Expression and Initial Characterization of a Soluble Glycine Binding Domain of the N-Methyl-d-aspartate Receptor NR1 Subunit*

Aleksandra Ivanovic‡, Helmut Reiländer‡, Bodo Laube‡, and Jochen Kuhse¶

From the Department of Neurochemistry, Max-Planck-Institute for Brain Research, Deutschordenstraße 46 and the Department of Molecular Membrane Biology, Max-Planck-Institute for Biophysics, Heinrich-Hoffmann-Strasse 7, D-60528 Frankfurt/Main, Germany

Glycine is an essential co-agonist of the excitatory N-methyl-d-aspartate (NMDA) receptor, a subtype of the ionotropic glutamate receptor family. The glycine binding site of this hetero-oligomeric ion channel protein is formed by two distinct extracellular regions, S1 and S2, of the NR1 subunit, whereas the homologous domains of the NR2 subunit mediate glutamate binding. Here, segments S1 and S2 of the NR1 polypeptide were fused via a linker peptide followed by N- and C-terminally tagging with Flag and His6 epitopes, respectively. Infection of High Five insect cells with a recombinant baculovirus containing this glycine binding site construct resulted in efficient secretion of a soluble fusion protein of about 53 kDa. After affinity purification to near-homogeneity, the fusion protein bound the competitive glycine site antagonist [3H]MDL105,519 with high affinity (Kd = 5.22 ± 0.13 nM) similar to that determined with rat brain membrane fractions. This high affinity binding could be competed by the glycine site antagonist 7-chlorokynurenic acid as well as the agonists glycine and d-serine but not by L-glutamate. This indicates that the S1 and S2 domains of the NR1 subunit are sufficient for the formation of a glycine binding site that displays pharmacological properties similar to those of the NMDA receptor in vivo.

Excitatory neurotransmission in the mammalian brain is predominantly mediated by members of the glutamate receptor family of ligand-gated ion channels, which have been grouped into three distinct subfamilies, the a-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors, the kainate receptors, and the N-methyl-d-aspartate (NMDA) receptors (1). Among the glutamate receptors, the NMDA receptor has gained particular interest because of a number of unique properties that classify this ligand gated ion channel as a coincidence detecting molecule in synaptic plasticity and memory formation (2). NMDA receptors are highly permeable to Ca2+ (3), they are regulated by a voltage-dependent Mg2+ block (4), and simultaneous binding of both glutamate and the co-agonist glycine is required for efficient activation of NMDA receptors (5, 6). Prolonged stimulation resulting in high Ca2+ influx causes neuronal cell death, which has been implicated in anoxic-ischemic injury and possibly other neurodegenerative disorders (7). Therefore, the binding site for the co-agonist glycine is a target for site-specific NMDA receptor antagonists, which are proved to be neuroprotective in animal stroke models (8).

Structural analysis of the NMDA receptor was initiated by expression cloning of the NMDA receptor subunit NMDAR1 (NR1 or a). Subsequently, additional cDNAs including eight NR1 splice variants (NR1a to NR1h) and different isoforms of a second type of NMDA receptor subunits (NR2A-D, e1–4) were identified (reviewed in Ref. 9). At the N terminus of both, the NR1 and NR2 subunits, a cleavable leader sequence is followed by a long extracellular domain and, in the second half of the polypeptides, four hydrophobic membrane domains (M1–M4). The M1, M3, and M4 segments are transmembrane spanning, whereas segment M2 is thought to form a reentrant loop that lines the ion channel (9). The NR1 subunit is expressed throughout the central nervous system, and immunohistochemistry has shown that the different NR2 subunits display more restricted, though overlapping, temporal and regional expression patterns (10). Functional and regional heterogeneity of NMDA receptors is therefore thought to result from differential assembly of the NR1 subunit with one or more members of the NR2 subunit family (11, 12).

Despite considerable progress in the molecular analysis of NMDA receptors, the structural basis for their properties is largely unknown, because the three-dimensional structure of these transmembrane proteins has not been solved. Therefore, mutational analysis combined with heterologous expression studies have been employed to identify channel-forming segments (13) and intracellular protein binding regions as discrete functional domains within NMDA receptor subunits (14). We and others have used site-directed mutagenesis to localize the glycine and glutamate binding sites of the NMDA receptor to a segment preceding M1 (S1) and a second region in between M3 and M4 (S2) of the NR1 and NR2 subunits, respectively (15–19). Our results indicate that these domains of NMDA receptor subunits are structurally homologous to soluble bacterial amino acid-binding proteins (15, 18, 20). As the corresponding regions of AMPA receptor channels could be successfully expressed as soluble fusion proteins, which specifically bound AMPA receptor ligands (21–23), we constructed a secreted protein composed of the S1 and the S2 domains of the NMDA receptor NR1 subunit. Upon expression, using the baculovirus system, this glycine-binding protein revealed properties resembling those of the native NMDA receptor glycine binding site. Thus, our experiments demonstrate that the NR1 subunit is sufficient for the formation of a glycine binding site and offer a potential strategy for determining the three-dimensional structure.
EXPERIMENTAL PROCEDURES

Materials—[3H]MDL105,519 (3.15 Tbq:mmol) and the ECL kit were purchased from Amersham Pharmacia Biotech (Braunschweig, Germany). The anti-Flag M1 and M2 antibodies were obtained from Integra Biosciences (Fernwald, Germany), and the secondary sheep anti-mouse IgG antibody (horseradish peroxidase-labeled) from Dianova (Hamburg, Germany). Benzozase was purchased from Merck (Darmstadt, Germany). All other chemicals were of reagent grade, obtained primarily from Sigma (Deisenhofen, Germany). Sf9 cells were supplied by ATCC (Rockville, MD). High Five cells were from Invitrogen.

Cell culture medium ingredients were either from Sigma or Life Technologies, Inc. Sf900 medium and ExCell405 medium was purchased from Life Technologies, Inc. or Biozol, respectively. Modified baculovirus DNA (BaculoGold) was obtained from PharcMingen (San Diego, CA).

Construction of Recombinant Baculoviruses—For the construction of the recombinant transfer vector pVMelFlagNR1S1S2H6, a BamHI/KpnI-DNA fragment generated by polymerase chain reaction encoding a His6-tagged NR1S1S2 receptor fragment was inserted into the vector pVMelFlag (24). The construct (see Fig. 1A) was verified by restriction analysis as well as DNA sequencing. Details for recombinant baculovirus production, insect cell culture, and immunoblot analysis are given in Refs. 25–27.

Purification of the Recombinant Protein by Immobilized Metal Ion Affinity Chromatography—High Five cells were grown in suspension culture to a cell density of 3 × 10^6 cells/ml and infected with recombinant baculovirus VLmelFlagNR1S1S2H6. Four days after infection, cells were pelleted by centrifugation, and the supernatant was dialyzed (1000 × volume) against buffer 1 (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10% (w/v) glycerol). Subsequently, 90–125 ml of the dialyzed protein solution was loaded onto a nitriotriacetic acid (NTA) column (2–10 ml; Qiagen, Hilden, Germany) that had been equilibrated with buffer 1. The column was washed with buffer 1 until the A_280 base line was reached. Elution of the bound proteins were performed with a gradient of 0–300 mM imidazole in buffer 2 (50 mM sodium phosphate, pH 6.0, 300 mM NaCl, 10% glycerol).

Molecular Mass Separation of Purified Protein—Oligomeric S1S2 proteins were separated by size exclusion chromatography or sucrose density gradient centrifugation on a 9–41% (w/v) sucrose gradient. This gradient was generated by underlaying 3.6 ml of 15% sucrose in 50 mM Tris acetate, pH 7.5, with 300 ml of 60% sucrose in a SW60 Ti tube followed by freezing (−70 °C) and thawing (4 °C), and the gradient was formed. After loading a sample of purified protein, the gradients were run in a SW60 Ti rotor at 48,000 rpm for 13.5 h. Fractions of 280 ml, each, were analyzed for S1S2 protein by immunoblot analysis with the anti-Flag M2 antibody. The positions of the following marker proteins run in parallel were determined by respective enzyme assays: β-galactosidase, 13.92 S; catalase, 11.3 S; lactate dehydrogenase, 6.95 S; malate dehydrogenase, 4.32 S; cytochrome c, 1.8 S. Gel filtration was performed on a Superose 12 PC column using the SMART system (Amersham Pharmacia Biotech, Uppsala, Sweden) under the following conditions: 50 mM NaPi, pH 8.0, 300 mM NaCl, 10% (w/v) glycerol, at 4 °C with a flow rate of 40 μl/min. Samples (50 μl) were applied in flow buffer at a protein concentration of 0.3 mg/ml.

Filtration Binding Assays—Binding assays were performed with volumes of 50–250 μl containing about 1.5 μg of protein of purified S1S2 protein. Saturation measurements with [3H]MDL105,519 were done at 22 °C for 60 min in 50 mM Tris acetate, pH 7.5. After incubation, free and bound ligand were separated by filtration through NC 45 membrane filters (Schleicher and Schuell, Dassel, Germany) and washed four times with 1 ml of binding buffer. Non-specific binding was determined in the presence of 100 μM glycine. All measurements were performed in duplicate and repeated two to three times. Ligand competition assays with glycine, β-merine, and 7-chlorokynurenic acid were performed in the presence of 10 μM [3H]MDL105,519 and increasing concentrations of unlabeled ligands at 4 °C for 20 min using the same filtration conditions as above. Binding data were analyzed using a nonlinear curve fitting procedure (KaleidaGraph, Abelbeck Software).

RESULTS

Production of S1S2 Protein—To examine whether the S1 and S2 domains of the NR1 subunit can generate a soluble glycine binding polypeptide, the expression vector pVL93MelFlagNR1S1S2H6 was constructed. As shown in Fig. 1A, the construct included amino acids Thr^{568} to Thr^{543} of the S1 domain and, interconnected via a 13-amino acid spanning linker (21), amino acids Asp^{640} to Asn^{794} of the S2 domain of the NR1 subunit. This S1S2 cassette was N-terminally fused to the prepromelitin signal peptide to ensure proper secretion through the endoplasmatic reticulum pathway. Additionally, the S1S2 construct was tagged with a Flag epitope at the N terminus, and with a His6-tag at the C terminus, for immunological detection and purification of the recombinant protein.

After co-transfection of Sf9 insect cells with the BaculoGold virus and pVL93MelFlagNR1S1S2H6 plasmid DNA, recombinant baculoviruses were identified and analyzed as described (25). The characterized virus was propagated, and infection of Sf9 cells with this recombinant baculovirus VLmelFlagNR1S1S2 resulted in efficient production of the fusion protein, as revealed by immunoblot analysis (Fig. 1B). Five bands in the molecular mass range of 41–53 kDa were detected in infected cells with the monoclonal antibody M1, whereas only one prominent band of about 53 kDa was detected in the cell culture supernatant. In addition, a protein band of about 90–100 kDa, which might represent a dimer of the expressed protein, could be detected in the cell extracts (Fig. 1B). The predicted molecular mass of the recombinant fusion protein is 41.4 kDa; to investigate whether the observed bands of 43–53 kDa might correspond to posttranslational modification products, we grew the infected insect cells in the presence of tunicamycin, which inhibits N-linked glycosylation. As shown in Fig. 1B, treatment with tunicamycin eliminated polypeptide species in the 43–53 kDa range; only a single band of 41–42 kDa and its putative dimer of about 90–100 kDa were detected by the anti-Flag M1 antibody. The size difference of about 11–12 kDa is in agreement with the expected presence of up to five oligosaccharide chains at the five potential N-linked glycosylation sites predicted by the amino acid sequence. We therefore conclude that the higher molecular mass bands seen in the infected cells represent different glycosylation states of the recombinant protein and that the fully glycosylated protein was secreted more efficiently into the cell culture medium.

Purification of the S1S2 Protein—For purification of the heterologously produced fusion protein, the cell culture superna-
tient of infected High Five cells was harvested 3 days after infection with baculovirus VLMelFlagNR1S1S2H6 and, after dialysis, loaded onto a nickel-NTA affinity column. After extensive washing, bound proteins were eluted with a linear imidazole gradient and fractions were analyzed by immunoblotting with M2 antibody. As shown in Fig. 2 the S1S2 fusion protein (53 kDa) was specifically enriched by the metal ion affinity chromatography. Immunoblot analysis disclosed the presence of multimerized S1S2 polypeptides and in particular putative SDS-resistant dimers in fractions with high S1S2 protein concentration. Further purification of the fusion protein was performed using a M1 antibody affinity column (data not shown).

**Homo-oligomerization of the S1S2 Polypeptide**—To further investigate the oligomerization of the S1S2 polypeptide indicated by the presence of SDS-resistant complex in SDS-PAGE, sucrose density gradient centrifugation and size exclusion chromatography was performed on the protein fractions eluted from the nickel-column. Sedimentation analysis on sucrose gradients (Fig. 3A) revealed different oligomerization states of the S1S2 protein. Besides a protein complex of high molecular mass (>450 kDa) sedimenting to the bottom of the gradient, an additional peak could be detected at gradient positions between the peak fractions of marker enzymes with S values of 4.32 S (malate dehydrogenase, 70 kDa) and 6.9 S (lactate dehydrogenase, 134 kDa). Interestingly, in fractions that corresponded to higher molecular masses, putative SDS-resistant S1S2 oligomers could be seen (Fig. 3A). Consistent with the results from sucrose gradient centrifugation, different oligomerization states were also found by size exclusion chromatography. Here, a high molecular mass fraction could be separated from a multiphasic peak fraction with a maximum at an elution volume corresponding to a molecular mass of about 100 kDa. The absorption spectra (280 nm) of fractions were nearly superimposable with column run in the presence or absence of 2 mM dithiothreitol (data not shown); thus intramolecular disulfide bridges seem not to be responsible for the oligomer formation by the S1S2 protein.

**Binding Properties of the Recombinant Fusion Protein**—To establish the functional integrity of the NR1 S1S2 fusion protein produced in insect cells, its binding affinity for the glycine site antagonist \[^{3}\text{H}]\text{MDL105,519}\) (28) was determined. For these assays, protein was purified by nickel-affinity chromatography as described above, and saturation analysis was performed using 0.78–50 nM \[^{3}\text{H}]\text{MDL105,519}\) (Fig. 4A). Binding was saturable with a \(K_d\) of 5.22 ± 0.13 nM and a \(B_{max}\) of 100 pmol/mg protein as calculated by nonlinear regression (\(n = 2\)) (Fig. 4A). Scatchard transformation of the data indicated that binding of the antagonist to the purified protein was best described by a model assuming a single class of equivalent and independent binding sites (Fig. 4A). The dissociation constant determined is in good agreement with results obtained for the binding of this glycine antagonist to membrane-bound NMDA receptor (\(K_d = 3.77 \text{ nM}\)) (28).

The ability of glycine, d-serine, L-glutamate, and 7-chlorokynurenic acid to compete for binding of \[^{3}\text{H}]\text{MDL105,519}\) to the purified protein is shown in Fig. 4B. L-Glutamate (1 and 10 mM) had no effect on radioligand binding (data not shown); inhibition constants (\(K_I\)) of 27 ± 5.2 \(\mu\text{M}\) for glycine, of 10.7 ± 7.3 \(\mu\text{M}\) for d-serine, and of 0.71 ± 0.15 \(\mu\text{M}\) for 7-chlorokynurenic acid were calculated from the inhibition curves. The \(K_I\) values determined for glycine and d-serine for the S1S2 fusion protein resembles those obtained for homomeric NR1 receptors expressed in CHO-K1 cells, i.e. \(K_I\) values of 2.6 and 1.2 \(\mu\text{M}\) for glycine and d-serine, respectively (29). In conclusion, our results demonstrate that the S1S2 protein displays specific binding of glycine, d-serine, MDL105,519, and 7-chlorokynurenic acid with affinities similar to those found with homomeric expressed NR1 receptors.

**DISCUSSION**

In this study, we show that the S1 and S2 domains from the NMDA receptor NR1 subunit can be produced as a soluble and secreted fusion protein in insect cells. Biochemical analysis of the produced fusion protein revealed heterogeneity, because of N-linked glycosylation. Highly glycosylated protein was secreted into the medium and could be purified by affinity chromatography. Several reports indicate that both the expression levels and subcellular localization of receptor subunits are critically dependent on glycosylation (30). Thus, glycosylation of
with the finding that the glycine binding affinities of different NR1/NR2 heteromeric NMDA receptors are significantly different. In other words, the NR2 subunits appear to importantly contribute to the conformation of the NR1 glycine binding site.

In earlier studies, we proposed a model for the glycine binding site (15, 17, 18) that was based on the known three-dimensional structure of the bacterial lysine/arginine/ornithine-binding protein from *Salmonella typhimurium* (32). Accordingly, the glycine binding site is formed by a deep cleft between two globular domains, which can be appositioned in either an “open” or a “closed” conformation. The presumed “opening-closing” movement of the two lobes around a connecting “hinge” region is thought to allow binding and dissociation of the ligated amino acid and has been described as the “Venus flytrap” model (33).

Mutagenesis experiments have identified several amino acid residues of the S1 and S2 segments that determine apparent glycine affinities, whereas competition with the glycine antagonist 7-chlorokynurenic acid was affected only by mutations within the S1 sequence (15, 17). Thus, high affinity glycine binding might implicate closure of the bilobate binding structure, whereas antagonistic binding seems to be restricted to domain S1, suggesting an open conformation of the binding pocket (15, 17, 18). The reduced agonist affinity of the S1S2 protein could reflect an open conformation of the binding fold, which we propose to be characteristic for homomeric glycine binding sites. This interpretation is consistent with the low affinity for glycine and α-serine of heterologously expressed, nonfunctional homomeric NR1 receptors (29). In conclusion, we propose a model of receptor function in which the opening of the ion channel of NMDA receptors can only be achieved when the NR1 glycine binding site switches to a closed conformation because of allosteric interactions in the hetero-oligomeric, but not in NR1 homo-oligomeric, receptors.

This model of the glycine binding pocket of the NR1 subunit is supported by experiments that revealed that the S1 and S2 segments of the homologous AMPA and kainate receptors form high affinity glutamate binding sites (34–36). Moreover, soluble fusion proteins have been produced from respective domains of AMPA receptor subunits (21–23) that bind glutamate-specific ligands. The finding that both glutamate- and glycine-binding proteins can be engineered by fusing the corresponding S1 and S2 segments corroborates the view that the overall structures of these different binding sites have to be similar in three-dimensional structure. This interpretation is consistent with molecular modeling of the glycine and glutamate binding sites of the NR1 and NR2B NMDA receptor subunits, respectively (18).

In conclusion, this study shows that the S1 and S2 domains of the NMDA receptor NR1 subunit can be joined into a soluble fusion protein capable of binding glycine site specific agonists and antagonists. This approach should allow insight into the functional organization of the NMDA receptor and provide a novel approach to disclose the molecular structure of agonist binding sites.

Acknowledgments—We thank Dr. S. Nakanishi for supplying the NR1 cDNA, G. Maul for excellent technical assistance, and H. Betz and J. Jacobsen for critical reading of the manuscript.

REFERENCES


**Soluble NR1 Glycine Binding Domain**
Expression and Initial Characterization of a Soluble Glycine Binding Domain of the 
N-Methyl-d-aspartate Receptor NR1 Subunit
Aleksandra Ivanovic, Helmut Reiländer, Bodo Laube and Jochen Kuhse

doi: 10.1074/jbc.273.32.19933

Access the most updated version of this article at http://www.jbc.org/content/273/32/19933

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 34 references, 11 of which can be accessed free at http://www.jbc.org/content/273/32/19933.full.html#ref-list-1