A method for the second-site screening of K-Ras in the presence of a covalently attached first-site ligand

Journal of Biomolecular NMR

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Supplementary Material

METHOD

Cloning, expression and purification of K-Ras protein

All K-Ras proteins described here contain a G12V oncogenic mutation and a C118S mutation for thiol-reaction selectivity. The DNA construct, cloning, expression and purification procedure was described previously[1]. Each of the 6 mutations (K5C, S39C, D54C, Q70C, T74C, G75C) was introduced by site-directed mutagenesis using the QuickChange Kit.

Screening for the appropriate combination between mutants and thio-reacted compounds

Each of the 6 mutant K-Ras proteins were uniformly ^{15}N labeled. $^{1}H/^{15}N$ SOFAST HMQC spectra were collected to ensure the mutants were properly folded. The protein was exchanged to a buffer containing 25 mM TrisHCl pH 7.0, 150 mM NaCl and concentrated to 5 mg/mL. The thiol-reactive compounds were added individually to 2-5 mM (The compounds are in 8-20 fold excess) The reactions were carried out at 4 °C overnight, and the reaction mixture was applied to a G25 desalting column to remove the excess compound. The modified protein was then subject to mass spectrometry analysis to confirm the covalent modification. Mutant proteins that were completely modified by thiol-reactive compounds were selected for further testing. In addition, the HMQC spectrum of modified protein was collected. Significant chemical shift changes for the residues near the binding pocket indicates binding. Finally, a probe compound **1** (N-[2-(1H-indol-3-ylmethyl)-1H-benzimidazol- 5-yl]-L-prolinamide) was added at a concentration of 2 mM. No additional chemical shift changes indicated that the primary pocket is fully blocked.

Fragment screen

The fragment screen of the second site was conducted using a K-Ras S39C mutant linked to compound **3**. The NMR screening procedure was describe previously.[1]

Protein Crystallization

GDP bound K-Ras (G12V/C118S) with a cysteine mutation (K5C, S39C, D54C, Q70C, T74C, G75C) was exchanged into a reaction buffer (20 mM Tris, 150 mM NaCl pH 8.0) and concentrated to 40 mg/mL. Protein-ligand complexes were prepared by adding a concentrated DMSO stock solution of the ligand to a protein:compound molar ratio of 1:5, incubated at 4 °C on a rocker overnight. The reaction mix was subject to a desalting column to remove excess ligand and exchanged into crystallization buffer (20 mM HEPES pH7.5, 150 mM NaCl, 5 mM $MgCl_2$, 2 mM GDP). All crystallization experiments were set up using either the sitting or hanging-drop vapor diffusion method at 18 °C. The GDP K-Ras T74C and S39C linked to compound **2** crystallized under the condition containing 25% PEG3350, 0.1 M Bis-Tris-HCl pH 5.5, The GDP K-Ras Q70C linked to compound **2** crystallized under the condition containing 30% PEG MME 2000 and 0.1 M KSCN. K-Ras S39C linked to compounds **3** and **6** crystallized from 24% PEG4000, 0.1 M MMT pH 4.0. K-Ras S39C linked to compounds **5** crystallized from 28% PEG4000, 0.1 M sodium acetate, and pH 5.0. K-Ras S39C linked to compounds **4** crystallized from 22% PEG4000, 0.1 M sodium acetate pH 4.5. All crystals were cryo-protected with 10% ethylene glycol addition for low temperature data collection.

X-ray Data Collection, Structure Solution, and Refinement

X-ray diffraction data were collected at 100K in the oscillation mode on single flash-cooled crystals using a Bruker-Nonius Microstar rotating anode X-ray generator equipped with a Proteum PT135 CCD area detector (Biomolecular Crystallography Facility in the Vanderbilt University Center for Structural Biology), as well as the synchrotron radiation (Beamline 21 LS-CAT, Advanced Photon Source, Argonne National Laboratory). Data were processed with HKL-2000[2], and the structures were determined by molecular replacement using the coordinates of GDP K-Ras G-domain (residues 1-169; PDB Entry 4EPY) with solvent and ligand molecules stripped off. The program package Phenix[3] was employed for phasing and refinement, and model fitting was performed with COOT[4]. Data collection and restrained refinement statistics are summarized in Table 1. The refined models were validated with Molprobity.

Figure S1 The structures of the thiol-reactive compounds used to identify the preferred mutant/compound combination for the second site screen

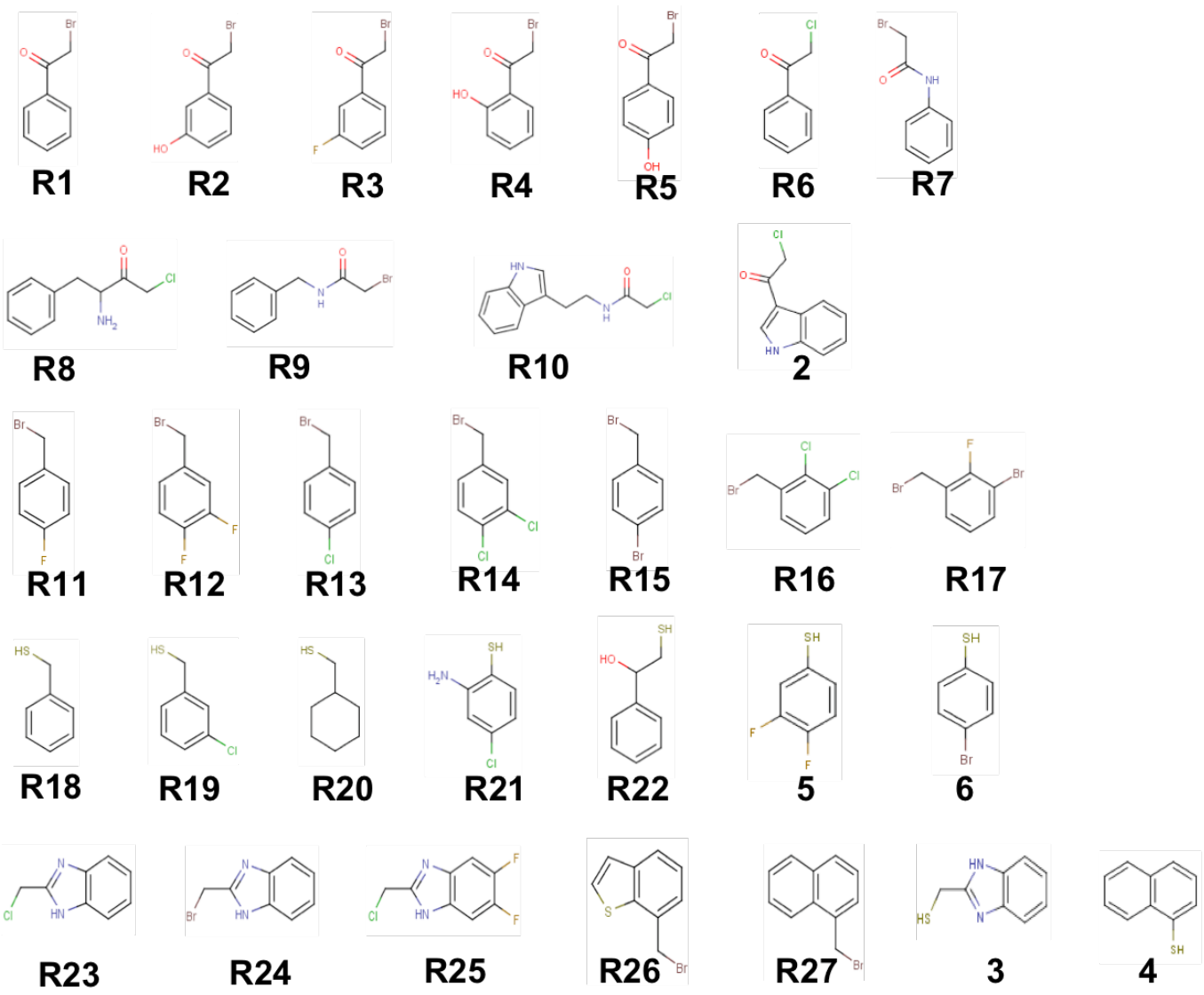

Table S1 X-ray data collection and refinement statistics

(Last resolution shell numbers are in parentheses)

GDP K-Ras	Q70C/**2**	S39C/**3**	S39C/**4**	S39C/**5**	S39C/**6**
PDB code	4PZY	4PZZ	4Q01	4Q02	4Q03
Data collection					
Space group	P 21 21 21	P 21 21 21	P 21 21 21	P 21 21 21	P 21 21 21
Cell dimensions					
a, b, c (Å)	44.10, 55.96, 120.76	39.08, 41.06, 91.68	36.31, 83.67, 104.84	39.19, 40.93, 92.70	39.04, 41.54, 91.76
α, β, γ (°)	90.00, 90.00, 90.00	90.00, 90.00, 90.00	90.00, 90.00, 90.00	90.00, 90.00, 90.00	90.00, 90.00, 90.00
Resolution (Å)	33.30 – 1.88	28.30 – 1.40	25.01 – 1.29	30.68 – 1.70	24.63 – 1.20
	(1.95 – 1.88)	(1.45 – 1.40)	(1.34 – 1.29)	(1.76 – 1.70)	(1.24 – 1.20)
R_{merge}	9.9 (47.6)	8.0 (15.2)	5.9 (74.3)	8.8 (13.3)	6.5 (24.3)
$I / \sigma I$	9.4 (1.9)	11.3 (4.3)	14.9 (2.7)	9.74 (5.27)	20.08 (9.11)
Completeness (%)	94.5 (60.90)	96.0 (74.3)	98.0 (93.7)	96.5 (62.7)	97.3 (96.0)
Redundancy	4.3 (1.3)	3.7 (1.5)	8.5 (7.4)	4.0 (1.8)	10.7 (10.5)
Structure Refinement					
No. reflections	23696	28457	79531	16347	46120
R_{work} / R_{free}	18.51 / 23.84	13.07 / 15.62	18.81 / 21.33	16.09 / 19.60	16.87 / 19.77
B-factors					
Protein	21.20	11.20	19.60	15.00	14.10
GDP / Mg	14.50 / 18.90	10.40 / 8.82	12.70 / 10.42	13.90 / 11.74	13.10 / 11.16
Water	28.70	22.70	32.00	24.50	27.80
R.m.s. deviations					
Bond lengths (Å)	0.009	0.010	0.012	0.008	0.010
Bond angles (°)	1.18	1.39	1.47	1.15	1.41

References

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