Cysteine Mutagenesis and Homology Modeling of the Ligand-binding Site of a Kainate-binding Protein*

(Received for publication, August 2, 1999, and in revised form, September 23, 1999)

Z. Galen Wo‡, Kamaldeep K. Chohan§, Haiying Chen‡, Michael J. Sutcliffe§, and Robert E. Oswald‡**

From the ‡Department of Molecular Medicine, Cornell University, Ithaca, New York 14853 and the §Department of Chemistry, University of Leicester, Leicester LE1 7RH, United Kingdom

Glutamate receptors comprise the most abundant group of neurotransmitter receptors in the vertebrate central nervous system. Cysteine mutagenesis in combination with homology modeling has been used to study the determinants of kainate binding in a glutamate receptor subtype, a low molecular weight goldfish kainate-binding protein, GFKARβ. A construct of GFKAR with no cysteines in the extracellular domain was produced, and single cysteine residues were introduced at selected positions. N-Ethylmaleimide or derivatized methanethiosulfonate reagents (neutral or charged) were used to modify the introduced cysteines covalently, and the effect on [3H]kainate binding was determined. In addition, cysteine mutants of GFKARβ transiently expressed in HEK293 cells were labeled with a membrane-impermeable biotinylating reagent followed by precipitation with streptavidin beads and specific detection of GFKARβ by Western blot analysis. The results are consistent with the proposal that the energy driving kainate binding is contributed both from residues within the binding site and from interactions between two regions (i.e. two lobes) of the protein that are brought into contact upon ligand binding in a manner analogous to that seen in bacterial amino acid-binding proteins.

Glutamate receptors (GluRs) are the major excitatory receptors in the vertebrate central nervous system and have been implicated in a number of normal and pathologic processes including synaptic plasticity, epilepsy, and ischemic cell death (1). GluRs consist of two large superfamilies of membrane-bound receptors: (i) metabotropic receptors, which are linked by G proteins to second messengers such as phospholipase C, and (ii) ionotropic receptors (iGluRs), which are ligand-gated ion channels. The iGluRs have been subdivided based upon agonist sensitivity and sequence homology into three subfamilies (2, 3): (i) N-methyl-D-aspartate receptors (R1–3), (ii) α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors (Glur1–4), and (iii) kainate receptors (Glur5–7, KA1–2, and kainate-binding proteins from nonmammalian vertebrates). For all these subtypes, some uncertainty exists over the subunit stoichiometry; each receptor is thought to be composed of either four (4–6) or five (7) subunits that form functional ion channels in the cell membrane.

iGluRs have a distinct modular structure (Fig. 1A and Ref. 8). This consists of (i) one (S1S2, also known as LAOBP-like) or two (both S1S2 and leucine, isoleucine, valine-binding protein-like) domains that are homologous to bacterial amino acid-binding proteins (9, 10), (ii) a channel domain that has some similarities to cyclic nucleotide-gated and potassium channels (8, 11), and (iii) a C-terminal domain that mediates regulation by phosphorylation (12) and binds intracellular proteins (13–15). In particular, the agonist and antagonist-binding domain (S1S2 domain; Refs. 8, 10, and 16) consists of a portion of the N terminus of the protein before the first transmembrane segment (M1) and a large portion of the protein between the third and fourth membrane associated segments (M3 and M4). The S1S2 domain has been modeled previously based on the relatively low homology with bacterial amino acid-binding proteins (16–22) and can be expressed independently in bacteria (23, 24) and insect cells (25, 26) with an apparently native fold. From homology modeling and site-directed mutagenesis, six regions (R1–R6; Fig. 1B) were suggested to contribute to the binding site. The three-dimensional structure of the S1S2 domain of Glur2 has been solved recently by x-ray crystallography (27). Although differences exist between the crystal structure and previous homology models, the overall structure and many of the contributions to the binding site were predicted correctly. With the availability of a three-dimensional structure of Glur2, mutagenesis studies are particularly interesting for comparing different subtypes and for testing the importance of specific interactions. In particular, the static crystal structure of the agonist-binding form suggests that the two lobes comprising the S1S2 domain may close to surround the ligand upon binding, but further evidence is required to demonstrate this experimentally.

Cysteine mutagenesis is an extremely useful tool for probing structural features of proteins (28, 29) if (i) individual mutations can be made in the absence of a cysteine background (to avoid both spurious disulfide bonds and adventitious interactions with sulphydryl reagents) and (ii) the mutation can be made with little or no change in the binding site. Postexpression modifications of the protein can then be made by treat-
Cysteine Mutagenesis of a Kainate-binding Protein

Cysteine Mutagenesis of a Kainate-binding Protein

Fig. 1. A, transmembrane topology of iGluRs showing the position of the S1S2 domain. The kainate-binding proteins, including GFKARβ, do not have the shaded N-terminal domain prior to S1S2. B, sequence of the S1S2 domains of GluR2 and βA5. The positions of R1–R6 are shown in gray. The three mutations of Cys to Ser are shown white on black. The positions of the 13 cysteine substitution mutants are boxed. Filled triangles indicate additional residues playing an important role in binding according to Armonth et al. (27). Those residues absent from the crystal structure are shown in lowercase letters. The figure was generated using ALSCRIPT 2.0 (44).

EXPERIMENTAL PROCEDURES

Materials—[3H]Kainic acid (60 Ci/mol) was purchased from NEN Life Science Products. The polyclonal anti-peptide antiserum Ab-β1 against GFKARβ was produced by Research Genetics (Huntsville, AL; Ref. 32). N-Ethylmaleimide was purchased from Sigma. The MTS reagents methyl methanethiosulfonate (MMTS), 2-aminoethyl methanethiosulfonate hydrobromide (MTEA), [2-(trimethylammonium)ethyl] methanethiosulfonate bromide (MTESET), and sodium (2-sulfonatoethyl) methanethiosulfonate hydrobromide (MTSEA) were purchased from Toronto Research Chemicals, Inc. (North York, Canada). Biotin-HPDP and streptavidin beads were purchased from Pierce. cDNA sequencing was performed by the Cornell DNA Sequencing Facility.

Expression in Cultured HEK 293 Cells—cDNAs were cloned from the pRBG4 vector into the expression vector, pcDNA1.1/Amp (Invitrogen). HEK 293 cells, grown to 6 × 10⁶ cells/10-cm dish, were transfected (20 μg of plasmid DNA/dish) using the calcium phosphate precipitation method, and cell membranes were prepared 48 h after transfection as described previously (31). Membrane pellets from each dish were resuspended in 0.5 ml (1 mg protein/ml) of 1 mM phenylmethylsulfonyl fluoride, lysed by sonication, and centrifuged (12,000 × g) for 25 min. The pellets containing membrane preparations were resuspended in 1 ml of solubilization buffer (1% Triton X-100 and 0.1% SDS in 1× PBS) and centrifuged. The supernatant contained solubilized membrane proteins that were used for subsequent analysis. For each sample, 800 μl of the solubilized membrane proteins were incubated with 120 μl of streptavidin-linked beads at 4 °C for 4 h. The beads were then centrifuged briefly in a microcentrifuge and washed three times with solubilization buffer. Proteins were eluted from the streptavidin-linked beads using 20-μl washes with 0.1 M glycine-HCl buffer (pH 2.2). The samples were mixed with 30 μl of 5× SDS gel loading buffer and heated for 5 min. Samples were compared using silver-stained gels before Western blotting. Each Western blot shown in Fig. 4 is representative of four to five independent replicates.

Construction of Cysteine Substitution Mutants—The cDNAs coding for the three Cys to Ser single point mutations βC278, βC305S, and
Cysteine Mutagenesis of a Kainate-binding Protein

RESULTS

Construction of βΔC5—Wild type GFKARβ contains a total of 10 cysteines in the fully processed protein, with an additional two in the signal sequence (30). Four of these cysteines are in membrane spanning regions (two in M1 and two in M3), and two in the signal sequence (30). Four of these cysteines are in the extracellular domain. A 30-residue truncation of the C-terminal domain was mutated to serine. Cys305 and Cys357 form a disulfide bond by reducing agents or mutation to serines resulting in a protein with higher affinity for kainate (31, 36). The S1S2 domain of GluR2 was therefore used as the only structural template in the homology modeling (18) of GFKARβ. Our previous sequence alignment (17), including both GFKARβ and GluR2, was used in conjunction with the program MODELLER (35) to generate a set of 10 models of the kainate-bound form of GFKARβ. To achieve this, kainate was added to the MODELLER topology library; this then allowed MODELLER to reproduce automatically in the models protein-kainate interactions that were present in the crystal structure of GluR2 and for which equivalent sequences are present in GFKARβ. Consistent stereochemical violations across this set of 10 models were removed by manual adjustment of the sequence alignment using CAMELEON (Oxford Molecular, Oxford, UK); the adjustment was determined by visual inspection, using InsightII (MSI, San Diego, CA), of the stereochemically strained regions of the models. In adjusting the sequence alignment, care was taken wherever possible not to introduce insertions and deletions into the crystallographically determined secondary structural elements of GluR2. Once the sequence alignment had been adjusted to remove all consistent stereochemical violations, the sequence alignment was used to produce a final set of 10 models. The model with the lowest energy was selected and analyzed in detail. In particular, the ability of the model to accommodate the sulfhydryl reagents was investigated using interactive molecular graphics (InsightII).

βC358S, Fig. 1B and Ref. 31) were used to create a mutant with all three extracellular cysteines mutated to serine. In addition, a 30-residue C-terminal deletion was introduced at the end of the cDNA coding sequence by PCR to remove Cys410 and Cys411. An antisense primer (5′-CCGCTCAGATTAGGGATCTCCTG-3′) was designed to encode a stop codon and a XhoI site after the Ser419, was used to amplify βC358S cDNA by PCR. The PCR DNA was cut with XhoI and XhoI and used to replace the corresponding DNA sequence (310 base pairs) of βC358S. Next, an EcoRI/EcoRV fragment (400 base pairs) was exchanged with the corresponding one of β278S. This resulted in a construct (βΔC5) with no extracellular cysteines and no cysteines in the C-terminal domain (i.e., βΔC5 provides a background free of reactive cysteines). The βΔC5 mutant served as a template for the introduction of thirteen individual cysteine substitution mutants. The overlapping PCR method was used to introduce the single-point mutations. In addition, a silent mutation, creating a new restriction site, was introduced with each point mutation through overlapping primers to facilitate identification of the mutant clones. The overlapping primer pairs were: βD13C, 5′-TCTCACTTTGCCGACAGTTGAGA-3′ (sense) and 5′-CTCCACATTGGTACGAGG-3′ (antisense); βD16C, 5′-GCCCGTTCGAGATCGGTGCG-3′ (sense) and 5′-CCGCTCAGATTAGGGATCTCCTG-3′ (antisense); βG5C, 5′-CCGCTCAGATTAGGGATCTCCTG-3′ (sense) and 5′-CCGCTCAGATTAGGGATCTCCTG-3′ (antisense); ββ271C, 5′-GGAACGACATAGACTACCGGCGGATCGTGTGCTGAGAAC-3′ (antisense); β286C, 5′-GATGAGCTGACGGGCCAAATACCCACCATTTGACG-3′ (antisense); β289C, 5′-GATGAGCTGACGGGCCAAATACCCACCATTTGACG-3′ (antisense); and β289C, 5′-GATGAGCTGACGGGCCAAATACCCACCATTTGACG-3′ (antisense) and β286C, 5′-GATGAGCTGACGGGCCAAATACCCACCATTTGACG-3′ (antisense) and 5′-CCGCTCAGATTAGGGATCTCCTG-3′ (antisense).

The six PCR fragments (lobe 1 mutations) containing the βD13C, βD21C, βR54C, βD17C, and βA84C mutations were cut with EcoRI and EcoRV, and the resulting cassettes of 400 base pairs were exchanged for the corresponding fragment of βΔC5 cDNA in the pcDNAI/Amp vector. The seven PCR fragments (lobe 2 mutations) containing the βS245C, βT247C, βS258C, βK282C, βS275C, and βS296C mutations were cut with EcoRV or XhoI, and the resulting cassettes of 900 base pairs were exchanged for the corresponding fragment of βΔC5 cDNA in pcDNAI/Amp vector. The selected clones containing the desired mutations were verified by DNA sequencing.

Molecular Modeling—The first step in the modeling procedure (18) was to derive the amino acid sequence of GFKARβ into domains and to extract the amino acid sequence corresponding to the S1S2 domain (17). Scanning through the proteins of known three-dimensional structure, the kainate-bound form of the S1S2 domain of GluR2 (27) had by far the highest sequence homology to the corresponding domain in GFKARβ (40% identity compared with a maximum of ~20% among other structures). The S1S2 domain of GluR2 was therefore used as the only structural template in the homology modeling (18) of GFKARβ. Our previous sequence alignment (17), including both GFKARβ and GluR2, was used in conjunction with the program MODELLER (35) to generate a set of 10 models of the kainate-bound form of GFKARβ. To achieve this, kainate was added to the MODELLER topology library; this then allowed MODELLER to reproduce automatically in the models protein-kainate interactions that were present in the crystal structure of GluR2 and for which equivalent sequences are present in GFKARβ. Consistent stereochemical violations across this set of 10 models were removed by manual adjustment of the sequence alignment using CAMELEON (Oxford Molecular, Oxford, UK); the adjustment was determined by visual inspection, using InsightII (MSI, San Diego, CA), of the stereochemically strained regions of the models. In adjusting the sequence alignment, care was taken wherever possible not to introduce insertions and deletions into the crystallographically determined secondary structural elements of GluR2. Once the sequence alignment had been adjusted to remove all consistent stereochemical violations, the sequence alignment was used to produce a final set of 10 models. The model with the lowest energy was selected and analyzed in detail. In particular, the ability of the model to accommodate the sulfhydryl reagents was investigated using interactive molecular graphs (InsightII).
TABLE I

| Mutant     | $K_D$ (−NEM) | $K_D$ (+NEM) |
|------------|--------------|--------------|
| βD13C     | 42 ± 8       | 88 ± 10      |
| βS21C     | 43 ± 4       | 42 ± 3       |
| βA51C     | 24 ± 1       | 61 ± 3       |
| βG71C     | 48 ± 6       | 48 ± 3       |
| βA84C     | 17 ± 2       | 13 ± 3       |
| βS245C    | 64 ± 13      | 63 ± 8       |
| βS258C    | 28 ± 3       | 19 ± 3       |
| βS271C    | 104 ± 23     | 102 ± 17     |
| βS275C    | 20 ± 2       | 20 ± 1       |
| βK393C    | 42 ± 4       | 29 ± 4       |
| βS296C    | 28 ± 10      | 114 ± 23     |

$B_{max}$ was decreased in NEM-treated membranes relative to untreated membranes.

roughly consistent with the 3-fold increase in kainate affinity when the disulfide bond alone is removed by mutation of either Cys305 or Cys307 to Ser (31) but with otherwise normal binding characteristics. The expression level was approximately 2–3-fold lower than wild type judging from the total binding and Western blot analysis.

**Design and Expression of Cysteine Substitution Mutations**

The strategy was to produce mutations to cysteine in or near the glutamate-binding site or at the interlobe interface. The two considerations were (i) to make relatively conservative changes and (ii) to place the mutations in positions that would not interfere with folding or of interest in interlobe interactions. βD13C is also near the domain interface. The βD13C mutation itself decreases affinity by 3-fold (Table I), and NEM decreases the binding by approximately 30% (Figs. 2B and 3B). The MTS series of sulfhydryl reagents (Fig. 3A) show results similar to that of NEM (Fig. 3B), suggesting that modification of this position most likely produces only a modest steric inhibition at the interlobe interface. The conserved Tyr52 is critical for ligand binding (31, 37), and two residues flanking Tyr52 were tested here. In a previous study (31), βA51K decreased kainate affinity (31), although in these studies the mutational analysis does not distinguish changes in folding from changes in direct interactions with ligand. The modest effects of NEM, MMTS, and MTSES on βA51C (Fig. 3B) may be consistent with a minor allosteric contribution of this residue to binding. The GluR2 structure and the homology model described below (see Fig. 5A) place this residue near the interlobe interface. Thus, the contributions of this residue to the binding energy would be due to allosteric interactions rather than a direct contact with the ligand. Notably, however, the positively charged MTSEA and MTSET had no effect on [3H]kainate binding to βA51C. In contrast, [3H]kainate binding to βS21C, βS54C, βG71C, and βA84C is unaffected by NEM (Fig. 2B). The residues corresponding to βS21C, βS54C, βG71C, and βA84C in GluR2 are solvent exposed in the crystal structure (27) and the GFKARβ homology model based on GluR2 and therefore predicted not to be involved in either agonist binding or the interlobe interface. In the absence of agonist and antagonist, all cysteine mutations (in both lobe 1 and lobe 2) could be labeled in situ with biotin-HPDP (Fig. 4, B and C; see below).

In lobe 2, binding to βS245C is strongly affected by NEM modification. βS245C is adjacent to two serines that are involved directly in agonist binding in GluR2. The βS245C mutation alone decreases the binding affinity relative to wild type by 2-fold (Fig. 2B and Table I), and modification by NEM reduces $B_{max}$ relative to unmodified βS245C by 2- to 5-fold. The decrease in $B_{max}$ (with no evidence for a second binding site) likely indicates that binding in all modified receptors is undetectable and that the binding that was detected may be to unmodified receptors. This is not unexpected given the predicted close proximity of Ser245 to kainate in the binding site (as judged from the homology model presented below). Interestingly, NEM, MMTS, MTSEA, and MTSET all significantly decrease binding to βS245C. In contrast, negatively charged MTSES has little or no effect.

Another mutation is strongly affected by NEM modification (βS296C). βS296C is near Glu293, which is predicted to interact with the amide of kainate and mutation of which severely affects ligand binding (38). NEM modification of βS296C could easily disrupt this interaction. However, it is more likely to interact directly with kainate; kainate comes within 6 Å of the side chain of S296, and the NEM could therefore partially block the binding site. The modification of βS296C by MTSEA, MTSET, and MTSES decreases binding to the same or a greater extent as NEM, MMTS, on the other hand, has little effect. This result suggests that the effects of the modification are likely to be steric rather than affected by charge.

Previous mutagenesis results and modeling studies (17, 18, 20, 21, 38) differed significantly in their interpretations of the
role of the sequence between R4 and R6 (Fig. 1B). The cysteine substitution mutations βS258C, βS271C, βS275C, and βK282C were introduced to explore the role of this region. βS258C was chosen as a site in lobe 2 that was not expected to be associated with the binding site. βS271C is homologous to Ser684 of GluR6, which was originally thought to be a site for cAMP-dependent protein kinase phosphorylation (39, 40). Ser275 is the position equivalent to Asn 721 in GluR6, the residue that seems to be involved in controlling affinity for AMPA (21). Finally, the conserved Lys282 was originally thought to be in the binding site based on modeling studies (17). All four mutants expressed normally (Fig. 2A) but are insensitive to NEM modification (Fig. 2B), suggesting that all four are outside of the binding site. This interpretation is entirely consistent with the GluR2 structure (and our GluR2-based model), which places these residues away from the binding cleft (27). However, although the mutation of Ser271 to Cys did not affect expression, it decreased noticeably affinity for [3H]kainate (104 ± 23 nM versus 13 ± 2 nM for βΔC5). As will be discussed below, this site is likely to be involved in a conformational change associated with ligand binding.

Biotinylation of Cysteine Substitution Mutants—Of the total of 12 cysteine mutants of GFKARβ, four (D13C, A51C, S245C, and S296C) showed sensitivity of [3H]kainate binding to chemical modifications by NEM or MTS derivatives. Labeling with the membrane-impermeable biotinylation reagent (biotin-HPDP) was used to address the question of whether introduced cysteine residues are solvent exposed and, more interestingly, whether ligand-binding would change the accessibility.

HER293 cells expressing single cysteine mutants were reacted with biotin-HPDP. After solubilization of the membrane proteins, proteins with covalently bound biotin were recovered on and subsequently eluted from streptavidin-linked beads. Western blots were then used to determine whether the mutant GFKARβ was among the proteins eluted from the streptavidin-linked beads, indicating labeling by biotin-HPDP. Wild type GFKARβ was not labeled by biotin-HPDP (Fig. 4A, lanes 1 and 2), consistent with the fact that of the three extracellularly located cysteines, Cys305 and Cys358 form a disulfide bond (31), and Cys27 is partially buried. The C305S mutant was readily labeled (Fig. 4B, lanes 3 and 4) because the disulfide can no longer form and Cys358 becomes a free and solvent-exposed cysteine. βΔC5, which has the three extracellularly located cysteines removed, showed no biotin-HPDP labeling, as expected (Fig. 4C, lanes 5 and 6). All cysteine substitution mutants on lobe 1 could be labeled with biotin-HPDP (Fig. 4B), indicating that they are all solvent exposed. Of these, the labeling of βD13C was inhibited by kainate. The modest effect of NEM on binding and the potent inhibition of biotin labeling by kainate supports the notion that

---

**Fig. 3.** A, structure of the sulfhydryl reagents used to modify the cysteine substitution mutants of βΔC5. The leaving group of the MTS reagents is shaded; only the unshaded portion of each molecule is added to the cysteine in place of the sulfhydryl hydrogen. For NEM, the point of attachment is denoted by an arrow. B, the effect of sulfhydryl reagents on the binding of [3H]kainate to five cysteine substitution mutants of βΔC5. Each bar is the mean of three independent determinations. (Note that for each mutant, “100%” corresponds to the binding of kainate to the respective mutant in the absence of thiol reagents.)
Cysteine Mutagenesis of a Kainate-binding Protein

was less potent (Fig. 4kainate and glutamate inhibited biotin labeling, but CNQX

versus

much higher sequence homology (sequence identity of 40% versus 40%) that likely corresponds to a more similar struc-
ture. The differences between the previous homology models and the crystal structure arise for two reasons: (i) difficulties in aligning the sequences of the iGluRs with the sequences of the LAOBP-like structures, leading to errors in mapping structural features from the templates to the models and (ii) the kainate-bound form of GluR2, unlike complexes of the LAOBP-like structures, is thought not to have undergone full domain closure (26). Solvent accessibility calculations on the model of GFKARβ (using NACCESS) reveal that, unlike in the previous models, the reactive thiol is exposed to solvent in all 13 introduced cysteine residues. The accuracy of the new GluR2-based models is supported further by the finding that all cysteine substitution mutants could be labeled with biotin-HPDP (Fig. 4), as predicted by the model.

DISCUSSION

The model of GFKARβ built based on the GluR2 S1S2 domain template can guide the interpretation of mutagenesis studies, help to demonstrate differences in subtypes, and provide information on conformational changes upon binding. Both mutagenesis studies and the GluR2 structure suggest that agonist affinity is controlled by two classes of residues: (i) those that are found in the binding site interacting with ligand and (ii) those that are involved in interlobe contacts, which in turn can affect the energetics of lobe closure and therefore indirectly affect ligand binding. Homology modeling of glutamate receptors based on bacterial amino acid-binding proteins identified six regions (R1–R6) thought to form the binding site (18, 19). Of those regions, R2, R3, R4, and R6 are found in the binding site. Amino acids in R1, R2, R4, and R5 are involved in interlobe contacts.

Binding Site—Considering first the residues that line the binding pocket (Fig. 5B), most determinants of binding seem to be well conserved across iGluR subtypes. In R2, Tyr562 interacts with the pyrrolidino ring and isopropenyl groups of kainate (27), and mutation to serine (but not phenylalanine) completely eliminates binding activity (31). The amino acid at this position is tyrosine or phenylalanine in all iGluRs. In R3, a highly conserved arginine residue (Arg86) forms a salt bridge to the α-carboxyl group of kainate. In an AMPA receptor, the modification of the homologous Arg (Arg2975) to Lys was sufficient to abolish binding (37, 38). The homologous mutation in the chick kainate-binding protein also completely abolished binding, although this residue was excluded from the binding site in the model of that protein (20).

In R4, T247 interacts with the carboxymethyl group of kainate, and mutation of this residue to cysteine, as described above, completely abolishes binding but not expression of the protein. In the model, Ser245 is also in a position to form a hydrogen bond with the carboxymethyl group of kainate. The equivalent position in GluR2 is Gly562. Mutation of Ser245 to cysteine decreases binding affinity by 4–5-fold relative to βΔC5, suggesting that this hydrogen bond could form but that its contribution to the binding energy is less than Thr247. Modification of βS245C with NEM and the MTS reagents reveals an interesting pattern (Fig. 5B). MMTS, MTSEA, and MTSET significantly reduce binding, but negatively charged MTSES has no effect. This is a bit surprising because the MTS-modified S245C would be in a position to interact with Arg86 (the residue in R3 that forms the salt bridge with the 2-carboxyl group of kainate) and the amide of Gly53. The negative charge could interfere with binding both because of its proximity to the kainate carboxyl and its putative interaction with R86. However, as shown in Fig. 5C, MTSES can be positioned so as to allow kainate to bind. In contrast, the positively charged MTSET can lie in the same position as the amide of kainate (Fig. 5D), thereby blocking the binding site. Interes-
ingly, βS245C can be labeled with biotin-HPDP both in the presence and absence of kainate. This would suggest that at least in the cysteine mutant, position 245 is solvent exposed in the bound complex. Thus, Ser245 is likely to contribute only a small amount of binding energy and, because the equivalent position is glycine in AMPA receptors as well as GluR5, GluR6, and GluR7, its role may be unique to kainate-binding proteins.

The highly conserved Glu293 in R6 coordinates the amino group of kainate. βS296C has no effect on kainate affinity, but modification by sulfhydryl reagents significantly affects binding. As shown in Fig. 3B, NEM and the MTS reagents (with the exception of MMTS) dramatically decrease kainate binding. Presumably, this is a steric effect because MMTS is the smallest reagent used, and both positively and negatively charged reagents have the same effect. The side chain is predicted to point directly into the binding site and could easily block the binding of kainate (Fig. 5E). As indicated by inhibition of biotin-HPDP labeling by kainate, glutamate, and CNQX, this site is no longer solvent exposed in the presence of agonist or antagonist.

**Interlobe Interface**—The interlobe interface consists of several points of contact that can affect the ligand specificity allosterically (21, 27). Two points of contact have been identified in the crystal structure of GluR2 (27) and consist of contacts between R1 and R5 and between R2 and R4. In GluR2, the corresponding contacts are Glu402-Thr686 and Lys449-Asp651-Ser652. The fact that GFKARβ has a Gln (Gln12) corresponding to Glu402 and an Ala (Ala51) corresponding to Lys449 could provide additional insight into this interface. Q12E results in an increase in kainate affinity (31), suggesting that, as with the
equivalent residue Glu402 in GluR2, this residue could be involved in interlobe interactions. Inspection of the model suggests that a hydrogen bond with the backbone amide of Met276 (the position equivalent to Thr686 of GluR2, Fig. 1B) is the most likely interaction and that interaction with Ser275 is unlikely. The latter is consistent with other experiments; with Glu in position 12, βS275C does not affect kainate affinity, nor does modification of βS275C by NEM. Both βD13C and βS275C are occluded upon agonist binding in that they are no longer available for biotin-HPDP labeling in the presence of either agonist or antagonist. This is consistent with the positioning of these residues outside of the binding site but at the domain interface. The fact that the residues are exposed in the absence but not the presence of agonist and antagonist is suggestive of a domain closure similar to that seen in bacterial amino acid-binding proteins. Because Ser275 is analogous to the position in GluR6 that controls AMPA affinity (21), the differences in agonist affinity between AMPA and kainate receptors maybe be at least partially because of allosteric interactions at the interlobe interface rather than to direct contact with ligand.

βA51K decreases the binding affinity for kainate by 2.5-fold (31). NEM treatment of βA51C decreases kainate affinity by approximately 3-fold (Fig. 2B). Perhaps surprisingly, however, MTSEA and MTSET have no effect on βA51C (Fig. 5B). This suggests that the aliphatic chain of these two reagents could produce a hydrophobic interaction that cannot be produced by the shorter aliphatic chain of lysine. Linked to this hypothesis, the model also suggests the possibility that the charged groups on the longer MTSEA and MTSET could interact with Asp56, whereas a lysine could not reach this far. Thus, at least in the case of kainate binding, the putative interaction between the lobes at this site does not extensively affect ligand affinity. This is supported by the finding that βA51C was accessible to biotin-HPDP in the presence and absence of kainate, suggesting that, at least in its unmodified form, conformational changes resulting from ligand binding do not affect the solvent accessibility of this residue.

βS271C—Position 271 is predicted to be neither in the binding site nor at the interdomain interface. Nevertheless, the βS271C mutation decreased binding affinity by more than 4-fold. Likewise, the labeling by biotin-HPDP is decreased in the presence of kainate and glutamate and consistently less so with CNQX. Similar results have been observed in GluR6 (34). Although this position is at the end of a stretch of relatively low homology between GFKAR and GluR2, it is almost certainly located in the loop between the G and H helices and is outside of the cleft that defines both the binding site and the interface between the two lobes. The postulated conformational change, which accompanies binding and which is based on homology with bacterial amino acid-binding proteins, is assumed to be a closure of the two lobes. Because the conformational change decreases the solvent accessibility of Ser271, which is neither at the binding site nor the interlobe interface, a modification of the structure of at least lobe 2 is likely to take place in addition to the lobe closure. Thus, conformational changes accompanying binding likely involve more than a rigid body movement of the two lobes relative to each other and may differ for agonists and antagonists. This position is of particular interest because in GluR6, a mutation to alanine abolishes the effects of cAMP-dependent protein kinase phosphorylation (39, 40).

Site-directed mutagenesis has been used to probe the ligand-binding site of various iGluRs. These included, among others, the AMPA receptor subunits (37, 38, 41), the kainate receptor subunits (21), chick (20, 42) and goldfish (31) kainate-binding proteins, and the N-methyl-D-aspartate receptor subunits (22, 43). The results of these studies have provided valuable information that was an indispensable component in the development of homology models (17–19). However, the interpretation of mutagenesis results is complicated by two factors: (i) In some cases, differential effects of mutations at homologous sites of different subtypes occurred. For example, E424Q and E424A of GluRD (GluR4; Ref. 38) did not affect kainate binding, but the homologous E33V mutation of chick kainate-binding protein decreased kainate binding by greater than 10-fold (20) and the homologous Q12E mutant of GFKARβ increased affinity for kainate and glutamate (31). (ii) The loss of binding activity cannot be unambiguously interpreted as a loss of a specific interaction in the binding site because it could equally be because of a large structural disturbance. In the case of a model of the chick kainate-binding protein (20), some key residues with dramatic effects on ligand binding (e.g. Arg307 and Glu316) were placed outside of the binding site, resulting in a model of the binding site with no positively charged residues and based on hydrogen bonding alone. Cysteine substitution mutagenesis can partially overcome some of the problems of standard mutagenesis. Assuming that the mutation to cysteine does not greatly affect binding and that the cysteine is accessible for modification, the effects of cysteine modification are less likely to result in misfolding of the protein. Furthermore, a single mutation can be modified by a variety of sulphydryl reagents to test the effects of positive and negative charges. Thus, cysteine mutagenesis is a useful complement to standard mutagenesis, homology modeling, and direct structural analysis.

Summary—With the availability of an atomic resolution structure of GluR2, which has considerable sequence identity to GFKARβ, and the subsequent production of a model based on the GluR2 structure, the interpretation of the cysteine mutants and their modification by sulphydryl reagents as well as site-directed mutagenesis can be made with a much higher level of confidence than has been previously possible. The results support the notion that the energy for binding kainate is contributed both by essential residues in the binding site as well as by allosteric interactions between two distinct regions of the protein. The use of biotin-HPDP to assess the solvent accessibility of each cysteine substitution provided additional information on the conformational changes associated with binding. The findings support previous suggestions that the closure of the two lobes accompanies binding (8, 16); however, the changes in the accessibility of position 271 suggest that the closure of the lobe likely involves more than a rigid body movement of the lobes. Furthermore, based on the inhibition of biotin labeling for residues 13 and 275, the lobe closure can occur both upon binding of agonist and antagonist, suggesting that a more subtle conformational change may be associated with gating.

Acknowledgments—We thank Michael Francis, Bruce Kornreich, Rob McFeeters, and Gregory Weiland (Cornell University) for helpful discussions.

REFERENCES
1. Monaghan, D. T., Bridges, R. J., and Cotman, C. W. (1989) Annu. Rev. Pharmacol. Toxicol. 29, 365–402.
2. Dingledine, R., Borges, K., Bowie, D., and Traynelis, S. F. (1999) Pharmacol. Rev. 51, 7–61.
3. Höllmén, M., and Heinemann, S. (1994) Annu. Rev. Neurosci. 17, 31–108.
4. Rosenmund, C., Stern-Bach, Y., and Stevens, C. F. (1998) Science 280, 1596–1599.
5. Laube, B., Kuhn, J., and Betz, H. (1998) J. Neurosci. 18, 2954–2961.
6. Mano, I., and Teichberg, V. I. (1998) Neuron 20, 327–331.
7. Premkumar, L. S., and Auerbach, A. (1997) J. Gen. Physiol. 110, 485–502.
8. Wo, Z. G., and Oswald, R. E. (1995) Trends Neurosci. 18, 161–168.
9. Nakashiba, N., Schneider, N. A., and Axel, R. (1990) Neuron 5, 569–581.
10. O’Hara, P. J., Sheppard, P. O., Thegersen, H., Venezia, D., Haldeman, B. A., McGrane, V., Houamed, K. M., Thomsen, C., Gilbert, T. L., and Mulvihill, E. R. (1993) Neuron 11, 41–52.
11. Wood, M. W., VanDongen, H. M. A., and VanDongen, A. M. J. (1995) Proc.
Cysteine Mutagenesis of a Kainate-binding Protein

12. Tingley, W. G., Roche, K. W., Thompson, A. K., and Huganir, R. L. (1993) Nature 364, 70–73
13. Dong, H., O’Brien, R. J., Fung, E. T., Lanahan, A. A., Worley, P. F., and Huganir, R. L. (1997) Nature 386, 279–284
14. Nishimune, A., Isaac, J. T., Mohar, E., Noel, J., Nash, S. R., Tagaya, M., Collingridge, G. L., Nakanishi, S., and Henley, J. M. (1998) Neuron 21, 87–97
15. Kornau, H. C., Schenker, L. T., Kennedy, M. B., and Seeburg, P. H. (1995) Science 269, 1737–1740
16. Stern-Bach, Y., Bettler, B., Hartley, M., Sheppard, P. O., O’Hara, P. J., and Heinemann, S. F. (1994) Neuron 13, 1345–1357
17. Sutcliffe, M. J., Smeeton, A. H., Wo, Z. G., and Oswald, R. E. (1998) Methods Enzymol. 293, 589–620
18. Sutcliffe, M. J., Smeeton, A. H., Wo, Z. G., and Oswald, R. E. (1998) Biochem. Soc. Trans. 26, 450–458
19. Paas, Y., Eisenstein, M., Medevielle, F., Teichberg, V. I., and Devillers-Thiery, A. (1996) Neuron 17, 979–990
20. Sutcliffe, M. J., Gereau, R. W., Green, T., and Heinemann, S. F. (1997) Neuron 19, 913–926
21. Swanson, G. T., Sturgess, M., Betz, H., and Kuhse, J. (1997) Neuron 18, 493–503
22. Chen, G. Q., and Gouaux, E. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13431–13436
23. Arvola, M., and Keinanen, K. (1996) J. Biol. Chem. 271, 15527–15532
24. Kousinen, A., Arvola, M., and Keinanen, K. (1995) EMBO J. 14, 6327–6332
25. Ivanovic, A., Reilander, H., Paas, Y., and Kuhse, J. (1998) J. Biol. Chem. 273, 19933–19937
26. Armstrong, N., Sun, Y., Chen, G. Q., and Gouaux, E. (1998) Nature 395, 913–917
27. Akahas, M. H., Stauffer, D. A., Xu, M., and Karlin, A. (1992) Science 258, 307–310
28. Kuner, T., Wollmuth, I. P., Karlin, A., Seeburg, P. H., and Sakmann, B. (1996) Neuron 17, 343–352
29. Wo, Z. G., and Oswald, R. E. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7154–7158
30. Wo, Z. G., and Oswald, R. E. (1996) Mol. Pharmacol. 50, 770–780
31. Wo, Z. G., and Oswald, R. E. (1998) J. Biol. Chem. 273, 2000–2009
32. Hall, R. A., Hansen, A., Andersen, P. H., and Soderling, T. R. (1997) J. Neurochem. 68, 625–630
33. Bastry, S. S., Mendoza, P., Lee, P. D., and Raymond, L. A. (1999) J. Neurosci. 19, 644–652
34. Sali, A., and Blundell, T. L. (1993) J. Mol. Biol. 234, 779–815
35. Abele, R., Lampinen, M., Keinanen, K., and Madden, D. R. (1998) J. Biol. Chem. 273, 25132–25138
36. Uchino, S., Sakimura, K., Nagahari, K., and Mishina, M. (1992) FEBS Lett. 308, 253–257
37. Lampinen, M., Pentikainen, O., Johnson, M. S., and Keinanen, K. (1998) EMBO J. 17, 4704–4711
38. Raymond, L. A., Blackstone, C. D., and Huganir, R. L. (1993) Nature 361, 637–641
39. Wang, L. Y., Taverna, F. A., Huang, X. P., MacDonald, J. F., and Hampson, D. R. (1993) Science 259, 1173–1175
40. Mano, I., Lamed, Y., and Teichberg, V. I. (1996) J. Biol. Chem. 271, 15299–15302
41. Paas, Y., Devillers-Thiery, A., Changeux, J. P., Medevielle, F., and Teichberg, V. I. (1996) EMBO J. 15, 1548–1556
42. Wafford, K. A., Kathoria, M., Bain, C. J., Marshall, G., Le Bourdelles, B., Kemp, J. A., and Whiting, P. J. (1995) Mol. Pharmacol. 47, 374–380
43. Barton, G. J. (1993) Protein Eng. 6, 37–40
44. Wallace, A. C., Laskowski, R. A., and Thornton, J. M. (1995) Protein Eng. 8, 127–134