On the Mechanisms of α-Amino-3-hydroxy-5-methylisoxazole-4-propionic Acid (AMPA) Receptor Binding to Glutamate and Kainate*[^1]

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The α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) subtype of ionotropic glutamate receptors mediates much of the fast excitatory neurotransmission in the central nervous system. The ability of these receptors to shape such responses appears to be due in part to dynamic processes induced by agonists in the ligand-binding domain. Previous studies employing fluorescence spectroscopy and whole cell recording suggest that agonist binding is followed by sequential transitions to one or more distinct conformational states. Here, we used hydrogen-deuterium exchange to determine the mechanisms of binding of glutamate and kainate (full and partial agonists, respectively) to a soluble ligand-binding domain of GluR2. Our results provide a structural basis for sequential state models of agonist binding and the free energy changes of the associated state-to-state transitions. For glutamate, a multi-equilibrium binding reaction was discerned involving distinct ligand docking, domain isomerization, and lobe-locking steps. In contrast, binding reaction was discerned involving distinct ligand docking states-to-state transitions. For glutamate, a multi-equilibrium of agonist binding and the free energy changes of the associated

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[^2]: The abbreviations used are: AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; GluR2, ionotropic glutamate receptor 2; HD, hydrogen-deuterium; HH, hydrogen-hydrogen; H^2, protein backbone NH proton; EPPS, 4-(2-hydroxyethyl)-1-piperazinopropanesulfonic acid; CHES, 2-(cy clohexylamino)ethanesulfonic acid; CAPS, 3-(cy clohexylamino)propane-sulfonic acid.

[^3]: http://www.jbc.org/

large extracellular domains, a membrane-spanning ion channel, and a C-terminal intracellular region (4, 7–12). One of the extracellular domains, S1S2, is a bilobed structure that binds agonists in a cleft between its lobes (13). Domain closure of S1S2 around the agonist leads to channel opening, and maximum currents have been attributed to complete lobe closure upon complex formation with full agonists (13–17). However, among the full agonists examined, those like glutamate, which are less potent and which bind to S1S2 with lower affinity, produce higher rates of channel deactivation and desensitization (18), despite inducing the same degree of cleft closure in S1S2. This suggests that AMPA receptor function is dependent not only on the extent of lobe closure caused by the agonist but also on the lobe closing and opening dynamics associated with agonist binding and dissociation (19).

A prevailing model for the binding reaction is

\[
S + G \rightleftharpoons SG \rightleftharpoons S^*G
\]

where \(SG\) represents a state in which glutamate \((G)\) has rapidly docked to sites in lobe 1 of S1S2 \((S)\) (20). The species \(S^*G\) establishes key contacts between glutamate and lobe 2, which brings about domain closure and triggers the opening of a gate in the ion channel. This has been referred to as the dock-isomerization model and is consistent with data from stopped flow (20) and time-resolved Fourier transform infrared spectroscopy experiments (21). However, the following model, which was recently proposed based on whole cell recordings of GluR2 channels, implies a related but more complicated binding reaction (18),

\[
S + G \rightleftharpoons SG \rightleftharpoons S^*G_1 \rightleftharpoons S^*G_2 \rightleftharpoons S^*G_3
\]

where \(O_i\) and \(D_i\) (where \(i = 1, 2, \) or 3) denote open and desensitized channel states, respectively. Although this model accounts for structural changes occurring throughout a subunit of the tetrameric receptor, \(SG, S^*G_1, S^*G_2\), and \(S^*G_3\) were suggested to represent unique conformational states in the binding cleft of S1S2, with \(S^*G_3\) possessing the highest degree of cleft closure stability (18, 19). The sequential nature of this mech-
nism was found to be critical to reproduce current responses for full agonists of varying potency and binding affinity for S1S2. For glutamate, receptors primarily occupy S∗G1, whereas higher potency full agonists show increased occupation of S∗G2 and S∗G3. Importantly, though, longer application of glutamate followed by its sudden removal under nondesensitizing conditions leads to slower current decays corresponding to increased sampling of S∗G2 and S∗G3. According to Equation 2, these deactivation rates are dependent on agonist binding affinity and application time because increases in either of these variables increase the probability that the binding cleft of S1S2 is closed and populating S∗G2 or S∗G3 (18). However, solution studies of S1S2 have yet to answer the more fundamental question of whether lobe opening and closure occur by way of a single domain isomerization event, according to Equation 1, or whether there are indeed additional conformational equilibria in the binding cleft distinct from lobe isomerization that have measurable free energies, as suggested by Equation 2.

In the present study, this question was addressed by performing hydrogen-deuterium (HD) exchange experiments on complexes of glutamate and kainate with S1S2J, a 30-kDa agonist-binding domain excised from a full-length GluR2 subunit (14) (Fig. 1A). To evaluate binding mechanisms, we determined free energy changes, ΔG = −RT ln K_E, associated with the solvent exposure of backbone NH protons (H^N) in S1S2J that are protected, in most cases by H-bonds, as a consequence of agonist binding and cleft closure. Assuming EX2-type Linderstrøm-Lang kinetics (22, 23), equilibrium constants, K_E, were estimated from K_E = k_HDX/k_HDX, where k_HDX is the measured HD exchange rate, and k_HDX is the exchange rate in solvent-exposed states (see “Experimental Procedures”). Importantly, K_E, in addition to providing a thermodynamic measure of structural stability, supplies mechanistic detail, because the OD^-catalyzed exchange reaction requires complete disruption of pre-existing H-bonds, including ones made to water molecules (24, 25). Previous studies have argued that small thermal fluctuations are not expected to lead to successful reactions but rather that separations of at least a few angstroms are required (25, 26). Based on this view, domain opening and agonist dissociation events were inferred from the detection of hydrogen exchange at key sites protected through ligand binding and lobe closure.

The key sites considered were H^N_Y450, H^N_G451, H^N_T480, H^N_S654, and H^N_T655 (Fig. 1B). H^N_Y450 in lobe 1 and H^N_S654 and H^N_T655 in lobe 2 are solvent-protected as a result of H-bonding to glutamate. In contrast, H^N_Y450 and H^N_G451 are protected via interlobe H-bonds with the backbone COs of Asp^651 and Ser^652, respectively, where the former interaction is mediated by a water molecule. However, these interlobe H-bonds are not made in all of the glutamate-bound S1S2J crystal structures because of the ability of Asp^651–Gly^652 to adopt distinct backbone conformations (14) (Fig. 1C). Notably, for full agonists that have higher binding affinity than glutamate, the Asp^651–Gly^652 peptide tends to favor more the conformer that enables interlobe H-bond formation (14–16), and this apparent shift in equilibrium may be related to the increase in occupation probabilities of S∗G2 and S∗G3 for such agonists over glutamate (18).

Understanding the relative conformational free energies associated with the interlobe H-bonds and the interactions between glutamate and sites on lobe 2 is central to determining the binding mechanism. Assuming that glutamate docking to
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lobe 1 is rapid, similar free energy changes for these equilibria would provide strong evidence for a single-step mechanism of domain opening and closure, as such that implied in Equation 1. On the other hand, distinct free energy changes for these equilibria would be more consistent with Equation 2. Below, we provide evidence for the latter view, namely, that the mechanism of cleft closure for glutamate binding comprises multiple equilibria. However, our results for kainate binding, in which the interlobe H-bonds are absent (13, 14, 28), are consistent with Equation 1 and hence with previous results reported for kainate binding to GluR4-S1S2 (20).

EXPERIMENTAL PROCEDURES

All experiments were performed on the 263-residue protein S1S2J (14) derived from the rat GluR2-flop subunit (4). S1S2J was expressed in BL-21(DE3) Escherichia coli grown in fully deuterated minimal medium, and perdeuterated samples of the native protein were prepared via refolding from inclusion bodies and extensive purification (29, 30). NMR spectroscopic measurements were made using a Varian 500 MHz spectrometer fitted with a cryoprobe. The spectra were processed using Lorentzian–Gaussian window functions and modeled using Gaussian line shapes for the estimation of peak volumes (31). 

HD Exchange Measurements—The HD exchange rates, k_HDX, for ~70 binding cleft H’s were determined by fitting V(t) = V_o exp(-k_HDX t) to measured V(t) results. The measured peak intensity profiles were obtained from spectra of 1H-15N heteronuclear single quantum coherence-transverse relaxation optimized (33) spectra acquired after purified samples were transferred into deuterated buffers. Uncertainties in V were estimated based on the spectral noise, and errors in the fitted parameters were calculated from Monte Carlo simulations (31). For each spectrum, 192 points were recorded with 32 scans performed per increment, which corresponded to ~2 h. HD exchange measurements were carried out with solutions of 0.3 mM S1S2J, 25 mM sodium acetate, 25 mM NaCl, 3 mM sodium azide, and either glutamate (0.45, 1, 4, or 10 mM) or kainate (4 or 10 mM).

In most experiments, single protein samples were used to collect multiple series of spectra at progressively increasing temperatures (T). This procedure helped identify conditions yielding appreciable intensity decays for accurately computing k_HDX. Furthermore, for sites experiencing modest peak intensity loss at lower temperatures, it enabled k_HDX determination for additional temperatures. Estimates for higher temperatures benefited from increased linesharpening. Spectra were acquired after equilibration for 2 h. HD exchange measurements were carried out with solutions of 0.3 mM S1S2J, 25 mM sodium acetate, 25 mM NaCl, 3 mM sodium azide, and either glutamate (0.45, 1, 4, or 10 mM) or kainate (4 or 10 mM).

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and the overall binding dissociation constant is

$$K_D = \prod_{i=1}^{M} K_i$$  \hspace{1cm} (Eq. 7)

where \([L]\) is the free ligand concentration. Equations 6 and 7 assume that \(k_{\text{HDX}} = k_{\text{HDX}_1} = k_{\text{HDX}_2} = \ldots = k_{\text{HDX}_M}\), exchange is slow relative to the conformational transition rates, and \(\Delta G_E \gg RT\) (or \(K_Y \ll 1\)), which implies that the HN is well protected from solvent exposure (see supplemental materials).

For the determination of \(K_D \cdot k_{\text{HDX}}\) was estimated using the random coil approximation, i.e., \(k_{\text{HDX}} = k_{D}[D^+] + k_{OD}[OD^-] + k_{D,OD}[D_O]\), where \(k_D\), \(k_{OD}\), and \(k_{D,OD}\) are tabulated, sequence-specific HD exchange rates for HNs in model peptides (36). For particular HNs in S1S2J, this approximation was found to be inaccurate, and corrected values were estimated as described under “Results.”

**HH Exchange Measurements**—Although this study focused primarily on HD exchange, we also conducted several HH exchange experiments to obtain an independent assessment of H-bond stability. For example, a CLEANEX-PM pulse sequence (37) was used to test whether \(^1\text{H}-^{15}\text{N}\) magnetization could be built up over millisecond time scale windows by HH exchange from water (for which coherent \(^1\text{H}\) magnetization was maintained) to S1S2J HN sites. Such experiments were applied to solutions of 0.3 mM S1S2J, 25 mM NaCl, 3 mM sodium azide, 10 mM sodium glutamate, and 25 mM of either sodium acetate (pH 5.1, 6.0, 6.9, 7.5, and 8.7), EPPS (pH 8.0), CHES (pH 9.5), or CAPS (pH 10.3). In general, as the buffer pH is increased typically (pH 5.1, 6.0, 6.9, 7.5, and 8.7), EPPS (pH 8.0), CHES (pH 9.5), or CAPS (pH 10.3) is slow relative to the conformational transition rates, and \(k_{\text{HHX}}\) is protected from solvent exposure (see supplemental materials). In general, as the buffer pH is increased typically (pH 5.1, 6.0, 6.9, 7.5, and 8.7), EPPS (pH 8.0), CHES (pH 9.5), or CAPS (pH 10.3) is slow relative to the conformational transition rates, and \(k_{\text{HHX}}\) is protected from solvent exposure (see supplemental materials).

**RESULTS**

**Key Binding Site HNs in Glutamate-bound S1S2J Under Go Exchange over a Wide Range of Time Scales**—HD exchange lifetimes, \(\tau_{\text{HDX}} = 1/k_{\text{HDX}}\) for S1S2J backbone HNs were measured over 10–25 °C, pH 5.1–6.68, and several glutamate concentrations. Under these conditions, exchange is base catalyzed \((k_{\text{HDX}} = k_{OD}[OD^-])\), and in the EX2 limit where \(k_{\text{HDX}}\) is proportional to \(k_{\text{HDX}}\), \(k_{\text{HDX}}\) changes by a factor of \(10^3\) for a change \(\Delta pD\) at a given \(T\). At the slowest exchange conditions considered (pD 5.1, 10 °C), all of the key HNs involved directly in binding and lobe closure were found to be sufficiently protected for HD exchange analysis.

Figs. 2 and 3 demonstrate that these sites exchange over distinct time scales. In particular, H\text{G653} and H\text{G451} undergo rapid exchange with \(\tau_{\text{HDX}}\) of 3.4 and 9.3 h, respectively, at pH 5.1 and 10 °C (Fig. 2A). As shown in Fig. 2F, exchange at these sites is fast enough to be detected from HH transfer experiments (37) performed under alkaline conditions at which \(^1\text{H}-^{15}\text{N}\) magnetization was built up over 50 ms via proton exchange from water. The \(k_{\text{HHX}}\) reported for pH 9.5 may be considered as lower bounds for the structural transition rates that expose H\text{G653} and H\text{G451} to solvent. In contrast, H\text{Y540} and H\text{T480} do not experience significant HD exchange for any \(T\) explored at pH 5.1. For these HNs, higher pDs of \(\approx 6.5,\) where the driving force for exchange is 25 \((10^{24})\) times greater, were required to obtain quantifiable \(\tau_{\text{HDX}}\) (Fig. 3). Finally, \(H\text{N}_{\text{S654}}\) and \(H\text{N}_{\text{T655}}\) exchange on intermediate time scales with \(\tau_{\text{HDX}}\) comparatively long at pH 5.1 (Fig. 2, A–E) and short at pDs near 5.6 (Fig. 3, A, D, and G).

\(\tau_{\text{HDX}}\) for HNs That Make H-bonds with Glutamate Depends Strongly on Ligand Concentration, whereas \(\tau_{\text{HDX}}\) for HNs That Form Interlobe H-bonds Shows an Apparent Independence—When glutamate is bound to both lobes of S1S2J through H-bonding as shown in Fig. 1B, H\text{S654} and H\text{T655} are protected from undergoing HD exchange. Hence, the \(\tau_{\text{HDX}}\) of these HNs should decrease significantly for decreasing concentrations of glutamate because, after dissociation, \(\tau_{\text{HDX}}\) is determined largely by a competition between ligand rebinding and HD exchange. We tested for such a dependence first through HD exchange measurements at pH 5.0 and 5.1 mm glutamate. Compared with pH 6.65 and 10 mm glutamate, \(\tau_{\text{HDX}}\) for sites insensitive to the ligand on-rate should increase by a factor of \(10^{24}\), whereas \(\tau_{\text{HDX}}\) for sensitive sites should increase less or show appreciable decreases. The results in Fig. 3 (A–F) demonstrate that such a distinction is observed for several HNs. Specifically, the \(\tau_{\text{HDX}}\) of H\text{Y450}, H\text{S654} and H\text{T655} are reduced by a factor of 2 or 4 when the difference caused by changes in pD is corrected) at 1 mm glutamate, indicating a sensitivity to glutamate concentration. This dependence is also seen in Fig. 3 (G and H), which plot intensity decays for pH 6.65, 25°C, and either or 4.05 mm glutamate, respectively. At 0.45 mm glutamate, \(\tau_{\text{HDX}}\) for H\text{Y450} is reduced by a factor of 7, and H\text{S654} and H\text{T655} exchange too rapidly to yield detectable peaks. Finally, Fig. 2 (A, B, D, and E) present results for pH 5.1 and either or 10 mm glutamate, which show 3–7-fold smaller \(\tau_{\text{HDX}}\) for H\text{S654} and H\text{T655} at 1 mm glutamate.

In striking contrast to what is observed for H\text{S654} and H\text{T655} the \(\tau_{\text{HDX}}\) of H\text{Y450} and H\text{G451} as well as H\text{G653} and H\text{T655} located directly across the cleft, exhibit essentially no dependence on glutamate concentration (Figs. 2 and 3).

**Estimated \(K_F\) Indicate That the Binding Reaction Involves Multiple Equilibria**—Fig. 4A presents \(K_F\) and \(\Delta G_E\) results for the key binding site HNs based on the \(\tau_{\text{HDX}}\) given in Figs. 2 and 3 and \(k_{\text{HHX}}\) set to random coil values (36). Notably, particular pairs of HNs that occupy similar regions of the binding site have similar \(K_F\), which suggests that their solvent exposure arises from related conformational opening events. For example, the \(K_F\) of H\text{G451} \((1.9 \times 10^{-3})\) and H\text{G653} \((2.9 \times 10^{-3})\) are relatively...
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large and comparable in magnitude. Structurally, H$_N^{653}$ resides directly across the cleft from H$_N^{451}$, and shares a peptide bond with the CO of Ser$_652$; this CO in turn forms an interlobe H-bond with H$_G^{451}$(Fig. 1, B and C). The $K_E$ results imply that this H-bond is relatively unstable and that its breakage is associated with backbone flexibility in lobe 2 near Gly$_653$, a view consistent with previous interpretations (14).

In addition, at several conditions tested, H$_N^{654}$ exchanges approximately two times faster than H$_N^{655}$ (Figs. 2 and 3), and such rates lead to similar $K_E$ because random coil predictions for H$_N^{654}$ are two times greater than those for H$_N^{655}$ (36). As these H$_N$s form the only direct interactions between glutamate and the backbone of lobe 2 (Fig. 1B), the results suggest that the HD transfer events emanate from a structural equilibrium involving detachment of glutamate from lobe 2. That the $K_E$ of H$_N^{654}$ and H$_N^{655}$ are larger than those of H$_N^{480}$ is suggestive of unique ligand docking and lobe isomerization equilibria (Fig. 4A). However, as discussed in the next subsection, the high degree of protection of H$_N^{480}$ appears to be due in part to departure of solvent-exposed states from random coil behavior.

Although the random coil approximation is commonly applied to solvent-exposed states of H$_N$s in proteins, it must be used carefully because departure from random coil behavior can clearly lead to erroneous $k_{HDX}$ and hence $\Delta G_E$. Furthermore, it may be difficult to identify and correct for such discrepancies when $\Delta G_E$ corresponds to local backbone unfolding events. However, because the key H$_N$s considered in the present study presumably become solvent-exposed upon ligand detachment or domain opening, it is possible in principle to estimate their $k_{HDX}$ from experiments employing either the

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**FIGURE 2.** A–E, $^1$H-$^15$N peak intensity versus time curves from two HD exchange experiments conducted at pD 5.1 with 0.3 mM GluR2-S1S2J and either 10 mM (A–C) or 1 mM (D and E) glutamate. In each experiment, runs at different temperatures were performed consecutively without removing the sample from the magnet (see "Experimental Procedures"). Fitted $\tau_{HDX}$ are shown in parentheses. The increasing peak intensity profiles presumably arise from reduced magnetic relaxation due to more rapid HD substitution at nearby sites, such as in the Arg$_{485}$ side chain for H$_N^{480}$ (see Fig. 1B). F, $^1$H-$^15$N magnetization build-up curves resulting from CLEANEX-PM exchange experiments (37). Intensity ratios ($V/V_{ref}$) are plotted as a function of the mixing time $\tau_{mix}$. $\tau_{HDX}$ estimated from initial slope analysis are given in parentheses.
Apo protein or agonists that stabilize the HNs in solvent-exposed states. As discussed below, the latter approach was found to be useful for evaluating the exchange behavior of HY450N.

Surprisingly, the $K_e$ of HY450N (based on random coil values for $k_{HDX}$) suggest a high degree of protection (Fig. 4A). The increased stability relative to HS654N and HT655N is particularly interesting given that the latter two HNs make H-bonds to glutamate that are buried beneath where HY450N forms an interlobe H-bond (Figs. 1B and 5A). In addition, the $K_e$ of HY450N are independent of glutamate concentration, unlike those of HS654N and HT655N. There are seemingly only two explanations for these results: either (i) the lobes of Apo S1S2J adopt closed conformations that protect HY450N in a way that leads to the observed glutamate concentration independence or (ii) HY450N can become solvent accessible in closed cleft conformations with glutamate bound but residual structure slows $k_{HDX}$ well below random coil estimates. Although solution x-ray scattering studies suggest that Apo S1S2J adopts a mixture of open and closed domain states (38), the rapid docking of agonist to the protein (20, 21) argues against explanation (i). Similar arguments against extended closed states in the Apo protein can be made from free energy calculations as a function of the degree of lobe closure (39, 40).

Further support for explanation (ii) was obtained from additional HD exchange experiments with kainate as the agonist. Relative to glutamate, the lobes of S1S2J are substantially more

**FIGURE 3.** $^1$H-$^1$N peak intensity-time profiles from four HD exchange experiments involving 0.3 mM GluR2-S1S2J. A–C, 10 mM glutamate, pD 6.65, and 14, 20, or 25 °C. D–F, 1 mM glutamate, pD 6.4, and 14, 20, or 25 °C. G, 4 mM glutamate, pD 6.68, and 25 °C. H, 0.45 mM glutamate, pD 6.68, and 25 °C. The dashed lines in H derive from fits that excluded the first six data points or approximately 12 h, over which time small intensity losses occurred for strongly protected sites. For sites that exchange substantially at these conditions (e.g. HY450N, HT450N, and HN651H), such losses do not alter $t_{HDX}$ significantly.
open in the presence of kainate, and formation of the interlobe 
H-bonds involving HY450N and HG451N is prevented (Fig. 5, A and 
B) (13, 14, 28). As a first approximation, we assigned KHDX 
for HY450N measured with kainate to \( \tilde{K}_{HDX} \) for HY450N in glutamate- 
bound S1S2J. Fig. 5 (C and D) shows that HY450N exchanges with 
\( \tau_{HDX,kai} \) of 33 and 17 h for pD 5.1, 10 mM kainate, and 10 or 
14°C, respectively. The \( \tau_{HDX,kai} \) for 14 °C, in combination with the 
\( \tau_{HDX,glu} \) in Fig. 3 (A and D), provides an estimate of \( K_{E,glu} \).
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This value of $K_{D_{kai}}$ was used to estimate $K_{1_{kai}}$ and $K_{2_{kai}}$ for comparison with $K_1$ and $K_2$ determined for glutamate. Figs. 2A and 5C show that $H_{N654}^N$ and $H_{G451}^N$ exchange 60–80 times faster for kainate relative to glutamate at pH 5.1, 10°C, and 1 mM agonist. Equation 10 assumes that exchange occurs from $P_2$ only and requires that $\tau_{HDX,glu}/\tau_{HDX,kai} = K_{D_{kai}}/K_{D_{glu}}$ for equal concentrations of glutamate and kainate. However, for $H_{N654}^N$ and $H_{G451}^N$, $\tau_{HDX,glu}/\tau_{HDX,kai} >> K_{D_{kai}}/K_{D_{glu}}$ suggesting that exchange occurs from $P_1$, i.e. from domain opening, more readily for kainate than glutamate. By applying the $K_{E}$ for $H_{N654}^N$ and $H_{G451}^N$ to Equation 8, we estimated that $K_{1_{kai}} = 0.0050 \pm 0.0005$ and $K_{2_{kai}} = 0.8 \pm 0.08$ mM. Thus, relative to kainate, these results suggest that glutamate binds to the open domain state of S1S2 with roughly five times lower affinity but shifts the domain isomerization equilibrium 60-fold toward cleft closure.

$M = 2$: Distinct Dock-isomerization Equilibria Detected for Glutamate- and Kainate-bound S1S2—We first applied the $K_{E}$ results to the dock-isomerization model given by Equation 1. In this case, $M = 2$ in Equation 3, and Equations 6 and 7 simplify to Equations 8 and 9.

$$K_{E} = K_1 + K_1K_2/\left[L\right] \quad (Eq. \ 8)$$

$$K_{D} = K_1K_2 \quad (Eq. \ 9)$$

$K_1$ and $K_2$ are the equilibrium constants for domain opening and ligand dissociation from lobe 1, respectively. In the next section, it is argued utilizing the $K_{E}$ of $H_{G451}^N$ and $H_{G451}^N$ that $M > 2$ for glutamate binding because of the presence of significant interlobe interactions. Here, these interactions are ignored, and the relative lobe closure stabilities of glutamate- and kainate-bound S1S2 are analyzed based solely on the $K_{E}$ of $H_{T480}^N$, $H_{N654}^N$, and $H_{T655}^N$.

Application of $K_{E}$ for $H_{N654}^N$ and $H_{T655}^N$ to Equation 8, which assumes exchange from both $P_1$ and $P_2$, results in $K_1 = 8.1 \times 10^{-5} \pm 4.1 \times 10^{-5}$ and $K_2 = 4.0 \pm 0.6$ mM for glutamate binding. This calculation was based on five pairs of $K_{E}$ in Fig. 4B corresponding to two glutamate concentrations, identical temperatures, and similar pDs. By Equation 9, $K_{D} = 320 \pm 120$ nM, which is consistent with competition binding results for this protein ($K_{D}$ 450 nM) (14) and for two related GluR2-S1S2 proteins that differ only in the regions where the domains are excised from the full subunit (120 and 250 nM) (41, 42).

It was also possible to estimate $K_{D}$ from the $K_{E}$ of $H_{T480}^N$. However, calculations assuming either exchange from both $P_1$ and $P_2$, or from $P_2$ only, lead to values of ~4 mM, which are too small. Thus, as with $H_{Y250}^N$, random coil HDX exchange rates (36) seemed overestimate $K_{D}$ for $H_{T480}^N$. This is not surprising though because, unlike $H_{N654}^N$ and $H_{T655}^N$, $H_{T480}^N$ resides more deeply in the binding cleft and in a more stable section of the protein (29, 43, 44).

Despite the apparent error in the random coil values of $H_{T480}^N$, its measured $\tau_{HDX}$ was used to correctly estimate the $K_{D}$ for kainate (based on the $K_{D}$ for glutamate determined above) via Equation 10.

$$K_{D_{kai}} = K_{D_{glu}}\tau_{HDX,glu}(pD,T)[kai] \quad \tau_{HDX,kai}(pD,T)[glu] \quad (Eq. \ 10)$$

In accordance with a dock-isomerization mechanism, this expression assumes that $H_{T480}^N$ exchanges only from $P_1$, i.e. $K_{E} = K_1/L$ [L]. $\tau_{HDX}$ does not appear in Equation 10 by $\tau_{HDX,glu} = \tau_{HDX,kai}$. Application of the $\tau_{HDX}$ in Figs. 3 (C and F–H) and 5E to Equation 10 leads to $K_{D_{kai}} = 4.1 \pm 2.3 \mu M$, which agrees well with the competition binding results of 8.0, 1.3, and 2.3 $\mu M$ (41, 42, 14).
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structure protomers lacking the H-bonds mediated by \( H^N_{\text{Y450}} \) and \( H^N_{\text{G451}} \) (14). Moreover, a number of mutagenic studies have shown that alteration of these side chain interactions impacts domain closure stability and receptor function (19, 20, 45–49).

**DISCUSSION**

The lobe dynamics occurring in S1S2 of AMPA receptors appear to be tailored for the efficacious binding and fast release of glutamate to produce large activating currents in combination with efficient channel deactivation and resensitization (14, 19, 18). Models based on channel recordings suggest a sequential multi-state binding mechanism comprising rapidly and slowly equilibrating components (18). Here, using hydrogen-deuterium exchange, we have provided evidence for such a mechanism for glutamate binding and have estimated the thermodynamic stabilities of the detected states. Furthermore, for kainate binding, which lacks the interlobe interactions constituting distinct states in the complex with glutamate, we have confirmed the two-state dock-isomerization mechanism first proposed from stopped flow experiments (20) and have shown that this reaction has a higher proclivity for sampling agonist-bound open domain conformations.

The HD exchange rates presented in this study have uncertainties typically much less than 10%. Yet the associated \( K_E \) were evaluated carefully due to the use of random coil exchange rates for estimating \( k_{\text{HDX}} \). Although random coil rates have been validated for \( ^2H \)'s in denatured proteins (36, 50, 51) and appear to be realistic measures for many solvent-exposed \( ^2H \)'s in native proteins (52), examples of poor predictions have been reported (53, 54). Thus, for the key binding site \( ^2H \)'s in S1S2], we either justified the use of the random coil approximation or provided corrections through experiments. It is important to note, however, that our main conclusions can also be justified without invoking this approximation. First, the significance of the lobe-locking equilibrium in the binding reaction for glutamate is evident by the much higher degree of protection of \( H^N_{\text{Y450}} \) and \( H^N_{\text{G451}} \) in complexes with glutamate compared with kainate. Second, the uniqueness of the lobe-unlocking and domain opening equilibria is established by the insensitivity of the HD exchange rates of \( H^N_{\text{Y450}} \) and \( H^N_{\text{G451}} \) to changes in glutamate concentration. For these equilibria to be unique, then these \( H^N \)'s would become solvent-exposed only upon domain opening and a strong glutamate concentration dependence similar to that exhibited by \( H^N_{\text{S654}} \) and \( H^N_{\text{S655}} \) would have been observed. Finally, the higher tendency for glutamate binding to induce closed cleft states in S1S2] relative to kainate is apparent in the 60–80-fold smaller exchange rates of \( H^N_{\text{S654}} \) and \( H^N_{\text{S655}} \) measured for glutamate.

We have demonstrated that the binding reaction for glutamate is composed of multiple states, but the question arises as to how this relates to the functional model in Equation 2 (18). One might speculate that, for the HD exchange model of Equation 3 with \( M = 3 \), \( P_0 \) is the state \( S \), and \( P_1 \) is the state \( S^*G \), i.e. glutamate docked to lobe 1 without H-bonding to \( H^N_{\text{S654}} \) and \( H^N_{\text{Y655}} \) in lobe 2. \( P_1 \) would then correspond to the state \( S^*G_1 \), for which glutamate interacts with lobe 2 in the absence of the interlobe H-bonds mediated by \( H^N_{\text{Y450}} \) and \( H^N_{\text{G451}} \). Whether the side chain interactions shown in Fig. 5A are made in this state or in \( S^*G \) or \( S^*G_2 \) is unclear (all of the crystal structure protomers of glutamate-bound S1S2) contain these side chain interactions, whereas only some of the protomers show the interlobe main chain H-bonds) (14, 27). Finally, \( P_0 \) would be associated with \( S^*G_2 \) and/or \( S^*G_3 \) in some way and include the H-bonds formed by \( H^N_{\text{Y450}} \) and \( H^N_{\text{G451}} \). In the present study, we could only detect the breakage of H-bonds made to backbone \( ^2H \)'s, but clearly the side chain interactions are an essential component of the binding mechanism and may be energetically and kinetically separable from the main chain interactions.

In the whole cell experiments for measuring deactivation upon which Equation 2 is partially based, glutamate was applied to GluR2 restrained in the nondesensitizing state by cyclothiazide (18). Seemingly, for this reason \( S^*G_1 \) was primarily populated with lower percentages observed for \( S^*G_2 \) and \( S^*G_3 \). In contrast, our HD exchange measurements were performed on S1S2], which lacks structural restraints present in the non-desensitizing state. Because the \( \Delta G^*_E \) of \( H^N_{\text{S654}} \) and \( H^N_{\text{S655}} \) are significant, free S1S2] appears to favor transitions to states with stronger interactions across the cleft, which may be active in the desensitized state. This is consistent with the finding that the \( EC_50 \) for glutamate activation of GluR2 (in cyclothiazide) is 300 \( \mu \)M (18) but that the binding affinity for the desensitized state is much higher (1 \( \mu \)M) (55). Likewise, glutamate binds to GluR2 S1S2] with a \( K_D \) of roughly 300 nM (41, 42, 14), suggesting that this complex represents the desensitized state, as proposed previously (14).

Thus, the process of successive H-bond disruption measured via HD exchange may account for structural changes in S1S2] that occur during recovery from desensitization. On the other hand, the responses of nondesensitizing (or resting) GluR2 to brief glutamate applications would seemingly be dominated by agonist docking and domain isomerization events (consistent with occupation of \( S^*G_1 \) (18) with less lobe locking than that observed in S1S2]. This would account for the ability of AMPA receptors to activate and deactivate rapidly. The lobe-locking equilibria presumably become more functionally active during extended glutamate applications (consistent with increased occupation of \( S^*G_2 \) and \( S^*G_3 \) (18) and aid in the stabilization of glutamate binding in desensitized states geared to abolish macroscopic currents.

Because full and partial agonists sample similar unitary conductance levels in single channels (17) and the extent of channel activation can be correlated with the degree of cleft closure in S1S2] (17, 28, 56), a more significantly closed cleft apparently acts to increase the probability that a channel gate opens rather than promote a gradually more open gate. Such a mechanism would also seemingly depend on the probability that the cleft is closed when agonist is bound, and this is consistent with recent studies showing that domain closure stability also influences activation (18, 19, 48). However, this form of structural stability is difficult to characterize energetically from crystal structures and ps–ms dynamics because domain opening in S1S2] occurs on slower time scales. Thus, the methods established in the present study, which probe ms and longer time scale motions and yield equilibrium con-
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...constants for lobe opening in agonist-bound S1S2J, should be useful for such characterizations.

As a case in point, kainate binding to nondenensitizing GluR2 generates currents that are 50 times smaller than those evoked by glutamate (45), and this has been attributed to the smaller degree of cleft closure in S1S2 induced by kainate (13, 14). However, a broader view that includes domain closure stability measures is obtained from the K1 of our two-state binding model (Equation 3; M = 2), which indicates that S1S2J adopts agonist-bound, open cleft states (identified by the HD exchange of HS654 and H1665) much more often for kainate than glutamate. Although it is difficult to assess the relative importance of cleft closure extent and stability on channel activation for kainate, these results support a dynamic view of agonism consistent with an activation mechanism that depends not only on the extent of cleft closure but also on the probability that the cleft is closed when agonist is bound.

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REFERENCES

On the Mechanisms of $\alpha$-Amino-3-hydroxy-5-methylisoxazole-4-propionic Acid (AMPA) Receptor Binding to Glutamate and Kainate

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