Supporting Information

6-azido-7-nitro-1,4-dihydroquinoxaline-2,3-dione (ANQX) Forms an Irreversible Bond To the Active Site of the GluR2 AMPA Receptor Ligand-Binding Core

Leslie A. Cruz[a], Eva Estébanez-Perpiña[b], Sam Pfaff[c], Ning Bao[d], Justin Blethrow[a], Robert J. Fletterick[b] and Pamela M. England[d,e]*

[a]Graduate Program in Chemistry and Chemical Biology, [b]Department of Biochemistry and Biophysics, [c]Graduate Program in Biophysics, [d]Department of Pharmaceutical Chemistry, [e]Department of Cellular and Molecular Pharmacology, University of California San Francisco.

Address correspondence to: Pamela M. England, Department of Pharmaceutical Chemistry and Molecular & Cellular Pharmacology, University of California, San Francisco CA 94158-2517. Tel. (415) 502-6606; Fax (415) 514-4070 USA; Email: england@picasso.ucsf.edu

Experimental

Expression and Purification of the GluR2 S1S2J Domain

The GluR2 ligand-binding core (S1S2J) construct, consisting of the GluR2 S1 segment linked to the GluR2 S2 segment via two amino acids (GT), was kindly provided by Dr. Eric Gouaux (HHMI Investigator, Oregon Health & Science University). The S1 and S2 domains correspond to amino acids 390–506 and 632–763, respectively, in the full-length GluR2 subunit. The S1S2J core was expressed in *E. coli* and purified to homogeneity as previously reported Gouaux and co-workers.[1]

Crystallization, Structure Determination and Refinement

The purified S1S2J protein was co-crystallized with the competitive antagonist ANQX. Apo-S1S2J was concentrated to 10 mg/mL in 10 mM HEPES buffer (10 mM HEPES, 20 mM NaCl, 1 mM EDTA, pH 7.0) and incubated with ANQX (6 mM final concentration). Crystals were grown at 4 °C using vapor-diffusion (hanging-drop) with a 1:1 ratio of protein to well solution. Crystals grew to full dimensions after one month in 12.5%-25% PEG 4000, 0.25-0.4 M ammonium sulfate, and 0.1 M NaOAc pH 5.0. Just prior to data collection, the crystals were soaked in 25% ethylene glycol as cryoprotectant and then flash-cooled in liquid nitrogen. While submerged in liquid nitrogen, S1S2J-ANQX co-crystals were exposed to ultraviolet light for 10 s using a Hg/Xe arc lamp (1000 Watt) outfitted with a UV bandpass filter (#51660, Oriel Instruments: transmitting 300–400 nm, peak 360 nm) and a heat absorbing filter (#51944, Oriel Instruments: transmitting 300-1000nm). Synchrotron data for S1S2J-FQX was collected at 100K on

the Advanced Light Source beamline 8.3.1 at Lawrence Berkeley National Laboratory. Data processing was performed using Elves[2] and HKL2000. For additional details see Table 1 (supplementary material).

A complete dataset from an S1S2J-FQX crystal diffracted to 1.87 Å and exhibits the space group $P2_12_12_1$ and contains four molecules per asymmetric unit (Table 1). Molecular replacement solutions for S1S2J-ANQX crystal structure was obtained using as a search model one of the monomers of S1S2J-DNQX dimer structure.[3] The molecular replacement solutions were obtained using rotation and translation functions from Crystallography & NMR Systems (CNS, http://cns.csb.yale.edu/v1.1/). Protein model refinement consisted of simulated annealing, group and individual B-factor refinement, and conjugate gradient minimization in CNS followed by model building (monitored by free-R factor) using COOT.[4,5] Visual inspection of electron density using COOT allowed identification of the ligand FQX bound to the S1S2J domain. Using CNS, a composite omit map was also calculated in which a different 5% of the model was omitted in an attempt to minimize model bias. Calculation of the electron density maps and crystallographic refinement was performed with CNS using the target parameters of Engh and Huber.[6] Several cycles of model building, conjugate gradient minimization and simulated annealing using CNS resulted in models with good stereochemistry. A Ramachandran plot shows that all but three of the residues fall into the favored regions. The statistics for data collection and refinement of each one of the data sets are in Table 1.

Photocrosslinking ANQX to S1S2J

A solution of Ni-NTA beads bound to the 6-HIS tagged S1S2J in 10 mM HEPES buffer was placed on a piece of filter paper (Whatmann) in a Petri dish outfitted with a perfusion inlet, supplying unphotolyzed ANQX (100 μM), and an outlet attached to an in house vacuum, removing photolyzed ANQX from the beads. Unphotolyzed ANQX in 10 mM HEPES buffer was continuously perfused (20 mL/min) over the S1S2J Ni-NTA beads while exposing the beads to ultraviolet light for 90 s (1000 Watt Hg/Xe arc lamp outfitted with a UV bandpass filter (#51660,Oriel Instruments: transmitting 300–400 nm, peak 360 nm) and a heat absorbing filter (#51944, Oriel Instruments: transmitting 300-1000nm). In control experiments S1S2J Ni-NTA beads were similarly irradiated in the absence of ANQX. The S1S2J Ni-NTA beads were washed with 10 mM HEPES buffer to remove any remaining photolyzed ANQX. The S1S2J domain was eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0), dialyzed against 10 mM HEPES, pH 7.0, 20 mM NaCl, 1 mM EDTA, and concentrated (4 mL 10,000 MWCO Amicon centrifuge tubes, Millipore). The samples were analyzed by high-throughput mass spectrometry using a CIT Analytics Autosampler and a LTC premier mass spectrometer (Waters Micromass).

Trpysin-digestion and Tandem Mass Spectrometric Analysis

The experimental (UV light, ANQX) and control (UV light only) S1S2J cores (2.5 μg) were treated with 8 M urea and sonicated for 10 min in a 37 °C water. The resulting denatured proteins were digested overnight at 37 °C with a mixture of

trypsin/chymotrypsin. Approximately five picomoles of each sample were then analyzed by nanoscale LC/MS^2 using a Q TRAP mass spectrometer (Applied Biosystems, Foster City, USA) coupled to an LC Packings Ultimate/Famos/Switchos liquid chromatography system (Dionex). Peptides were resolved over a 75 micron x 150 mm C18 column using a two hour gradient at a flow rate of 150 nL/min. Tandem mass spectra were acquired automatically in IDA mode, and the resulting data were analysed with MASCOT (Matrix Science).

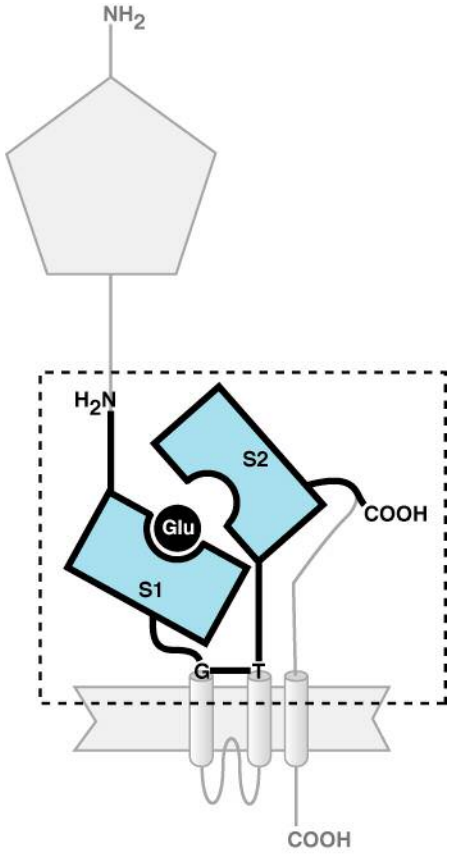

Figure S1. Cartoon depicting the AMPAR ligand-binding core (S1S2J) with respect to the subunit topology. The portion of the subunit corresponding to the S1S2J (dashed box) is comprised of the S1 segment linked to the S2 segment via two amino acids (GT). The S1 and S2 segments correspond to amino acids 390–506 and 632–763, respectively, in the full-length GluR2 subunit.

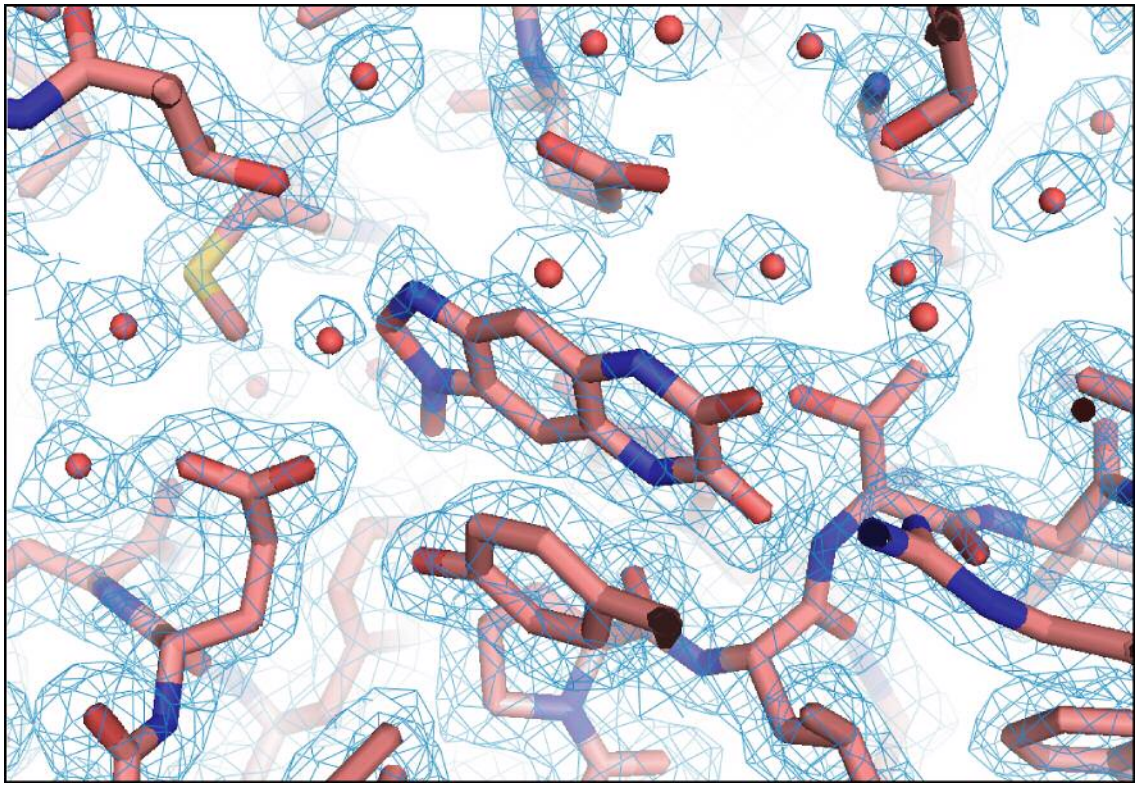

Figure S2. Fo-Fc omit electron density for FQX and surrounding ligands contoured at 1.0 sigma. At 1.5 sigma, the electron density shows the good shapes for the side chains and aromatic rings and has reliable solvent peaks. At 3.0 sigma contouring, there is significant loss of information.

Table 1. Statistics for X-ray Data Collection and Refinement.

	S1S2J-FQX
Data Collection Statistics	
Space group	$P2_12_12_1$
Cell constants a/b/c (Å)	62.34
	92.26
	195.24
# Molecules/ASU	4
Total Reflections	191553
Unique Reflections	90300
Reflections used refinement	90300
R-merge(%)[a,b]	5.6 (51.2)
Redundancy	2.1
I/ σ (I)[b]	8.0 (2.0)
Completeness (%)[b]	97.0 (92.8)
Refinement Statistics	
Resolution (Å)	1.87
R-factor (%)[c]	21.2
R-free (%)[d]	22.0
B-factors	
Protein	17.677
Ligand	23.172
Solvent	24.521
R.M.S.D bonds	0.017
R.M.S.D angles	1.7
Waters	875
Matthews Coefficient (Da-1)	2.34
Solvent content (%)	47.4
Ramachandran plot (%)	
Most Favored	94%
Allowed	6%
PDB code	3BKI

ASU=asymmetric unit

[a] R merge (%)= $\sum_{hkl} |<I>-I| / \sum_{hkl} |I|$

[b] Values in parentheses refer to the highest resolution shell (1.87-1.97 Å).

[c] R factor (%)= $\sum_{hkl} ||Fo| - |Fc| / \sum_{hkl} |Fo|$

[d] 5% of the reflections were set aside for the calculation of the R free value.

References

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