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AMPA receptors and bacterial periplasmic amino acid-binding proteins share the ionic mechanism of ligand recognition

Milla Lampinen[1,2], Olli Pentikäinen[3,4], Mark S.Johnson[3,4] and Kari Keinänen[1,2,5]

[1]Department of Biosciences (Division of Biochemistry) and Institute of Biotechnology, P.O.Box 56, FIN-00014 University of Helsinki, Helsinki, [2]VTT Biotechnology and Food Research, FIN-02044 VTT, Espoo, [3]Department of Biochemistry and Pharmacy, Åbo Akademi University and [4]Turku Center for Biotechnology, University of Turku, FIN-20521, Turku, Finland

[5]Corresponding author
e-mail: kari.keinanen@helsinki.fi

In order to identify key structural determinants for ligand recognition, we subjected the ligand-binding domain of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-selective glutamate receptor GluR-D subunit to site-directed mutagenesis. Based on the analysis of the [^{3}H]AMPA-binding properties of the mutated binding sites, we constructed a revised three-dimensional model of the ligand-binding site, different in many respects from previously published models. In particular, our results indicate that the residues Arg507 and Glu727 represent the structural and functional correlates of Arg77 and Asp161 in the homologous bacterial lysine/ornithine/arginine-binding protein and histidine-binding protein, and directly interact with the α-carboxyl and α-amino group of the bound ligand, respectively. In contrast, Glu424, implicated previously in ionic interactions with the α-amino group of the agonist, is unlikely to have such a role in ligand binding. Our results indicate that glutamate receptors share with the bacterial polar amino acid-binding proteins the fundamental mechanism of amino acid recognition.

Keywords: glutamate/ligand binding/neurotransmission/quinoxalinedione antagonists/site-directed mutagenesis

Introduction

Ionotropic glutamate receptors (iGluRs) mediate excitatory neurotransmission in the mammalian central nervous system, and are involved in the control of synaptic strength and in the pathological mechanisms leading to excitotoxic neuronal damage. Like other ligand-gated channels, iGluRs are oligomeric complexes composed of homologous subunits. The subunits encompass 900–1300 residues and, according to the current model for the transmembrane topology, the subunits harbor an extracellular N-terminus, an intracellular C-terminus, three transmembrane segments (M1, M3 and M4) and one pore loop (M2), which inserts into the membrane from the intracellular side and lines the interior of the channel pore (Hollmann *et al*., 1994; Bennett and Dingledine, 1995; Wo and Oswald, 1995).

According to the model, the extracellular part of the receptor comprises the ~550 residues preceding M1 and the segment between M3 and M4, including two regions S1 and S2 which are sequence-related to bacterial polar amino acid-binding proteins (Nakanishi *et al*., 1990; O'Hara *et al*., 1993; Stern-Bach *et al*., 1994). This relationship between glutamate receptors and the bacterial proteins, considered together with the similarity in the chemical nature of their cognate ligands (amino acids), suggests that (i) the S1 and S2 segments form the neurotransmitter-binding site in iGluR subunits, and (ii) the mechanism of amino acid binding is similar in these two classes of proteins. Importantly, three-dimensional structures of several bacterial amino acid-binding proteins have been determined to high resolution, providing detailed information on their ligand-binding mechanism (Kang *et al*., 1991; Oh *et al*., 1993, 1994a,b; Yao *et al*., 1994; Hsiao *et al*., 1996). In the absence of direct structural information on glutamate receptors, the bacterial proteins may thus serve as templates for modeling the ligand-binding site. Furthermore, in bacterial proteins, ligand binding is accompanied by closing of the two-lobed binding domain around the ligand, a mechanism which in iGluRs may be exploited to couple ligand binding to the transmembrane movements leading to channel opening (Wo and Oswald, 1995; Laube *et al*., 1997; Paas, 1998).

The postulated structural relationship between the ligand-binding sites of iGluRs and the bacterial proteins is supported by some experimental evidence. Studies on chimeric and mutated receptors indicate that the S1 and S2 segments indeed determine the ligand pharmacology of iGluRs (Kuryatov *et al*., 1994; Stern-Bach *et al*., 1994; Kuusinen *et al*., 1995). Site-directed mutagenesis studies on α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) (Uchino *et al*., 1992; Li *et al*., 1995; Mano *et al*., 1996; Kawamoto *et al*., 1997) and *N*-methyl-D-aspartate (NMDA) receptors (Kuryatov *et al*., 1994; Wafford *et al*., 1995; Hirai *et al*., 1996; Laube *et al*., 1997; Wood *et al*., 1997; Anson *et al*., 1998), and the kainate-binding proteins of chick (Paas *et al*., 1996b) and goldfish (Wo and Oswald, 1996) have identified a number of residues in the S1 and S2 segments which contribute to ligand pharmacology. In homology-based structural models, these residues largely localize along the cleft between the predicted two lobes of the ligand-binding domain, supporting similarity between the binding sites. (Paas *et al*., 1996b; Sutcliffe *et al*., 1996; Laube *et al*., 1997). In the bacterial lysine/arginine/ornithine-binding protein (LAOBP; Oh *et al*., 1993, 1994a) and histidine-binding protein (HBP; Oh *et al*., 1994b; Yao *et al*., 1994), ligand recognition is mediated by multiple hydrogen bonds and by salt bridges (Oh *et al*., 1994a,b; Yao *et al*., 1994). In particular, the ionized side chains of conserved arginine (Arg77) and aspartate (Asp161) residues neutralize the

charged α-carboxylate and α-amino groups of the bound amino acid ligand, respectively. Interestingly, bacterial leucine/isoleucine/valine-binding protein (LIVBP; Sack *et al*., 1989), which has a structural fold similar to that of LAOBP and HBP, uses exclusively hydrogen bonds and hydrophobic stacking for ligand binding, indicating that ionic interactions are not a universal feature of amino acid binding. It may be noteworthy, however, that no clear sequence similarity exists between LIVBP and the polar amino acid-binding proteins (including the S1 and S2 segments of iGluRs).

The possible contribution of ionic interactions in ligand recognition by glutamate receptors is still unclear. An arginine residue in the S1 segment of iGluR subunits (equivalent to Arg507 in the GluR-D AMPA receptor) which corresponds to Arg77 has been proposed to interact either with the α-carboxylate (Kawamoto *et al*., 1997; Laube *et al*., 1997) or with the γ-carboxylate (Sutcliffe *et al*., 1996) of glutamate. In contrast, the identity and even the existence in the S2 segment of iGluRs of an anionic residue corresponding to Asp161 has remained unclear, and the molecular models presented for iGluR–ligand interaction differ from the bacterial binding mode in this respect. As the interactions between the receptor and α-carboxyl and α-amino groups are expected to be the most highly conserved in amino acid-binding proteins, the above-mentioned results raise serious doubts on the validity of the structural models of iGluRs reported so far.

In the present study, we provide evidence that Arg507 and Glu727 in the AMPA receptor subunit GluR-D are functionally and structurally equivalent to Arg77 and Asp161 in the bacterial proteins, indicating that the fundamental mechanism of amino acid binding is conserved. In addition, our results demonstrate that structural determinants important for the binding of the competitive antagonists are present in both lobes of the predicted two-lobed binding site.

Results

As the invariant (charged) α-amino and α-carboxyl groups represent a structural feature which is common to iGluR agonists and the ligands of the bacterial polar amino acid-binding proteins, we examined whether the interactions between the receptor and these functional groups in the ligand are mediated by homologous charged residues in these two classes of proteins. In a multiple alignment of iGluR subunits and bacterial polar amino acid-binding proteins (Figure 1A), Arg507 (numbering herein refers to the GluR-D subunit and starts from the first translated residue; Keinänen *et al.*, 1990) and Glu727 show up as the potential correlates of Arg77 and Asp161 of LAOBP and HBP, which interact with the α-carboxyl and α-amino group of the bound amino acid. We examined the role of these and other, mostly charged residues in ligand binding by introducing site-directed mutations into the ligand-binding domain of the AMPA-selective GluR-D subunit, produced as a soluble, secreted S1–S2 fusion protein (Kuusinen *et al*., 1995). By selecting this model system, which reproduces the ligand-binding characteristics of the parental membrane-bound receptor, we wished to minimize the indirect contributions of the mutations to the assembly of the receptor, transit to the cell surface, coupling of ligand binding with channel gating, and desensitization.

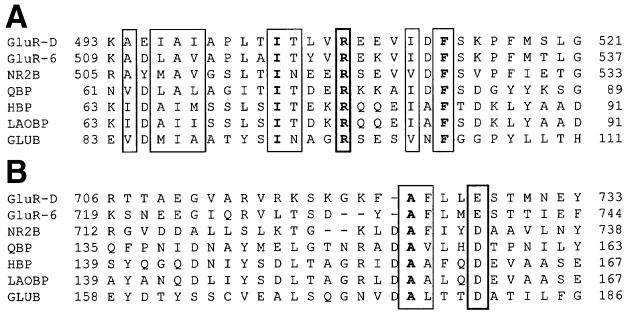

Fig. 1. Sequence alignment of three GluR subunits with four bacterial proteins illustrating the regions around LAOBP residues Arg77 (**A**) and Asp161 (**B**) involved in binding of the α-aminocarboxyl group of the amino acid ligand. The shading indicates sequence identity (bold type) or isofunctionality. The sequences used in the alignment: rat iGluR subunits GluR-D (DDBJ/EMBL/GenBank accession No. M36421), GluR-6 (Z11548) and NR2B (M91562); *Escherichia coli* glutamine-binding protein (QBP; X14180), *Salmonella typhimurium* histidine-binding protein (HBP; V01373) and lysine/arginine/ornithine-binding protein (LAOBP; V01368), and *Corynebacterium glutamicum* glutamate-binding protein (GLUB; X81191).

Role of Arg507

The functional importance of the arginine residue corresponding to Arg507 in GluR-D has been demonstrated in NMDA (Kuryatov *et al*., 1994; Wafford *et al*., 1995) and AMPA receptors (Uchino *et al*., 1992; Kawamoto *et al*., 1997) and in the chick kainate-binding protein (Paas *et al*., 1996b). This residue is conserved in all iGluR subunits and aligns with Arg77 of LAOBP and HBP (Figure 1A), which forms a salt bridge with the negatively charged α-carboxylate of the bound amino acid and participates in a hydrogen bonding network within the binding pocket (Oh *et al*., 1993). Expression of the GluR-D S1–S2-binding site carrying the conservative mutation R507K resulted in the secretion into the culture medium of a polypeptide with the expected size (Figure 2A), suggesting that the mutant protein was not grossly misfolded. The culture supernatants containing the mutant polypeptide were dialyzed extensively and tested for [^{3}H]AMPA binding (Figure 2A). In contrast to the wild-type S1–S2 polypeptide, which bound the radioligand with a high affinity (K_d 37 nM), no specific binding was observed with the R507K mutant even at high radioligand concentrations (Figure 2A).

Role of other cationic residues

As glutamate agonists share, in addition to the α-carboxylate, another distal anionic group (γ-carboxylate of L-Glu), the role of additional positively charged residues in the predicted ionic interactions was examined by subjecting three lysine residues in the S1 segment, Lys431, Lys432 and Lys471, that could be located in the predicted ligand-binding pocket depending upon the alignment, to mutagenesis. Substitutions of lysine residues at positions 431 and 432 by serine did not result in any significant alterations in the [^{3}H]AMPA-binding affinity or pharmacological profile (Figure 2A; Table I). The role of the lysine residue at position 471, previously shown to contribute to ligand pharmacology of AMPA (Uchino *et al*., 1992;

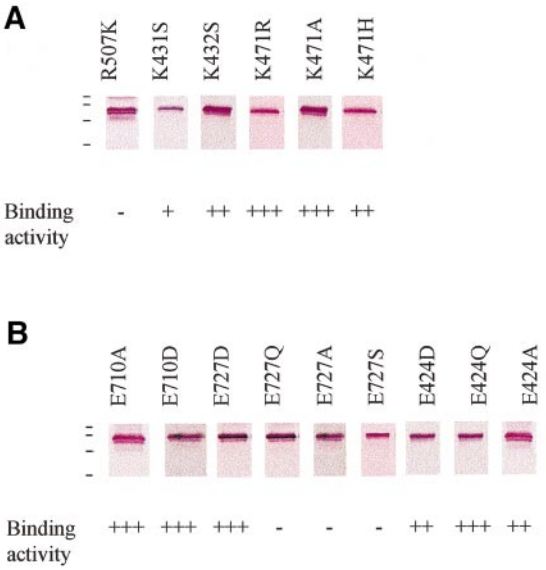

Fig. 2. Site-directed mutagenesis of charged residues. (**A**) Cationic residues Arg507, Lys431, Lys432 and Lys471. (**B**) Anionic residues Glu710, Glu727 and Glu424. Expression and [^{3}H]AMPA-binding activity of the mutant binding sites are shown. The upper panel shows an anti-Flag Western blot of the insect cell supernatants. The dashes on the left indicate the positions of the mass markers: 55.6, 42.7, 36.5 and 26.6 kDa. The lower panel indicates the specific binding of 5 nM [^{3}H]AMPA as ranked qualitatively in four categories: –, no radioligand binding above background (500 d.p.m.); +, 1000–4000 d.p.m./25 µl sample; ++, 5000–14 000 d.p.m.; +++, > 15 000 d.p.m./25 µl.

Li *et al*., 1995) and NMDA receptors (Wafford *et al*., 1995), and of chick kainate-binding protein (Paas *et al*., 1996a,b), was investigated by analyzing the ligand-binding properties of site-directed mutants K471R, K471H and K471A. All three were secreted into the culture medium at levels comparable with that of the wild-type binding domain, and bound [^{3}H]AMPA with essentially unchanged properties except for the K471H mutant which showed decreased affinity both for the radioligand and for unlabeled L-glutamate (Figure 2A; Table I).

Role of Glu727

A conserved anionic residue in the S2 segment of iGluR subunits, Glu727 in GluR-D, aligns with Asp161 (Figure 1B). Although the overall sequence conservation between iGluRs and the polar amino acid-binding proteins is very low in this region, contributing to the differences in many earlier published alignments, this anionic residue invariably is preceded by a non-polar aliphatic or aromatic residue at the –3 and an alanine residue at the –4 position. Asp161 is involved in charge neutralization of the positively charged primary amino group of the bound amino acid in both LAOBP and HBP (Oh *et al*., 1993, 1994b; Yao *et al*., 1994). Accordingly, we chose Glu727 as a target for site-directed mutagenesis. As a safeguard against ambiguities in the alignment, another nearly conserved negatively charged residue, Glu710, was also mutagenized. In order to test the importance of the polarity and charge of the side chain, mutations E727D, E727Q, E727S and E727A were studied. At position 710, two mutations (E710D and E710A) were studied. The level of expression and secretion into the culture medium were similar to the wild-type control for each of the mutants

Table I. Ligand-binding properties of the mutated binding sites

Mutation	K_d (nM)	K_i (µM)		
		L-Glutamate	Kainate	DNQX
Wild-type	37 ± 13	0.19 ± 0.09	1.9 ± 0.47	0.49 ± 0.18
E424D	29 ± 2.2	1.3 ± 0.53[a]	3.3 ± 0.83	1.0 ± 0.11
E424Q	28 ± 7.0	1.1 ± 0.16[b]	7.0 ± 3.6	0.48 ± 0.13
E424A	35 ± 6.2	0.25 ± 0.04	11 ± 1.6[b]	1.3 ± 0.10[a]
K431S	60 ± 17	0.30 ± 0.13	2.2 ± 0.23	0.35 ± 0.14
K432S	33 ± 4.8	0.38 ± 0.20	2.8 ± 0.69	ND
K471R	29 ± 7.9	0.29 ± 0.07	3.7 ± 2.1	0.66 ± 0.31
K471H	90 ± 22[a]	0.77 ± 0.15[a]	1.9 ± 0.38	0.48 ± 0.12
K471A	37 ± 14	0.31 ± 0.04	2.9 ± 0.58	0.55 ± 0.26
Y472F	380 ± 80[b]	0.20 ± 0.07	2.2 ± 0.33	2.01 ± 0.47[a]
R507K	NB	–	–	–
E710D	24 ± 7.2	0.23 ± 0.07	1.6 ± 0.30	0.33 ± 0.15
E710A	50 ± 21	ND	2.6 ± 0.49	ND
E727D	4.8 ± 0.7[b]	1.7 ± 0.39[b]	1600 ± 740[a]	0.013 ± 0.006[a]
E727Q	NB	–	–	–
E727S	NB	–	–	–
E727A	NB	–	–	–

[^{3}H]AMPA-binding affinity and the relative affinities (K_i values) of unlabeled L-glutamate, kainate and DNQX are indicated. The numbers represent average values ± SD from 3–10 independent experiments. ND not determined; NB, no measurable binding.
The mutants which differ significantly from the wild-type are indicated by superscripts: [a]P <0.02, [b]P <0.002.

(Figure 2B). In the [^{3}H]AMPA-binding assay, however, mutations at position 727 resulted in dramatic effects on the ligand-binding properties whereas mutations at position 710 had no effect (Figure 2B; Table I).

The presence of an uncharged residue (aliphatic or polar) at position 727 always resulted in the total loss of measurable [^{3}H]AMPA binding. In contrast, the E727D mutant which preserved the negative charge of the side chain (and, thus, the possibility to participate in ionic interactions), bound [^{3}H]AMPA and displayed a highly interesting ligand-binding phenotype (Figure 3; Table I). The radioligand, [^{3}H]AMPA, bound to the E727D mutant with a 5- to-10-fold increased affinity (K_d 5 nM), but in the displacement assay, unlabeled agonists L-glutamate and kainate bound with at least 10- and 1000-fold decreased affinities, respectively.

Role of Glu424

Glu424 is located in the N-terminal 'hot spot' region of the S1 segment and aligns with the Tyr14 in LAOBP and HBP, a residue which contacts the distal end of the side chain of the bound amino acid. Although in the bacterial proteins this residue is not involved in recognition of the α-amino group of the ligand, a recent molecular model was used to suggest that Glu424 forms an ionic bond with the α-ammonium group of L-glutamate in AMPA and kainate receptors (Sutcliffe *et al*., 1996), with glutamate in the opposite orientation opposite to that seen in defined three-dimensional structures of both the polar amino acid-binding proteins, the model proposed by Paas and coworkers (Paas *et al*., 1996b) and in our model (see below). Furthermore, mutations at the corresponding residue have been reported to exert effects on the ligand pharmacology of glutamate receptors (Uchino *et al*., 1992; Kuryatov *et al*., 1994; Mano *et al*., 1996; Wo and Oswald, 1996; Laube *et al*., 1997). Therefore, we prepared and

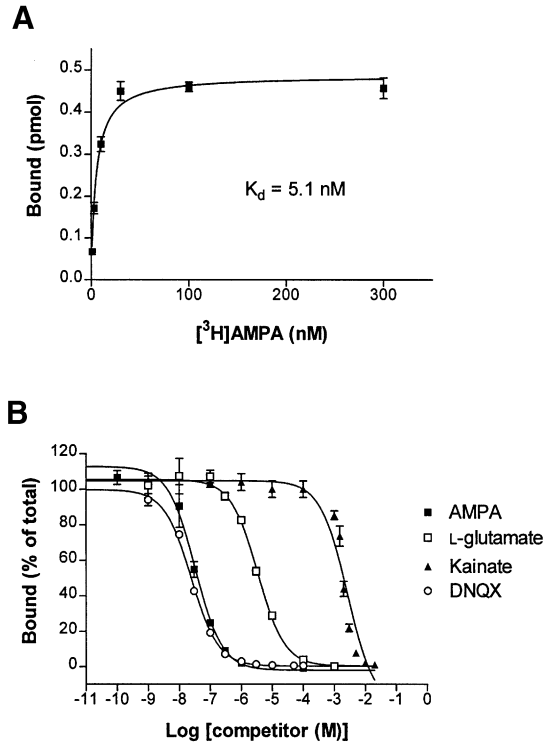

Fig. 3. Binding of [^{3}H]AMPA to GluR-D/S1S2 E727D. (**A**) Saturation binding isotherm; (**B**) displacement of 5 nM radioligand by unlabeled L-Glu, AMPA, kainate and DNQX.

characterized three different Glu424 mutants, E424D, E424Q and E424A. These were all expressed and secreted at a level comparable with that of the wild-type ligand-binding domain (Figure 2B; Table I). In the radioligand-binding assay, all three mutants displayed [^{3}H]AMPA binding with an affinity at the wild-type level, which, considering the loss of charge with the E424Q and E424A mutants, argues against a role for a coulombic interaction with the ligand α-amino group. In a ligand displacement analysis, the E424D and E424Q mutants showed a slightly (~5-fold) decreased affinity for L-glutamate, whereas the E424A mutant displayed wild-type glutamate affinity (Table I).

Interaction with quinoxalinedione antagonist

We have shown previously that the structural determinants responsible for binding of the quinoxalinedione antagonists are also present within the S1–S2 ligand-binding domain (Kuusinen *et al*., 1995). Therefore, it was interesting to test the potency of DNQX to inhibit [^{3}H]AMPA binding to the mutated binding sites. Of all the mutations described above, only two, E424A and E727D, showed alterations: DNQX interacted with the E424A mutants with a slightly decreased affinity, and with the E727D mutant with an increased affinity (Table I). As the chick kainate-binding protein residue Tyr71, corresponding to Tyr472 in GluR-D, has been implicated in antagonist binding, we characterized the ligand-binding properties of an additional mutant Y472F (Paas *et al*., 1996b). In agreement with the results of Paas and co-workers (Paas *et al*., 1996b), mutation Y472F resulted in a decrease in the antagonist interaction. At the same time, however, binding of the radioligand was even more severely affected (Table I).

Molecular model of the binding site

A three-dimensional model of the structure of the S1–S2 polypeptide was constructed on the basis of homology to the bacterial polar amino acid-binding proteins (Figure 4). This model structure was used to visualize and interpret interactions of GluR-D with ligand (L-glutamate) as indicated by mutagenesis of the receptor. Arg507 and Glu727 occupy the corresponding positions of Arg77 and Asp161 of the HBP and LAOBP, illustrated in Figure 4B and C, showing the striking similarity between the ligand complexes of the HBP and the proposed model of GluR-D, especially as regards the α-amino and α-carboxylate groups of the bound amino acid. Key stabilizing interactions in the GluR-D model include hydrogen and ionic bonds (all $<$3.0 Å) between the side chain of Arg507 and the α-carboxylate oxygens of the ligand; the side chain of Glu727 and the α-amino group of the ligand; hydrogen bonds between the main-chain NH groups of Thr502 and Thr677 and one oxygen each of the α-carboxylate of the ligand; and a hydrogen bond between the main-chain carbonyl of Thr502 and the side chain of Arg507. Mutagenesis of the semi-conserved cationic residues Lys431, Lys432 and Lys471 in the S1 segment did not drastically alter the ligand-binding behavior of the receptor, excluding any substantial contribution of these residues to ionic stabilization of the proximal ('α') or distal ('γ') anionic group of the ligand. The structural basis for the subclass-selective agonist pharmacologies of AMPA, NMDA and kainate receptors is likely to be related to the interaction of the ligand's distal anionic group with the receptor. To address this problem in detail, a more extensive mutagenesis study, currently in progress, is needed.

Discussion

Our results indicate that GluR-D residues Arg507 and Glu727 are functionally equivalent to Arg77 and Asp161 of LAOBP and HBP, interacting with the oppositely charged α-carboxyl and α-amino groups, respectively, of the bound amino acid.

The importance of Arg507 to ligand binding agrees with previous studies showing that substitutions of the corresponding arginine residue in GluRs invariably lead to loss of function (Uchino *et al*., 1992; Wafford *et al*., 1995; Hirai *et al*., 1996; Kawamoto *et al*., 1997; Laube *et al*., 1997). In our model, Arg507 interacts with the agonist's α-carboxylate as observed in the crystal structures of LAOBP and HBP and in the model presented for the glutamate site of the NR2B subunit (Laube *et al*., 1997), and not with the γ-carboxylate of the ligand as suggested by Sutcliffe and co-workers (1996). We consider the interaction with the α-carboxylate, rather than with the γ-carboxylate, more likely for two reasons. First, the invariant structure (carboxylate) of the α-anionic group contrasts with the diversity of distal anionic structures of natural and synthetic iGluR agonists and, therefore, is more in agreement with the absolute conservation of Arg507. Secondly, the orientation of glutamate in the binding site model presented by Sutcliffe and co-workers (1996) differs from amino acids bound to LAOBP and HBP, in contrast with what would be expected from the similarities in the sequences and chemical structures of

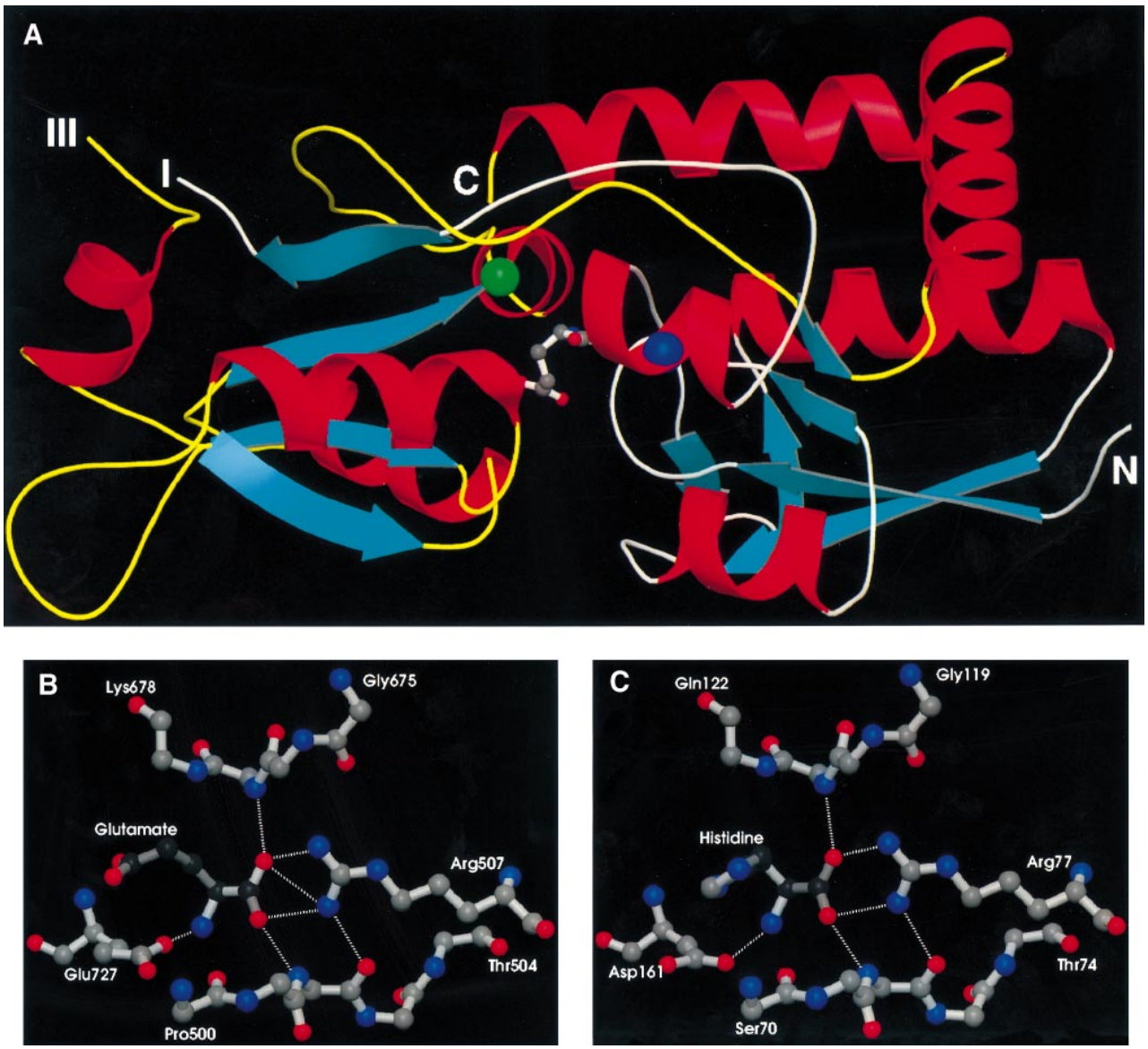

Fig. 4. (**A**) Model of the closed-form of the S1–S2 ligand-binding domain of GluR-D based on the three-dimensional structures of two bacterial periplasmic binding proteins: LAOBP with bound lysine (Protein Data Bank structure code: 1lst) and HBP with bound histidine (1hsl). N and C refer to the N- and C-terminal ends, respectively, of the portion of the receptor encompassing the S1 and S2 domains; I and III indicate the N- and C-terminal ends of the membrane-spanning units, I–III, not a part of the model and located between domains S1 and S2. These regions are replaced by a linker peptide in the recombinant S1–S2 construct. Bound glutamate is shown as a ball and stick figure; Arg507, blue sphere; Glu727, green sphere. α-Helices are shown in red, and β-strands in cyan, the S1 connecting regions in white, and the S2 connecting regions in yellow. (**B**) Detailed view of interactions between the α-amino and α-carboxylate groups of bound glutamate (Glutamate) and main-chain and side-chain atoms of the GluR-D model. Only the side chains of Arg507 and Glu727 are shown. Predicted hydrogen bonds are indicated by dashes. Oxygen atoms (red), nitrogen atoms (blue), carbon (gray). (**C**) Corresponding interactions between bound histidine in the X-ray structure of HBP. Only the side chains of Arg77 and Asp161 are shown. The experimental evidence and the model reported here show that the analogs of the HBP residues Arg77 and Asp161 are GluR-D residues Arg507 and Glu727, respectively. This figure was prepared using MOLSCRIPT (Kraulis, 1991), RASTER3D (Bacon and Anderson, 1988) and RENDER (Merritt and Murphy, 1994).

the ligands of these two classes of proteins (i.e. iGluRs and the bacterial proteins).

The major new finding of the present study is the functional similarity between Glu727 (in GluR-D) and Asp161 in bacterial LAOBP and HBP in neutralizing the positive charge of the ligand's α-ammonium group. Previous findings on inactivation of the receptor due to non-conservative mutations introduced at this position (Mano *et al.*, 1996; Paas *et al.*, 1996b) have been interpreted as indirect structural alterations rather than direct effects on ligand recognition. Williams and co-workers (1996) reported that the replacement of Asp732 (equivalent to Glu727) of the NR1 subunit receptor by a glutamate residue resulted in a drastic (>4000-fold) decrease in the glycine sensitivity of the NR1/NR2A NMDA receptor expressed in *Xenopus* oocytes, and speculated that possible mechanisms might be direct ligand interaction and indirect effects on channel gating. In the present study, the mutation of Glu727 to an aspartate, in contrast to three mutations that neutralized the negative charge (Glu→Gln, Glu→Ser and Glu→Ala), not only retained high-affinity AMPA binding but produced a ligand-binding profile which differs

from the wild-type receptor for all agonist and antagonist ligands tested. The binding affinity for AMPA was increased 5- to 10-fold, whereas the affinities for glutamate and quisqualate were decreased by one order of magnitude. For kainate, the drop in the binding affinity was more dramatic, ~1000-fold, corresponding to a standard free energy change of 17 kJ/mol. The fact that glutamine and serine at position 727 do not, in contrast to glutamate and aspartate, support high-affinity agonist binding indicates the importance of the negative charge. We therefore predict that the ionized γ-carboxylate of Glu727 is in the vicinity (~2.8 Å) of the positively charged α-amino group of the agonist and, in addition to coulombic attraction, forms a hydrogen bond with it. The opposite effects of the E727D mutation on binding affinities of the three agonists may illustrate the different stereochemical interactions between the ligand and the slightly altered binding site.

Previously, Glu424 or a corresponding residue has been suggested to interact directly via coulombic attraction and hydrogen bonds with the α-amino group of the ligand in the NR1 and NR2B subunits of the NMDA receptor (Laube *et al*., 1997), chick kainate-binding protein (Paas *et al*., 1996b) and in AMPA and kainate receptors (Sutcliffe *et al*., 1996). In support of this, substitutions of the glutamate at this position in the NR2B subunit of the NMDA receptor and in the GluR-A AMPA receptors resulted in decreased agonist sensitivities (Uchino *et al*., 1992; Mano *et al*., 1996; Laube *et al*., 1997). In a ligand-binding study of chick kainate receptor, a charge-neutralizing mutation of the glutamate at this position to valine led to a 100-fold decrease in the affinity for kainate but did not influence affinity for glutamate (Paas *et al*., 1996b). The three different substitutions at residue 424 characterized in this study, including one (Glu→Ala) with an uncharged aliphatic side chain, all displayed high-affinity AMPA binding, and in two cases, slightly altered binding of unlabeled glutamate. This indicates that ionic interactions or hydrogen bonds between the ligand and Glu424 of the AMPA receptor, if present at all, do not contribute markedly to ligand binding. The reported effects of substitutions at this position on agonist sensitivity may be due to effects on the activation pathway rather than to binding *per se* or, alternatively, differences between iGluR subclasses.

As an anionic distal group is an invariant and necessary part of the pharmacophore of the glutamate agonist, we expected the binding site to contain a complementary positively charged residue, lysine, arginine, or perhaps histidine. A close inspection of the alignments of iGluR sequences reveals only a few conserved positively charged residues which in LAOBP-based models would line the binding pocket. Our results suggest that the cationic side chains of lysine residues at positions 431, 432 and 471 in the S1 segment are not necessary for ligand binding. Alternatively, the distal anionic group of the ligand may be stabilized by multiple hydrogen bonds from uncharged donor groups or by an as yet unidentified positively charged group elsewhere in the molecule. Accordingly, more data are needed to model the interactions of the distal anionic group of the ligand with the receptor.

Interestingly, three binding site mutations, E424A, Y472F and E727D, affected the binding of DNQX, a competitive AMPA/kainate receptor antagonist (Honore *et al*., 1988). The apparent affinity for DNQX, as determined from the IC_{50} values in the inhibition of [^{3}H]AMPA, was slightly lower than wild-type for the E424A and Y472F mutants, but was ~20-fold higher for the E727D mutant. We speculate that there is a direct interaction between the γ-carboxylate of Glu727 and the imino group of quinoxaline dione antagonists, whereas Tyr472 may stack with the aromatic ring (π-bonding) of the antagonist. The improved binding of CNQX and DNQX to the binding site containing an aspartate instead of glutamate may be due to a better fit of the rigid antagonist molecule to the slightly increased binding pocket. Thus far, only residues in the S1 segment corresponding to Glu424 and Tyr472 in GluR-D have been identified as structural determinants for antagonist action. Previously, Paas and co-workers have reported that in the chick kainate-binding protein, replacement of Tyr71 (corresponding to Y472 of GluR-D) by an isoleucine residue results in decreased affinity for CNQX, a structurally very similar quinoxalinedione antagonist (Paas *et al*., 1996b).

In conclusion, Arg507 and Glu727, predicted to be located in opposite lobes of a bilobate ligand-binding domain of AMPA receptor, play key roles in ionic interactions with the charged α-amino and α-carboxyl groups of the agonist.

Materials and methods

Site-directed mutagenesis

Single amino acid replacements in the ligand-binding domain of GluR-D were generated by PCR using the plasmid pK503-4 as a template (Kuusinen *et al*., 1995). The resulting mutation-carrying DNA fragments were then digested with appropriate restriction endonucleases and used to replace the corresponding fragment in the plasmid pK503-4, encoding the wild-type S1–S2-binding site of GluR-D under the polyhedrin promoter (Kuusinen *et al*., 1995). With mutations in the S1 segment, the unique *Nco*I (near the 3′ end of the sequence encoding GluR-D signal peptide) and *Sal*I (at the 5′ end of the linker sequence) sites in pK503-4 were used for cloning. With S2 mutations, *Eco*RI (encoding residues 679–680) and *Sac*I (at the 5′end of the sequence coding for the C-terminal myc tag) sites were used. The presence of all the designed mutations was confirmed by DNA sequencing.

Expression in insect cells

Recombinant baculoviruses were generated by using the Bac-to-Bac system (Gibco-BRL) based on targeted transposition of the expression cassette to the baculovirus genome maintained in *Escherichia coli* as a bacmid (Luckow *et al*., 1993). The resulting recombinant bacmids were used to transfect Sf21 cells by lipofection (Insectin, Invitrogen), and the presence of recombinant virus was verified 3–4 days after the transfection by Western blotting using anti-Flag M1 antibody. The amplified virus stocks were generated according to standard protocols (O'Reilly and Miller, 1989) and used to infect Sf21 and High Five insect cells growing in 25 cm^2 tissue culture flasks. The cells were harvested 3–4 days post-infection and the culture media were dialyzed against a >100-fold volume of 30 mM Tris–HCl, pH 7.2, 100 mM KCl with three changes, and finally against 30 mM Tris–HCl, pH 7.2, 100 mM KSCN, 2.5 mM $CaCl_2$ ('AMPA binding buffer') (Kuusinen *et al*., 1995).

Radioligand binding

For saturation binding analysis, 20–50 μl samples were incubated in AMPA binding buffer containing 1–300 nM [^{3}H]AMPA for 1 h on ice, followed by filtration through polyethyleneimine-treated GF/B (Whatman) glass fiber filters as described previously (Kuusinen *et al*., 1995). The ^{3}H radioactivity in the filters was measured by liquid scintillation counting in OptiPhase (Wallac). For ligand competition assays, dialyzed supernatant samples (25–100 μl) were incubated with 5 nM [^{3}H]AMPA in the presence of unlabeled ligands at the concentrations indicated in the figure legends. The binding data were analyzed by

using GraphPad Prism non-linear curve-fitting software. For statistical analysis, a non-paired Student's *t*-test was used.

Homology modeling of the ligand-binding domain

Molecular modeling studies were made on a Silicon Graphics O2 computer. The three-dimensional structures of the HBP complexed with L-histidine [Protein Data Bank code: 1hsl (Yao *et al*., 1994)], and the LAOBP complexed with L-lysine [1lst (Oh *et al*., 1993)] were obtained from the Brookhaven Protein Data Bank (Bernstein *et al.*, 1977). The sequence of the ligand-binding domain of GluR-D was aligned with the known structures with the programs MALIGN and MALFORM (Johnson and Overington, 1993; Johnson *et al*., 1996). The sequences of 1hsl and 1lst are ~21% identical with the sequence of S1–S2. Based on this alignment, the S1–S2 fusion protein was modeled using the program MODELLER 4 (Šali and Blundell, 1993). The models produced by MODELLER were energy minimized in SYBYL 6.3 (Tripos, St Louis, MO) using the TRIPOS forcefield. In the first application of energy minimization, the backbone was kept rigid and only the side chains were allowed to move. In the second step, all atoms were allowed to move. Energy minimization was performed until all short contacts and inconsistencies in geometry were rectified. The electrostatic term was not included as the main purpose was to remove steric hindrances and to correct bad geometry.

Other methods

SDS–PAGE and Western blotting were performed as described previously (Kuusinen *et al*., 1995). Protein concentrations were measured by using the Pierce Bicinchoninic Acid assay in a microplate format as described by the manufacturer.

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References

Anson,L.C., Chen,P.E., Wyllie,D.J.A., Colquhoun,D. and Schoepfer,R. (1998) Identification of amino acid residues of the NR2A subunit that control glutamate potency in recombinant NR1/NR2A NMDA receptors. *J. Neurosci*., **18**, 581–589.

Bacon,D.J. and Anderson,W.F. (1988) A fast algorithm for rendering space-filling molecule pictures. *J. Mol. Graphics*, **6**, 219–220.

Bennett,J.A. and Dingledine,R. (1995) Topology profile for a glutamate receptor: three transmembrane domains and a channel-lining reentrant membrane loop. *Neuron*, **14**, 373–384.

Bernstein,F.C., Koetzle,T.F., Williams,G.J.B., Meyer,E.E.,Jr, Brice,M.D., Rodgers,J.R., Kennard,O., Shimanouchi,T. and Tasumi,M. (1977) The Protein Data Bank: a computer based archival file for macromolecular structures. *J. Mol. Biol.*, **112**, 535–542.

Hirai,H., Kirsch,J., Laube,B., Betz,H. and Kuhse,J. (1996) The glycine binding site of the *N*-methyl-D-aspartate receptor subunit NR1: identification of novel determinants of co-agonist potentiation in the extracellular M3–M4 loop region. *Proc. Natl Acad. Sci. USA*, **93**, 6031–6036.

Hollmann,M., Maron,C. and Heinemann,S. (1994) *N*-glycosylation site tagging suggests a three transmembrane domain topology for the glutamate receptor GluR1. *Neuron*, **13**, 1331–1343.

Honore,T., Davies,S.N., Drejer,J., Fletcher,E.J., Jacobsen,P., Lodge,D. and Nielsen,F.E. (1988) Quinoxalinediones: potent competitive non-NMDA glutamate receptor antagonists. *Science*, **241**, 701–703.

Hsiao,C.D, Sun,Y.J., Rose,J. and Wang,B.C. (1996) The crystal structure of glutamine-binding protein from *Escherichia coli*. *J. Mol. Biol.*, **262**, 225–242.

Johnson,M.S. and Overington,J.P. (1993) A structural basis for sequence comparisons. An evaluation of scoring methodologies. *J. Mol. Biol.*, **233**, 716–738.

Johnson,M.S., May,A.C., Rodionov,M.A. and Overington,J.P. (1996) Discrimination of common protein folds: application of protein structure to sequence/structure comparisons. *Methods Enzymol.*, **266**, 575–598.

Kang,C.H., Shin,W.C., Yamagata,Y., Gocken,S., Ames,G.F. and Kim,S.H. (1991) Crystal structure of the lysine-, arginine-, ornithine-binding protein (LAO) from *Salmonella typhimurium* at 2.7-Å resolution. *J. Biol. Chem.*, **266**, 23893–23899.

Kawamoto,S., Uchino,S., Xin,K.Q., Hattori,S., Hamajima,K., Fukushima,J., Mishina,M. and Okuda,K. (1997) Arginine-481 mutation abolishes ligand-binding of the AMPA-selective glutamate receptor channel α1-subunit. *Brain Res. (Mol. Brain Res.)*, **47**, 339–344.

Keinänen,K., Wisden,W., Sommer,B., Werner,P., Herb,A., Verdoorn,T.A., Sakmann,B. and Seeburg,P.H. (1990) A family of AMPA-selective glutamate receptors. *Science*, **249**, 556–560.

Kraulis,P.J. (1991) MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. *J. Appl. Crystallogr.*, **24**, 946–950.

Kuryatov,A., Laube,B., Betz,H. and Kuhse,J. (1994) Mutational analysis of the glycine-binding site of the NMDA receptor: structural similarity with bacterial amino acid-binding proteins. *Neuron*, **12**, 1291–1300.

Kuusinen,A., Arvola,M. and Keinänen,K. (1995) Molecular dissection of the agonist binding site of an AMPA receptor. *EMBO J.*, **14**, 6327–6332.

Laube,B., Hirai,H., Sturgess,M., Betz,H. and Kuhse,J. (1997) Molecular determinants of agonist discrimination by NMDA receptor subunits: analysis of the glutamate binding site on the NR2B subunit. *Neuron*, **18**, 493–503.

Li,F., Owens,N. and Verdoorn,T.A. (1995) Functional effects of mutations in the putative agonist binding region of recombinant α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors. *Mol. Pharmacol.*, **47**, 148–154.

Luckow,V.A., Lee,S.C., Barry,G.F. and Olins,P.O. (1993) Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in *Escherichia coli*. *J. Virol.*, **67**, 4566–4579.

Mano,I., Lamed,Y. and Teichberg,V.I. (1996) A venus flytrap mechanism for activation and desensitization of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors. *J. Biol. Chem.*, **271**, 15299–15302.

Merritt,E.A. and Murphy,E.P. (1994) Raster3D version 2.0, a program for photorealistic molecular graphics. *Acta Crystallogr.*, **D50**, 869–873.

Nakanishi,N., Shneider,N.A. and Axel,R. (1990) A family of glutamate receptor genes: evidence for the formation of heteromultimeric receptors with distinct channel properties. *Neuron*, **5**, 569–581.

Oh,B.H., Pandit,J., Kang,C.H., Nikaido,K., Gokcen,S., Ames,G.F. and Kim,S.H. (1993) Three-dimensional structures of the periplasmic lysine/arginine/ornithine-binding protein with and without a ligand. *J. Biol. Chem.*, **268**, 11348–11355.

Oh,B.H., Ames,G.F. and Kim,S.H. (1994a) Structural basis for multiple ligand specificity of the periplasmic lysine-, arginine-, ornithine-binding protein. *J. Biol. Chem.*, **269**, 26323–26330.

Oh,B.H., Kang,C.H., De Bondt,H., Kim,S.H., Nikaido,K., Joshi,A.K. and Ames,G.F. (1994b) The bacterial periplasmic histidine-binding protein. Structure/function analysis of the ligand-binding site and comparison with related proteins. *J. Biol. Chem.*, **269**, 4135–4143.

O'Hara,P. *et al*. (1993) The ligand-binding domain in metabotropic glutamate receptors is related to bacterial periplasmic binding proteins. *Neuron*, **11**, 41–52.

O'Reilly,D. and Miller,L.K. (1989) A baculovirus blocks insect molting by producing ecdysteroid UDP-glucosyl transferase. *Science*, **245**, 1110–1112.

Paas Y. (1998) The macro- and microarchitectures of the ligand-binding domain of glutamate receptors. *Trends Neurosci*., **21**, 117–125.

Paas,Y., Devillers-Thiery,A., Changeux,J.P., Medevielle,F. and Teichberg,V.I. (1996a) Identification of an extracellular motif involved in the binding of guanine nucleotides by a glutamate receptor. *EMBO J.*, **15**, 1548–1556.

Paas,Y., Eisenstein,M., Medevielle,F., Teichberg,V.I. and Devillers-Thiery,A. (1996b) Identification of the amino acid subsets accounting for the ligand binding specificity of a glutamate receptor. *Neuron*, **17**, 979–990.

Sack,J.S., Saper,M.A. and Quiocho,F.A. (1989) Periplasmic binding protein structure and function. Refined X-ray structures of the leucine/isoleucine/valine-binding protein and its complex with leucine. *J. Mol. Biol*., **206**, 171–191.

Šali,A. and Blundell,T.S. (1993) Comparative protein modelling by satisfaction of spatial restraints. *J. Mol. Biol.*, **234**, 779–815.

Stern-Bach,Y., Bettler,B., Hartley,M., Sheppard,P.O., O'Hara,P.J. and Heinemann,S.F. (1994) Agonist selectivity of glutamate receptors is specified by two domains structurally related to bacterial amino acid-binding proteins. *Neuron*, **13**, 1345–1357.

Sutcliffe,M.J., Wo,Z.G. and Oswald,R.E. (1996) Three-dimensional models of non-NMDA glutamate receptors. *Biophys. J.*, **70**, 1575–1589.

Uchino,S., Sakimura,K., Nagahari,K. and Mishina,M. (1992) Mutations in a putative agonist binding region of the AMPA-selective glutamate receptor channel. *FEBS Lett.*, **308**, 253–257.

Wafford,K.A., Kathoria,M., Bain,C.J., Marshall,G., Le Bourdelles,B., Kemp,J.A. and Whiting,P.J. (1995) Identification of amino acids in the *N*-methyl-D-aspartate receptor NR1 subunit that contribute to the glycine binding site. *Mol. Pharmacol.*, **47**, 374–380.

Williams,K., Chao,J., Kashiwagi,K., Masuko,T. and Igarashi,K. (1996) Activation of *N*-methyl-D-aspartate receptors by glycine: role of an aspartate residue in the M3–M4 loop of the NR1 subunit. *Mol. Pharmacol.*, **50**, 701–708.

Wo,Z.G. and Oswald,R.E. (1995) A topological analysis of goldfish kainate receptors predicts three transmembrane segments *J. Biol. Chem.*, **270**, 2000–2009.

Wo,Z.G. and Oswald,R.E. (1996) Ligand-binding characteristics and related structural features of the expressed goldfish kainate receptors: identification of a conserved disulfide bond and three residues important for ligand binding. *Mol. Pharmacol.*, **50**, 770–780.

Wood,M.W., VanDongen,H.M. and VanDongen,A.M. (1997) An alanine residue in the M3–M4 linker lines the glycine binding pocket of the *N*-methyl-D-aspartate receptor. *J. Biol. Chem.*, **272**, 3532–3537.

Yao,N., Trakhanov,S. and Quiocho,F.A. (1994) Refined 1.89-Å structure of the histidine-binding protein complexed with histidine and its relationship with many other active transport/chemosensory proteins. *Biochemistry*, **33**, 4769–4779.

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