Fluorescent competition assay for retinoic acid binding proteins Supplementary Information

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EXPERIMENTAL PROCEDURES

CRABP-II Expression

CRAPBII protein was prepared from BL21 cells transformed with a pGEX 4T1 vector, generated as 25 ml starter cultures (25 g/L lysogeny broth, 100 µg/ml ampicillin) and transferred to 1 L expression cultures after overnight incubation at 37 °C. Expression was induced after 4 hr shaking (37 °C, 150 rpm) using IPTG (1 mM in culture), before a 20 hr overnight shake. The resulting cultures were spun down using an Avanti Hi-Speed centrifuge (JLA 8.1000, 4000 rpm, 25 min, 4 °C), before removal of supernatant and freezing of pellets at -80 °C.

CRABP-II Purification

Pellets were resuspended with 15 ml cold lysis buffer (10 mM DTT, 100 mM $MgCl_2$, in PBS pH 7.5) and lysed with the addition of 0.5 mg/ml lysozyme for 30 min. The resulting suspension was sonicated on ice (40% power) and centrifuged (20,000 rpm, 50 min, 4 °C). The supernatant collected was loaded onto a GST column (1 ml, GE healthcare) and washed using 30 ml PBS pH 7.5. The column was then loaded with 1 ml thrombin (GE Healthcare) used to cleave the GST tag and reserved at room temperature for 16 hrs. The cleaved protein was eluted using 3 ml PBS pH 7.5, and bound GST tag was eluted using 5 ml reduced glutathione (10 mM). The resulting protein fractions were analysed by SDS PAGE and mass spectrometry, and concentration was estimated using Thermo Scientific Nanodrop A280.

CRABP-II Crystallisation

Purified CRABPII (5 mg/ml) in PBS was combined with DC271 freshly dissolved in EtOH, in a 1:1 molar ratio. Crystallisation conditions were prepared from 0.2 µm filtered buffer and PEG stocks. Trays were loaded with 500 uL reservoir solution, and hanging drops prepared with varying protein: reservoir ratios. Hanging droplets were inspected under Leica microscope, and periodically reviewed for crystal formation.

Data Processing and structural determination

Data was collected at the Diamond light source on the I-03 beamline, equipped with a Pilatus Detector.[1]

Crystallography data for CRAB-II bound to DC271 was processed using the CCP4i2 suite of programs following successful auto processing by XIA2 (XDS)[2-3]. AIMLESS and POINTLESS[4-5] were used for scaling and data reduction. MR BUMP (PHASER)[6-7] was used to perform molecular replacement based on the 2FR3 structure of CRABP-II (PDB 2FR3), and to generate a protein structure. Manual building was performed using COOT[8], and refinement carried out in REFMAC5[9] using jelly-body refinement and adding anisotropic B-factors. Further details are summarised in Table 1.

CRABPII assay validation

DC271 (300 nM in 1% EtOH) and CRABPII (300 nM in 20 mM K_2HPO_4, 100 mM KCl, pH 7.4 + 0.05% Pluronic F127) solutions were prepared and mixed before use. A Corning, Black Non-binding Surface (NBS), 96 well plate was cleaned using compressed air and Maximum and Median wells were loaded with 60 µl CRABPII/DC271 solution. Minimum wells were loaded with 60 µl control solution of DC271 mixed with assay buffer. Median wells were then loaded with 30 µl EC23 (600 nM in 1% EtOH), and Maximum and Minimum wells with 30 µl of a 1% EtOH solution. Plates were spun at 1700 rpm, 2 mins to ensure mixing. Plates were read using a Synergy H4 plate reader, at ex/em 355/460 nm. The total well volume is 90 µl, the on plate concentration of CRABPII and DC271 is 100 nM.

CRABPII competitive binding assay

DC271 (300 nM in 1% EtOH) and CRABPII (300 nM in 20 mM K_2HPO_4, 100 mM KCl, pH 7.4 + 0.05% Pluronic F127) solutions were prepared and mixed before use. A Corning, Black Non-binding Surface (NBS), 96 well plate was cleaned using compressed air and Maximum, Median and all test compound wells were loaded with 60 µl CRABPII/DC271 solution. Minimum wells were loaded with 60 µl control solution of DC271 mixed with assay buffer. Median wells were then loaded with 30 µl EC23 (600 nM in 1% EtOH), and Maximum and Minimum wells with 30 µl of a 1% EtOH solution. Test compounds were prepared as dilution series and applied to the plate as 30 µl aliquots. Plates were spun at 1700 rpm, 2 mins to ensure mixing. Plates were read using a Synergy H4 plate reader, at ex/em 355/460 nm. The total well volume is 90 µl, the on plate concentration of CRABPII and DC271 is 100 nM.

CRYSTALLOGRAPHIC DATA

Table 1: Crystallographic statistics for DC271 CRABPII co-cyrystal structure

	Overall	Inner	Outer
Low resolution limit	40.52	40.52	1.53
High resolution limit	1.50	8.22	1.5
Rmerge(within I+/I-)	0.114	0.111	0.637
Rmerge(all I+ and I-)	0.122	0.114	0.710
Rmeas (within I+/I-)	0.136	0.133	0.803
Rmeas (all I+ & I-)	0.134	0.126	0.792
Rpim (within I+/I-)	0.073	0.072	0.484
Rpim (all I+ & I-)	0.053	0.053	0.348
Rmerge in top intensity bin	0.085		
Number of observations	172350	1081	6693
Number unique	27224	211	1310
Mean((I)/sd(I))	8.9	15.5	2.3
Half-set correlation CC(1/2)	0.991	0.990	0.778
Completeness %	100.0	99.8	100.0
Multiplicity	6.3	5.1	5.1
Anomalous completeness %	99.8	100.0	99.8
Anomalous multiplicity	3.3	3.4	2.6
Statistic			
Resolution	40.55-1.50		
No. reflections all/free	27170/1374		
R-factor/R-free	0.143/0.175		
R.M.S.D Bonds	0.0147		
RMSD Angles	1.903		

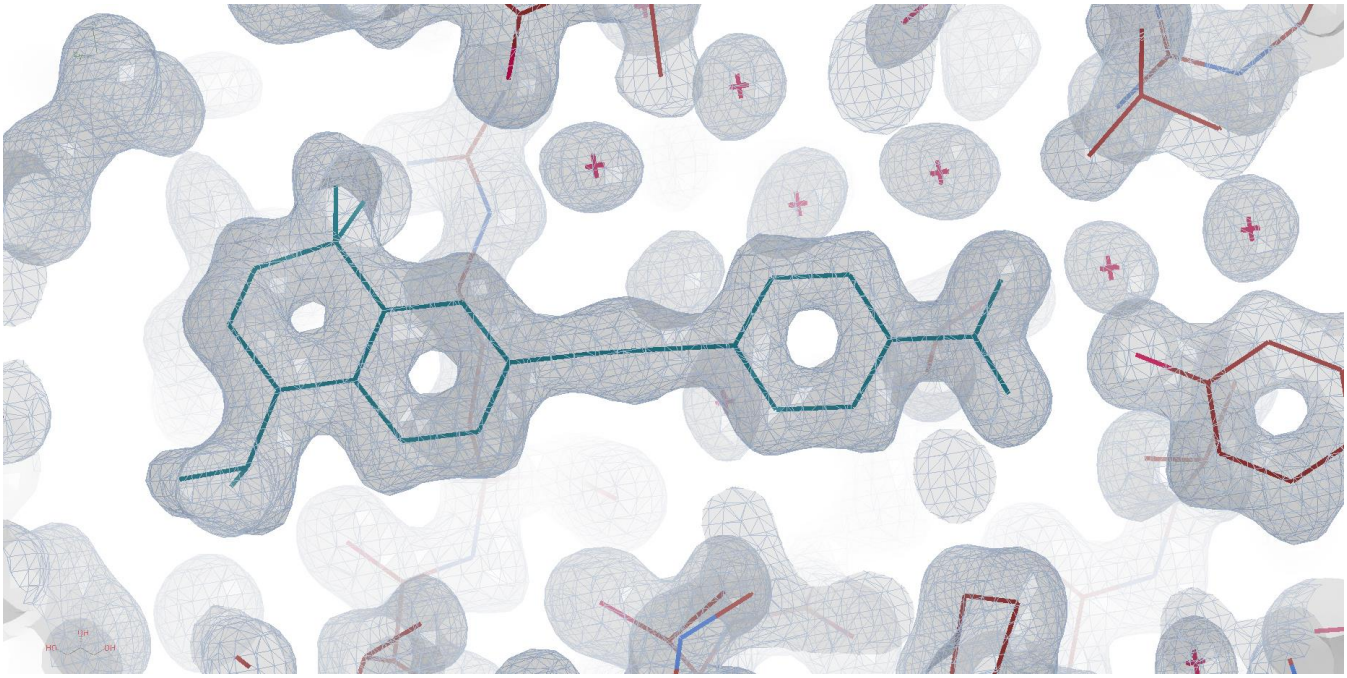

Figure 1: Unbiased electron density map ($\sigma = 1$) of DC271 in the binding site of CRABPII at 1.5 A resolution, R/R_{free} 0.14/0.17. The binding triad ARG111 ARG132 TYR134 can be seen off to the right of the image.

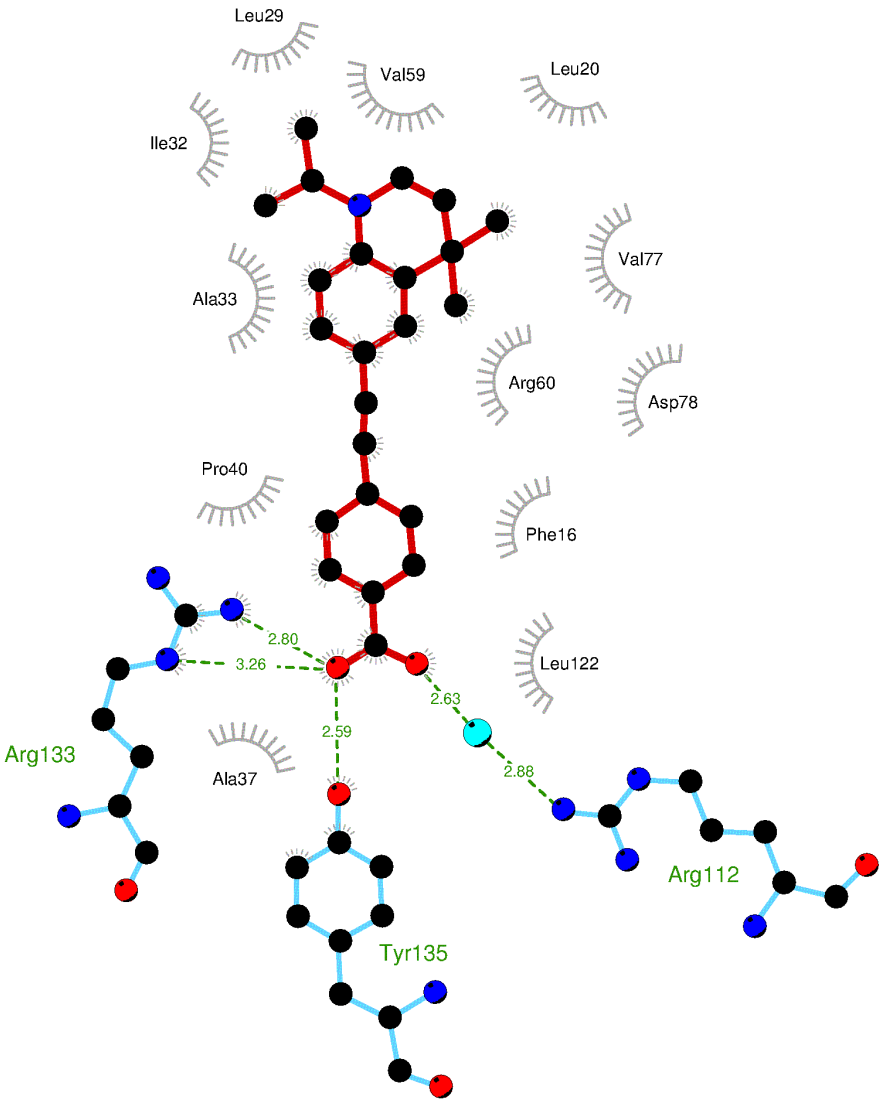

Figure 2: Ligplot[10] schematic of 6HKR binding site – clearly shown is the binding triad Arg112 Arg133 Tyr135 and the relevant hydrogen bonds to the ligand.

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