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Ligand−Protein Interactions in the Glutamate Receptor[†]

Vasanthi Jayaraman,*[,‡] Robert Keesey,[‡] and Dean R. Madden[§]

Chemistry Department, Marquette University, Milwaukee, Wisconsin 53233, and Ion Channel Structure Research Group, Max Planck Institute of Medical Research, Jahnstrasse 29, 69120 Heidelberg, Germany

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ABSTRACT: Fourier transform infrared spectroscopy was used to investigate ligand−protein interactions in the ligand-binding domain of the GluR4 glutamate receptor subunit. Glutamate binding induces more extensive secondary structural changes in the ligand-binding domain than does kainate binding. Glutamate also alters the hydrogen bonding strength of the single free cysteine side chain in the domain, while kainate does not. On the other hand, the interaction of a binding site arginine residue with kainate appears to be stronger than that with glutamate. These results identify chemical and structural differences that may explain the different functional characteristics of the two agonists acting on ionotropic glutamate receptors. In doing so, they complement and extend recent crystallographic structures of the ligand-binding domain.

Excitatory synaptic transmission in the central nervous system is mediated predominantly by ionotropic glutamate receptors (GluR),[1] ligand-gated transmembrane ion channels that open upon agonist binding and subsequently enter a closed, desensitized state that is unresponsive to the continued presence of agonist (*1*−*4*). Different agonists produce distinct gating and desensitization profiles, presumably mediated by specific ligand−protein interactions. For instance, glutamate activation of the α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) subtype of the glutamate receptor is followed by rapid and extensive desensitization (*5*−*8*). On the other hand, kainate binding to AMPA receptors exhibits a higher EC_{50} value and leads to desensitization that is more rapid and incomplete than that seen with glutamate (*5*−*8*). We have investigated the structural basis for these functional differences by studying ligand and protein vibrational modes, using Fourier transform infrared spectroscopy (FTIR).

Glutamate and kainate (Figure 1) bind to an extracellular domain in GluR subunits (Figure 2) (*9*, *10*). This ligand-binding domain has been expressed as a soluble fusion protein for two AMPA receptor subunits, namely, GluR2 and GluR4 (*10*−*12*). The fusion protein, known as S1S2, retains ligand binding parameters quite similar to those of the intact receptor, indicating that it can be used as a model to study the ligand−protein interactions of the complete molecule (*10*−*13*). Furthermore, the X-ray crystal structure of kainate-bound S1S2 from GluR2 has been determined recently (*14*), which provides a foundation for detailed structure−function analyses.

The S1S2 crystal structure revealed a bilobate molecule with kainate bound in the cleft between the two lobes (Figure 3). On the basis of a comparison with the homologous

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* To whom correspondence should be addressed: Chemistry Department, Marquette University, Milwaukee, WI 53233. Telephone: (414) 288-7859. Fax: (414) 288-7066. E-mail: vasanthi.jayaraman@marquette.edu.

[‡] Marquette University.

[§] Max Planck Institute of Medical Research.

[1] Abbreviations: GluR, glutamate receptors; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; FTIR, Fourier transform infrared spectroscopy.

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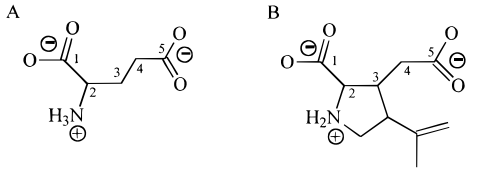

FIGURE 1: Chemical structures of (A) glutamate and (B) kainate.

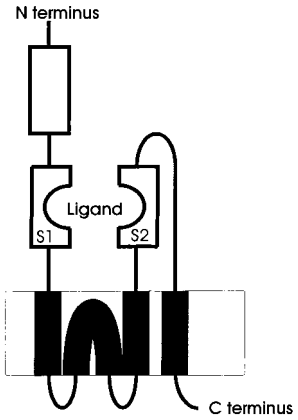

FIGURE 2: Schematic representation of the GluR4 subunit topology. The S1S2 protein contains the S1 and S2 domains joined by a linker (*10*).

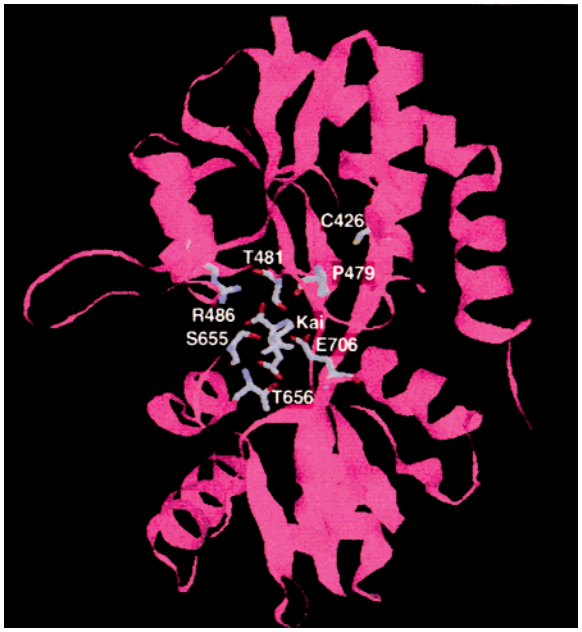

FIGURE 3: Structure of the GluR2 S1S2 protein in complex with kainate (*14*). The 5C carboxylate of kainate is hydrogen bonded to the backbone amine of T656 and S655, and the 1C carboxylate group is hydrogen bonded to R486 and T481. The amine group of the kainate is hydrogen bonded to the backbone carbonyl of P479, which is in proximity (4 Å) to C426. C426 is hydrogen bonded to the backbone carbonyl of either I477 or A478 (residues not shown). The residue numbering is based on the GluR4 subunit amino acid sequence.

structure of the prokaryotic glutamine-binding protein, it has been suggested that the S1S2 protein may exist in an open form in the unligated state. According to this hypothesis, kainate binding should induce an intermediate cleft closure, whereas that of glutamate should induce a more complete closure. The variable degrees of closure might correspond to functional differences between the agonists. On the other hand, solution-scattering studies failed to detect a cleft

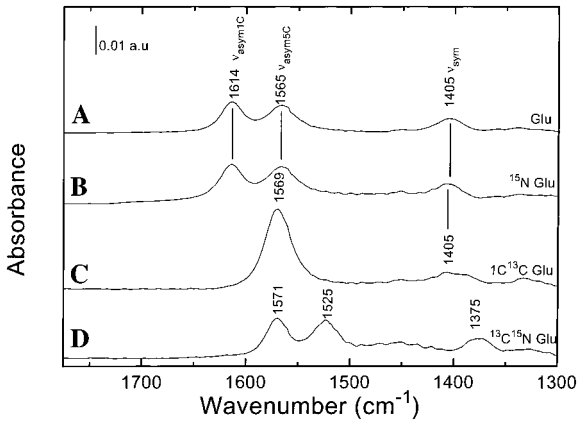

FIGURE 4: Difference FTIR spectrum between (A)glutamate and D_2O (Glu), (B) [^{15}N]glutamate and D_2O (^{15}N Glu), (C) [1C-^{13}C]-glutamate and D_2O (1C^{13}C Glu), and (D) [^{13}C,^{15}N]glutamate and D_2O (^{13}C^{15}N Glu).

closure of the expected magnitude in S1S2 from GluR4 upon glutamate binding (*15*). To analyze further the basis for the different effects of kainate and glutamate on GluR, we have used infrared vibrational spectra, which are extremely sensitive to the strength and nature of inter- and intramolecular interactions and which therefore provide detailed structural information about the protein in solution (*16*−*19*).

MATERIALS AND METHODS

Protein Preparation and Characterization. The GluR4 S1S2 protein was expressed, purified, and characterized as described previously (*15*). In brief, S1S2 was expressed as a secreted construct in the baculovirus system. Following clarification and concentration of the cell-culture supernatant, it was purified to homogeneity by immunoaffinity and ion exchange chromatography. Protein (0.25−0.5 mM) in 25 mM phosphate buffer containing 250 mM NaCl and 0.02% NaN_3 was used for the FTIR measurements. D_2O was used as the solvent to obtain the spectra in the 1450−1800 cm^{-1} region, since water has a large infrared absorption band at ∼1600 cm^{-1}. However, water was used as the solvent in studying the S−H stretching vibration.

FTIR Difference Spectroscopy. The FTIR spectra were obtained using a Nicolet Magna 560 apparatus, using an FTIR cell with CaF_2 windows, with a 50 μm spacer. Spectra were collected at 4 cm^{-1} spectral resolution. A modified variable-length sample holder (Aldrich, Milwaukee, WI) with CaF_2 windows was used to obtain the spectra. The modification of the sample holder allowed liquid from a constant-temperature bath to be circulated around the holder, which ensured that the protein solutions were kept at a constant temperature of 15 °C. A 50 μm path length was used to obtain the spectra in the 1450−1800 cm^{-1} region, and a 75 μm path length was used in studying the S−H vibration. The difference spectra, in Figures 5 and 6, were generated by subtracting the spectra of the unligated form of the protein from the spectra of the ligated from of the protein. The subtraction was performed using the band at 2045 cm^{-1} arising from the sodium azide present in the buffer as an internal standard. Furthermore, the peaks that arise from the unbound glutamate (or isotopes of glutamate, or kainate) were subtracted using a spectrum of glutamate (or isotopes of glutamate, or kainate) in D_2O.

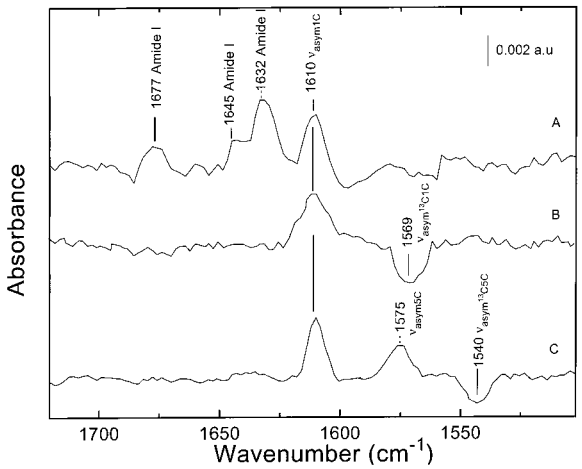

FIGURE 5: Difference FTIR spectrum (*26*) between (A) the glutamate-bound and unligated forms of the GluR4 S1S2 protein (*27*) in D_2O, (B) the glutamate-bound and [1C-^{13}C]glutamate-bound form of the GluR4 S1S2 protein in D_2O, and (C) the glutamate-bound and [^{13}C,^{15}N]glutamate-bound forms of the GluR4 S1S2 protein in D_2O.

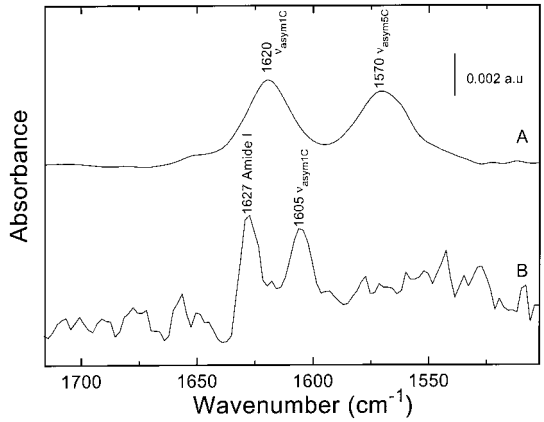

FIGURE 6: Difference FTIR spectrum (*26*) between (A) kainate and D_2O and (B) the kainate-bound and unligated forms of the GluR4 S1S2 protein in D_2O.

RESULTS AND DISCUSSION

Ligand-Binding Environment. To understand the different chemistries of interaction of these agonists with S1S2, we have compared their infrared spectra before and after binding to S1S2, using isotopically labeled reagents. Since the agonist asymmetric carboxylate vibrations are particularly sensitive to changes in electrostatic potential and hydrogen bonding strength (*20*−*22*), they provide an excellent monitor of differences in the chemical environments experienced by glutamate and kainate.

Figure 4 shows the vibrational spectra of glutamate and various isotopically labeled glutamates in D_2O. On the basis of the well established α-carbon substituent effect on the carboxylate frequency (*20*), the band at 1614 cm^{-1} can be assigned to the asymmetric stretching mode of the 1C carboxylate group (ν_{asym1C}; see Figure 1), while the band at 1565 cm^{-1} can be assigned to the asymmetric stretching vibration of the 5C carboxylate group (ν_{asym5C}). The symmetric stretching vibrations of both carboxylate groups give rise to the mode at 1405 cm^{-1} (ν_{sym}). These assignments (Table 1) are corroborated by the spectral shifts due to ^{13}C and ^{15}N isotopic labeling of the glutamate. ^{15}N substitution does not have an effect on the spectra (Figure 4B); ^{13}C substitution at the 1C position downshifts the band from 1614 to 1571 cm^{-1}, where it overlaps with and reinforces the unlabeled band at 1565 cm^{-1}. This substitution also downshifts part of the band at 1405 cm^{-1} (shoulder on the peak labeled 1405 cm^{-1}). All the bands (ν_{asym1C}, ν_{asym5C}, and ν_{sym}) are downshifted for glutamate in which all the carbons and the nitrogen are labeled with ^{13}C and ^{15}N, respectively (Figure 4D). Since the 1C and 5C carboxylates exhibit different frequencies for the asymmetric carboxylate vibrations, this mode is used to study the environment of these carboxylates in the S1S2 protein.

Table 1: Asymmetric Carboxylate Vibrations of the 1C and 5C Carboxylates

	ν_{asym1C} (cm^{-1})		ν_{asym5C} (cm^{-1})	
ligand	free	bound to S1S2	free	bound to S1S2
glutamate	1614	1610	1565	1575
[1C-^{13}C]glutamate	1571	1569	1565	1575
[^{13}C,^{15}N]glutamate	1571	1569	1525	1540
kainate	1620	1605	1570	not assigned

The difference spectrum between the glutamate-bound and unligated forms of the protein is shown in Figure 5A. In this difference spectrum, the bands that arise due to the bound glutamate have been determined using isotopically labeled glutamate. The difference spectrum between the protein bound to glutamate and the protein bound to [1C-^{13}C]-glutamate (Figure 5B) exhibits a positive band at 1610 cm^{-1} and a negative band at 1569 cm^{-1} (see Table 1 for frequencies and assignments). Thus, the difference feature in Figure 5A at 1610 cm^{-1} can be attributed to the ν_{asym1C} mode from the bound glutamate. Similarly, the difference spectrum between the protein bound to glutamate and the protein bound to [^{13}C,^{15}N]glutamate (Figure 5C) exhibits positive bands at 1610 and 1575 cm^{-1}, and a negative band at 1540 cm^{-1}. As in parts A and B of Figure 5, the peak at 1610 cm^{-1} reflects the unlabeled ν_{asym1C} mode. The negative peak at 1540 cm^{-1} is the ν_{asym5C} mode downshifted by isotopic labeling. The second positive band at 1575 cm^{-1} presumably represents a composite of a positive band at 1575 cm^{-1} (from the unlabeled ν_{asym5C} mode) and a negative band at 1570 cm^{-1} due to the downshifted ^{13}C-labeled ν_{asym1C} mode (as seen in Figure 5B). The assignments described above indicate that the ν_{asym5C} mode of the bound glutamate should give rise to a positive band at 1575 cm^{-1} in the difference spectrum in Figure 5A. However, this band cannot be clearly distinguished from the protein difference features. Compared to the frequencies of these modes in free glutamate (Figure 4), the ν_{asym1C} mode is downshifted by 4 cm^{-1} and the ν_{asym5C} mode is upshifted by 10 cm^{-1} upon binding to S1S2.

Similar difference spectra between free kainate and kainate bound to the S1S2 protein are shown in Figure 6. On the basis of the α-carbon substituent effect on the carboxylate frequency (*20*), and the observed frequencies for the carboxylate vibrational modes of amino acids, the ν_{asym1C} and ν_{asym5C} modes for kainate can be assigned to the bands at 1620 and 1570 cm^{-1}, respectively (Figure 6A). Since the crystal structure of the GluR2 S1S2 protein in complex with kainate shows that the 1C carboxylate is hydrogen bonded to a positively charged arginine, the mode is expected to be downshifted from 1620 cm^{-1} (*20*−*22*) (see below). Hence, in the difference spectrum between the kainate-bound and

unligated S1S2 protein (Figure 6B), the difference feature at 1605 cm^{-1} is assigned to the bound kainate. This assignment is also consistent with that for the glutamate-bound form of the protein. The frequency of the ν_{asym1C} band is downshifted by 15 cm^{-1} in bound kainate relative to its frequency in free kainate. This S1S2-induced shift is larger than that observed for the glutamate mode. The ν_{asym5C} mode has not been assigned for S1S2-bound kainate, since it presumably occurs in a region masked by protein bands, as seen in the difference spectrum for glutamate (Figure 5A).

The frequency of the asymmetric carboxylate stretching vibration is extremely sensitive to the overall electrostatic environment of the carboxylate and to a lesser extent to changes in hydrogen bonding strength (*20*−*22*). An increase in the frequency of this vibration can be caused by a more negative electrostatic potential and/or by a decrease in hydrogen bonding strength. In the crystal structure of kainate-bound S1S2 (*14*), the 1C carboxylate group of the kainate forms hydrogen bonds with protein side chains T481 and R486, while the 5C carboxylate forms hydrogen bonds with S655 and T656 and is within 4 Å of the carboxylate group of side chain E706 (the residues are numbered here according to the GluR4 subunit mature sequence; corresponding GluR2 residue numbers are smaller by 1) (Figure 3). Since the carboxylate groups of glutamate are bound in the same orientation as those of kainate in the S1S2 protein (*23*), the interpretation of the observed chemical shifts is straightforward. The upshift in the ν_{asym5C} vibrational mode of glutamate upon binding S1S2 is consistent with the proximity of the 5C carboxylate to the side chain carboxylate group of E706, which provides both a negative electrostatic environment and a less favorable hydrogen bonding environment for the similarly charged ligand carboxylate. Conversely, the downshift in the ν_{asym1C} mode for both glutamate and kainate can be attributed to the positively charged R486, which is hydrogen bonded to the 1C carboxylate. Furthermore, since the shift in the ν_{asym1C} mode is larger for the kainate mode than for the glutamate mode, it can be concluded that the kainate 1C carboxylate is closer to the arginine residue than the corresponding glutamate moiety and/or that it enjoys a more favorable hydrogen bonding geometry.

Structural Differences between the Kainate- and Glutamate-Bound Forms of S1S2. To gain insight into the protein tertiary structural changes induced by ligand binding, secondary structure changes were monitored using amide I signals, and the environment of the single non-disulfide-bonded cysteine residue in S1S2 was probed using the S−H stretching mode.

Secondary Structure. The frequencies of the features at 1677, 1645, and 1632 cm^{-1} in the difference spectrum between the glutamate-bound and unligated forms of the protein (Figure 5A) are characteristic of amide I modes arising from turns, α-helices, and β-sheet secondary structures, respectively (*24*). The positive bands at these frequencies indicate that there is a modest increase in the content of all three ordered secondary structure elements in the protein when it binds to glutamate. On the other hand, in the difference spectrum between the kainate-bound and unligated forms of the protein (Figure 6B), there is only one positive band (1627 cm^{-1}) not accounted for by the asymmetric carboxylate vibration of kainate. This suggests an increase in the β-sheet content of the protein, and no changes in the α-helical content or turns in the protein, upon binding kainate.

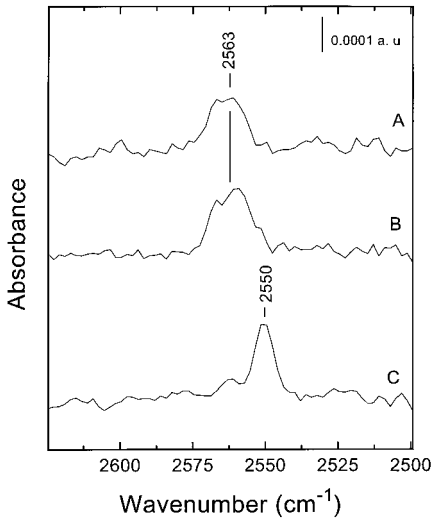

FIGURE 7: FTIR spectra of (A) unligated, (B) kainate-bound, and (C) glutamate-bound forms of the GluR4 S1S2 protein in H_2O.

Environment of Cysteine 426 in S1S2. The crystal structure of kainate-bound S1S2 (*14*) indicates that the single free cysteine residue in the domain (C426) is within 4 Å of proline 479. Since the backbone carbonyl of P479 is in turn hydrogen bonded to the ligand kainate (Figure 3), the S−H stretching mode of the C426 should be an excellent probe for studying changes in the GluR4 S1S2 protein induced by ligand binding. Furthermore, the S−H stretching mode occurs in a region that is well-separated from the vibrational bands of other protein moieties, and is extremely sensitive to even slight changes in hydrogen bonding strength that may not be able to be detected by X-ray crystallography (*25*, *26*).

The S−H stretching vibration of C426 in the S1S2 protein was monitored for the unligated, kainate-bound, and glutamate-bound forms in H_2O (Figure 7). The frequency of the S−H stretching band at 2563 cm^{-1} in the unligated state (Figure 7A) indicates that the S−H group forms a medium-strength donor hydrogen bond (*25*, *26*). On the basis of the GluR2 crystal structure, the possible hydrogen bond acceptor atoms that are closest to this Cys residue are the carbonyl oxygen atoms of A478 or I477. The crystal structure shows that both these atoms are ∼4 Å from the cysteine sulfur atom.

The C426 S−H stretching frequency is unaltered upon kainate binding (Figure 7B), indicating that the environment of this cysteine residue is unchanged in the presence of the ligand kainate. On the other hand, the frequency of this mode is lowered to 2550 cm^{-1} upon binding glutamate (Figure 7C). This decrease in frequency implies that the S−H hydrogen bond is stronger in the glutamate-bound form of the protein than in the unligated and kainate-bound forms (*25*, *26*). Cys426 is located at the junction of S1S2's two lobes. The different effects of glutamate and kainate on Cys426 may be mediated by Pro479, which is hydrogen bonded to the amine groups of the ligands. P479 is adjacent to the hydrogen bonding partners of C426 (I477 and A478), and its cyclized side chain is in proximity to the sulfhydryl group. The absence of an effect by kainate on the sulfhydryl environment suggests that the interactions of the amine group of kainate with the protein may be weaker than that of glutamate. This difference may reflect the steric constraints of the pyrrolidine ring on the kainate amine moiety, which are absent in

glutamate. It may also reflect the fact that R486 interacts more strongly with the kainate 1C carboxylate than with that of glutamate, which could be coupled to a weakening of the amine interaction for kainate. In either case, in this scenario, C426 would play the role of a sensor that helps to distinguish glutamate binding from kainate binding and that participates in the conformational changes that underlie the observed differences in desensitization behavior. A second possibility is that the C426 hydrogen bonding environment is altered as a result of conformational changes that are mediated by the interaction of other side chains with glutamate. In this case, C426 would function as a passive "monitor" of the different conformational changes.

This report identifies, for the first time, differences in the chemical interactions of specific kainate and glutamate functional groups with the ligand-binding domain of the glutamate receptor. Kainate interacts more strongly with R486 than does glutamate, whereas the hydrogen bonding of C426 is strengthened by glutamate binding, but not by kainate binding. At the level of secondary structure, kainate induces changes in the number of residues forming β-sheet but not α-helical or turn structures; glutamate induces changes in all three. Receptor function is mediated by induced conformational changes such as those detected here. Our data therefore provide candidate spectroscopic correlates of observed electrophysiological differences. The chemical information thus obtained may also serve as a bridge between crystallographic structure on one hand and electrophysiology and mutagenesis experiments on the other.

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