Disulfide Bonding and Cysteine Accessibility in the α-Amino-3-hydroxy-5-methylisoxazole-4-propionic Acid Receptor Subunit GluRD

IMPLICATIONS FOR REDOX MODULATION OF GLUTAMATE RECEPTORS*

(Received for publication, April 24, 1998, and in revised form, June 16, 1998)

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Redox agents elicit a wide variety of effects on the ligand affinity and channel properties of ionotropic glutamate receptors and have been proposed as potential therapeutic agents for neuropathological processes. One such effect is the dithiothreitol (DTT)-induced increase in agonist affinity of certain ionotropic glutamate receptors (GluRs), presumably due to reduction of a disulfide bridge formed between cysteine residues conserved among all GluRs. Using biochemical techniques, this disulfide is shown to exist in the ligand-binding domain of the α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor subunit GluRD, although GluRD homomeric receptors are not modulated by DTT. The disulfide is inaccessible to DTT, explaining the insensitivity of the intact receptor. Single mutants C260S and C315S show a 2–3-fold higher ligand affinity than wild-type, as observed for several intact GluRs, indicating that the affinity switch is completely contained within the ligand-binding domain. Also, mutants lacking the native disulfide show non-native oligomerization and dramatically reduced specific activity. These facts suggest that the disulfide bridge is required for the stability of the ligand-binding domain, explaining its conservation. A third cysteine residue in the ligand-binding domain exists as a free thiol, partially sequestered in a hydrophobic environment. These results provide a framework for interpreting a variety of GluR redox modulatory phenomena.

The ionotropic GluRs1 are the predominant excitatory ligand-gated ion channels in the central nervous system (1–3). In some GluR subfamilies, both reducing and oxidizing (labeling) agents have been observed to modulate agonist affinity and channel properties, and it has been proposed that such agents could have therapeutic value in the treatment of various neuropathological processes (4). For example, in the NMDA receptor subfamily, NR1 is regulated in this way when co-assembled with subunits NR2B–NR2D. In the presence of DTT, there is a reversible potentiation of channel currents, slower desensitization, and a decreased EC50 value (5, 6). Similar effects are seen with the smaller GFKAR (7). This site may also be the target of nitric oxide regulation of NMDA receptors, which may have both neuroprotective and neurodestructive effects (8, 9).

The sensor for this DTT sensitivity has been localized by mutagenesis studies to homologous pairs of cysteine residues that are presumed to form disulfide bonds: Cys726 and Cys780 (Cys744 and Cys798 in the immature sequence) of NR1 (10) and Cys722 and Cys776 (Cys730 and Cys780 in the immature sequence) of GFKARβ (7). Based on sequence alignments, homologs of these two cysteines are found in all GluRs, and both are located in the loop between transmembrane segments 2 and 3. This region, known as S2, forms the GluR ligand-binding domain together with the region S1, consisting of approximately 150 amino acids N-terminal to the first transmembrane domain. Together, these regions are predicted by threading algorithms to fold similarly to bacterial periplasmic binding proteins (11–14). Connected by a linker peptide, the S1 and S2 domains of GluRD can be expressed as a soluble protein (S1S2) that reproduces the pharmacology of intact GluRD (15). Sutcliffe et al. (16) have created a molecular model of the corresponding domain of GluR6 incorporating the proposed disulfide bridge. They suggest that it stabilizes the open, unliganded form of the molecule by inhibiting a conformational change seen in the periplasmic binding proteins upon ligand binding (17). This is consistent with the observation that reducing agents increase the affinity of certain GluRs for agonists.

Despite the apparent conservation of the putative disulfide bond across GluR subfamilies, the effects of DTT are quite variable. The AMPA receptors are not potentiated by DTT, although it has recently been shown that mutation of Cys726 (Cys726 of NR1) to alanine in the AMPA GluR subunit GluRC does lead to higher affinity for glutamate and a decrease in channel conductivity (18). In NR1/NR2B–NR2D heteromeric NMDA receptors, only NR1 cysteines appear to be the target of DTT potentiation, although the NR2 subunits are required for phy; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid.

* This work was supported by the Departments of Biophysics and Cell Physiology at the Max Planck Institute for Medical Research, European Union Grant B104-CT96-0589, the Academy of Finland (to K. K.), and the Supercomputing Resource for Molecular Biology (European Union Human Capital and Mobility Contract ERBCHGECT940062) (to D. R. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The abbreviations used are: GluR, ionotropic glutamate receptor; NMDA, N-methyl-D-aspartate; NR1/2, NMDA receptor subunit 1/2; DTT, 1,4-dithiothreitol; GFKAR, gold fish kainate receptor; IAM, iodamine; PM, N-1-pyrenylmaleimide; NaP, sodium phosphate; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); ODNB, n-octyl-5-dithio-2-nitrobenzoic acid; MALDI-MS, matrix-assisted laser desorption ionization mass spectroscopy; HPLC, high performance liquid chromatogra-
potentiation, since NR1 homomers expressed in oocytes are not potentiated by DTT (5, 10, 19). Furthermore, potentiation in NR1/NR2A heteromers has two components, one of which is mediated by the NR1 disulfide and one of which is not (6, 10). NR1/NR2A heteromers also exhibit different susceptibilities to reducing and oxidizing agents other than DTT, compared with NR1/NR2B-D channels (6).

To clarify the role of the extracellular cysteine residues in the redox modulation of GluR responses, we have addressed the following questions. Is there a disulfide bridge in GluRligand-binding domain homologous to that identified in NR1 and GFKAR? Is the ligand-binding domain alone sufficient to reproduce the disulfide-controlled agonist affinity shifts seen in intact GluRs? What is the basis for the insensitivity of AMPA receptors to DTT? Given this insensitivity, what explains the conservation of the disulfide bridge among GluRs?

**EXPERIMENTAL PROCEDURES**

**Baculovirus Constructs**—The three cysteine residues in the ligand-binding domain of GluRD were replaced individually by serines by using overlap extension PCR (20). The plasmid pK503-4 (15) served as the following oligonucleotides were used as primers: 371, 5'-GGAGACCTTATCAATGCTATCCATAGGCGAGGCTCC-3'; 1413, 5'-GGGTGTCGATTATACGATATTTTGCTGTCGACGGAGAGAATGGTCTCCCCCAT-3'; 1680, 5'-GGTTGGAGCTCGGTTGTCAGGCTTCGGTGGAAGGGCCCAAGGACTCGGGA-3'; 2416, 5'-GGTTGTCCAGCGAGGAAATGGTCTTCCCATCAT-3'; 2806, 5'-TACCAGAAACTTCCTCCCCATGAGGACTGAGCGAAGAATTT-3'; 2906, 5'-GCAGTTTGCTACCTTGATTGCGGAGGAAATTT-3'; 2707, 5'-AAAGGTAAGCTGGCCCAAGGGTCTCGCCAGAAGGCTT-3'; 2708, 5'-CTACGGTGTCCAGAAGGCTTTGCTGTCGATG-3'; 2709, 5'-CTGGGCCCAGGGATCGGAATCGTACCAACCCAT-3'.

For the C56S mutation, the PCR products generated with primer pairs 2416/2709 and 2707/1680 using the C260S PCR products were gel-purified and combined to serve as a template in the second PCR reaction with primers 1413 and 371. The product was digested with Nhel and SacI and cloned into similarly treated pK503-4. For the C260S and C315S mutations, the first PCR reaction was performed with primer pairs 2416/2708 and 2706/1680 and primer pairs 2416/2709 and 2707/1680, respectively, and the second PCR reaction was performed with primers 2416 and 1680. The PCR products were digested with EcoRI and SacI and cloned into similarly treated pK503-4 vector.

To generate the double mutant C260S/C315S, the first PCR reaction was performed with primers 2416/2709 and 2707/1680 using the C260S mutation—carrying plasmid as a template. The presence of the predicted mutations was verified by DNA sequencing. Recombinant baculoviruses for expression of the mutated S1S2 domains were generated by using the Bac-to-Bac system of Life Technologies, Inc. as described previously (15).

**Expression and Purification**—All constructs were expressed in *Trichoplusia ni* High Five cells in Excell-400 medium (JRH) as soluble protein secreted into the extracellular medium. The insect cells were infected with recombinant baculovirus at a cell density of 2.0 × 10⁶ cells/ml and a multiplicity of infection of 4. After 66 h of infection at 27 °C, the extracellular medium was cleared of cells and viruses in a two-step centrifugation (4000 × g for 30 min; 185,000 × g for 1 h). CoCl₂ was added to the supernatant to a final concentration of 3 mM. The supernatant was loaded onto a 25-ml anti-FLAG M1 affinity gel column (Kodak) preequilibrated with washing buffer (10 mM Tris·HCl, pH 7.4, 140 mM NaCl, 3 mM CaCl₂). The column was washed with washing buffer until the protein concentration of the wash was less than 10 μg/ml. Bound protein was eluted with 10 mM Tris·HCl, pH 7.4, 140 mM NaCl, 2 mM EDTA. Protein-containing fractions of the FLAG column were loaded onto an anion exchange column (Sephadex Fast Flow Q-Sepharose, Amersham Pharmacia Biotech) preequilibrated with 10 mM Tris·HCl, pH 8.0, 140 mM NaCl. The bound protein was eluted with 10 mM Tris·HCl, pH 8.7, 10 mM urea, 1 mM EDTA (6). The protein was then incubated with DTT at a final concentration of 10 mM for 60 min at 37 °C.

To remove excess DTT after reduction, gel filtration chromatography was performed with a Sephadex G-25F column (Amersham Pharmacia Biotech). For denatured protein, urea buffer (100 mM NaF, pH 7.3, 10 mM urea, 1 mM EDTA) was used as the elution buffer, and for native protein, phosphate buffer (100 mM NaF, pH 7.3, 1 mM EDTA) was used. The fractions (0.5 ml each) were titrated individually. To ensure separation of DTT from the protein, gel filtration purifications were used only if they had clearly resolved protein and DTT peaks separated by fractions without any absorption at 280 nm (and 412 nm in the presence of DTNB). The Ellman reaction (22, 23) was recorded with a Shimadzu UV260 spectrophotometer that has two optical pathways. The reference cuvette contained 1 ml of urea buffer and 40 μl of 10 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (10–100-fold excess of cysteines) in urea buffer for denatured protein and 600 μl of urea buffer, 40 μl of DTNB, and 400 μl of phosphate buffer for native protein. The sample cuvette contained 600 μl of urea buffer, 400 μl of protein, and 40 μl of 10 mM DTNB in urea buffer. To test the accessibility of the SH group under native conditions, untreated protein was diluted in 1 ml of phosphate buffer, and 40 μl of 10 mM DTNB or ODNB were added to the sample cuvette. The reference cuvette contained 1 ml of phosphate buffer and 40 μl of DTNB or ODNB. The absorption was measured as the absorption difference between the sample and reference cuvette at 412 nm.

The concentration of SH groups (ε NH) was determined by the equation,

\[ \text{ε NH} = \frac{A}{14,150 \times \text{m}_1 \times \text{c}_1 \times \text{d}} \]  

where \( A \) is the absorbance, \( d \) is the path length of the cuvette, and \( \epsilon_{NH} = 13,700 \text{ M}^{-1} \text{ cm}^{-1} \) for titration with urea buffer (for urea concentrations between 6 and 10 M) and \( \epsilon_{NH} = 14,150 \text{ M}^{-1} \text{ cm}^{-1} \) for titration with phosphate buffer.

**Protein Labeling**—The protein was lyophilized and then dissolved in reducing buffer (50 mM NaF, pH 7.3, 8 mM urea, 1 mM EDTA) or labeling buffer (50 mM NaF, pH 6.0, 8 mM urea, 1 mM EDTA) depending on which cysteines were to be labeled. To label all cysteines, the protein was dissolved at a final concentration of 2 mg/ml in reducing buffer and incubated at 37 °C for 5 min DTT. After 30 min, a further 5 mM DTT was added, and the protein was incubated for another 30 min at 37 °C. Then the protein was precipitated in a 10× volume of acetic/1 mM HCl (98/2), centrifuged 10 min at 10,000 × g. The pellet was washed three times in aceton, 1 mM HCl, H₂O (98:2:10) to remove excess DTT. It was then resuspended in labeling buffer, and 2 mol of PM was added per mol of denatured and oxidized free cysteine. Consistent with these assumptions, no more than 50% of the initial PM was detected as having reacted with protein. The labeling reaction was performed for 1.5 h at room temperature. To purify S1S2 from excess PM, the protein was precipitated with aceton as above.

To label only the cysteines participating in a disulfide bridge, S1S2 was dissolved in reducing buffer, and the free SH group was blocked by a 10-fold excess at 5 min at 37 °C. After a further 5 mM DTT was added, the cysteines were labeled, and the protein was separated from excess PM. For labeling with PM and the purification from excess PM, cysteines were labeled, and the protein was separated from excess PM by gel filtration with acetone/HCl as above and resuspended in labeling buffer. The cysteines were labeled, and the protein was separated from excess PM as above. To label only the free cysteine, S1S2 was dissolved in labeling buffer, and the labeling with PM and the purification from excess PM were performed as above. Then the protein was resuspended in reducing buffer and reduced with 5 mM DTT for 30 min at 37 °C. The reduced cysteines were blocked with a 6-fold molar excess of IAM, and the sample cuvette contained 1 μg of protein in 1 ml labeling buffer (50 mM NaF, pH 8.0, 5 M urea, 1 mM EDTA) and 400 μl of 10 mM DTNB and was monitored at both 210 and 342 nm were analyzed with MALDI-MS (Voyager Elite Workstation, Perseptive Biosystems). Using the CCG package program PEPTIDESORT (24), a list of all predicted proteolytic cleavage sites in S1S2 was determined for the V8-protease. On this basis, all possible peptide fragments were computed for a partial proteolysis allowing the identification of the one whose mass was closest to the experimental value. The identification was tested by N-terminal sequencing by Ed-
man degradation (25).

**Equilibrium Dialysis**—Equilibrium dialysis half-chambers with a volume of 60 μl each were separated by a dialysis membrane with a cut-off pore size of 6–8 kDa (Spectrapor). One half-chamber was filled with 10 mM NaPi, pH 7.3, and different concentrations of l-glutamate (NEN Life Science Products). For l-glutamate concentrations greater than 1.5 μM, a mixture of 1.5 μM l-glutamate and different concentrations of cold l-glutamate was used. The other half-chamber contained the same solution supplemented with 0.5–1 μM S1S2. The chambers were allowed to equilibrate for 15 h at 4°C. Then 10 μl from each half-chamber was mixed with 5 ml of scintillation fluid (Packard Instruments), and the radioactivity was determined against ABB, was incubated with 5 nM [3H]AMPA in the presence of increasing concentrations of unlabeled ligands (kainate and 6,7-dinitroquinoxaline-2,3-dione) in a total volume of 500 μl of unpurified protein, dialyzed against ABB, was incubated with 5 nM [3H]AMPA in the presence of increasing concentrations of unlabeled ligands (kainate and 6,7-dinitroquinoxaline-2,3-dione) in a total volume of 500 μl of ABB.

**Results**

**SH Group Titration**—S1S2 possesses three cysteine residues. To determine whether there is a disulfide bridge in the glutamate binding region of the AMPA receptors, SH group titrations were performed.

**Table I**

| Concentration | Cysteines/S1S2 |
|---------------|---------------|
| 10 mM DTT     |               |
| 10 mM urea*   |               |
| -             | 0.94 ± 0.02   |
| +             | 0.97 ± 0.15   |
| -             | 1.23 ± 0.06   |
| +             | 3.20 ± 0.22   |

*Conditions prior to Ellman reaction.

The Ellman reaction was performed under denaturing conditions. The Cheng-Prusoff (26) equation was applied to calculate the kinetic parameters. The difference between non-reduced and reduced S1S2 was 0.33 cysteine/mol of S1S2 and thus in the same range as that observed with the Ellman reaction.

**Disulfide Arrangement**—To localize the disulfide bridge, S1S2 was labeled with PM and digested with *S. aureus* V8-protease. Peptide fragments were separated by reversed phase HPLC and analyzed by MALDI-MS and N-terminal sequencing. The results were compared with peptide fragments predicted for V8-protease digestion.

S1S2 was denatured prior to reduction and labeling with PM, because it was not possible to fully reduce S1S2 in the native state. The labeling yield was approximately 83%. Different proteases were tested under different conditions, and the best chromatographic separation of peptides was obtained with partial proteolysis using the *S. aureus* V8-protease (data not shown). After digestion, the sample was immediately loaded onto the reversed phase column, because it was not possible to inhibit the V8-protease completely either by denaturation (incubation at 95°C for 5 min; addition of SDS to a final concentration of 5%) or by inhibitor (monovalent anions). S1S2 was labeled in three different reactions to determine the position of the disulfide bridge (Fig. 1).

Fig. 1, a and b, shows the elution profile of the free cysteine labeled with PM. There are two prominent peaks with an absorption at 342 nm with a retention time of approximately 55 min in one fraction (D). Using MALDI-MS and N-terminal sequencing, the nonapeptide from Gly54 to Glu62 was identified (Table II). This peptide contains Cys56, which corresponds to Cys493 in the full GluRD sequence. With MALDI-MS, a second, PM-labeled peptide (residues 45–62) could be detected, although the corresponding N-terminal sequence was not found by Edman degradation.

To confirm the disulfide bridge position, a second labeling reaction was performed in which only the cysteines that form the disulfide bridge were labeled with PM (Fig. 1, c and d). Small peaks at 342 nm are detected in one fraction at 50 min (C) and in another at 55 min that corresponds to position D in Fig. 1b. The peak at 50 min could not be analyzed, due to insufficient material. The peaks at 55 min correspond to the free cysteine, Cys56, which was not blocked completely by IAM. There were two prominent peaks at 342 nm with retention times of 44 min (A) and 46 min (B). The peak at position B corresponds to the peptide Ala298 to Glu334 as determined by MALDI-MS and N-terminal sequencing (Table II). This fragment contains Cys315, which corresponds to Cys774 of the intact GluRD. The MALDI-MS profile of the fraction at position A shows two pairs of masses. Within each pair, the mass difference equals that of PM, reflecting incomplete labeling (Table II). The fragment Ala298 to Glu334 containing Cys315 fits well to one pair of masses and was confirmed by N-terminal sequencing. The mass of the second fragment does not fit closely to the calculated mass of any partial V8-proteolytic peptide of S1S2. The closest match is to peptide Tyr251 to Glu297 containing Cys260. The mass deviation of 12 daltons is, however, greater...
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Cysteine-containing peptides of the disulfide arrangement analysis

The fractions that showed an absorption at 210 and 342 nm were analyzed by MALDI-MS (experimental mass) and N-terminal sequencing. The mass deviation of MALDI-MS is smaller than 1 Da. The peptide in fraction C could not be determined (ND), because there was insufficient material.

| Fraction | Fragment | Experimental mass (with/without PM) | Calculated mass (with/without PM) | Da |
|----------|----------|-----------------------------------|----------------------------------|----|
| A        | 232YTEQRKPCC 260..LKLSF 297 | 4822.1/5119.3, 4810.5/5106.8 | 4822.1/5119.3, 4810.5/5106.8 | 1580.7 |
| B        | AGVLD.C 315..SLSNE 334 | 4056.3/4353.8, 4056.6/4352.9 | 4056.3/4353.8, 4056.6/4352.9 | 2070.3 |
| C        | AGVLD.C 315..SNELE 335 | /4595.6, 4595.2 | /4595.6, 4595.2 | |
| D        | 54GYC 456VDASE 472 | 956.4/1253.3, 956.4/1253.3 | 956.4/1253.3, 956.4/1253.3 | 1580.7 |
| E        | MFEGN..C 456VDLA 472 | /2367.7, 2366.6 | /2367.7, 2366.6 | |

The single mutants C260S and C315S showed a clear reduction in expression relative to the wild type protein (Table III); this also applied to the double mutant C260/315S (1.3 mg/liter versus 3.0 mg/liter for WT S112). The single mutants C260S and C315S formed covalent oligomers that disappeared following treatment of the sample with reducing agents such as DTT or β-mercaptoethanol (data not shown). The double mutant also formed oligomers that are retained by a 300-kDa cut-off filter. However, these oligomers appear to be noncovalent, since they are not present on SDS-polyacrylamide gels even in the absence of reducing agents. Thus the double mutant seems to form aggregates perhaps from incorrectly folded molecules.

All three mutants show a reduced expression level and a tendency to oligomerize. In the single mutants, the oligomerization is probably mediated by the remaining cysteine, although the disulfide bridge is sequestered in the wild type S112. In contrast, in the double mutant, noncovalent, probably hydrophobic interactions are responsible for the formation of oligomers. Thus, the correct folding and/or the stability of the protein appears to require both of these cysteines.

The mutation of either cysteine to a serine results in a 2-fold higher affinity for agonist compared with S112, as predicted. Thus, the disruption of the disulfide bridge does appear to stabilize the liganded state relative to the unliganded state. Unfortunately, it was not possible to obtain reproducible agonist binding data from the double mutant, perhaps due to its tendency to aggregate.

Comparison of S112 with Cysteine Mutants—Since it was not possible to reduce S112 in the native state, cysteine mutants were designed to establish whether this disulfide bridge has any influence on the glutamate binding affinity as predicted by Sutcliffe et al. (16) and as shown for GFKAR (7).

The single mutants C260S and C915S both show a similar, slightly higher affinity for [3H]AMPA (Table III) than does S112. C915S has also a 2.4-fold higher affinity for L-[3H]glutamate compared with S112 (Table III). For kainate, the single mutants have a roughly 1.7-fold lower affinity than expected from the uncertainty of this technique (±1 dalton) and does not correspond to any common modification (e.g., methylation). This fragment also could not be sequenced by Edman degradation. Together, these results suggest that the fragment is chemically modified. When all cysteines are labeled with PM, the elution profile at 342 nm (Fig. 1, e and f) is approximately equal to the sum of Fig. 1, a and b, and Fig. 1, c and d.

Taken together, these results demonstrate the presence of a single disulfide bridge in S112, formed between cysteines Cys260 and Cys315.

Discussion

S112 expressed as a soluble secreted protein in insect cells contains one disulfide bridge as shown by titration with DTNB. It is formed between Cys260 and Cys315 of S112, corresponding to Cys719 and Cys774 of GluRD, respectively (Cys740 and Cys795 in the immature sequence). This disulfide bridge was predicted by sequence homology to NR1 (Cys236 and Cys270) and GFKAR (Cys305 and Cys385) (7, 10, 13, 14, 16). In NR1 and GFKAR, these residues are expected to form a disulfide bridge, because site-directed mutants lacking either cysteine are insensitive to DTNB and DTNB, unlike the wild-type molecules, which show clear redox modulation. This work presents the first biochemical evidence that the target of such DTT potentiation is indeed an intramolecular disulfide bridge. Taken together with mutational studies of NMDA and kainate receptors, there is now evidence for conservation of this disulfide bridge across iono-
The \( K_d \) values for AMPA were determined by filter binding with \(^3\text{H}\)AMPA in the presence of KSCN and fitted as a hyperbolic curve fit. The \( K_d \) values for L-glutamate were determined by equilibrium dialysis and analyzed in the same way. The \( K_d \) values for kainate and DNQX were determined by ligand competition assays as described under “Experimental Procedures.” The expression yield was calculated for a 1-liter insect cell preparation after the last purification step.

| Constructs | \( K_d \) of AMPA \( \mu \text{M} \) | \( K_d \) of Glu \( \mu \text{M} \) | \( K_d \) of kainate \( \mu \text{M} \) | \( K_d \) of DNQX \( \mu \text{M} \) | Activity of Glu % | Expression mg |
|------------|-----------------|-----------------|-----------------|-----------------|---------------|----------|
| S1S2       | 13.3 ± 1.7      | 999 ± 88        | 1.94 ± 0.47     | 432 ± 181       | 106.0 ± 2.5   | 3        |
| C315S      | 7.2 ± 2.2       | 336 ± 24        | 3.22 ± 0.42     | 257 ± 78        | 17.5 ± 0.4    | 1.4      |
| C260S      | 7.8 ± 0.9       | ND*             | 3.40 ± 0.40     | 321 ± 70        | ND            | 0.9      |

\* ND, not determined.

**TABLE III**

**Equilibrium constants, activities, and expression yield of S1S2 and its cysteine mutants**

These results are qualitatively consistent with the modeling predictions of Sutcliffe et al. (18). Their model was developed based on the putative structural homology between the GluR ligand-binding domains and the bacterial periplasmic binding proteins, for which several apo and holo structures have been determined at atomic resolution (17, 29–31). By analogy to the periplasmic binding proteins, the two conserved cysteines would be close enough to form a disulfide bridge in the unbound conformation but would be separated by more than 20 Å in the bound conformation (there is no homologous disulfide bridge in the periplasmic binding proteins). This led to the prediction that disrupting the disulfide bridge should increase the affinity of GluRs for ligand, as observed. The small size of the observed effect (2–3-fold decrease in \( K_d \), corresponding to approximately 0.5 kcal/mol), indicates, however, that the coupling between ligand binding and the predicted conformational shift of >20 Å is not strong and/or that the magnitude of the shift may be considerably smaller than predicted.

Furthermore, several lines of evidence suggest that elimination of the disulfide bridge not only relaxes a stereochemical restraint but also disrupts the native structure. This is true even for the isosteric substitution of serine for cysteine; although the disulfide bridge is poorly accessible to reducing agents and therefore presumably buried, single cysteine mutants form covalent oligomers, and the double mutant forms noncovalent aggregates, indicating that normally sequestered structural features are exposed for inappropriate interactions.

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Fig. 2. A, the binding of \(^3\text{H}\)glutamate to S1S2 and the single mutant C315S. Binding was determined by equilibrium dialysis. The data were fitted as a hyperbolic curve fit. The corresponding parameters are presented in Table III. Data were obtained from duplicate measurements. B, Scatchard Plot of the data presented in A.

With our finding that the mutants C260S and C315S have 2-fold higher affinity for ligands than wild-type S1S2.

Our results provide the first evidence that the DTT modulation of agonist affinity in GluR requires only the ligand-binding domain S1S2, i.e. that it does not depend on interactions with the remainder of the protein or with the membrane. This means that the observed changes in EC\(_{50}\) or agonist affinity of intact receptors upon reduction/elimination of the disulfide bridge predominantly reflect a more favorable free energy of binding rather than a less unfavorable free energy of, for example, gating. Interestingly, in NR1, Cys\(^{726}\) and Cys\(^{780}\) have also been shown to influence cooperative gating by glutamate and glycine (28), implying a possible additional role in NMDA receptors.

These structural changes or others may well also be reflected in the reduced expression level of the mutants and the significant loss of activity. Taken together, these lines of reasoning suggest that the disulfide bridge is required for the overall stability and functionality of the GluR ligand-binding domains. As a result, particular care is required in the interpretation of site-directed mutant data, since physiological effects may be due to elimination of the disulfide bridge and/or to the disruption of native structure. In addition, the observed structural role of the disulfide, if applicable to the intact receptor (see below), would be sufficient to explain the conservation of the cysteine residues involved, regardless of the in vivo relevance of DTT-like modulation.

tropic GluR subfamilies, despite the observed lack of redox modulation of AMPA receptors (10, 27).

This disulfide bridge influences the affinity for ligand as shown by electrophysiological and ligand binding experiments with NR1, GF2AR, and GluRC (7, 10, 18). In NR1 the two single mutations caused a decrease in the EC\(_{50}\) value of 3–6-fold (10). The treatment of GF2ARα and β with DTT increases their affinity for kainate about 2-fold, and this can be reversed by incubation with DTNB. Further, Wo and Oswald (7) have shown that single cysteine mutants of GF2ARβ also have a 2-fold smaller \( K_d \) for kainate than wild-type GF2ARβ. The higher affinity of the single cysteine mutants of GF2ARβ, the decrease of the EC\(_{50}\) value of NR1 in the presence of DTT, and the decrease of the EC\(_{50}\) value of the cysteine mutant GluRC C722A corresponding to Cys\(^{260}\) of S1S2 are in good agreement
The at least partial inaccessibility of both the disulfide-bonded and the free cysteines in S1S2 has significant consequences for the sometimes controversial interpretation of the numerous effects of reducing and labeling agents on the properties of various GluRs (4, 32, 33). The fact that some but not all GluRs exhibit DTT potentiation has led to the proposal that the subunit composition of the receptors determines whether or not the disulfide bridge is formed (4). Instead, the observed lack of DTT potentiation in AMPA receptors is probably due to the fact that the disulfide bridge is poorly accessible to the reducing agent in these subunits, as it is in S1S2. Considered together with the structural importance of the disulfide, it now appears more likely that the disulfide exists but is inaccessible to particular reducing agents in certain GluR subunits. Subunit interactions can also be imagined to influence the accessibility of the disulfide to different reducing agents, either directly or via induced conformational changes, as in the case of the NMDA receptors. Here, DTT can modify a NR1 disulfide in NR1/NR2B-NR2D heteromers but not in NR1 homomers (5, 6, 10, 19). In NR1/NR2A heteromers, a second potentiation phenomenon appears to be mediated by a different redox site, which is also influenced by the redox compounds GSH and mercaptoethylamine (6). This may involve a disulfide in NR2A that is inaccessible to DTT, GSH, and mercaptoethylamine in the other NR2 subunits, perhaps even the homologue of the Cys726/Cys780 disulfide in NR1.

Furthermore, it has been suggested that the redox state of the receptors is regulated in vivo, in order to maintain the receptors in a state that is neither fully oxidized nor fully reduced (4). While such regulation is possible in principle, it is not necessary to explain the existence of an intermediate redox state: here we have shown that S1S2 exists as an extracellular protein with one disulfide bridge and one free thiol. This requires only the appropriate local stereochemistry (e.g. presence or absence of a partner cysteine, accessibility of a free thiol to oxidizing agents). Finally, since the accessibility of a cysteine or disulfide bridge can be GluR subunit-dependent, the variable susceptibilities of different tissue preparations to a given redox agent may well simply reflect the presence of different subunit combinations; they need not be the result of differential regulation of the redox state of GluR in different tissues (4). Thus, the hypothesis that GluRs are subject to active redox regulation in vivo needs to be supported by direct biochemical evidence.

Despite these questions concerning GluR redox regulation in vivo, redox agents remain interesting as potential therapeutics for the treatment of a variety of neuropathological conditions. Our results suggest that future experiments with both reducing and oxidizing agents must consider not only the redox state of individual cysteine residues but also the accessibility of such residues or disulfides to the particular reducing, oxidizing, or labeling agent involved. As we have shown, the free thiol in S1S2 is poorly labeled by DTNB but well labeled by the more hydrophobic ODNB. This is consistent with the observation that S1S2 does not form covalent dimers even at the high protein concentrations typical of crystalization experiments. In general, both the steric and chemical properties of the redox agent will have to be considered. This means that even saturating concentrations of labeling agents may not produce a “fully oxidized” state in the receptor; free cysteines inaccessible to the labeling agent may remain in the reduced state and available to other oxidizing agents (33).

The possibility must be considered that S1S2 is not structurally identical to intact GluRD. Due to the presence of other domains in the subunit, the lipid bilayer and/or other subunits in the oligomeric channel, the significance of the disulfide bridge may be altered in intact channels relative to S1S2. In particular, it is difficult to compare the structural stabilization contributed by the disulfide to that found in intact channel. Homologous cysteine mutants of NR1 and the smaller GFKARβ mutants have been successfully expressed in Xenopus oocytes (10) and human embryonic kidney 293 cells (7), respectively. On the other hand, the specific activity of the mutant protein was not quantitated in these systems; nor was the overall level of expression reported for NR1, so that it remains possible that the disulfide is as important for the structural integrity of the intact receptor as for S1S2.

As far as the pharmacological results are concerned, substantial evidence argues that S1S2 is an excellent surrogate for the intact protein. S1S2 and the intact GluRD have identical pharmacology (15). The change of affinity for single S1S2 mutants and single GFKARβ mutants (7) is in the same range. The inaccessibility of the disulfide bridge between Cys660 and Cys115 to DTT accounts for DTT insensitivity of the intact GluRD (5, 27). The decrease of the EC50 value and a significantly lower activity of the mutant GluRC C722S (18) agrees also with our observations. In every case where measurements on intact AMPA receptors can be compared with equivalent ones in S1S2, the results are very similar. Thus, it is highly likely that S1S2 represents the physiological structure of the glutamate binding domain of GluRD, and our measurements provide additional evidence that it contains the determinants of agonist affinity of intact receptors.

In this paper, we have used a variety of redox agents both to characterize the disulfide topology in the AMPA receptor GluRD ligand-binding domain and to establish the basis for the lack of DTT potentiation in the AMPA receptor family. The ligand-binding domain alone has been shown to be sufficient to reproduce the disulfide modulation of agonist affinity seen in intact GluRs. In addition, we have shown that loss of the disulfide bridge not only has a weak effect on agonist affinity but leads as well to a disruption of native structure and a dramatic loss of specific activity. This stabilizing function of the disulfide bridge may explain its conservation across the GluR family.

Acknowledgments—We thank U. Reygers and A. Pallas for skilful technical assistance. We thank H. Faustlich, D. Heintz, and W. Kliche (Max Planck Institute for Medical Research; MPIMF) for providing technical assistance. We thank H. Faulstich, D. Heintz, and W. Kliche (Max Planck Institute for Medical Research; MPIMF) for help in HPLC, M. Rentzea (MPIMF) and D. Waiderlich (PerSeptive Biosystems) for the MALDI-MS, and R. Kellner (Johannes Gutenberg University, Mainz, Germany) for N-terminal sequencing.

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