Is the Isolated Ligand Binding Domain a Good Model of the Domain in the Native Receptor?*

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Numerous studies have used the atomic level structure of the isolated ligand binding domain of the glutamate receptor to elucidate the agonist-induced activation and desensitization processes in this group of proteins. However, no study has demonstrated the structural equivalence of the isolated ligand binding fragments and the protein in the native receptor. In this report, using visible absorption spectroscopy, we show that the electronic environment of the antagonist 6-cyano-7-nitro-2,3-dihydroxyquinoxaline is identical for the isolated protein and the native glutamate receptors expressed in cells. Our results hence establish that the local structure of the ligand binding site is the same in the two proteins and validate the detailed structure-function relationships that have been developed based on a comparison of the structure of the isolated ligand binding domain and electrophysiological consequences in the native receptor.

**Glutamate receptors are the predominant mediators of excitatory synaptic signals in the central nervous system and play an important role in the regulation of synaptic strength and in diverse neuropathologies, including epilepsy and ischemia (for reviews, see Refs. 1 and 2). Based on agonist affinity profiles, they can be subdivided into three subfamilies: α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), N-methyl-D-aspartate, and kainate receptors (1, 3). The AMPA subtype of the glutamate receptors are homo- or hetero-oligomers composed of GluR1–GluR4 subunits. The recent determination of the structure of the soluble ligand binding domain (S1S2) for the glutamate receptors are homo- or hetero-oligomers composed of GluR1–GluR4 subunits. The recent determination of the structure of the soluble ligand binding domain (S1S2) for the glutamate receptors is the same in the two proteins and validate the detailed structure-function relationships that have been developed based on a comparison of the structure of the isolated ligand binding domain and electrophysiological consequences in the native receptor.

**Experimental Procedures**

**S1S2 Protein Preparation—**The GluR2-S1S2 construct containing the S1 segment (α-amino acids 390–506 in the GluR2 sequence) and the S2 segment (α-amino acids 632–763 in the GluR2 sequence) with two domains being linked together via an amino acid linker (GT) was kindly provided by Dr. Gouaux (Columbia University, New York, NY) (5). The protein was expressed, purified, and characterized as described by Chen et al. (13). The digestion and purity of the protein was tested using SDS-PAGE, and the activity (ligand binding) was tested by measuring the Kᵢ values using fluorescence spectroscopy. GluR4-S1S2 was kindly provided by Dr. Madden (Dartmouth College, Hanover, NH).

**Human Embryonic Kidney 293 (HEK-293) Cell Cultures—**HEK-293 (ATCC CRL 1573) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen) and 2 mM glutamine, 50 μg/ml penicillin, and 50 μg/ml streptomycin. One day before transfection, cells were replated in 35-mm culture dishes coated with rat tail collagen (Sigma). Transfections were performed using FuGENE 6 (Roche Applied Science) transfection reagent with 1–2 μg of glutamate receptor cDNA. The plasmid encoding for the GluR4-Zip subunit of the glutamate receptors used for the transfection was generously provided by Dr. Seeburg (Max Planck Institute, Heidelberg, Germany). Transfected cells were allowed to grow for 1–3 days before use.

**Absorption Measurements—**The UV–visible absorption spectra were obtained using Agilent 8453 or Shimadzu UV 2501 spectrometers (photometric reproducibility of 0.0003 absorbance units) and analyzed using Gram’s Spectral Notebase (Thermo Galactic, Salem, NH). The spectra were collected in the range of 500–250 nm with a sampling interval of 1 nm using a 1-cm quartz cuvette. The absorption spectra for GluR2-S1S2 and GluR4-S1S2 were obtained using 10 μM protein and 20 μM 6-cyano-7-nitro-2,3-dihydroxyquinoxaline (CNQX). For the transfection experiments with HEK-293 cells the number of cells used was such that the background due to scattering from cells was 0.7 absorbance units at 310 nm; this ensured that the total absorption was less than 1 absorption unit upon adding 20 μM CNQX.

When determining the difference absorption spectra for GluR4-S1S2 and GluR2-S1S2 using competitive ligands, parallel control experiments were performed by adding the corresponding concentrations of ligands to 20 μM CNQX in buffer to ensure that no spectral changes were observed due to ligand-CNQX interactions. Similarly for the HEK-293 cells the difference spectra have been corrected for any differences arising due to nonspecific changes in the CNQX spectrum that arise due to the addition of the agonist ligands by performing parallel control experiments using HEK-293 cells that have not been transfected. Specifically, difference spectra were obtained between the spectra for homogenized non-transfected HEK-293 cells with 20 μM CNQX in the presence and...
The dissociation constant for the agonist. The bound and free concentrations and therefore depletion of CNQX was small.

expression of glutamate receptor S1S2 versus native.

RESULTS AND DISCUSSION

A number of ligands bind with high specificity to the glutamate family of receptors. Of particular interest is CNQX, which exhibits electronic transitions in the visible region as evidenced by its orange/red color. Moreover CNQX exists in a unique electronic configuration when bound to the S1S2 protein as established by the dramatic changes in its infrared vibrational modes (9). The difference in the electronic configurations of the free and protein-bound forms of CNQX is expected to be reflected in its absorption spectra.

The UV-visible absorption spectra of CNQX bound to GluR2-S1S2 and in the free form are shown in Fig. 2. A decrease in the absorption is observed at 330 and 343 nm upon CNQX binding to the protein. Based on the wavelength as well as on the structure of CNQX (extended conjugation and presence of a nitro group) these absorbance bands are expected to arise from Π-Π* or n-Π* transitions. The change in the absorption spectral features are highlighted by examining the difference absorption spectrum (shown in Fig. 3). These changes in the absorption and the underlying changes in the electronic environment could arise due to specific and nonspecific binding of CNQX. By conducting a series of competitive binding studies we have identified that the changes in the absorption spectra are mainly due to specific binding of CNQX to the GluR2-S1S2. Specifically we have examined the competition with glutamate receptor agonists such as glutamate (Fig. 3B), AMPA (Fig. 3C), kainate (Fig. 3D), and a non-binding compound, γ-aminobutyric acid (E). The difference spectra were obtained at pH 7.4 using a phosphate buffer and at room temperature. a.u., absorbance units. 

![Figure 1](image1.png)

**Fig. 1.** The schematic representation of the topology of the AMPA receptor (shown on the left) and the isolated ligand binding domain (S1S2 protein, shown to the right, contains the S1 and S2 domains joined by a linker). AMPA receptors are thought to be tetramers, each subunit with three transmembrane domains, a pore-loop sequence that lines the channel, and an extracellular domain with two lobes (S1 and S2) forming a ligand-binding cleft.

![Figure 2](image2.png)

**Fig. 2.** The top panel shows the structures of CNQX in the free (A) and S1S2-bound (B) forms (9); the bottom panel shows the absorption spectrum of free CNQX (A) (dashed line), CNQX bound to S1S2 (B) (solid line), and S1S2 (C) (solid line).

![Figure 3](image3.png)

**Fig. 3.** Difference absorption spectra between 20 μM CNQX in buffer and CNQX in the presence of GluR2-S1S2 (A) and 20 μM CNQX bound to GluR2-S1S2 in the presence and absence of 500 μM AMPA (B), 500 μM glutamate (C), 500 μM kainate (D), and 500 μM γ-aminobutyric acid (E). The difference spectra were obtained at pH 7.4 using a phosphate buffer and at room temperature. a.u., absorbance units.
Dose-Response Curves—The absorbance bands shown in Fig. 3A are not only useful for qualitative investigations of electronic environment of the ligand binding site, but they also allow the quantitative determination of the fraction of bound CNQX. Specifically, since the absorbance is directly proportional to the concentration of the chromophore (CNQX), a quantitative measure of the concentration of CNQX dissociated from the binding site can be obtained from the integrated area of the difference spectral features (320–353 nm). Based on this, dose-response curves were obtained for the displaced CNQX at various concentrations of competitive agonists, and the dissociation constants of the competitive agonists were determined. A plot of the integral areas as a function of the concentration of the competitive ligands is shown in Fig. 5 for various ligands added to a solution containing CNQX bound to GluR2-S1S2. Using a logistic fit for these dose-response curves the IC$_{50}$ values were determined to be 7 ± 2, 22 ± 5, and 115 ± 25 μM for AMPA, glutamate, and kainate, respectively. Based on these IC$_{50}$ values, using the corrected Cheng-Prusoff equation (14) (corrected for ligand depletion; see “Experimental Procedures” for details), the $K_d$ values were determined for AMPA, glutamate, and kainate (shown in Table I). These values are in good agreement with the values previously reported based on radioactive ligand binding experiments (Table I) (13). The ability to use the differences in the absorbance of CNQX to determine the affinities of various ligands makes this an excellent assay to screen for compounds that selectively bind to this receptor.

Structure of S1S2 Versus the Ligand Binding Domain in the Full Receptor—Since the difference spectrum provides a fingerprint of the local electronic structure of the ligand binding site, we used this to compare the structure of the isolated ligand binding domain with the same domain in the full receptor. For these investigations we have compared the difference absorption spectra for CNQX binding to the GluR4-S1S2 (isolated ligand binding domain of GluR4 subunit) and homomeric GluR4 receptors expressed in HEK-293 cells (Fig. 6). The difference spectra are identical for the isolated domain and the native receptor. Additionally no spectral features were observed in displacement studies with a non-binding compound γ-aminobutyric acid, confirming that the spectral features shown in Fig. 6 arise due to specific binding of CNQX. Finally for a quantitative comparison a dose-response curve was obtained for competitive displacement of CNQX by glutamate from homomeric GluR4-flip receptors expressed in HEK-293 cells (Fig. 5, open squares). The IC$_{50}$ value determined using a logistic fit was 20 ± 5 μM, similar to that obtained for GluR2-S1S2, and the corresponding $K_d$ value for glutamate was 450 ± 110 nM assuming the $K_d$ for CNQX is 460 nM (15–17). This value is in reasonably good agreement with previously published values for glutamate binding based on radioactive ligand binding (15–17).

The results presented here provide definitive evidence that the electronic environment of CNQX is the same in the homomeric GluR4 glutamate receptors transiently expressed in HEK-293 cells as that in the isolated ligand binding domain, therefore clearly indicating that the GluR2-S1S2 and GluR4-S1S2 are good models for the ligand binding domain in the native receptor, thus providing additional validation for the structure-function insights currently available based on the atomic level structure of GluR2-S1S2.

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