Chemistry and Conformation of the Ligand-binding Domain of GluR2 Subtype of Glutamate Receptors

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In the present report, using vibrational spectroscopy we have probed the ligand-protein interactions for full agonists (glutamate and α-amino-5-methyl-3-hydroxy-4-isoxazole propionate (AMPA)) and a partial agonist (kainate) in the isolated ligand-binding domain of the GluR2 subunit of the glutamate receptor. These studies indicate differences in the strength of the interactions of the α-carboxylates for the various agonists, with kainate having the strongest interactions and glutamate having the weakest. Additionally, the interactions at the α-amine group of the agonists have also been probed by studying the environment of the non-disulfide-bonded Cys-425, which is in close proximity to the α-amine group. These investigations suggest that the interactions at the α-amine group are stronger for full agonists such as glutamate and AMPA as evidenced by the increase in the hydrogen bond strength at Cys-425. Partial agonists such as kainate do not change the environment of Cys-425 relative to the apo form, suggesting weak interactions at the α-amine group of kainate. In addition to probing the ligand environment, we have also investigated the changes in the secondary structure of the protein. Results clearly indicate that full agonists such as glutamate and AMPA induce similar secondary structural changes that are different from those of the partial agonist kainate; thus, a spectroscopic signature is provided for identifying the functional consequences of a specific ligand binding to this protein.

Glutamate receptors are the predominant mediators of excitatory synaptic signals in the central nervous system (1–5). These receptors form cation-specific channels on binding glutamate and later enter a desensitized state in which the channel is closed, despite the presence of an agonist. Significant insights into the mechanism of agonist-induced activation and desensitization of this receptor have been obtained from the recent x-ray structures of the isolated, extracellular ligand-binding domain (S1S2) of the α-amino-5-methyl-3-hydroxy-4-isoxazole propionate (AMPA) subtype of the glutamate receptors (6–9). The x-ray structures of the S1S2 protein indicate a bilobed structure with the ligand-binding site located in the cleft between the lobes. The protein exists in an open structure in the apo state and undergoes varying degrees of cleft closure on binding various agonists. This cleft closure is believed to be a rigid body motion of one lobe relative to the other. Furthermore, the degree of cleft closure has been shown to be directly proportional to the extent of activation of these receptors, suggesting cleft closure in the S1S2 protein as the mechanism of activation of the receptor (7, 8).

The x-ray structures of the S1S2 protein in various ligated states have provided the first structural insights into the structure-function correlations in the glutamate receptor. These insights, however, are low temperature static images that are limited by the constraints of crystal-packing forces. For a more detailed investigation of specific interactions, it becomes essential to complement these x-ray structures with spectroscopic investigations that probe the dynamic state of the protein with significantly higher resolution. Recent NMR studies, for instance, suggest that the protein is not a rigid structure and that domain 2 of the S1S2 protein, which interacts with the γ substituents of the agonists, exhibits considerable mobility on the microsecond to millisecond time scale (5, 10, 11). Because this region of the protein is directly connected to the transmembrane segments, these dynamic motions are expected to be involved in the subtleties of channel gating. These results suggest that the mode of agonist-induced activation may be more complex than the rigid body domain closure suggested by x-ray structures.

The complexity of the allosteric mechanism of agonist action in glutamate receptors is further exemplified in investigations of the GluR4-S1S2 protein by using vibrational spectroscopic studies, which allow a detailed analysis of specific noncovalent interactions of the full and partial agonists and antagonists of the glutamate receptor (12, 13). In this report, we have performed a similar investigation using the GluR2-S1S2 protein, which allows us to compare the vibrational spectroscopic data with the x-ray and NMR structures that were obtained using this protein (7, 8). Additionally, the signal-to-noise ratio of the spectra has also been improved. This improvement allows a more detailed quantitative analysis of the differences in the strength of the interactions of the α-carboxylates of the various ligands with the protein, which have not been observed in the crystal structures. The higher resolution of the vibrational spectra also indicates changes at the single non-disulfide-bonded Cys residue, which is in close proximity to the α-amine groups of the agonists and is, therefore, an indirect probe of the strength of the interactions between this group of agonists and the protein. The differences that occur between the interactions of the protein and the α substituents of the various agonists (glutamate, AMPA, and kainate) contribute to differences in affinity and most likely would also be factors in the observed differences in the degree of domain closure induced by...
these ligands and thus play a role in the differences in activation/function.

**Materials and Methods**

**Protein Preparation and Characterization**—The plasmid S12S2, used to express the GluR2-S1S2 protein, was kindly provided by Eric Gouaux (Columbia University, New York). The GluR2-S1S2 protein was expressed, purified, and characterized as described by Armstrong et al. (14). In brief, the protein was expressed in *Escherichia coli* Origami-B (DE3) cells. Following clarification and concentration, the cell culture supernatant was purified by ion exchange chromatography (nickel-nitrilotriacetic acid) column. The E705D mutant was obtained by using the QuikChange site-directed mutagenesis kit and expressed and purified using the same methodology as outlined for GluR2-S1S2 wild type protein. 0.25–0.5 mM protein in 25 mM phosphate buffer containing 250 mM NaCl and 0.02% NaN₃ was used for the buffer as an internal standard. Furthermore, the peaks that arise from the unbound agonists were subtracted using a spectrum of the agonist buffer. Differences between protein bound glutamate in buffer (Fig. 1, trace B) were obtained for the E705D mutant in the presence and absence of glutamate (Fig. 2). Additionally, the frequency of the ∆v̇asym mode was established using the 13C-labeled glutamate corresponding to D₂O (hydrogen ~ 3.6 kcal/mol). The x-ray structures of GluR2-S1S2 in complex with glutamate show two possible hydrogen bond donors (Ser-654 and Thr-655) and a negatively charged side chain (Glu-705, unfavorable interaction) in close proximity to the γ-carboxylate (6). To further investigate the role of residue Glu-705, a difference FTIR spectrum was obtained for the E705D mutant in the presence and absence of glutamate (Fig. 2). Additionally, the frequency of the ∆v̇asym mode was established using the 13C-labeled glutamate at the γ position (Fig. 2, trace B). The FTIR spectra indicate a 7-cm⁻¹ downshift in the ∆v̇asym mode for the glutamate bound to the E705D mutant, relative to the free glutamate frequency (Table I). This downshift suggests that the unfavorable interactions at the γ-carboxylate of glutamate bound to the wild type protein is eliminated in the E705D mutant. Therefore, a downshift is observed in the frequency of the ∆v̇asym mode, which could arise because of the favorable interactions with the two possible hydrogen bond donors (Ser-654 and Thr-655).

The environments of the carboxylate moieties have also been investigated for the ligand kainate and AMPA by obtaining similar difference FTIR spectra of free ligand and ligand bound to the S1S2 protein (Fig. 3). Based on the α-carbon substituent effect on the carboxylate frequency (17) and the observed frequencies for the carboxylate vibrational modes of amino acids, the ∆v̇asym and ∆v̇asym isoazole modes have been assigned for kainate and AMPA (shown in Table I). These assignments indicate that the frequency of the ∆v̇asym band is down-shifted by 18 cm⁻¹ in bound kainate, relative to its frequency in free kainate, and by 9 cm⁻¹ in bound AMPA, relative to its frequency in free AMPA. The S1S2-induced downshift in the ∆v̇asym band follows the order kainate > AMPA > glutamate. It can be concluded, therefore, that the hydrogen bond to the arginine residue in the protein follows the same order, namely, kainate > AMPA > glutamate.

The x-ray structures indicate identical environments for the α substituents (amine and carboxylate groups) of the agonists glutamate, kainate, and AMPA, leading to the conclusions that this region of the protein is rigid and functions as a lock and key fit. The vibrational spectra, however, show that the
strength of the interactions of the α-carboxylates of the agonists with the GluR2-S1S2 is different for the various agonists, with the difference in enthalpy of 4.7 kcal between the strongest (kainate) and weakest (glutamate) interactions. This suggests that, in the dynamic state of the protein, the α-substituents of the agonists undergo slight adjustments to the lock and key fit for the various agonists and these small variations play a role in the differences in the affinity and function associated with these agonists.

**Protonation State of the α-Amine Group of Glutamate in the Ligand-binding Pocket**—The frequency of the α-carboxylate group acts not only as a probe of the noncovalent interactions at this moiety but can also be used as an indicator for the protonation state of the α-amine group of the ligand. As shown in Fig. 1, traces D and E, the frequency of the α-carboxylate is significantly up-shifted (1614 cm⁻¹) from the typical frequencies of the asymmetric carboxylate vibrational mode (1565 cm⁻¹) only when the α-amine group is positively charged. When the pH is increased such that the α-amine group is deprotonated, the frequency of the α-carboxylate is similar to that of the γ-carboxylate, and the bands for the two moieties overlap (Fig. 1, trace E). Because the frequency of the asymmetric stretching vibration of the α-carboxylate of glutamate bound to S1S2 protein is at 1610 cm⁻¹, closer to the 1614-cm⁻¹ value of the free glutamate, it can be concluded that the α-amine group of the ligand glutamate is protonated in the protein. The implication of this observation is that the amine group can be involved in three donor hydrogen bonds, and there are a number of groups (Glu-705, Pro-478, and Thr-480) within hydrogen bonding distance in the x-ray structure that can be the hydrogen bond acceptors. Additionally, the positive charge on the α-amine group would have favorable electrostatic interactions with the negatively charged Glu-705 residue, thus justifying the position of the Glu-705 residue, despite unfavorable interactions with the γ-carboxylate of the ligand glutamate.

**Conformational Differences between the Partial and Full Agonist-bound Forms of S1S2**—The crystal structures of the GluR2-S1S2 protein indicate that the degree of domain closure exhibits a linear dependence on the extent of activation of the channel. For instance, the protein S1S2 in complex with the partial agonist kainate exhibited intermediate closure of the cleft, whereas full agonists such as glutamate and AMPA exhibited larger closure. To identify analogous correlations between the conformational and functional changes in the infrared signals, we have investigated the secondary structure changes using amide I signals and probed the environment of the single non-disulfide-bonded cysteine residue in S1S2, using the SH stretching mode.

**Secondary Structure**—The features at 1673, 1643, and 1630 cm⁻¹ in the difference spectrum between the glutamate-bound and unligated forms of the protein (Fig. 4, top trace), are characteristic of amide I modes rising from the protein backbone. Based on the frequencies, the 1673 and 1630 cm⁻¹ features can be assigned to changes in turns and β-sheet secondary structures, respectively (18). On the other hand, the feature at the 1643-cm⁻¹ frequency could arise from changes in random structures (19), irregular β-sheet (20), and/or solvent-exposed helices (21).

The difference features observed on glutamate binding to GluR2-S1S2 are also observed in the difference spectrum because of AMPA binding to the protein (Fig. 4, middle trace). This indicates that the conformational changes induced by AMPA binding to GluR2-S1S2 are similar to those resulting from glutamate binding. Therefore, these difference features in the amide I region could be used as a signature for full agonism.

On the other hand, the difference features caused by the binding of partial agonist kainate are significantly more varied compared with those caused by glutamate and AMPA. Although the increase in intensity of the band arising from the β-sheet secondary structure on binding kainate is similar in magnitude to that observed for full agonist binding, the frequency of this mode is lower (1625 cm⁻¹ versus 1630 cm⁻¹). Additionally, no differences are observed at 1643 cm⁻¹ on kainate binding to GluR2-S1S2, unlike the case for full agonists, and a new feature is observed at 1650 cm⁻¹. Based on the frequency, the features at 1650 cm⁻¹ can be assigned to perturbations in α-helical structures. Overall, these results indi-

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**Table I**

| Ligand         | $\nu_{\text{asym}}$ (cm⁻¹) Free | $\nu_{\text{asym}}$ (cm⁻¹) Bound to S1S2 | $\nu_{\text{sym}}$ (cm⁻¹) | Visoxamide |
|---------------|----------------------------------|----------------------------------------|--------------------------|------------|
| Glutamate     | 1614                             | 1610 (−1.3 kcal)                        | 1565                     | 1576 (3.7 kcal) |
| Kainate       | 1620                             | 1602 (−6 kcal)                          | 1570                      | Not assigned |
| AMPA          | 1614                             | 1605 (−3 kcal)                          | 1659                      | 1660 (tentative) |
| Glutamate (E705D) | 1614                              | 1608 (−2 kcal)                         | 1565                      | 1568 (−2.3 kcal) |

**Fig. 2. Difference FTIR spectrum between glutamate-bound and unligated form of E705D-S1S2 protein in D₂O (A), and glutamate-bound and [13C]glutamate-bound form of E705D-S1S2 protein in D₂O (B). a.u., absorption unit.**

**Fig. 3. Left, difference FTIR spectrum between kainate and D₂O (Kai) and kainate-bound and unligated form of GluR2-S1S2 protein (S1S2-Kai). Right, AMPA and D₂O (Kai) and AMPA-bound and unligated form of GluR2-S1S2 protein (S1S2-AMPA).**
cater that the perturbations in the protein backbone are different for full agonists versus partial agonists. The exact nature of these differences, in terms of structural changes in the protein, however, cannot be determined without further experiments in which the backbone carbonyls of specific parts of the protein are isotopically labeled.

Irrespective of the differences in specific frequencies, the binding of all three ligands leads to an increase in intensities at specific frequencies, with no concomitant negative features resulting from a loss in specific types of secondary structure. This indicates that the observed changes caused by agonist binding do not arise because of conversion from one type of secondary structures to another, e.g., α-helices to random coil, but because of changes in the molar absorptivities of the specific secondary structural changes. Such changes in molar absorptivities reflect changes in the dipole moment arising from the small structural changes. Such changes in molar absorptivities reflect changes in the dynamics of the protein on agonist binding. This would be consistent with the NMR structure of the glutamate-bound form of S1S2 that indicates interesting dynamics of specific β-sheet, α-helical, and turn structures, which has been suggested to be a possible pathway for channel gating (10). A more detailed comparison between the results observed here and the dynamics of the protein should be possible once the NMR structures to another, e.g., β-sheets to random coil, but because of changes in the molar absorptivities of the specific secondary structural changes.

The frequency of the SH stretching band at 2560 cm\(^{-1}\) has only one free cysteine residue (Cys-426; SH stretching frequency is unaltered on kainate binding (Fig. 5, trace A)) and Cys-436, on the other hand, is at 2550 cm\(^{-1}\), which is indicative of a strong donor hydrogen bond (6). Based on the crystal structure, the backbone carbonyl of Ala-745 is 3.3 Å from the Cys-436 and, hence, the most likely hydrogen bond partner. The orientation of the possible hydrogen bond partners for Cys-425 and Cys-436 also accounts for the intensity differences observed for the two (6). The torsion angle between the O group of the hydrogen bonding partner was chosen to determine the SH bond and C=Ca (α and β with respect to the peptidyl CO) bonds are 130° and 67° for Cys-425 and Cys-436 respectively. These torsion angles and the observed intensities for the SH stretching vibration for Cys-425 and Cys-436 are consistent with the vibrational spectra of ethane thiols, which indicates significantly smaller intensities for thiols that exist in cis configuration (torsion angle of 30°), relative to those that exist in trans configuration (torsion angle of 180°) (6).

The frequency of the SH stretching vibration of Cys-436 is not significantly altered on addition of kainate, AMPA, or glutamate, thus, suggesting that the environment of this residue is not significantly different among the apo, kainate, AMPA, or glutamate-bound states. On the other hand, the Cys-425 SH stretching frequency is unaltered on kainate binding (Fig. 5, trace B) but lowered to 2542 cm\(^{-1}\) on binding glutamate (Fig. 5, trace C) and AMPA (Fig. 5, trace D). The lack of any significant effect of the frequency on kainate binding suggests that the environment of this residue is not significantly altered. The downshift of \(\Delta 18\) cm\(^{-1}\) on binding of a full agonist to the protein implies that the SH hydrogen bond is stronger in this form of the protein, relative to the unligated and partial agonist-bound forms. Based on the studies of Li and Thomas (16),
in which a correlation was obtained between the SH stretching frequency and the SH distance, the \( \Delta 18\text{cm}^{-1} \) shift observed in the SH stretching vibration between the apo and full agonist can be correlated to a change in distance of the SH bond by 0.1 Å. This change is far beyond the resolution of the x-ray structures, and hence no changes were observed at the Cys-425 residue between the apo and various ligated forms of GluR2-S1S2. This result also highlights the ability of vibrational spectroscopy to probe specific interactions of interest in finer detail than most other structural methods.

Can the SH stretching mode be used as an indicator for partial versus full agonism? The different effects of full and partial agonists on Cys-425 do not correlate with the degree of domain closure observed for the various agonists in the x-ray structures (full agonists vs partial agonist vs apo) or with the extent of secondary structural changes observed in this study. It is more likely, therefore, that the changes at the Cys-425 residue reflect local perturbations such as differences in the strength of the interactions of the ligand \( \alpha \)-amine group with the protein. Pro-478, which is hydrogen-bonded to the amine groups of the ligands, is adjacent to the hydrogen-bonding partners of Cys-425 (Ile-476 and Ala-477). Therefore, based on the downshifts observed for glutamate and AMPA binding and the lack of an effect because of kainate binding, it can be concluded that the interactions between the \( \alpha \)-amine group of glutamate and AMPA with the protein are stronger than the interaction of the \( \alpha \)-amine group of kainate with the protein. This difference in the amine interaction could be one of the mechanisms by which full agonists induce larger conformational changes relative to partial agonists; however, additional interactions of the ligand, such as those at the \( \gamma \) substituents, are most likely involved in determining the final degree of domain closure.

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