TCEP (4 mM), DMSO (5%), and glucose (200 mM). After overnight dialysis at 4°C, dialysis buffer was used to dissolve titrant (GKA22/Cpd A). DMSO was included in the dialysis buffer to increase activator solubility, and did not impact enzyme activity. We found that slightly heating solutions further assisted solubility to yield our desired titrant concentration (100 μ M). To ensure saturation, enzyme concentration (10 μ M) was maintained at a concentration 10-fold lower than the titrant concentration. ITC experiments were run at 25° C using a MicroCal VP-ITC with a 1.441 mL reaction cell volume. The concentration of glucokinase was determined spectrophotometrically at 280 nm using the calculated extinction coefficient of 32,500 $\text{M}^{-1} \text{cm}^{-1}$. Experimentally determined molar absorptivities of 16,500 $\text{M}^{-1} \text{cm}^{-1}$ and 6,200 $\text{M}^{-1} \text{cm}^{-1}$ at 280 nm were used to calculate the concentration of GKA22 and Cpd A, respectively.

Docking Studies

To investigate possible conformations of activating molecules in the glucokinase allosteric site, docking studies were carried out using PyMOL with Autodock Vina (1, 2). Each activator utilized in the experimental work was initially drawn and 3D-rendered in MarvinSketch. Docking simulations were performed using two independent sources of crystallographic data (PDB entries 1V4S and 3IMX). Both crystal structures had been obtained with the assistance of synthetic activators, each of which were removed from the binding site prior to initiating the simulation. Results for the top ten ligand poses for

each run were ranked by favorability of the free energy of binding (ΔG). The only further constraint imposed on docked molecules was the requirement of bidentate hydrogen bonding between the ligand and backbone of Arg63, a known motif among bound activators.

Synthesis of GKA22

An overview of the synthetic route is provided in Figure S1. All solvents and reagents were obtained from Sigma-Aldrich or VWR and were used without further purification. Thin layer chromatography was performed on plastic EMD TLC silica gel 60 F₂₅₄ and column chromatography was performed on EMD silica gel 60 (230-400 mesh ASTM). NMR analysis was performed on a Bruker 500 MHz spectrometer and mass spectra were obtained using EOL AccuTOF JMS T-100LC for ESI.

Methyl 3-hydroxy-5-isopropoxybenzoate (A)

Methyl 3,5-dihydroxybenzoate (2.00 g, 11.9 mmol) was dissolved in 10 mL of N, N-dimethylformamide, followed by addition of potassium carbonate (2.46 g, 17.9 mmol), and then the solution was heated to 60°C. After approximately fifteen minutes, 2-bromopropane (1.68 mL, 17.9 mmol) was added dropwise to the mixture and the solution stirred at 60°C for three hours under an argon atmosphere. After cooling to room temperature, the reaction mixture was partitioned between ethyl acetate and water. The aqueous layer was extracted with ethyl acetate (3 x 20 mL) and the combined organic layers were dried with MgSO₄, filtered, and concentrated to yield a dark brown oil. Purification via column chromatography (3:1 hexanes: ethyl acetate) gave a white

solid (mp 81 – 82 °C) in 46% yield (1.15 g, 5.48 mmol). Spectral data for **A**: *Rf* 0.40 (33% ethyl acetate in hexanes); ¹H NMR (500 MHz, CDCl₃) δ 7.15 (m, 2H), 6.62 (t, 1H, *J* = 2.5 Hz), 6.03 (s, 1H), 4.56 (m, 1H), 3.91 (s, 3H), 1.33 (d, 6H, *J* = 6.0 Hz); ¹³C NMR (75 MHz, CDCl₃) δ 167.3, 159.2, 156.9, 131.9, 109.0, 108.4, 70.4, 52.4, 22.0; HRMS (EI+) calculated for C₁₁H₁₄O₄ 210.0892; found 210.0894.

Methyl 3-isopropoxy-5-(2-(3-thienyl)ethoxy)benzoate (B)

Purified benzoate **A** (760 mg, 3.62 mmol) was dissolved in 10 mL of acetonitrile in a round-bottom flask at room temperature. Potassium carbonate (749 mg, 5.43 mmol) was added to the flask and allowed to stir at room temperature for 45 minutes. In 3 mL of acetonitrile, 3-(2-bromoethyl)thiophene (1.04 g, 5.43 mmol) was added dropwise to the reaction mixture and the flask refluxed overnight under an argon atmosphere. Upon cooling to room temperature, the reaction mixture was partitioned between water and ethyl acetate. The aqueous layer was extracted with ethyl acetate (3 x 20 mL) and the combined organic layers were dried with MgSO₄, filtered, and concentrated to give a tan oil. Purification via column chromatography (2:1 hexanes: ethyl acetate) gave **B** as a clear oil (830 mg, 72%). Spectral data for **B**: *Rf* 0.80 (33% ethyl acetate in hexanes); IR (neat) ν 2981, 1719, 1604, 1451, 1243, 1157 cm⁻¹. ¹H NMR (500 MHz, CDCl₃) δ 7.28 (dd, 1H, *J* = 5.0, 3.0 Hz), 7.19 – 7.17 (m, 2H), 7.10 (dt, 1H, *J* = 2.5, 1.5 Hz), 7.05 (dd, 1H, *J* = 5.25, 1.5 Hz), 6.65 (t, 1H, *J* = 2.5 Hz), 4.59 (m, 1H), 4.20 (t, 2H, *J* = 7.0 Hz), 3.90 (s, 3H), 3.14 (t, 2H, *J* = 7.0 Hz), 1.35 (d, 6H, *J* = 6.0 Hz); ¹³C NMR (75 MHz, CDCl₃) δ 167.0, 159.8, 159.7, 138.4, 131.9, 128.5, 125.6, 121.7, 109.1, 107.9, 107.7,

70.2, 68.3, 52.3, 30.2, 22.0; HRMS (EI+) calculated for C₁₇H₂₀O₄S 320.1082; found 320.1082.

3-Isopropoxy-5-(2-(3-thienyl)ethoxy)benzoic acid (C)

To a solution of ester **B** (1.03 g, 3.22 mmol) in 15 mL THF, 12.9 mL (12.9 mmol) of 1M NaOH was added and the mixture refluxed under an argon atmosphere for five hours or until starting material disappeared via TLC. After cooling to room temperature, the reaction mixture was diluted with water and the flask was cooled to 0°C, then acidified with 2M HCl. Upon reaching a pH of approximately 2, the mixture was allowed to reach room temperature and then approximately 20 mL of ethyl acetate was added. The aqueous layer was extracted with ethyl acetate (3 x 20 mL) and the combined organic layers were dried with MgSO₄, filtered, and concentrated to give a light white solid (mp 104 – 105°C) in 90% yield (880 mg, 2.88 mmol). Spectral data for **C**: *R_f* 0.36 (33% ethyl acetate in hexanes); IR (neat) ν 2976, 2930, 1691, 1594, 1162, 1048 cm⁻¹. ¹H NMR (500 MHz, CDCl₃) δ 7.30 (dd, 1H, *J* = 4.5, 3.0 Hz), 7.23 (m, 2H), 7.11 (m, 1H), 7.05 (d, 1H, *J* = 5.0 Hz), 6.69 (t, 1H, *J* = 2.5 Hz), 4.60 (m, 1H), 4.21 (t, 2H, *J* = 6.5 Hz), 3.15 (t, 2H, *J* = 6.5 Hz), 1.68 (bs, 1H), 1.35 (d, 6H, *J* = 6.0 Hz); ¹³C NMR (75 MHz, CDCl₃) δ 172.0, 159.1, 159.1, 138.4, 131.1, 128.6, 125.7, 121.8, 109.7, 109.4, 108.9, 108.1, 70.4, 68.4, 30.2, 22.1; HRMS (EI+) calculated for C₁₆H₁₈O₄S 306.0926; found 306.0929.

Methyl 6-(3-isopropoxy-5-(2-(3-thienyl)ethoxy)benzamido)nicotinate (e)

Carboxylic acid **C** (2.87 g, 9.38 mmol) was dissolved in 20 mL of dichloromethane, which was then cooled to 0°C. Oxalyl chloride (4.09 mL, 46.9 mmol) was slowly added

to the cooled mixture via syringe, followed by four drops of DMF. After the noticeable exothermicity subsided, the reaction was allowed to reach room temperature. After stirring for two hours, the solvent removed to afford a brown oil (2.92 g, crude).

The methyl 6-aminopyridine-3-carboxylate (2.37 g, 15.6 mmol) was added portion-wise to a solution of the acid chloride (2.53 g, 7.80 mmol) in 20 mL of THF. The reaction mixture was cooled to 0°C, followed by the dropwise addition of pyridine (1.25 mL, 15.6 mmol). After addition, the ice bath was removed and the mixture stirred for 2 hours at 40°C under argon. After cooling to room temperature, the mixture was diluted with water and the aqueous layer was extracted with ethyl acetate (3 x 20 mL) and the combined organic layers were dried with MgSO₄, filtered, and concentrated to give 4.66 g of the crude product. Following flash chromatography (20% ethyl acetate in hexanes), white solid **E** (mp 88 – 91°C) was obtained in 44% yield (1.50 g, 3.41 mmol). Spectral data for **E**: *R_f* 0.44 (33% ethyl acetate in hexanes); IR (neat) ν 3314, 2973, 1723, 1686, 1594, 1300 cm⁻¹. ¹H NMR (500 MHz, CDCl₃) δ 8.93 (dd, 1H, *J* = 2.5, 0.5 Hz), 8.69 (s, 1H), 8.44 (dd, 1H, *J* = 8.5, 1.0 Hz), 8.35 (dd, 1H, *J* = 9.0, 2.5 Hz), 7.30 (dd, 1H, *J* = 5.0, 3.0 Hz), 7.11 (dt, 1H, *J* = 1.5, 1.0 Hz), 7.05 (dd, 1H, *J* = 5.0, 1.5 Hz), 7.03 (t, 1, *J* = 2.0 Hz), 7.01 (t, 1H, *J* = 1.8 Hz), 6.65 (t, 1H, *J* = 2.0 Hz), 4.61 (m, 1H), 4.22 (t, 2H, *J* = 6.5 Hz), 3.95 (s, 3H), 3.15 (t, 2H, *J* = 6.5 Hz), 1.35 (d, 6H, *J* = 6.0 Hz); ¹³C NMR (75 MHz, CDCl₃) δ 165.6, 165.4, 159.5, 154.4, 150.1, 139.9, 138.2, 135.8, 128.4, 125.7, 122.2, 121.7, 113.0, 107.1, 106.9, 105.4, 70.5, 68.4, 52.3, 30.2, 22.2; HRMS (ESI+) calculated for C₂₃H₂₅N₂O₅S 441.1491; found 441.1491 [M+H]⁺.

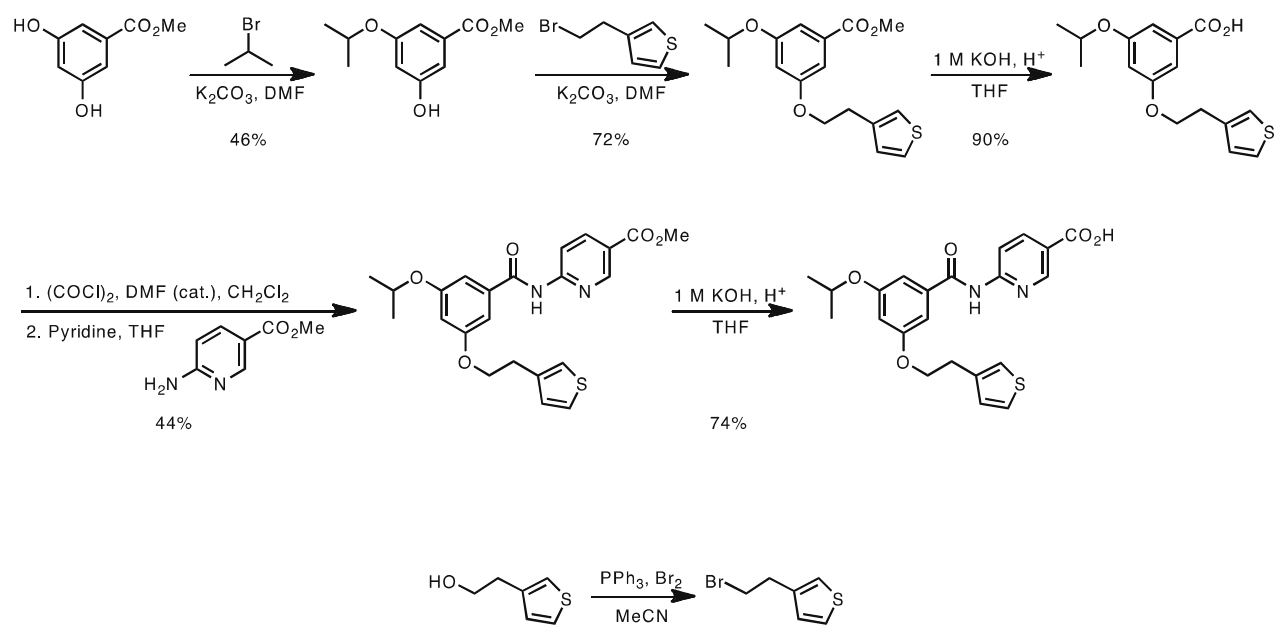
6-(3-isopropoxy-5-(2-(3-thienyl)ethoxy)benzamido)nicotinic acid (GKA22)

To a solution of amide **E** (1.50 g, 3.41 mmol) in 12 mL of THF, 4 M KOH (4.41 mL, 17.6 mmol) was added and the reaction stirred at room temperature overnight under argon. Reaction progress was followed by TLC (25% ethyl acetate in hexanes) and showed gradual disappearance of starting material. Upon full consumption, the reaction mixture was cooled to 0°C followed by an aqueous work-up and acidification with 2M HCl until an approximate pH of 2 was reached. After warming to room temperature, the acidified mixture was extracted four times with 10 mL portions of ethyl acetate. The organic layers were combined and dried with MgSO₄, filtered, and concentrated to give an off-white solid. This crude product was recrystallized from isopropanol to afford a white powder, **GKA22** (1.07 g, 74% yield). Spectral Data for **GKA22**: R_f 0.16 (1:1 hexanes: ethyl acetate), IR (neat) ν 3292, 1693, 1606, 1294 cm⁻¹. ¹H NMR (500 MHz, DMSO) δ 13.2 (s, 1H), 11.6 (s, 1H), 9.31 (t, 1H, J = 1.5 Hz), 8.73 (d, 2H, J = 1.5 Hz), 7.91 (dd, 1H, J = 5.0, 3.0 Hz), 7.63 (dd, 1H, J = 2.0, 1.5 Hz), 7.60 (t, 1H, J = 2.0 Hz), 7.55 (dd, 1H, J = 3.3, 1.5 Hz), 5.15 (m, 1H), 4.67 (t, 2H, J = 7.0 Hz) 3.76 (s, 2H), 3.48 (t, 2H, J = 7 Hz), 2.91 (dt, 2H, J = 3.5, 1.5 Hz), 1.69 (d, 6H, J = 6.0 Hz); ¹³C NMR (75 MHz, DMSO) δ 166.0, 165.8, 159.6, 158.6, 155.3, 149.5, 139.4, 138.5, 135.6, 128.8, 125.9, 121.9, 121.9, 113.7, 107.5, 106.1, 69.6, 68.0, 29.5, 21.8 HRMS (ESI+) calculated for C₂₂H₂₃N₂O₅S 427.1323; found 427.1328 [M+H]⁺.

Figure Legends

Figure S1. Synthetic route for the production of GKA22

FIGURE S1.



References Cited

- (1) DeLano, W.L. (2002) The PyMOL molecular graphics system, DeLano Scientific, San Carlos, CA.
- (2) Trott, O., and Olson, A.J. (2010) AutoDock vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.* **31**, 455-461.