Hydrophobic Side Chain Dynamics of a Glutamate Receptor Ligand Binding Domain*

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Ionotropic glutamate receptors are ligand-gated ion channels that mediate much of the fast excitatory neurotransmission in the central nervous system. The extracellular ligand binding core (S1S2) of the GluR2 subtype of ionotropic glutamate receptors can be produced as a soluble protein with properties essentially identical to the corresponding domain in the intact, membrane-bound protein. Using a variety of biophysical techniques, much has been learned about the structure and dynamics of S1S2 and the relationship between its ligand-induced conformational changes and the function of the receptor. It is clear that dynamic processes are essential to the function of ionotropic glutamate receptors. We have isotopically labeled side chain methyls of GluR2 S1S2 and used NMR spectroscopy to study their dynamics on the ps-ns and μs-ms time scales. Increased disorder is seen in regions that are part of the key dimer interface in the intact protein. When glutamate is bound, the degree of ps-ns motion is less than that observed with other ligands, suggesting that the physiological agonist binds to a preformed binding site. At the slower time scales, the degree of S1S2 flexibility induced by ligand binding is greatest for willardiine partial agonists, least for antagonists, and intermediate for full agonists. Notable differences among bound ligands are in the region of the protein that forms a hinge between two lobes that close upon agonist binding, and along the β-sheet in Lobe 2. These motions provide clues as to the functional properties of partial agonists and to the conformational changes associated with lobe closure and channel activation.

The majority of excitatory synaptic transmission in the vertebrate central nervous system is mediated by ionotropic glutamate receptors (iGluRs, Ref. 1). These receptors also play important roles in neuronal development and the formation of synaptic plasticity underlying higher order processes such as learning and memory (2). In addition, iGluRs are associated with neurologic disorders including epilepsy and ischemic brain damage and neurodegenerative disorders such as Huntington chorea, Parkinson, and Alzheimer diseases. iGluRs are membrane-bound receptor ion channels composed of four subunits surrounding a central ion channel in which each subunit contributes to pore formation. Subunits are categorized by pharmacological properties, sequence, functionality, and biological role into those that are sensitive: 1) to the synthetic agonist α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA; GluR1–4); 2) to the naturally occurring neurotoxin kainate (GluR5–7, KA1,2); and 3) to the synthetic agonist N-methyl-D-aspartic acid (NMDA; NR1, NR2A-D, NR3A-B).

The extracellular agonist binding domain (S1S2) can be expressed as a soluble protein in bacteria, and a great deal of structural and functional detail concerning the mechanisms of receptor activation has been determined using this isolated domain. This has included a large number of crystal structures (e.g. 3–9), analysis of dynamics by NMR spectroscopy (10–13), fluorescence measurements (14, 15), ITC measurements (16, 17), and IR spectroscopy (18–20). The agonist binding domain is a bilobed structure that, under some conditions, can form dimers. The agonist binding site is in a crevice between the two lobes, and the closure of the lobes around the agonist is associated with the activation of the ion channel in the full-length, membrane-bound receptor (4). The receptor is a tetramer, built as a dimer of dimers (21). The dimer interface of the binding domain is intact in the activated form, but the dissociation of the interface is associated with desensitization (22).

Although the large scale mechanisms (e.g. lobe closure is associated with channel activation) are relatively clear from the crystal structures, the kinetic and thermodynamic details of activation and desensitization require analysis by methods that can accurately detect dynamic processes (NMR, IR, etc.). This has led to additional insights into the function of these important neurotransmitter receptors. For example, studies using NMR spectroscopy suggest that partial agonists show increased dynamics in the μs-ms time scale that is associated not only with specific motions within the agonist binding site but also seems to report on large scale relative motions of the two lobes (11, 23) that may be related to mechanisms of channel activation. Although backbone motions are of considerable interest, side chain methyl groups have the potential to report on motions not detected by other methods (24), because methyls tend to participate in the packing of the hydrophobic core of the protein.

We report here on the motions of isoleucine, valine, and leucine methyl groups on the ps-ns and μs-ms time scales studied using NMR spectroscopy in combination with specific 13C and 1H labeling of the GluR2 S1S2 domain. On the
faster time scale, binding of glutamate was found to induce a higher average order parameter of the methyl groups compared with all other ligands, possibly reflecting the fact that it is the natural ligand for this receptor and produces the least perturbation of the hydrophobic packing. On the longer time scale, motions were observed along the β-sheet of Lobe 2 for agonists and most partial agonists, but not antagonists. These motions as well as those near the hinge axis may provide additional details concerning processes associated with channel activation.

**EXPERIMENTAL PROCEDURES**

**Materials**—AMPA, kainate, DNQX, IW, CIW, UBP282, and FW were obtained from Tocris (St. Louis, MO) or Ascent Scientific (Princeton, NJ). The GluR2 S1S2J construct was obtained from Eric Gouaux (Vollum Institute; Ref. 4). Isotopically labeled substrates were obtained from Cambridge Isotopes (Andover, MA), except for sodium 2-ketobutyrate-4-^{13}C,4,4-d_2 and sodium α-ketoisovalerate-3-(methyl-^{13}C, d_2)A-^{13}C,d_2, which were obtained from Sigma-Aldrich.

**Protein Preparation and Purification**—The GluR2 (flop) S1S2 domain consists of residues Asn-392—Lys-506 and Pro-632—Ser-775 of the full-length rat subunit (25), a GT linker connecting Lys-506 and Pro-632 (4) and a GA segment at the N terminus. The polyhistidine-tagged protein was expressed in inclusion bodies (BL-21(DE3) Escherichia coli), refolded, treated with trypsin for histidine tag removal, and purified (12, 27, 28). Two different labeling schemes were used for the carbon HSQC peak assignments and for relaxation experiments, although in both cases α-ketoacids were used for ILV labeling as described by Tugarinov and Kay (29).

For the ^1H,^{13}C-HSQC peak assignments, bacteria were grown in fully deuterated M9 medium with 4 g/liter of [^{13}C,2H]glucose, and 1 h prior to induction, 50 mg/liter of [^{13}C,2H]glucose as the sole carbon source and [^{15}N]ammonium chloride as the sole nitrogen source. Upon purification and refolding in H_2O-based buffers, protons and methyls of isoleucines (81 only), leucines, and valines, with deuterons exchanged into the β-positions as described above. The resulting protein is uniformly ^13N-labeled with protons only in the methyl positions. This was achieved by exchanging deuterons into the β-position of α-ketoisovalerate by incubation in D_2O at 45 °C, pH 10.5, for 20 h (30) and into the same position of α-ketoisovalerate by incubation in D_2O at 45 °C, pH 12.4, for 3 h (31). The protein was also uniformly labeled with ^15N using [^{15}N]ammonium chloride as the sole source of nitrogen. Upon purification and refolding in H_2O-based buffers, protons are exchanged back on the amides, so that the resulting protein is uniformly ^15N- and ^13C-labeled with protons on amide nitrogens and methyls of isoleucines (81 only), leucines, and valines, with deuterons elsewhere. This preparation was used to assign methyl group protons and carbons using the HCC-TOCSY and CCC-TOCSY experiments (32).

To perform carbon relaxation experiments, the desired methyl group configuration was ^13CH_2. Bacterial culture was initially grown in fully deuterated medium containing [^{12}C,2H]glucose as the sole carbon source and [^{15}N]ammonium chloride as the sole nitrogen source. Approximately 1 h prior to induction, 50 mg/liter of sodium α-ketoisovalerate-4-^{13}C,4,4-d_2 and 100 mg/liter of sodium α-ketoisovalerate-3-(methyl-^{13}C, d_2)A-^{13}C,d_2 were added to the growth medium (deuterons were exchanged into the β-positions as described above). Protein production was induced by adding isopropyl-1-thio-β-d-galactopyranoside to 0.7 mM, and induction was allowed to proceed for ~16 h. The protein was refolded and purified as described previously (11) using H_2O-based buffers. The resulting protein had protons at amide positions, and methyls in isotope configuration ^13CH_2 in Ile 61, Leu 6, and Val γ positions, with deuterons and ^13C in all other positions.

Protein yield was 3–4 samples of 0.25–0.35 mM purified protein per 1.25 liters of the growth medium. The NMR buffer was an aqueous solution of 25 mM sodium acetate, 25 mM sodium chloride, 1 mM sodium azide, 100% D_2O, pH 5.0. Final concentrations of kainate, fluorowillardiine (FW), chlorowillardiine (CIW), and UBP282 were 3 mM, of AMPA and DNQX were 1 mM, and of glutamate was 10 mM. Ligands were exchanged using successive dilution and concentration using an Amicon Ultra-4 (10K) filter.

**NMR Spectroscopy**—All measurements were made on a Varian Inova 500 spectrometer equipped with a triple resonance, z-gradient cryogenic probe at a sample temperature of 14 °C. T_1 and T_1p experiments were performed on ^1CH_2. ILV-labeled S1S2 using pulse sequences similar to those described by Ishima et al. (33) and written by Tugarinov and Kay (34). Delays of 3, 15, 30, 50, 70, and 90(2) ms were used in T_1p experiments and delays of 0.04, 0.2(2), 0.4, 0.6, 0.8, 1.2, 1.6 s were used in T_1 experiments. Spectra with different delays were acquired in interleaved fashion to avoid the effects of potential loss of protein during the course of the experiment. The resulting data file was separated into the appropriate number of files each containing a single two-dimensional spectrum, which was then processed using NMRPipe (35). The two-dimensional spectra were analyzed in Sparky (36). The internal Sparky function for fitting to exponential decay was used to obtain values of R_1 and R_1p, and their error estimates (500 Monte-Carlo iterations were performed to obtain standard deviations). The transverse relaxation rate was calculated using Equation 1.

\[
R_2 = \frac{(R_{1p} - R_1 \cos^2 \theta)}{\sin^2 \theta},
\]

(Eq. 1)

where \( \theta = \arctan(\omega_t / \Delta \omega) \) (37, 38). The values of R_1 and R_2 were used to extract the order parameter S_2^axis and the methyl internal rotation correlation time \( \tau_c \) closely following the procedure described in Tugarinov and Kay (34).

CPMG experiments on ^13CH_2 ILV-labeled S1S2 used a pulse sequence modified from the T_1p, spin-lock sequence by substituting a train of 180° carbon pulses, separated by delays of 2\( \tau_{cpmg} \) in place of the carbon spin-lock period as described by Ishima et al. (33). The duration of the relaxation delay was set to 40 ms, and the number of pulses was varied. Spectra were obtained with 8, 12, 16, 20, 24, 32, and 48 CPMG pulses (setting the pulse spacing as \( \tau_{cpmg} = 40 \text{ ms}/2 \times \text{(number of pulses)} \)). A reference spectrum completely omitting the relaxation delay was also obtained. Spectra for the same ligand were obtained in interleaved fashion and transformed as described for \( T_1 \) and \( T_{1p} \) experiments. Peak heights, measured in Sparky, were used to calculate relaxation dispersion profiles. Relaxation rates as a function of effective CPMG frequency were calculated according to Mulder et al. (39) using Equation 2.
where \( T \) is the fixed duration of the relaxation period (40 ms), \( I_o \) is the peak height measured when omitting the relaxation period, and \( I(\nu_{\text{cpmg}}) \) is the peak height measured for a given frequency of CPMG pulses \((\nu_{\text{cpmg}} = 1/4\tau_{\text{cpmg}})\). The range of CPMG frequencies covered in these experiments was from 100 to 600 Hz.

Because relaxation dispersion experiments were performed at only one value of static magnetic field (500 MHz), quantitation of the parameters of motion is limited. The chemical exchange contribution in a two-site exchange model is dependent on three parameters: fractional population of the minor (major) state, chemical shift difference between the two states, and exchange rate \((k_{ex})\). With the data from only one magnetic field, the values of the first two parameters cannot be reliably extracted; however, the value of \( k_{ex} \) can be determined. To improve quality of fits used to interpret CPMG data, it was supplemented with \( R_2 \) values measured from spin-lock experiments that correspond to CPMG frequency of \( \sim 2200 \text{ Hz} \).

Note that the lowest frequency of CPMG pulses used in the present experiments was 100 Hz. Skrynnikov et al. (40) found that the presence of the directly bound deuterons in CHD\(_2\) isotopomers at low CPMG frequencies interferes with decoupling and leads to a modulation of relaxation profiles not related to exchange. We observed aberrant relaxation rates at 50 Hz, but flat relaxation profiles for methyls not undergoing chemical exchange were observed when using CPMG frequencies of 100 Hz and above.

**RESULTS**

Assignments—Based on the backbone assignments previously reported (41), CCC-TOCSY and HCC-TOCSY experiments were used to assign peaks in the \(^{1}H,^{13}C\)-HSQC spectrum of the ILV-labeled S1S2 protein (Fig. 1A). Only the 61 methyl of Ile is protonated, and the resulting Ile peaks occupy an isolated portion of the spectrum. All 16 of the possible resonances were assigned. Val and Leu have two protonated methyl groups, but in some cases, only one was observed either due to peak overlap or fast relaxation. Peaks belonging to 35 of 38 Val and Leu were identified in the \(^{1}H,^{13}C\)-HSQC spectrum. No attempt was made to obtain stereospecific assignments. In all, 88% of the observed peaks (76 of 86) in the \(^{1}H,^{13}C\)-HSQC spectrum were assigned. Fig. 1B shows the distribution of the assigned ILV methyls within the structure of GluR2 S1S2 (glutamate-bound). In Lobe 1, all 36 residues were represented in assignments, and in Lobe 2, 15 of 18 were represented (only Leu-639, Leu-650, and Val-68 had no assignments; colored green in Fig. 1B). Assignments of the \(^{1}H,^{13}C\)-HSQC spectrum for other ligands were obtained by comparison with glutamate assignments. This resulted in little ambiguity. In the few cases in which the assignment could not be made with certainty, the peak was omitted from consideration.

FIGURE 1. A, \( T_1 \) experiment with a relaxation delay of 40 ms for GluR2 S1S2 bound to AMPA with the assignments shown. B, distribution of methyl groups in the GluR2 S1S2 protein bound to glutamate (3dp6, 3). Isoleucine is shown in blue, valine is shown in red, and leucine is shown in yellow. Unassigned residues are shown in green. The S1 segment is colored cyan, and the S2 segment is colored green. The yellow segment in Lobe 2 is the position of the artificial GT linker that replaces the membrane-spanning regions.
ps-ns Dynamics—The most conspicuous differences in order parameters ($S^{\text{axis}}_2$) are seen at different parts of the protein rather than among the different ligands tested. Clusters of lower order parameters are found in two positions in Lobe 2 (Ile-664/Val-666, Val-723/Leu-727) and in Lobe 1 (Leu-483/Val-484, Leu-401/Leu-442/Ile-444). The most striking cluster, however, is in the last two helices (J and K helices) that are involved in dimerization of the protein (Fig. 2). Although the protein is largely monomeric under these conditions (12), it is this part of the protein that forms the dimer interface at higher concentrations or in the presence of allosteric modulators. Also, allosteric modulators bind to a complex surface formed by the dimer interface, which includes the K helix. The intact receptor is a tetramer (dimer of dimers) and in two of the four subunits (one from each dimer), the C-terminal end of the K helix forms part of the interface with the other dimer (21). This apparent loose packing of the helices against the remainder of the protein may be reflective of the increased dynamics typically observed in regions of proteins that participate in protein-protein interactions (42).

One unexpected aspect of these measurements is that the order parameters for bound glutamate are consistently somewhat higher than for the other ligands. These relatively small differences become much more significant when conformational entropies of methyls are calculated according to Yang and Kay (Ref. 43 and Table 1). This would suggest that the packing of the hydrophobic cores of the protein is relatively unaffected by the bound ligand, with glutamate allowing somewhat tighter packing.

μs-ms Dynamics—The presence or absence of chemical exchange, and the rate of chemical exchange can be inferred from dispersion plots of the type shown in Fig. 3. Methyl groups showing exchange ($R_{\text{ex}} \approx 3 \text{ s}^{-1}$; where $R_{\text{ex}}$ is defined as $R_2(100 \text{ Hz}) - R_2(2200 \text{ Hz})$) are displayed in Fig. 4. Overall, the weaker partial agonists of the substituted willardiine series (IW and CIW) show the greatest number of residues exhibiting chemical exchange and the antagonists (DNQX and UBP282), and the very weak partial agonist kainate, show the least (Fig. 4). This is similar to the trend seen with ITC, in which, relative to glutamate, binding of antagonists is entropically less favored (16, 44), but the binding of partial agonists is entropically more favored (16).

The specific methyl groups undergoing exchange differ depending upon the ligand bound and may shed light on the function of the protein. In particular, a series of three methyl-containing side chains (Ile-481, Leu-498, and Ile-712) along the hinge axis for lobe closure show exchange for the willardiine partial agonists (Fig. 5A). I481 is in Lobe 1 and contacts the interlobe linkers. In this position, exchange is only observed for ClW and IW ($R_{\text{ex}}$ of 3.2 and 5.6 Hz, respectively), with different exchange rates (2000 and 1200 s$^{-1}$, respectively). Leu-498 is part of the interlobe linkers and, with the exception of DNQX, shows some exchange for all bound ligands tested. However, the largest $R_{\text{ex}}$ was observed for CIW and IW. The only ligands that induce exchange in Ile-712 are FW, ClW, and IW. Relaxation dispersion data reveal that the exchange contributions are 9.2 Hz, 3.9 Hz, 2.8 Hz, and the values of $k_{\text{ex}}$ are 2000 s$^{-1}$, 2900 s$^{-1}$, and 3400 s$^{-1}$ for IW, CIW and FW, respectively. The chemical exchange seen in methyl groups along the hinge axis is consistent with previous studies (10, 11, 23), which suggests.

### TABLE 1

| Ligand   | Entropy $S$ | $-T^2S$ | $-T^2S$ relative to glutamate |
|----------|-------------|---------|--------------------------------|
| Glutamate| -238        | 68.2    | 0                              |
| AMPA     | -185        | 53.0    | -15.2                          |
| FW       | -182        | 52.2    | -16.0                          |
| IW       | -166        | 47.6    | -20.6                          |
| Kainate  | -168        | 48.3    | -19.9                          |
| DNQX     | -179        | 51.3    | -16.9                          |
| UBP282   | -167        | 47.8    | -20.4                          |

FIGURE 2. Distribution of methyl groups showing the position of methyls with $S^{\text{axis}}_2$ less than 0.5 for GluR2 S1S2 bound to all of the ligands tested. Backbone coloring is the same as in Fig. 1B. Methyls with $S^{\text{axis}}_2$ less than 0.5 are shown in red.
that the willardiine partial agonists exhibit large scale lobe motions due at least in part to the steric hindrance from the halogen in the 5-position. This is much more pronounced for IW and CIW than FW, consistent with the more extensive exchange contributions along the hinge axis.

A large number of hydrophobic contacts are made between the helices and the β-sheet in Lobe 1, creating what could be effectively considered a single rigid core. Exchange is observed throughout the hydrophobic cores of Lobe 1, particularly in the cases of the partial agonists, IW and CIW. In Lobe 2, the prominent hydrophobic cluster formed almost exclusively by helices F and G (Core A), makes only scarce hydrophobic contacts with the β sheet (Fig. 5C). Depending upon the bound ligand, exchange is seen at the surface of the β-sheet contacting Core A (Ile-500, Ile-502, Ile-504, Ile-633, Leu-727; Fig. 5B). This suggests a possibility of subtle movements of helices F and G as a whole with respect to the rest of Lobe 2. Core A is a critical part of the structure both in the formation of the dimer interface and the interaction between the two dimers (see “Discussion” and Sobolevsky et al. (21)). For agonists, the methyl groups facing Core A along the two interlobe β-strands show exchange, whereas this is much less apparent for the antagonists and the weak partial agonist kainate, for which most of the exchange is associated with proximity to the artificial linker replacing the ion channel domain. The reason for this may be related to strain in forming the closed lobe conformation. The closure of the two lobes brings the loop containing Asp-651 and Ser-652 (“flip region”) into close contact with Lobe 1 such that the backbone flips and hydrogen bonds can be made with Tyr-450 and Gly-451. The hydrogen bonds are relatively labile, partially relieving the strain, so that the exchange observed may reflect small-scale motions of Core A relative to β-sheet. In the cases of bound UBP282, DNQX, and kainate, these hydrogen bonds are not present and the lobes are more open, possibly removing most strain between Core A and the β-sheet.

Along this interface between the two hydrophobic cores, I500 is closest to the ligand binding site (within 7 Å of the bound ligand), and the methyl group exhibits exchange in all ligands tested except DNQX. Furthermore, it exhibits the most pronounced chemical shift changes among the different ligands. The magnitude of $R_{ex}$ varies between 2.6 and 18.8 Hz, depending upon the ligand, which is apparently a reflection of the difference in chemical shift. However, $k_{ex}$ values ($2100 \pm 500 \text{ s}^{-1}$) are consistent across ligands, suggesting that the motions observed for this residue are independent of bound ligand and possibly reflect the native conformational behavior of the interlobe linker as described above for Leu-498.

DISCUSSION

The availability of the isolated binding domain (S1S2 domain) of ionotropic glutamate receptors has led to numerous crystal structures and the characterization of the protein by NMR, fluorescence, and IR spectroscopy. These approaches have provided unprecedented detail of the binding of agonists, partial agonists, antagonists, and allosteric modulators, and have suggested clues as to how the activation signal is transmitted to the ion channel domain. Understanding the activation of the protein and its modulation by drugs and toxins requires knowledge of the dynamic properties in conjunction with the structure. The dynamics of backbone amides are most commonly studied by NMR due to the wide distribution of amide protons and the sensitivity of backbone amide groups to even minute structural perturbations. Experiments have been devised to measure backbone dynamics on time scales ranging from ps to seconds (45).

Measurements of side chain methyl dynamics provide a unique window into protein dynamics due to the fact that the side chain motions are often not coupled to backbone motions (46). Methyl groups are typically part of the hydrophobic cores
of a protein that link together helices and $\beta$-sheets. In previous studies of backbone dynamics (11–13) and H/D exchange, different time scales provide clearly different insights into the dynamic processes of the protein. Backbone (11, 13) and tryptophan sidechain (10, 11) studies suggest that, except for several specific locations in the protein, the S1S2 bound to AMPA, kainate, FW, and glutamate show only minor chemical exchange on the $\mu$s-ms time scale. Based on backbone dynamics measurements, IW-bound S1S2, on the other hand, is much more dynamic on this time scale (10, 11).

Measurements of longitudinal ($R_1$) and transverse ($R_2$) relaxation rates are sensitive to ps-ns motions, and provide a dominant contribution to conformational entropy (24). In this work, values of $R_2$ were measured using a $T_1p$ experiment with a spin-lock field of 2.4 kHz to remove the exchange contribution. Slower motions ($\mu$s-ms) were detected using CPMG experiments in which the effective field was varied and ranged as low as 110 Hz ($\nu_{cpgm} = 100$ Hz). The two types of experiments provide a view of side chain dynamics in two distinct time domains: ps-ns and $\mu$s-ms.

**ps-ns Dynamics**—Experiments using competition ITC (16) showed that the entropic contribution to binding compared with glutamate is: (A) more favorable for FW and IW ($-T\Delta S$ of 2.8 and -5.7 kcal/mol, respectively), (B) decidedly less favorable for DNQX and UBP282 ($-T\Delta S$ of 7.6 and 11 kcal/mol, respectively), (C) slightly less favorable for AMPA ($-T\Delta S$ of 0.4 kcal/mol). The conformational entropy based on the side chain dynamics measured relative to glutamate for all ligands tested is favorable ($-T\Delta S$ between -15 and -21 kcal/mol). This would suggest that other contributions to the overall entropy would offset those measured here and would give rise to the characteristic differences due to binding of partial agonists and antagonists. As compared with glutamate-bound form, the hydrophobic cores of the protein are less ordered when bound to all the other tested ligands.

This uniqueness of glutamate appears to be real, as the calculated conformational entropies for all other ligands fall within a narrow range of $\sim$5 kcal/mol in extent, while they on average...
differ from glutamate by 18 kcal/mol (Table 1). This finding was unexpected, as glutamate has a lower affinity than AMPA and more chemical exchange in the μs-ms time scale (10, 13). It appears that the only distinction that might explain the uniqueness of glutamate is the fact that it is the only natural agonist of GluR2. One possibility is that glutamate receptors evolved their structure for the sole purpose of functionally exploiting glutamate binding. The optimal structure would not necessarily have the highest affinity, but rather function as an efficient signaling molecule that can bind and release glutamate with rates optimal for synaptic function. Perhaps GluR2 S1S2 evolved so that the hydrophobic cores of the apo-form are “pre-ordered” to accommodate glutamate. In this light, synthetic agonists may produce distortions of the structure of S1S2, decreasing the quality of hydrophobic packing and increasing the conformational entropy of the hydrophobic cores of the protein.

The more noticeable differences in the faster time scale dynamics are between different parts of the protein. The most dramatic cluster of side chains that show decreased order parameters is on and in the vicinity of the J and K helices. This is an extremely important portion of the protein that is involved in the formation of the dimer interface and the binding of allosteric modulators. An important difference between the flip and flop isoforms is residue 754, which is an Asn in flop and a Ser in flip. This is a major determinant for the specificity of glutamate receptors. An important difference between the flip and flop isoforms is residue 754, which is an Asn in flop and a Ser in flip. This is a major determinant for the specificity of glutamate receptors.

As with other proteins, the flexibility of the interface would contribute to the thermodynamics of the protein-protein interaction. Thus, it is possible that the flexibility seen in the monomer contributes energetically to the formation and stability of the dimer interface, which is in turn associated with the activation and desensitization of the receptor.

**μs-ms Dynamics**—Identification of sites undergoing chemical exchange is of considerable interest. The crystal structures of the GluR2 S1S2 domain clearly show that agonist binding is associated with the closure of the two lobes, which envelopes the ligand and leads to channel activation in the intact tetrameric protein (4). NMR and IR spectroscopy have provided a more dynamic picture of the binding domain (10–13, 18, 50, 51) in which differences in agonists and antagonists lead to changes in strengths of interactions and dynamic processes in the binding site and near the disulfide bond. The process of glutamate binding involves first an interaction with Lobe 1 followed by the closure of the lobes and multiple interactions with Lobe 2 (14). Hydrogen bonds can form across the lobe interface between Gly-451 and Ser-652 and between Tyr-450 and Asp-651 (mediated by water). To form these H-bonds, the backbone has to flip by 180° relative to the apo state. In the glutamate-bound form, the backbone is flipped in a position favorable for making these H-bonds in ~90% of the population (23). Hydrogen-deuterium exchange studies suggest that the H-bond across the lobe interface and interactions of Lobe 2 with the ligand are two distinct kinetic processes. This would suggest that binding consists of three steps: docking (interaction with Lobe 1), isomerization (interaction with Lobe 2), and locking (H-bonds across the lobe interface). The locking step is the most labile, and this formation and breaking of H-bonds in the binding site may subtly change the interactions in the core of the protein, such that, in particular, Core A of Lobe 2 could move relative to the β-sheet. In the case of the antagonists, UBP282 and DNQX, and the weak partial agonist, kainate, the hydrogen bonds are not formed across the lobe interface and the lobes are open much more than with the other ligands used in this study (4, 5).

In the case of these ligands, very little chemical exchange is observed, which is likely a reflection of the fact that little or no strain is placed on Lobe 2 to form the closed lobe conformation. IW is a partial agonist and the degree of lobe opening relative to the glutamate-bound structure measured by crystallography ranges from 2.4° to 9.4°, with an average opening
of 5.3° measured in solution (23). The backbone is flipped in a position favorable for making H-bonds across the lobe interface ~30% of the time (23). Overall, the IW-bound form seems to exchange between a closed and open lobe form, with an H-bond between a tryptophan sidechain in Lobe 1 (Trp-767; helix K) and a backbone carbonyl in Lobe 2 (Thr-707; helix I) showing considerable chemical exchange (11, 23). Likewise, the methyl of Ile-712, which is also on Helix 1, shows chemical exchange only for the substituted willardiines, with the degree of exchange increasing in the order FW<CIW<1W. The exchange observed here might be a reflection of the same process observed for Thr-707, which has been interpreted as large scale opening and closing of the two lobes (23).

As described earlier, glutamate appears to be a unique ligand for GluR2 from the point of view of the observed entropy of side chain methyls. Careful consideration of the slow motions of S1S2 lends additional support to the idea of uniqueness of glutamate. Indeed, glutamate induces negligible $R_{ex}$ in Ile-502 and Ile-504, which show very substantial $R_{ex}$ for all other ligands. Ile-502 and Ile-504 are located on the surface of the β-sheet of Lobe 2, facing Core A. Perhaps the side chains of these residues can be comfortably packed only when S1S2 is bound to glutamate, while all the other ligands disturb the structure and result in local motions. On the other hand, Ile-664 shows exchange only with glutamate bound. This residue is on the edge of the dimer interface observed in x-ray crystal structures. It is possible that glutamate binding creates conformational changes around the dimer interface that prime GluR2 for desensitization. Ile-633, which is positioned next to the artificial linker replacing the transmembrane part of GluR2, shows by far the largest exchange contribution with glutamate bound. This behavior may reflect on the conformational changes that would normally propagate to the ion channel part of GluR2. The fact that glutamate induces the largest exchange contribution for Ile-633 may imply that glutamate binding results in application of certain optimal forces on the channel pore of GluR2.

GluR2 S1S2 and the ligand binding domain of ionotropic glutamate receptor from bacteria, GluR0 (9) differ in the organization of the hydrophobic cores of Lobe 2. All eukaryotic iGluRs have an extra helix in Lobe 2 compared with GluR0 (helix G of GluR2). Also, one β-strand is lost in GluR2 compared with GluR0 (strand 9 of GluR0). These changes lead to a drastic redistribution of hydrophobic interactions within Lobe 2. Indeed, while in GluR2 helix F forms extensive contacts with helix G (creating Core A) and only minor contacts with the β-sheet, in GluR0 helix F forms much more extensive contacts with the β-sheet. One of the most obvious features of GluR0 is that its activation is more than 10-fold slower than for GluR2 (26), and whereas GluR2 desensitizes almost completely within a few milliseconds following glutamate binding, GluR0 desensitizes in several seconds and less efficiently (steady state/peak current of around 0.3). Clearly, the evolution of eukaryotic AMPA receptors was tuned to produce a much rapid response (both activation and desensitization) than prokaryotic glutamate receptors. Although GluR0 differs from the eukaryotic receptors in several major features (e.g. lack of an N-terminal domain and one transmembrane helix), the additional helix and reorganization of the hydrophobic cores may be a factor in the speed of the response in that the effective decoupling of the hydrophobic cores of Lobe 2 may allow for greater flexibility, which might accompany the locking of the lobes in the activated state.

The full structure of the GluR2 tetramer recently reported by Sobolevsky et al. (21) presents new evidence for the importance of the F and G helices. In addition to the role of the F/G helices in the formation of the dimer interface (loop between the two helices and the C-terminal end of the F helix), the interaction between the two dimers is mediated in part by one of the G helices from each set of dimers. The G helix on one dimer contacts the N-terminal part of the G helix of one monomer in the other dimer and the C-terminal part of the K helix on the other monomer. In addition to the suggestions above concerning the role of Core A, the new structure suggests that Core A could be involved in cooperative interactions between the two dimers as well as in the formation and dissociation of the dimer interface.

Conclusions—Side chain methyl dynamics provide a unique view of the GluR2 S1S2 domain, which is consistent with but extends previous studies using backbone dynamics (11–13). Fast time scale motions are likely reflective of portions of the protein that are more loosely packed than others; in this case, the regions surrounding the kink between the J and K helices and Leu-483 that are involved in desensitization and binding of allosteric modulators. One of the most interesting findings is that the hydrophobic cores of S1S2 are most ordered when bound to the only natural agonist, glutamate. This may be a reflection of the fact that AMPA receptors evolved to bind glutamate and that artificial ligands may perturb the structure and induce somewhat looser packing of the hydrophobic cores of the protein. The slower motions differ to a greater extent among ligands and may reflect strain within the cores of the two lobes. Those agonists that allow the lobes to close fully or almost so, result in chemical exchange within the core of the protein. These constraints are loosened, reducing the chemical exchange, in antagonists and the weak partial agonist, kainate.

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