Activation of G protein-Coupled Receptor Kinase 1 Involves Interactions Between its N-terminal Region and its Kinase Domain

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SUPPLEMENTAL METHODS

Nucleotide binding assay– The K_D of BODIPY^{TR}-ADP (Invitrogen) was determined using 0.02 μM BODIPY^{TR}-ADP and 0 to 6.4 μM bGRK1$_{535}$H$_6$ variants in 20 mM HEPES-NaOH (pH 7.5), 0.15 M NaCl, and 5 mM $MgCl_2$ at 25 °C ($\lambda_{excitation}$=575 nm and $\lambda_{emission}$=620 nm). Competition assays were then conducted using 0.02 μM BODIPY^{TR}-ADP, 1 μM bGRK1$_{535}$H$_6$ protein, and 0 to 10 μM ADP in the same buffer at 25 °C. 20 mM EDTA was added for background subtraction. The K_D of BODIPY^{TR}-ADP was obtained by fitting to equation 2, and data from competition assays were fitted to equations 3 and 4 to obtain the K_D of ADP.

$$mP = \frac{mP_{\max} \times [P]_t}{K_{D1} + [P]_t} \qquad \text{(Eq. 2)}$$

$$mP = \frac{mP_{\max}}{(1 + K_{D1}/[P]_{free})} + mP_{\min} \qquad \text{(Eq. 3)}$$

$$[P]_{free} = [P]_t - \frac{b - \sqrt{b^2 - 4 \times [P]_t \times [L_2]_t}}{2} \qquad \text{(Eq. 4)}$$

where mP_{max} is the maximal millipolarization (mP), $[P]_t$ is the total protein concentration, K_{D1} and K_{D2} are K_Ds of BODIPY^{TR}-ADP and ADP, respectively, mP_{min} is the minimal mP, $[P]_{free}$ is the free protein concentration, $[L_2]_t$ is the total ADP concentration, and $b=[P]_t+[L_2]_t+K_{D2}$.

Crystallization and structure determination of bGRK1$_{535}$H6 T8C/N480C. Cross-linked bGRK1$_{535}$H6 T8C/N480C purified by size-exclusion chromatography was incubated with 2 mM ADP and 2 mM $MgCl_2$ on ice for 30 minutes prior to crystallization. The protein (~7 mg/ml) was crystallized in 14-19% PEG 3350, 0.1 M Bis-Tris (pH 5.5), 0.15 M $MgCl_2$, and 0.02% n-dodecyl-β-D-maltoside at 4 °C using the hanging-drop method by mixing equal volumes of protein and precipitant. Crystals appeared after one day and grew to 0.1 - 0.2 mm after five to seven days. Crystals were flash frozen in liquid nitrogen using precipitant containing 30% glycerol as a cryoprotectant, and then diffraction data were collected at 100 K at beam line 21 ID-G, LS-CAT, Argonne National Laboratory. Data were processed by HKL2000. The crystal diffracted to 2.7 Å and belongs to space group P1. The structure was solved by molecular replacement in PHASER using the structure of bovine GRK1 (PDB entry 3C4Z) as the search model. Modeling was carried out using O and the structure was refined using PHENIX. Final crystallographic statistics are listed in Supplementary Table 2. Coordinates and structure factors are deposited in the Protein Data Bank as entry 3QC9.

SUPPLEMENTAL TABLES

Table S1. Identification of the cross-linked disulfide bond of bGRK1$_{535}$H$_6$ T8C/N480C by tandem mass spectrometry[a].

chain A														
#	**b$^{'++}$**	**b^{*++}**	**b^{++}**	**b$^{'+}$**	**b^{*+}**	**b^{+}**	**seq**	**y$^{'++}$**	**y^{*++}**	**y^{++}**	**y$^{'+}$**	**y^{*+}**	**y^{+}**	**#**
1	56.52	--	65.53	112.04	--	130.05	**E^{7}**	911.93	912.42	920.93	1822.85	1823.83	1840.86	**M**
2	558.74	559.24	567.75	1116.48	1117.47	1134.49	**C^{8}**	**847.41**	**847.90**	856.41	1693.80	1694.79	1711.81	**8**
3	**608.28**	**608.77**	617.28	**1215.55**	1216.53	1233.56	**V^{9}**	345.18	345.68	354.19	689.36	**690.35**	**707.37**	**7**
4	**657.81**	**658.30**	666.82	**1314.62**	**1315.60**	**1332.63**	**V^{10}**	295.65	296.14	304.66	590.29	591.28	**608.30**	**6**
5	**693.33**	**693.82**	**702.34**	1385.66	**1386.64**	**1403.67**	**A^{11}**	246.12	246.61	255.12	**491.22**	492.21	**509.24**	**5**
6	**750.35**	**750.84**	**759.36**	1499.70	**1500.68**	1517.71	**N^{12}**	210.60	211.09	219.60	420.19	421.17	**438.20**	**4**
7	**793.87**	**794.36**	**802.87**	1586.73	1587.71	1604.74	**S^{13}**	153.58	--	162.58	306.14	--	324.16	**3**
8	**829.39**	**829.88**	**838.39**	1657.77	1658.75	1675.78	**A^{14}**	110.06	--	119.07	219.11	--	237.12	**2**
	--	--	--	--	--	--	**F^{15}**	74.54	--	83.55	148.08	--	166.09	**1**
chain B														
#	**b$^{'++}$**	**b^{*++}**	**b^{++}**	**b$^{'+}$**	**b^{*+}**	**b^{+}**	**seq**	**y$^{'++}$**	**y^{*++}**	**y^{++}**	**y$^{'+}$**	**y^{*+}**	**y^{+}**	**#**
1	--	--	36.53	--	--	72.04	**A^{478}**	--	--	--	--	--	--	
2	--	92.06	100.57	--	183.11	200.14	**K^{479}**	**876.41**	**876.90**	885.41	1751.81	1752.79	1769.82	**8**
3	611.27	611.77	620.28	1221.54	1222.52	**1239.55**	**C^{480}**	812.36	812.85	**821.37**	1623.71	1624.70	1641.72	**7**
4	667.82	668.31	**676.82**	**1334.62**	1335.61	**1352.63**	**I^{481}**	292.66	293.15	301.66	584.30	585.29	602.31	**6**
5	731.84	732.34	**740.85**	1462.68	1463.67	**1480.69**	**Q^{482}**	236.11	236.61	245.12	471.22	472.20	**489.23**	**5**
6	**789.36**	**789.85**	**798.36**	1577.71	1578.69	1595.72	**D^{483}**	172.08	--	181.09	343.16	--	361.17	**4**
7	**838.89**	**839.38**	**847.90**	1676.78	1677.76	1694.79	**V^{484}**	114.57	--	123.58	228.13	--	246.14	**3**
8	**867.40**	**867.89**	**876.41**	1733.80	1734.78	1751.81	**G^{485}**	65.04	--	74.04	129.07	--	147.08	**2**
	--	--	--	--	--	--	**A^{485}**	36.53	--	45.53	72.04	--	90.05	**1**

[a] The numbers in red are the ions identified by mass spectrometry that were found to be identical to theoretical ions of the peptide after collision-induced differentiation.

Table 1. Crystallographic data and refinement statistics

X-ray Source:	APS beam line 21 ID-G
Wavelength (Å)	0.97856
D_{min} (Å)	2.7 (outer shell: 2.75-2.70)
Space group	*P1*
Cell constants (Å; °)	*a*=58.7, *b*=90.0, *c*=122.6
	α=88.1, β=90.3, γ=68.8
Unique reflections	61717 (3087)
Average redundancy	1.9 (1.9)
R_{sym} (%)	6.4 (45.1)
Completeness (%)	95.5 (95.8)
$\langle I \rangle / \langle \sigma_I \rangle$	15.0 (1.6)
Refinement resolution (Å)	30 – 2.7 (outer shell: 2.80-2.70)
Total reflections used	57246 (4726)
Protein atoms	15565
Non-protein atoms	241
RMSD bond lengths (Å)	0.008
RMSD bond angles (°)	1.4
Estimated coordinate error (Å)	0.34
Average B-factor (Å^2)[‖]	60
Ramachandran plot statistics:	
Most favored, disallowed (%)	89.4, 0
R_{work}	19.3 (30.7)
R_{free}[†]	22.8 (38.3)
R_{final}[‡]	19.5

[‖] B-factor does not include the TLS contribution.

[†] 5% of the data set was excluded from refinement to calculate R_{free}.

[‡] All reflections were used in the final rounds of refinement.

SUPPLEMENTAL FIGURES

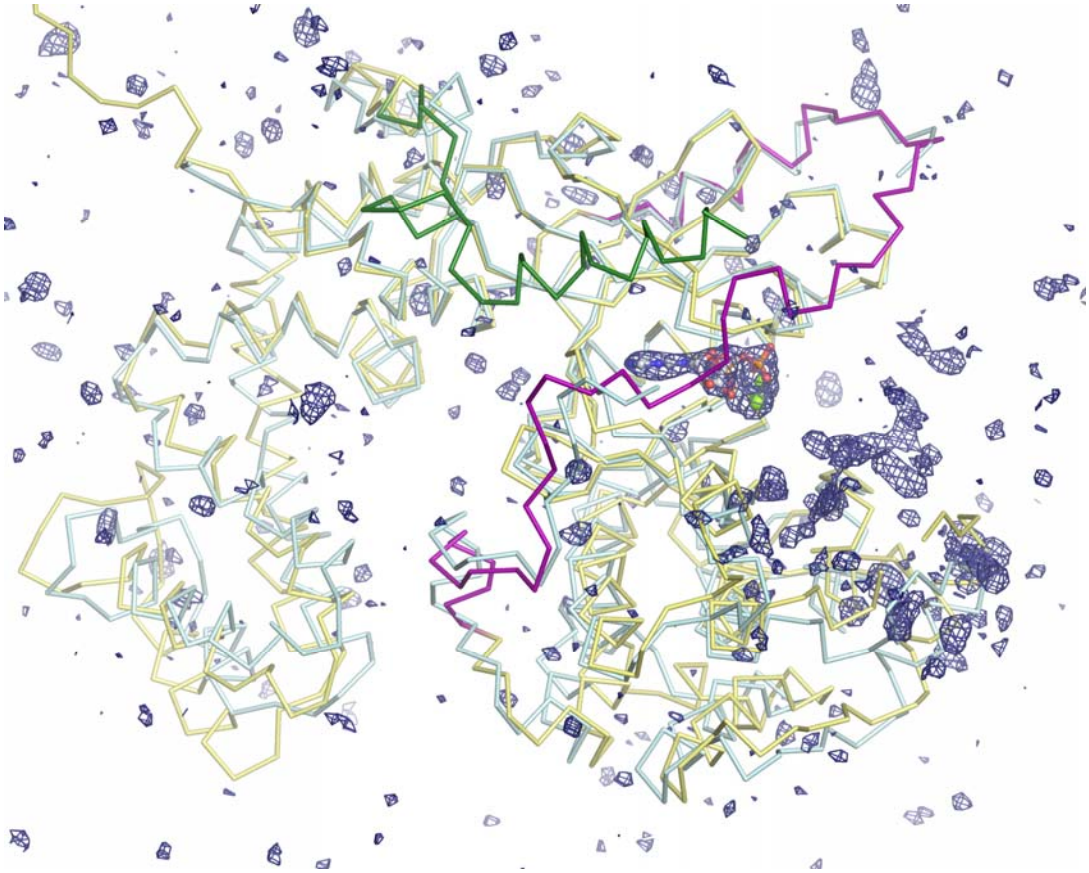

Figure S1. Crystal structure of the cross-linked GRK1 T8C/N480C mutant. One of the four chains of the asymmetric unit in the final model of cross-linked GRK1 T8C/N480C mutant is shown in light blue, and the superimposed, closed GRK6 structure is shown in yellow with its N-terminal region, including the αN helix, colored in green, and the C-tail of the kinase domain in purple. An $|F_o|$-$|F_c|$ map generated after rigid-body refinement is shown at a contour level of 3 σ (dark blue wire cage). One ADP molecule and two Mg^{2+} ions, which were omitted from the model, are clearly seen in the density. However, the αN helix and the C-tail are not ordered.

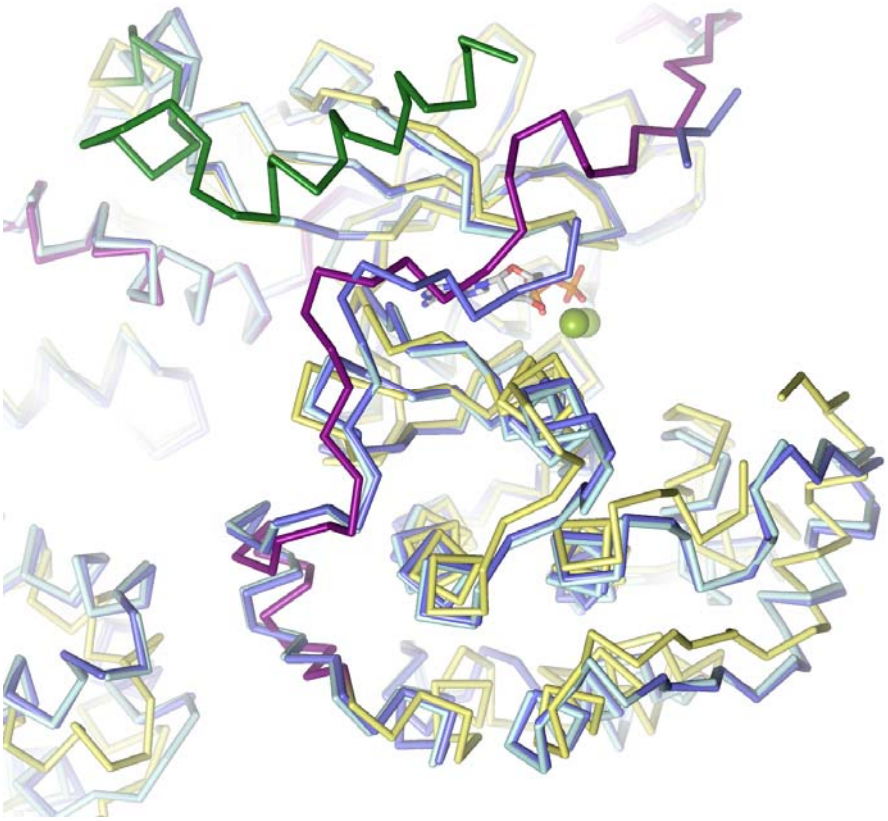

Figure S2. The cross-linked GRK1 T8C/N480C mutant adopts an open conformation similar to previously determined GRK1 structures. One of the four chains of the asymmetric unit in the final model of cross-linked GRK1 T8C/N480C mutant (light blue) was superimposed with the closed GRK6 structure (yellow with N-terminal region green, and C-tail in purple) and open GRK1 structure (slate, PDB entry 3C4Z) using their kinase N-lobes. The ADP molecule and Mg^{2+} ions in the active site are shown as sticks and spheres, respectively.