Dynamics of Cleft Closure of the GluA2 Ligand-binding Domain in the Presence of Full and Partial Agonists Revealed by Hydrogen-Deuterium Exchange*

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Background: Glutamate receptors are essential proteins for transmitting information in the CNS.

Results: The stability of H-bonds at multiple points within the ligand-binding domain varies with the efficacy of the bound agonist.

Conclusion: H-bonds inside and outside of the binding pocket contribute to channel activation and desensitization.

Significance: Fine-tuning of glutamate receptor responses is dependent upon electrostatic interactions and H-bonds outside of the binding pocket.

The majority of excitatory neurotransmission in the CNS is mediated by tetrameric AMPA receptors. Channel activation begins with a series of interactions with an agonist that binds to the cleft between the two lobes of the ligand-binding domain of each subunit. Binding leads to a series of conformational transitions, including the closure of the two lobes of the binding domain around the ligand, culminating in ion channel opening. Although a great deal has been learned from crystal structures, determining the molecular details of channel activation, desactivation, and desensitization requires measures of dynamics and stabilities of hydrogen bonds that stabilize cleft closure. The use of hydrogen-deuterium exchange at low pH provides a measure of the variation of stability of specific hydrogen bonds among agonists of different efficacy. Here, we used NMR measurements of hydrogen-deuterium exchange to determine the stability of hydrogen bonds in the GluA2 (AMPA receptor) ligand-binding domain in the presence of several full and partial agonists. The results suggest that the stabilization of hydrogen bonds between the two lobes of the binding domain is weaker for partial than for full agonists, and efficacy is correlated with the stability of these hydrogen bonds. The closure of the lobes around the agonists leads to a destabilization of the hydrogen bonding in another portion of the lobe interface, and removing an electrostatic interaction in Lobe 2 can relieve the strain. These results provide new details of transitions in the binding domain that are associated with channel activation and desensitization.

Determining the kinetic properties of various subtypes of glutamate receptors is key to understanding important neuronal processes such as learning, memory, and development of synaptic connections (1). These subtypes include 1) AMPA (GluA1–4), 2) kainate (GluK1–5) and 3) NMDA (GluN1, GluN2A–D, GluN3A, and GluN3B) receptors. On the postsynaptic membrane, AMPA receptors are rapidly activated by glutamate. In turn, the resulting excitatory postsynaptic potential releases the more slowly activating NMDA receptors from Mg2+ blockade. Following activation, AMPA receptors rapidly desensitize while NMDA receptors remain open.

Activation of AMPA receptors requires the formation of a dimer interface between subunits (two dimers per tetrameric receptor). The dimer interface is along one lobe (Lobe 1) of the bilobed ligand-binding domain (LBD), the structure of which is well characterized (2–4). The other lobe (Lobe 2) contains linkers to the ion channel. Upon binding agonist, the lobes close around the ligand. The stability of the dimer interface provides a platform to do work on the channel structure, which in turn controls channel opening. The strain associated with this process apparently leads to the dissociation of the dimer interface and desensitization (5). The dimer interface can be stabilized by allosteric modulators such as cyclothiazide (5, 6) and 4-[2-(phenylsulphonylamino)ethylthio]-2,6,-difluorophenoxy acetalamide (7, 8), which in turn slow desensitization. The strain placed on the dimer interface is likely related to the time that the LBD exists in the fully closed state, which is related to the energetics associated with the equilibrium distribution of possible lobe orientations and the stability of the contacts across the cleft between the two lobes of the LBD.

Partial agonists are useful tools for understanding the mechanisms of activation and desensitization (9–13) because their binding results in differences in cleft closure stability, which can be compared with functional measurements on the intact membrane-bound receptor. In this study, we investigated the stability of cleft closure of the GluA2 LBD using a series of agonists with different affinities and efficacies. Specifically, our studies employed the full agonists glutamate and AMPA and...
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the partial agonists kainate, (S)-5-fluorowillardiine (FW), (S)-5-nitrowillardiine (NW), and (S)-5-iodowillardiine (IW). Although glutamate is the physiological agonist, the affinity for AMPA is severalfold higher (2). On the other end of the spectrum, kainate is a partial agonist with low affinity and efficacy at AMPA receptors (14). The series of willardiine derivatives (FW, NW, and IW) have intermediate efficacies. FW exhibits the lowest EC50 and the highest efficacy of the series, and IW has the highest EC50 and the lowest efficacy (10, 12).

We used NMR measurements of hydrogen-deuterium (HD) exchange to probe the equilibrium between the formation and breakage of hydrogen bonds (15) that stabilize GluA2 LBD lobe closure (16) and to illustrate the differences caused by the binding of full and partial agonists. We found that binding of agonist involves not only formation of new H-bonds within the binding site but also changes in the stability of H-bonding outside of the binding site that affect activation, deactivation, and desensitization of the receptor.

EXPERIMENTAL PROCEDURES

Materials—The receptor construct consists of Asn-392–Lys-506 and Pro-632–Ser-775 of the full-length rat GluA2 flop subunit (17) with a GA segment at the N terminus and a GT linker connecting Lys-506 and Pro-632 (2). Agonists and partial agonists were purchased from Toecris Bioscience and Abcam. The GluA2 LBD (flop) construct (S1S2J) was obtained from Eric Gouaux (Vollum Institute) (2). Two alternatively spliced forms of the GluA2 LBD are found in the CNS (flip and flop) (18). The LBD of the flop form was used in these studies because all NMR assignments for the partial agonists (19) were done using this form, and direct comparisons can be made with crystal structures of the GluA2 LBD (flop) bound to the full and partial agonists used here (2, 10, 20). Stable isotopes were purchased from Cambridge Isotopes (Cambridge, MA). [3H]AMPA (40.6 Ci/mmol) was purchased from Perkin-Elmer Life Sciences.

Protein Preparation and Purification of the LBD—The pET-22b(+) plasmid containing the coding region for the GluA2 LBD was transformed in Escherichia coli (Origami B(DE3)) and grown at 37 °C in LB medium supplemented with antibiotics (ampicillin or kanamycin) to A600 = 0.9–1.0. Cultures were cooled to 20 °C for 20 min, and isopropyl β-d-thiogalactopyranoside was added to a final concentration of 0.5 mM. Cultures were then allowed to grow at 20 °C for an additional 20 h. The bacteria were pelleted, and the LBD was purified using a nickel-nitritotrriacetic acid column. The His6 tag was cleaved with trypsin, and the protein was further purified with a sizing column (Superose 12, XK 26/100) followed by a HiTrap SP HP ion exchange column (GE Healthcare). Glutamate (1 mM) was maintained in all buffers throughout purification. The protein was then concentrated and stored in 20 mM sodium acetate, 25 mM sodium chloride, 1 mM sodium azide, and 10 mM glutamate at pH 5.5. The protein used for the glutamate (10 mM) and kainate experiments was prepared as described previously (16). AMPA (5 mM), kainate (10 mM), IW (2 mM), FW (2 mM), and NW (2 mM) were exchanged for glutamate by successive concentration and dilution. The GluA2 LBD protein containing the E713T mutation was prepared identically to the wild-type GluA2 LBD protein.

NMR Spectroscopy—Spectra were acquired on a 500-MHz Varian Inova spectrometer with a triple resonance z-gradient cryogenic probe. A 1H,15N heteronuclear single quantum coherence/transverse relaxation-optimized spectroscopy (HSQC/TROSY) experiment (BioPack software) was used with 32 scans of 192 × 2048 total (real plus imaginary) points (2-h total acquisition time). After an initial control spectrum was taken in water, the protein was lyophilized and resuspended in D2O. A series of 1H,15N HSQC/TROSY experiments (generally 12 sequential experiments) was performed at each of several temperatures, starting at 10 °C up to 25 °C (16).

HD Exchange Measurements—HD exchange lifetimes were measured using SPARKY (21) at five temperatures (10, 14, 18, 20, and 25 °C) at pD 5.1 (glutamate and kainate) or pD 5.5 (AMPA and willardiines); these pD differences correspond only to a 2.5-fold difference in intrinsic exchange rate. Under these conditions, the exchange is in the EX2 limit and is base-catalyzed (22, 23). In the EX2 limit, HD exchange rates can be used to measure the stability of a hydrogen bond. The data were analyzed as described previously (16).

Thermal Denaturation—Thermal unfolding was monitored by intrinsic tryptophan fluorescence with a Varian Cary Eclipse spectrofluorometer using an excitation wavelength of 280 nm and an emission wavelength of 336 nm. The temperature was monitored in the cuvettes and adjusted at a rate of 1 °C/min between 10 and 75 °C. The protein concentrations were 5–10 μM in buffer (25 mM sodium acetate (pH 6.0), 25 mM sodium chloride, and 1 mM sodium azide) including 10 mM glutamate. The data were analyzed as described by Madden et al. (24).

Radioligand Binding—The binding of [3H]AMPA to the GluA2 LBD was determined as described by Chen and Gouaux (25). Binding buffer contained 30 mM HEPES, 100 mM KSCN, 2.5 mM CaCl2, and 10% glycerol (pH 7.2), maintained at all times at 4 °C. Glutamate was removed by successive concentration and dilution and diluted to a final concentration of 0.4 mM. After the addition of varying concentrations of glutamate, [3H]AMPA (10 nM) was added, and the reaction (200 μl) was allowed to proceed for 1 h, followed by filtration through Millipore GSWP filters and two 2-ml washes with binding buffer.

RESULTS

Using HD exchange, we showed previously (16) that the binding of glutamate to GluA2 (flop) is at least a three-step process (Fig. 1): 1) binding to Lobe 1, 2) lobe closure and interaction with Lobe 2, and 3) formation of H-bonds across the lobe interface. A flexible region in Lobe 2 makes Step 3 possible through the formation of interlobe H-bonds between the amide of Gly-451 and the carbonyl of Ser-652 and between the amide of Tyr-450 and a water molecule, which in turn H-bonds with the carbonyl of Asp-651. In the case of kainate binding to GluA2 (flop), Step 3 is much less probable and was not detected by HD exchange for the pH and temperature conditions tested. By using additional partial agonists and including the analysis of H-bonds outside of the binding site, we extend our understanding of partial agonism and the forces involved in lobe closure.
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This in turn adds to our understanding of the details of receptor activation and desensitization.

**Step 1: Interactions with Lobe 1**—The initial docking of agonist with Lobe 1 involves an electrostatic interaction with the side chain of Arg-485 and H-bonds with the carbonyl of Pro-478 and the amide of Thr-480 (Fig. 2B). As described previously (16), the exchange of the amide proton of Thr-480 is extremely slow at high concentrations of glutamate and kainate but shows an increase in the exchange rate at lower concentrations (16). Likewise, the exchange rate is extremely slow for all agonists used in this study at high concentration, with the rate for the IW-bound protein being slightly faster than the remainder of the agonists tested (Fig. 2A). Thus, the initial step in binding is essentially the same for both full and partial agonists.

**Step 2: Interactions with Lobe 2**—Following the interaction between agonist and Lobe 1, the lobes close, and the agonist interacts with Lobe 2. The important questions have to do with the stability of this state, which may be related to the efficacy of the agonist. Three amide protons in Lobe 2 are positioned to make H-bonds with agonist (Fig. 3). The Glu-705 NH makes a water-mediated H-bond with the γ-carboxyl of both glutamate and kainate (Fig. 3, D and E). As shown previously (16), an ~10-fold higher exchange rate at Asp-705 was observed for kainate relative to glutamate, which is likely due to a weaker interaction of kainate with Lobe 2. The NH of Ser-654 and that of Thr-655 form hydrogen bonds with the α- and γ-carboxyls of glutamate and kainate (Fig. 3, D and E). At both positions, exchange is faster in the kainate-bound form than in the glutamate-bound form, again reflecting a weaker interaction with Lobe 2 for kainate (16). In both cases, the Ser-654 site exhibits a 2-fold faster exchange rate than the Thr-655 site, which is a consequence of the difference in intrinsic random coil exchange rates for those positions (16). In the case of AMPA, water is displaced, and the isoxazole nitrogen makes an H-bond with the NH of Glu-705 (Fig. 3C) (2). An NH peak for Glu-705 was not observed in the first spectrum taken at 10°C, suggesting that the exchange rate is faster than that observed for glutamate. The NH peak for Ser-654 is not well resolved when AMPA is bound, and Thr-655 exchanges faster when bound to AMPA than when bound to glutamate (Fig. 3A). When AMPA is bound, the carbonyl on the isoxazole ring interacts with the side chain of Thr-655 in a manner similar to one of the carboxyl oxygens in the side chain of glutamate, and a water molecule fills the position taken by the other carboxyl oxygen of glutamate (Fig. 3C). This water molecule makes AMPA a biospecific mimic of glutamate (2), but the backbone amide proton of Thr-655 is not protected from exchange to the same extent when AMPA is bound than when glutamate is bound.

In the case of the willardiines, the amide protons of Glu-705 and Thr-655 may make H-bonds with the carbonyls of the uracil ring (Fig. 3F). However, the charge state of the ring depends on the pH of the solution. For IW and FW, the pK_a is well above the pH (8.25 and 8, respectively) (26), so the NH of the ring is largely protonated. In these cases, the amide protons of Glu-705 and Thr-655 exchange rapidly before the first spectrum is taken at 10°C. The pK_a of the uracil of NW is much lower (6.74) (27), so at least some of the interaction is with the charged form of the ring (Fig. 3B). The change in charged state of the uracil ring changes the thermodynamics of binding of willardiines, with an increased enthalpy relative to entropy in the charged versus the uncharged form. In this case, NW apparently makes stronger interactions with Lobe 2, resulting in measureable exchange of the amide protons for Glu-705 (a peak is observed for the first three datasets at 10°C for NW, but not for IW and FW in the first dataset at 10°C) and Thr-655 (Fig. 3A). Ser-654 interacts with the α-carboxyl (Fig. 3F), and the exchange is too fast to measure for all willardiine derivatives, suggesting a weaker protection of these sites.

For the interactions of agonist with Lobe 2, the HD exchange data suggest a somewhat more complicated picture than originally seen with glutamate and kainate (16). The H-bonds formed by AMPA may be weaker than those with glutamate, despite the higher affinity, suggesting that other interactions may be more important. For the willardiine compounds, the charged state of the ring may play an important role in the interaction with Lobe 2.

**Step 3: Interactions in the Flip Region**—The last step in binding consists of the formation of two interlobe H-bonds following a rotation of the peptide bond between Asp-651 and Ser-652 (Fig. 4, D and E). The flip region is a loop between strand 9 and helix F (Fig. 4D) that occupies two distinct conformations due to a 180° rotation of the peptide bond (unrelated to the flip-flop splice variant notation). In the “unflipped” form, the
backbone carbonyls of Asp-651 and Ser-652 are rotated away from the lobe interface. However, in the "flipped" form, the carbonyls rotate toward the interface and form H-bonds with the amides of Lobe 1 residues Tyr-450 and Gly-451, respectively (the interlobe Asp-651–Tyr-450 H-bond is mediated by a water molecule). This can be thought of as the last step in the stabilization of lobe closure. Previous results have suggested that the percentage of time that the peptide bond is in the flipped form (i.e. suitable for H-bonding) is correlated with efficacy (11). Crystal structures of bound antagonists, partial agonists, and the apo form are largely in the unflipped form, and full agonists tend to give the flipped form (although, in some cases, the unflipped form has been observed). In the case of at least partial agonists, the orientation of the Asp-651–Ser-652 peptide bond is likely to be in equilibrium between the two forms. Maltsev et al. (11) used residual dipolar couplings to estimate that ~50% of the population was in each of the two forms in the FW-bound LBD. The unflipped form was favored by >2-fold for the IW- and NW-bound LBDs, and the fully unflipped bonds could be found for the kainate-bound LBD. We showed previously (16) that the amide proton of Gly-451 exchanges much more rapidly than Tyr-450 when the protein is bound to glutamate, but this difference does not appear to be due to a difference in protection but rather in intrinsic exchange rate. Only for AMPA and glutamate could the exchange of Gly-451 be measured at 10 °C (it was fully exchanged at all other temperatures); however, the exchange rate of the amide proton of Tyr-450 could be measured for all agonists except IW, for which peak overlap precluded quantitation. This provides us with a measure of the stability of this interlobe H-bond and, by inference, that of Gly-451–Ser-652. At 14 °C, the amide proton of Tyr-450 for the kainate-bound protein exchanges the most rapidly, with exchange rates for glutamate and AMPA much slower (Fig. 4A). The proteins bound to NW and FW were intermediate, suggesting that the stability of the H-bond is intermediate between kainate and the full agonists, as is the efficacy of the willardiine partial agonists. This is consistent with previous findings that ~50% of the protein is in the flipped form when bound to willardiine partial agonists (11).

Side Chain Tryptophans (Trp-766 and Trp-767)—Although lobe closure is presumed to be the signal that initiates the process of channel opening, the signal must be propagated from the binding site to the channel domain. Changes in H-bonding outside of the binding site in the presence of agonists of different
efficacy can shed light on some of the relevant changes that may be involved in channel activation. With the exception of the two H-bonds in the flip region near the agonist-binding site, interactions between the two lobes are either through side chains or mediated by the ligand in the binding site. The lobe interface is fixed by a disulfide bond (Cys-718–Cys-773) at the base of helix I in Lobe 2 and helix K in Lobe 1 (Fig. 4E). In addition, however, an interesting H-bond is made between the indole nitrogen of Trp-767 and the carbonyl of Thr-707 (Fig. 1C). As Thr-707 is part of helix I, it is a shared H-bond with the amide proton of Tyr-711. Likewise, helices J and K, which loop back into Lobe 1 from Lobe 2 and then exit the LBD to form helix M4 (M1, M3, and M4 refer to transmembrane helices in the full-length protein; M1 and M3, along with the reentrant loop, M2, line the ion channel), are tethered in this region to Lobe 1 by the H-bond between the side chain of Trp-766 and the side chain of Asp-427 (Fig. 4E). For the side chain of Trp-766, the exchange rate is fastest when glutamate is bound and slower for bound willardines, kainate, and AMPA (Fig. 4C). In the case of Trp-767, the fastest exchange rate is seen with bound AMPA (the proton is fully exchanged after the first spectrum taken at 10 °C) and glutamate, with slower exchange observed for bound kainate and the willardines (Fig. 4C). The HD exchange for the LBD bound to IW for the side chain of Trp-767 is not shown in Fig. 4B, as it is exchange-broadened at that temperature (i.e. line broadening due to chemical exchange between two or more environments), but the proton exchanges with solvent slowly even at 25 °C. Particularly for glutamate, the changes in HD exchange are the reverse of what is seen in the flip region. One possibility is that for Trp-766, Trp-767, or both, the increased exchange rate is due to a strain induced by the closure of the lobes. Alternatively, for Trp-767, the changes in exchange rate may be a more local phenomenon because the H-bond to Thr-707 is next to Met-708, which is involved in the binding site (2). Met-708 can assume different conformations (and, in some cases, multiple conformations) depending upon the substituent in the 5-position of the willardine structure (10, 13).

**E713T Mutation**—Because major lobe-lobe interactions occur at opposite ends of the cleft, the core of Lobe 2 may experience stress upon lobe closure. Recently, Carbone and Plested (28) showed that mutations (E713T and Y768R) in the lower portion of the LBD of GluA2 near Trp-766 and Trp-767 slowed both deactivation and recovery from desensitization. Glu-713 and Tyr-768 are involved in helix-strand (Glu-713–Lys-722) or helix-helix (Tyr-768–Glu-710) interactions with helix I. This portion of the protein is sandwiched between the linkers to M1/M3 and the linkers to M4. Lobe closure that forms the H-bonds across the interface at the “flip region” is likely accompanied by strain in this region of the protein (as judged by the changes in HD exchange rate for the side chains of Trp-766 and Trp-767). These electrostatic and H-bonding interactions are likely to be important for the transmission of the signal to the ion channel for both channel activation and recovery from desensitization. Given the phenotype of the E713T mutation (mutations in the region of Tyr-768 were not done because they tend to affect stability of the LBD), we reasoned that the exchange rate for Trp-766, Trp-767, and perhaps other portions of the protein could be affected, possibly due to relaxing constraints on packing of Lobe 2. The faster exchange rate for the side chains of Trp-766 and Trp-767 for glutamate relative to the partial agonists might be explained by strain imposed by the tightly closed lobes, i.e. a twisting motion that brings the two halves of the binding site together but puts strain on the lower portion of the binding domain near the disulfide bond. Relaxing the constraints on the packing of Lobe 2 could potentially modify the degree of strain.

When bound to glutamate, the E713T mutation of the GluA2 LBD had no effect on the HD exchange of the side chain amide proton of Trp-767 but significantly slowed the exchange of the side chain amide proton of Trp-766 (Fig. 5A).
region, the mutation had no effect on the HD exchange of the backbone amide proton of Tyr-450 (Fig. 5B). However, considering the remainder of the LBD, the rates of amide proton exchange tend to be increased at the ends of helices and β-sheets (Fig. 5E), possibly suggesting that the loss of the glutamate side chain at position 713 in Lobe 2 may change the packing of Lobe 2. The mutation also gives rise to chemical shift changes throughout Lobe 2 (Fig. 5F), suggesting possible changes in structure and/or dynamics. These changes in the protein increased the unfolding temperature of the mutant relative to the wild-type protein by ~3°C (Fig. 5C), suggesting that the mutant protein is more stable. Likewise, the binding affinity for glutamate was increased by ~4-fold in the mutant relative to the wild-type protein (Fig. 5D). Taken together, the E713T results illustrate the importance to agonist binding and channel function of interactions within the core of Lobe 2. These interactions effectively connect the flip region to the two tryptophans near the linker to M4.

**DISCUSSION**

The LBDs of glutamate receptors function as signal transducers, with complex interactions that give rise to finely tuned signals. The signal begins at the agonist-binding site, where the agonist docks with one lobe and the other lobe closes upon it. A rotation of a peptide bond then promotes the formation of additional H-bonds that lock the closed lobe in place (Fig. 1).
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This general mechanism is very likely common to all AMPA receptors (see also Ref. 29) but has not been well characterized in NMDA or kainate subtypes. The forces generated by this process are transmitted through the LBD to linkers that connect the LBD to the ion channel domain. Agonists of different efficacies can be used as tools to assay changes in the protein that may be related to channel activation. We focused here on changes in hydrogen bonding (HD exchange) in the presence of a range of agonists with different efficacies. At low pH, HD exchange provides a measure of the stability of a hydrogen bond. Our previous work (16) on the binding of kainate and glutamate provided a view of the steps in the binding of agonist within the binding site but did not address the remainder of the domain or agonists with intermediate efficacy.

The results of this study shed light on the three steps in binding of agonist, as well as some of the downstream changes that occur upon binding. In the first step (docking with Lobe 1), the results for all agonists were the same, suggesting that the first step is common to both full and partial agonists. In the second step (interaction with Lobe 2), the partial agonists exhibited a weaker interaction with Lobe 2 than did glutamate, but the binding of the high affinity full agonist AMPA exhibited faster HD exchange than expected. For the third step (locking the lobes with H-bonds across the lobe interface in the flip region), the binding of willardiine partial agonists revealed the transient presence of the interlobe H-bonds, with HD exchange intermediate between glutamate and the very weak partial agonist kainate. Moving away from the binding site, HD exchange at two tryptophan side chains suggested that the stable lobe closure seen in partial agonists may affect the stability of H-bonds at other points in the protein, possibly related to activation or desensitization. Carbome and Plested (28) recently described a mutation (E713T) that decreases the rate of deactivation, suggesting perhaps that the agonist is released from the binding site at a slower rate compared with the wild-type protein. Glu-713 seems to participate in a crucial electrostatic interaction that is involved in the packing of Lobe 2 (Fig. 5G). One mechanism for a change in deactivation would be to decrease the strain induced by lobe closure, suggesting that the mutation would be an avenue for testing the idea that a stable lobe closure places strain on other portions of the protein. As discussed below, this seems to be the case. Overall, the results show that the binding of full agonists induces a stable lobe closure that presumably requires a tight packing of Lobe 2. Lobe closure induced by partial agonists can result in similar interactions, but the interactions are less stable and are manifested by stronger H-bonds in the tryptophan side chains outside of the binding site. These results provide a preliminary view of the propagation of the signal beyond the binding site. Further discussion of AMPA binding, the flip region, and the effects of the E713T mutation is presented below.

AMPA Binding—Although H-bonding is clearly important for each step in the binding interaction, the results for AMPA suggest that other factors may be equally important. AMPA binds to GluA2 with high affinity; radioligand binding assays indicate that the affinity is in the nanomolar range for the GluA2 LBD (25). However, the backbone amide protons involved in H-bonding to agonist show markedly faster exchange when bound to AMPA than to glutamate. Both AMPA and glutamate make H-bonds with the backbone NH and side chain hydroxyl of Thr-655 (Fig. 3, C and D). In the case of AMPA, it is the carbonyl of the isoxazole ring, and in the case of glutamate, it is the side chain carboxyl. In the case of bound AMPA, the H-bond to the backbone NH of Thr-655 is through a water molecule. The two additional H-bond donors are present in the Lobe 2 side of the binding site (backbone amide protons of Ser-654 and Glu-705). In the case of glutamate, H-bonds are formed through a water molecule to Glu-705 and directly to Ser-654. For AMPA, the H-bonding is directly to the backbone NHs of Glu-705 and Ser-654. For both Glu-705 and Ser-654, the exchange of the backbone amide proton is more rapid when AMPA is bound than when glutamate is bound. The higher affinity for AMPA would likely arise from factors other than H-bonding.

Flip Region—The last step in agonist binding involves the formation of two H-bonds across the lobe interface that stabilizes the closed lobe state of the LBD. Crystal structures (2, 10) have demonstrated the existence of these H-bonds when full agonists are bound, but in the presence of partial agonists or antagonists, the peptide bond between Asp-651 and Ser-652 rotates by 180°, and the H-bonds are not present. Our dipolar coupling results are consistent with an equilibrium between the two orientations of the Asp-651–Ser-652 peptide bond such that the orientation favorable for H-bonding is populated about half of the time (11) when the protein is bound to the willardiine partial agonists. Also, strategic placement of cysteine residues can trap the LBD in a closed and H-bonded form even in the presence of partial agonists and the antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (9). Likewise, the protection of the amide proton of Tyr-450 (Fig. 4A) suggests that the H-bond is populated to some extent in the presence of partial agonists. This varies from kainate, where little protection is seen, to the willardiine series, for which a slower exchange rate is observed. This would support the view that full lobe closure and stabilization can be observed with partial agonists, although a dynamic equilibrium is present, so both a more open cleft and a closed cleft can exist. If one assumes that the fully closed form is that which activates the channel, then this partial stabilization of the closed lobe form may account for the single channel behavior of partial agonists (9).

Impact of Lobe Closure—Changes in the line width of the NH peak for the side chain of Trp-767 have been used to infer the presence of intermediate exchange when partial agonists are bound, suggesting the possibility of large-scale motions of the two lobes (11). This is further supported by the fact that the motions measured are unlikely to arise from the side chain itself because no intermediate exchange in the presence of partial agonists was measured in the 19F spectrum when the 5-position of the Trp-767 indole ring was labeled with fluorine (30). HD exchange measurements at this site provide new information that supplements measurements of the axis of lobe closure in different agonists.

The hinge axis for lobe rotation to a closed state seems to shift for weak agonists (e.g. kainate (2) and IW (31)) versus full agonists, and the bend of helix I increases with decreasing efficacy in a series of willardiines (31). In addition, Fenwick and
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Oswald (31) showed that the most significant backbone chemical shift deviations that arise for a series of willardines occur within and near helices I and K of the GluA2 LBD. These results indicate that differences in cleft closure are associated with a redistribution of internal energy in this region, as can be implied by chemical shift differences that are directly related to changes in strength of H-bonds in these helices. The rate of HD exchange for the indole NH of Trp-767 is much more rapid when glutamate and AMPA are bound than when kainate or the willardine derivatives are bound. Thus, although considerable chemical exchange and weaker interlobe H-bonding (as predicted from chemical shift deviations) occur in the region of the indole ring in the presence of some partial agonists such as IW (31), the H-bond is actually more protected when partial agonists are bound than when full agonists are bound. This would not be predicted from the crystal structures that show very little difference in the indole Trp-767–Thr-707 H-bond length for the apo form and for the LBD bound to members of the willardine series, glutamate, kainate, or AMPA. Also, the chemical shift dependence is consistent with a weaker H-bond for the weaker partial agonists (31).

The side chain of Trp-766 also plays a potentially interesting role in the process of lobe closure. If one assumes that the lower portion of the cleft between the two lobes (i.e. between helices I and K) is largely stable due to the disulfide bond, the Trp-767–Thr-707 H-bond, and various interactions between side chains (Fig. 5G), then the H-bond between the side chains of Trp-766 and Asp-427 may be an important interaction that stabilizes helix K relative to the remainder of Lobe 1. Although the agonist-dependent changes in exchange rate are relatively small, the change observed in the E713T mutation is of interest. Losing this side chain apparently removes some of the strain on the H-bond between helix K and the remainder of Lobe 1 and perhaps decreases the packing of Lobe 2. These changes in turn increase the stability of the protein (measured by thermal unfolding) (Fig. 5C). Carbone and Pledsted (28) showed that this mutation in GluA2 decreased the rate of recovery from desensitization while slowing the rate of deactivation. The correlate in the isolated LBD of recovery from desensitization is difficult to assess, as recovery presumably involves reformation of the dimer interface. In the soluble LBD, dimer formation is less favored due to a lower local concentration and requires either very high concentrations in solution or an allosteric modulator such as cyclothiazide (5). However, slower deactivation may be associated with a bound state of high stability, which was observed here as a 4-fold increase in affinity for glutamate (Fig. 5D).

Our previous work on the dynamics of methyl side chains provides some context for understanding the effects of the E713T mutation. As shown in Fig. 5H, Lobe 2 consists of two hydrophobic cores (Core A, blue spheres; and Core B, green spheres) on either side of a β-sheet (yellow spheres) (32). The flip region is associated with Core A. The hinge axis for lobe closure runs through Glu-713, and possible interactions of the side chain with the β-sheet (Glu-713–Lys-722) could affect the packing of Lobe 2, particularly because it constitutes a major interaction between Core B and the β-sheet. Closure of the lobes brings Core A closer to Lobe 1, and the interactions between the two cores and the β-sheet maintain the packing of Lobe 1. This in turn may add strain to the lower portion of the domain (i.e. Core B near the lobe interface) when the lobes are closed. Because multiple interactions exist between the two lobes below the binding site (Fig. 5G), the strain may be sensed by the Trp-766 H-bond in Lobe 1 such that the E713T mutation would relieve that strain and slow the rate of HD exchange. This suggests that the details of the interactions between the hydrophobic cores of Lobe 2 contribute to the fine-tuning of the activation and desensitization of AMPA receptors, which is in turn crucial for normal synaptic function in the central nervous system (28).

Understanding the details of specific H-bonds within the LBD can provide a view of the mechanism of agonist binding and the transmission of the binding interaction to the linkers that lead to the ion channel domain. HD exchange measurements have provided insights into not only binding of full and partial agonists but also changes in other parts of the protein that are likely to be involved in channel activation, deactivation, and desensitization.

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