Conserved High Affinity Ligand Binding and Membrane Association in the Native and Refolded Extracellular Domain of the Human Glycine Receptor α1-Subunit*

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The strychnine-sensitive glycine receptor (GlyR) is a ligand-gated chloride channel composed of ligand binding α- and gephyrin anchoring β-subunits. To identify the secondary and quaternary structures of extramembraneous receptor domains, the N-terminal extracellular domain, the large intracellular TM3–4 loop (α1(309–392)) of the human GlyR α1-subunit were individually expressed in HEK293 cells and in Escherichia coli. The extracellular domain obtained from E. coli expression was purified in its denatured form and refolding conditions were established. Circular dichroism and Fourier-transform-infrared spectroscopy suggested ~25% α-helix and ~48% β-sheet for the extracellular domain, while no α-helices were detectable for the TM3–4 loop. Size exclusion chromatography and sucrose density centrifugation indicated that isolated glycine receptor domains assembled into multimers of distinct molecular weight. For the extracellular domain from E. coli, we found an apparent molecular weight compatible with a 15mer by gel filtration. The N-terminal domain from HEK293 cells, analyzed by sucrose gradient centrifugation, showed a bimodal distribution, suggesting oligomerization of ~5 and 15 subunits. Likewise, for the intracellular domain from E. coli, a single molecular mass peak of ~49 kDa indicated oligomerization in a defined native state. As shown by 3Hstrychnine binding, expression in HEK293 cells and refolding of the isolated extracellular domain reconstructed high affinity antagonist binding. Cell fractionation, alkaline extraction experiments, and immunocytochemistry showed a tight plasma membrane association of the isolated GlyR N-terminal protein. These findings indicate that distinct functional characteristics of the full-length GlyR are retained in the isolated N-terminal domain.

The inhibitory glycine receptor (GlyR) is a neurotransmitter-gated ion channel, mediating rapid synaptic transmission in the central nervous system (1–3). The GlyR is a member of the ligand-gated ion channel superfamily, which also includes the nicotinic acetylcholine receptor (nAChR), serotonin 5-HT3 receptor, as well as GABA_A/C receptors. Members of this protein family share a quaternary structure of five subunits surrounding a central ion-conducting pore (4–6). The GlyR isoform prevalent in adult mammalian central nervous system is thought to comprise three α1-subunits and two structural β-subunits, which are thought to contribute to synaptic anchoring of receptor complexes. Generally, GlyR α-subunits possess the ability to form homomeric receptor channels. The subunit topology of the nAChR superfamily, as deduced from hydropathy analysis, consists of a large N-terminal extracellular domain, followed by four hydrophobic stretches of sufficient length to span the plasma membrane, and the extracellular C terminus (7). Of all transmembrane regions, TM2 forms the inner lining of the central ion channel. A large cytosolic loop, flanked by TM3 and TM4, is thought to mediate intracellular interactions. Based on NMR studies of corresponding synthetic peptides, TM2 of the nAChR and GlyR has been demonstrated to be α-helical (8, 9). Several attempts have been made to elucidate the structure of the nAChR extracellular domain, which is widely seen as a prototype of the structural homologous GlyR. Recently, the chicken α7 extracellular domain was expressed in Xenopus oocytes (10), while a soluble fusion protein of the rat α7 nAChR N-terminal domain and mouse binding protein has been generated in Escherichia coli (11). In addition, the extracellular domain from the human muscle AChR α subunit was expressed as a soluble protein in the yeast Pichia pastoris (12), while the α7 nAChR extracellular domain fused to GST was expressed in E. coli and subsequently refolded (13). In an alternative approach, the x-ray structure of a molluscan ACh binding protein was solved at atomic resolution (14), and revealed structural similarity to the extracellular domain of nAChRs, as determined by electron microscopy (15).

The extracellular domain of the GlyR α1-subunit comprises ~50% of the entire subunit sequence, and contains determinants of ligand binding and subunit assembly (16). Six of eight key residues that form the putative intersubunit contact surface are located within the stretch of amino acid residues 1 to 100 (17), a segment that was not associated with high affinity ligand binding (18). Site-directed mutagenesis studies suggest that glycine and strychnine bind to distinct, but overlapping, sulfonic acid; GST, glutathione S-transferase; DORA, dot blot receptor assay; TM, transmembrane; mAb, monoclonal antibody; HEK, human embryonic kidney.

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The abbreviations used are: GlyR, glycine receptor; PEI, polyethyl-eneimine; ATR, attenuated total reflection; FTIR, Fourier-transform-infrared; CHAPS, 3-(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonic acid; GST, glutathione S-transferase; DORA, dot blot receptor assay; TM, transmembrane; mAb, monoclonal antibody; HEK, human embryonic kidney.

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sites on recombinant receptors (16). In the GlyR α1-subunit, residues Ala103–Asn109, Gly140–Tyr143, Lys183, and Lys259, Thr294 represent determinants of ligand binding and agonist-antagonist antagonism. Recently, an alternative model of the GlyR topology was proposed, based on limited proteolysis and chemical modification of reconstituted α1 GlyR (19). In particular, this alternative model suggested the presence of one or more membrane-associated stretches within the postulated extracellular N-terminal domain as well as a new topology of TM1 and TM3.

Here, we describe the large scale recombinant expression and purification of the isolated GlyR α1 extracellular and intracellular domains. Strichyne affinity in the nanomolar range was retained for both, eukaryotic and refolded N-terminal domains. Surprisingly, the isolated extracellular domain showed a strong membrane association when expressed in HEK293 cells. Secondary structure analysis suggested a significant contribution of α-helices to the extracellular domain, while no α-helical structure was detectable in the intracellular TM3–4 loop. Our results show that other extracellular regions of the GlyR, such as the TM2–3 loop and the C terminus may be important for signal transduction and channel gating (16, 42), but are not essential for ligand binding.

**EXPERIMENTAL PROCEDURES**

**Construction of Vectors, Expression, and Purification of Fusion Proteins—**Domains of interest were amplified by PCR using has1 DNA as template. Oligonucleotides were custom-synthesized (Invitrogen, Karlsruhe, Germany). PCR primers contained appropriate restriction sites, allowing ligation into the pET30a (Novagen, Darmstadt, Germany) and pRk5 vectors. Successful mutations were verified by DNA sequencing (ABI Systems, Weiterstadt, Germany). After transformation and expression, BL21 cells (Novagen, Darmstadt, Germany) were harvested by centrifugation, resuspended in 50 mM Tris-HCl, 0.5% EDTA, pH 7.4, treated with lysozyme (0.1 mg/ml, 30 min, 0 °C) and sonicated (eight 10-s pulses) on ice. Sonication was repeated and the supernatant collected for native purification. For purification under denaturing conditions, the pellet was washed four times with 50 mM Tris-HCl, 2.5 mM EDTA, 0.5% Triton X-100, pH 8.2, and finally dissolved in 100 mM NaPi, 10 mM Tris-HCl, 8 M urea, pH 8.0. The denatured protein was applied to a Ni-agarose column (Qiagen, Hilden, Germany), washed at pH 5.9 and eluted at pH 4.5. Native purification, performed on a Ni-agarose column, was achieved by washing and eluting with increasing imidazole concentrations. To avoid precipitation of these protein constructs in the presence of protease substrate buffers, tags were not removed.

**Refolding—**A variety of folding conditions was tested, following published folding screens (20, 21). After 2 h of incubation in denaturing buffer, 10 μl of the dissolved inclusion bodies (1 mg/ml) were added to 990 μl of folding screen solution. Samples were incubated overnight at 4 °C, dialyzed for 6 h against 100 mM of proteolysis buffer (20 mM Tris-HCl, pH 8.0), concentrated and proteolyzed using subtilisin (1 μg/ml), and sonicated (eight 10-s pulses) on ice. Sonication was repeated and the supernatant collected for native purification. For purification under denaturing conditions, the pellet was washed four times with 50 mM Tris-HCl, 2.5 mM EDTA, 0.5% Triton X-100, pH 8.2, and finally dissolved in 100 mM NaPi, 10 mM Tris-HCl, 8 M urea, pH 8.0. The denatured protein was applied to a Ni-agarose column (Qiagen, Hilden, Germany), washed at pH 5.9 and eluted at pH 4.5. Native purification, performed on a Ni-agarose column, was achieved by washing and eluting with increasing imidazole concentrations. To avoid precipitation of these protein constructs in the presence of protease substrate buffers, tags were not removed.

**Mass Spectrometry—**For matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and surface enhanced laser desorption ionization (SELDI), protein samples were diluted to 2 mg/ml and mixed (1:1) with a CCA-Matrix (3-hydroxy-cyano-cinnamic acid; saturated solution in 30% acetonitrile/0.1% trifluoroacetic acid), dotted onto a steel target, and air-dried. MALDI-TOF-MS analysis was performed on an Autoflex spectrometer (Bruker Daltonics, Bremen, Germany) in the reflector mode while the SELDI analysis was carried out on a PBS II spectrometer (Ciphergen, Göttingen, Germany). Desorption of the samples was carried out using a nitrogen laser (337 nm). For the MALDI-TOF-MS analysis, the samples were accelerated with 19 kV after a delay of 3500 ns and several individual spectra of the respective samples were averaged. A peptide standard mix served as an external calibration. For SELDI analysis, time-of-flight spectra were generated by averaging 360 laser shots collected in the positive mode at laser intensity 259, detector sensitivity 10, and a focus lag time of 900 ns.

**Transfection of HEK 293 Cells, Alkaline Extraction, and Immunocytochemistry—**HEK293 cells were cultured and transfected, and crude membrane fractions were prepared as described (22). For Western blot analysis (23) the monoclonal antibody mAb4a, a mouse monoclonal His-tag antibody (Novagen, Darmstadt, Germany) or a mouse monoclonal gephyrin antibody (BD Sciences, Heidelberg, Germany) were used, followed by a Cy5-coupled goat anti-mouse antibody gdMlgG-Cy5 (Dianova, Hamburg, Germany), and visualized on a Storm 880 Fluorimeter (Molecular Dynamics, Krefeld, Germany). To test for membrane association, membrane preparations from transfected HEK293 cells (0.5 mg/ml) were incubated for 60 min at pH 7.4 or pH 11.0 at 4 °C and centrifuged (180,000 × g, 4 °C) for 90 min as described (24). The pellets and supernatants were then subjected to Western blot analysis. HEK293 cells, grown on cover slips, were transferred to a 24-well plate 2 days after transfection. After fixation in paraformaldehyde for 10 min, cover slips were washed twice in PBS (1.5 mM K2HPO4, 6.5 mM Na2HPO4, 3 mM KCl, 137 mM NaCl), and blocked with 5% sheep serum for 30 min. For permeabilization, cells were treated with 0.1% Triton X-100. Primary (mAb4a, anti-Gephyrin) and secondary antibody, Cy3 goat anti-mouse Piab3 (Dianova, Hamburg, Germany) were applied for 60 min, every stop followed by three washes with phosphate-buffered saline. Cover slips were transferred to slides and examined on a confocal microscope (Leica, Bensheim, Germany).

**References**

**Histchrynine Binding—**Different radioligand filtration assays were performed to determine [3H]histchrynine binding of soluble protein, i.ii and membrane-associated protein (iii): (i) Polyethylene glycol (PEG) precipitation: 15 μg of refolded protein, 0.13% γ-globulin, [3H]histchrynine (PerkinElmer Life Sciences, Zaventem, Belgium: specific activity, 47.8 mCi/mol), and binding buffer B (25 mM KPi pH 7.4, 200 mM KCl) in the presence and absence of cold strychnine were incubated on ice for 30 min. PEG400 (15%) in buffer B was added and the mixture incubated on ice for another 30 min. The precipitated protein was applied to GF/C filters (Whatman) soaked in 0.1% Triton X-100, washed twice with cold buffer B. (ii) Adsorption on polyethylene-enameine (PEI) coated filters: Total cell extract preparation (50 μg) and [3H]histchrynine in the presence or absence of cold strychnine were incubated on ice for 30 min, before applying to 0.3% PEI-soaked GF/B filter and washing twice with buffer B. (iii) Filter adsorption of membrane fractions: Protein obtained from membrane preparations was treated as described previously (23). For all methods, dried filters were subjected to scintillation counting (Roth, Karlsruhe, Germany). Binding data were fitted to Equation 1.

\[ dp_{\text{spec}} = B_{\text{max}} \frac{1}{1 + K_d/S_{\text{tr}}} \]  

(Eq. 1)

Here, \( dp_{\text{spec}} \) represents specific binding expressed as scintillator counts (\( dp_{\text{spec}} \) was determined by subtracting unspecific counts from total counts), \( B_{\text{max}} \) = total number of agonist binding sites, \( K_d \) = binding constant, \( S_{\text{tr}} \) = [3H]histchrynine concentration. For each concentration, unspecific binding was determined in the presence of 1 μM cold strychnine. Determinations were carried out in triplicate.

**Size Exclusion Chromatography and Sucrose Gradient Centrifugation—**Secapryl S400 (30 ml) and Sephacryl S200 (120 ml) columns were chosen to determine the native molecular weight of the GlyR α1 subunit. Fractions were collected and their elution volume \( V_e \) (Eq. 2) was determined by subtracting unspecific counts from total counts, \( B_{\text{max}} \) = total number of agonist binding sites, \( K_d \) = binding constant, \( S_{\text{tr}} \) = [3H]histchrynine concentration. For each concentration, unspecific binding was determined in the presence of 1 μM cold strychnine. Determinations were carried out in triplicate.

\[ K_d = \frac{V_e - V_i}{V_i - V_i} \]  

(Eq. 2)

where \( V_e \) is the elution volume of the protein, \( V_i \) is the column void volume, and \( V_i \) is the total bed volume. Runs were performed at 4 °C in 50 mM KPi, pH 7.4, 100 mM NaCl for Sephacryl S200 and 50 mM Tris-HCl, 50 mM NaCl for Sephacryl S400. For molecular weight determinations, a continuous sucrose gradient, 1 ml of total cell extract (concentrated in a continuous sucrose gradient, 1 ml of total cell extract containing ~5 μg of protein) was layered on a continuous 10–40% sucrose gradient (26 ml) in 25 mM Tris-HCl, pH 7.4, 25 mM NaCl, 1% Triton X-100 on top of a 60% sucrose cushion (3 ml). After centrifugation for 14 h in a Sorvall SW-28 rotor at 27,000 rpm GlyR antigen was quantified using a dot blot receptor assay (DORA).
Sucrose fractions (1.5 ml) were collected, and 300 μl of the fraction was taken up in 300 μl of uptake buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 2% sodium deoxycholate, 40% methanol, 5 mM EDTA, 5 mM EGTA), and directly spotted onto a nitrocellulose membrane. GlyR content was then determined as described for Western blot analysis, mAb4a immune signal was quantified on a Storm860 fluoroimager. The following protein markers were used: cytochrome c (12.3 kDa), 1.83 Svedberg (S1), ovalbumin (45 kDa), 3.55 S, malate dehydrogenase (70 kDa, 4.32 S), lactic dehydrogenase (140 kDa, 6.95 S), and catalase (232 kDa, 11.3 S).

CD Spectroscopy—Purified intra- and extracellular GlyR domains, expressed in E. coli and buffered in 10 mM KP, pH 7.4, were subjected to CD analysis. Measurements were performed on a JASCO-J810 spectrometer (JASCO, Groß-Umstadt, Germany) in a 0.1-cm analytical cell. All spectra were baseline corrected by subtracting buffer runs. Eight individual scans were taken at room temperature in a range of 320 to 180 or 200 nm, with a 0.5-nm step size and averaged. Protein concentrations were determined by measuring the absorbance at 280 nm using the equation $c = \frac{A_{280 \text{ nm}}}{\text{v (ml)} 	imes 1 \times 10}$. The path length 1 was 1 cm, the extinction coefficients $\epsilon_{280 \text{ nm}}$ for GlyR 1-subunit in aqueous solution, 20 μM of a solution containing 4–15 mg protein/ml in 10 mM sodium phosphate buffer pH 7.4 were placed on the silicon elements (31–33) using the Microcal Origin and OPUS software. The corresponding peak positions were restricted within 2 cm$^{-1}$ to the original spectra. Band widths were restricted to 30–32 cm$^{-1}$. The integrals of the individual gaussian bands were taken as a relative measure of the type of secondary structure that corresponds with the center frequency of the resolved band.

RESULTS

For analysis of GlyR α1-subunit extra- and intracellular domains, three different cDNA constructs were designed and expressed in both E. coli and in HEK293 cells. The vector pET30a, containing a variety of functional sites, was prepared for high yield protein expression in E. coli. In particular, a His-tag, a thrombin site, an S-tag (i.e. a 15-residue part of the ribonuclease S protein), and an enterokinase cleavage site preceded the fusion protein. To control for a potential interference with the S-tag, the truncated vector pET30aSΔ-tag was designed, lacking the S-tag and the enterokinase cleavage site of the pET30a vector. The vector pRK5 was chosen for eukaryotic expression, followed by purification on Ni-NTA agarose. When the large intracellular loop was expressed in E. coli, a soluble protein was obtained which was efficiently purified under native conditions using a Ni-agarose column. In contrast, E. coli expression of the extracellular domain almost exclusively resulted in inclusion body formation. As shown for other receptors, e.g. the N-terminal domain of the α7 AChR (13), proteins from inclusion bodies may properly refold, but the refolding procedure does not necessarily result in a high percentage of correctly folded protein (35). While we made considerable efforts to avoid inclusion body formation by testing different E. coli strains and varying the expression conditions, we never obtained sufficient amounts of the target protein in its soluble form (not shown). Nevertheless, expression in form of inclusion bodies yielded high amounts of almost pure N-terminal protein, which was further purified on a Ni-NTA column under denaturing conditions. Refolding conditions were established following a modified protocol of Heiring and Muller (21) (Table I). Since correctly folded protein is expected to be more stable against degradation than a non- or incorrectly folded structure, resistance to a protease digest served as an indicator of correct folding (21). Refolded protein, prepared by 100-fold dilution into folding buffer, therefore, was treated with subtilisin and subjected to Western blot analysis (Fig. 2). Proper protein refolding was assessed by comparing signal intensities of intact full-length protein and degradation products. Large scale preparation of pure protein was carried out by stepwise dialysis against refolding buffer. Under these conditions, best refolding was obtained with 50 mM Tris-HCl and 7 μM CuCl$_2$ (Fig. 2, lane 16). Several other conditions resulted in

**Protein Expression, Purification, and Refolding of Pure GlyR Protein Constructs**—For large scale production of pure protein,
efficient refolding during the initial test analysis (Fig. 2, lanes 8, 12, and 13), but were hampered by precipitation of the desired protein during final large-scale preparations. This appeared to be due to elevated salt concentrations in combination with protein concentrations that were 2000–5000-fold higher than under the initial test conditions. High purity and integrity of the GlyR α1 fragments was verified by Coomassie gel staining (Fig. 3A) and Western blot analysis (Fig. 3B) using mAb4a for the extracellular domain and His-tag antibody for the intracellular domain. Mass spectrometry analysis by MALDI-TOF-MS and SELDI of pure protein constructs from *E. coli* verified the molecular weights of 12,115 kDa for the intracellular domain and 30,685 kDa for the N-terminal construct Sα1-(1–219) (Fig. 3C). All N-terminal constructs and the full-length receptor showed significant levels of expression in HEK293 cells (Fig. 3B).

**Membrane Association of the GlyR N-terminal Domain**—Lacking all the transmembrane domains present in GlyR subunits, one would expect the N-terminal domain to be stable in aqueous solution. Surprisingly, throughout all preparation steps of extracellular constructs, we observed a strong tendency toward precipitation. Therefore, low-salt buffer conditions were chosen to ensure stable protein solutions. Construct Sα1-(1–219) showed increased protein solubility as compared with construct α1-(1–219) (not shown), indicating a stabilizing effect of the N-terminal S-tag. Ionic detergents (SDS and deoxycholate) increased the solubility of the N-terminal domain, while non-ionic and zwitterionic detergents (Twee 20, Triton X-100, 3-[3-Cholamidopropyl]dimethylammonio]-1-propanesulfonate CHAPS, octylglucoside or dodecylmaloside) had no detectable effect. The observation that the N-terminal domain was less soluble than expected, led to the assumption that protein aggregation and precipitation might be attributed to hydrophobic patches in this domain. To test for possible interactions between putative hydrophobic surface stretches of this domain and lipid membranes, we expressed the eukaryotic glycoprotein HEK293 cells (Fig. 3, lane 9–12), total cell extract; lane 5, HEK α1-(1–219), total cell extract; lane 6, HEK hsal, total cell extract. C, SELDI and MALDITOF-MS analyses verifying the molecular masses of the extracellular domain Sα1-(1–219) and the TM3–4 loop α1-(309–392), respectively.

**Table 1.** Refolding screen for the GlyR extracellular domain.

| No. | Buffer | Salt | Cation/no cation | PEG | Detergent | Chaotr. | Polar/non polar addit. | Redox comp. |
|-----|--------|------|------------------|-----|-----------|---------|-----------------------|------------|
| 1   | Tris-HCl | High | EDTA             | +   | –         | –       | –                     | –          |
| 2   | MES    | Low  | Mg₂/₃CaCl₂       | –   | +         | LMT     | –                     | –          |
| 3   | MES    | Low  | EDTA             | +   | –         | –       | –                     | –          |
| 4   | Tris-HCl | High | Mg₂/₃CaCl₂       | –   | +         | Sucr/arg | –                     | –          |
| 5   | Tris-HCl | High | Mg₂/₃CaCl₂       | –   | +         | Sucr/arg | –                     | –          |
| 6   | Tris-HCl | Low  | EDTA             | +   | –         | Sucrose  | –                     | –          |
| 7   | Tris-HCl | Low  | Mg₂/₃CaCl₂       | –   | +         | Arginine | –                     | –          |
| 8   | MES    | High | EDTA             | +   | –         | Arginine | –                     | –          |
| 9   | MES    | High | Mg₂/₃CaCl₂       | +   | –         | –       | Sucr/arg              | –          |
| 10  | Tris-HCl | Low  | EDTA             | –   | –         | Sucr/arg | –                     | –          |
| 11  | Tris-HCl | Low  | Mg₂/₃CaCl₂       | +   | –         | Arginine | –                     | –          |
| 12  | MES    | High | EDTA             | –   | –         | Arginine | –                     | –          |
| 13  | Tris-HCl | High | EDTA             | –   | –         | –       | –                     | –          |
| 14  | MES    | Low  | Mg₂/₃CaCl₂       | +   | –         | Sucr/arg | –                     | –          |
| 15  | MES    | Low  | EDTA             | –   | –         | Sucr/arg | –                     | –          |
| 16  | Tris-HCl | –    | CuCl₂           | –   | –         | –       | –                     | –          |

**Fig. 2.** Western blot analysis of results obtained from refolding assays. Refolded proteins (folding conditions 1–16) before (X) and after (X+) subtilisin treatment are shown.

**Fig. 3.** Purification and verification of GlyR constructs. A, Coomassie gel of purified proteins: lane 1, SDS–7 low molecular weight markers (Sigma); lane 2, *E. coli* α1-(1–219), refolded; lane 3, *E. coli* Sα1-(1–219), refolded; lane 4, *E. coli* α1-(309–392). B, Western blot analysis (mAb4a): lane 1, *E. coli* α1-(1–219), refolded; lane 2, *E. coli* Sα1-(1–219), refolded; lane 3, *E. coli* α1-(309–392); lane 4, HEK-His α1-(1–219), total cell extract; lane 5, HEK α1-(1–219), total cell extract; lane 6, HEK hsal, total cell extract. C, SELDI and MALDITOF-MS analyses verifying the molecular masses of the extracellular domain Sα1-(1–219) and the TM3–4 loop α1-(309–392), respectively.

Formulations of the reagents: Tris-HCl, 55 mM, pH 8.2; MES, 50 mM, pH 6.5; salt high, 250 mM NaCl, 10 mM KCl; salt low, 10 mM NaCl, 0.5 mM KCl; EDTA, 1 mM; MgCl₂/CaCl₂, 2 mM MgCl₂, 2 mM CaCl₂; CuCl₂, 7 μM; PEG, 0.05% (w/v) PEG 4000, present (+) or absent (−); detergent, 0.3 mM β-lauryl maltoside (LM), no detergent (−); Chaotr., 0.5 mM guanidine-HCl, present (+) or absent (−); sucrose, 0.5 M; arginine, (arg) 0.55 M; dithiothreitol (DTT), 1 mM; GSSG/GSH, 0.1 mM oxidized glutathione (GSSG), 1 mM reduced glutathione (GSH).
immune signal of the construct SP α1(1–219) co-distributed exclusively with the membrane fraction, indeed indicative of a tight attachment. To check whether the N-terminal domain behaves like a peripheral or an integral membrane protein, we performed alkaline extraction experiments. Controls were made using the entire GlyR, and a coexpression of GlyR α1- and β-subunits, and gephyrin (1:4:4). While gephyrin could be extracted at alkaline pH, the entire GlyR remained in the membrane fraction as shown before (24) (Fig. 4A). Under these conditions the N-terminal domain SP α1(1–219) was found in the pellet at pH 7.4 and also at alkaline conditions (pH 11.0). Only minimal amounts of wild-type and truncated GlyR could be observed in the supernatant at pH 11.0 (Fig. 4A). This result would be expected for an integral membrane protein or a protein characterized by tight membrane association. To confirm this observation, the subcellular distribution of the SP α1(1–219) antigen was analyzed by immunocytochemistry. We hypothesized that an unspecified aggregation of the N-terminal domain inside the HEK293 cells, or its trapping in intracellular membranes, should restrict GlyR immune signals to the cell interior. However, immunostaining of HEK293 cells transfected with the N-terminal construct SP α1(1–219) showed a membrane distribution indistinguishable from the full-length α1 subunit. Additionally, permeabilization of transfected cells by Triton X-100 had no influence on immune signal distribution (Fig. 4, B–E). As control for an intracellular protein we chose gephyrin, which binds to the intracellular TM3–4 loop of the GlyR β subunit. HEK293 cells were cotransfected with GlyR α1, GlyR β, gephyrin (1:4:4) and probed with a gephyrin antibody. Here, the immune signal was only present in HEK293 cells that were permeabilized with Triton-X100 (Fig. 4, F–I). Taken together, these results consistently indicated a tight membrane association of the GlyR α1 extracellular domain, despite the complete absence of all putative transmembrane domains.

High Affinity [3H]Strychnine Binding of GlyR Extracellular Domains—The N-terminal domain of the GlyR α1 subunit carries well characterized determinants of ligand binding. To analyze whether ligand binding was preserved in the truncated receptor subunit, we investigated various GlyR N-terminal constructs obtained from both, expression in HEK293 cells and refolded inclusion bodies. Efficient binding of [3H]strychnine was observed for all of the constructs generated, yielding affinities similar to the wild type (Fig. 5). Total cell extracts, performed for constructs lacking the signal peptide, yielded apparent KD values of 115 ± 24 nM for the full-length GlyR, 107 ± 58 nM for construct α1(1–219), and 66 ± 16 nM for construct His-α1(1–219). Furthermore, membrane preparations were analyzed for the α1 wild-type construct and the membrane-associated construct SP α1(1–219), producing KD values of 23 ± 4 nM and 42 ± 3 nM, respectively, compared with a KD of 135 ± 67 nM for the refolded construct S α1(1–219). It should be noted that endogenous glycine could not be separated from the total cell extracts. Thus, the decrease in apparent affinity...
observed for these preparations as compared with membrane preparations, may be attributed to the endogenous amino acid agonist competing for $^3$H]strychnine binding. However, when analyzed under identical conditions, the full-length GlyR α1-subunit and the extracellular domain displayed similar $K_D$ values indicative of high affinity $^3$H]strychnine binding (Table II).

Oligomerization and Secondary Structure—To study the oligomerization states of the isolated protein constructs from E. coli, we performed size exclusion chromatography under native conditions. Each GlyR-derived protein eluted in a single peak at high molecular weight that indicated oligomerization into complexes of defined stoichiometry. For construct Sα1–(1–219) at high molecular weight that indicated oligomerization into.

| Expression | HEK293 | HEK293 | HEK293 | Membrane preparation | Membrane preparation | E. coli |
|------------|--------|--------|--------|----------------------|----------------------|---------|
| Preparation | Total cell extract | Total cell extract | Total cell extract | Membrane preparation | Membrane preparation | Refolding |
| App $K_M$[M] | 115 ± 24 | 107 ± 58 | 66 ± 16 | 23 ± 4 | 42 ± 3 | 135 ± 67 |
| $B_{max}$ [cpm]$^a$ | 5511 ± 570 | 2163 ± 570 | 595 ± 58 | 8211 ± 418 | 308 ± 9 | 1383 ± 357 |

$^a B_{max}$ under experimental conditions.

Discussion
The inhibitory GlyR is a member of the acetylcholine receptor superfamily. Each of its subunits consists of an extracellular domain, four transmembrane regions, and a large cytosolic TM3–4 loop. Here, the GlyR α1 extramembrane domains were generated and expressed in E. coli and eukaryotic HEK293 cells. The isolated eukaryotic and refolded extracellular domain retained its characteristic high affinity.
Oligomerization of GlyR extramembraneous domains

| Construct                  | MW monomer calculated | MW determined | Method          |
|----------------------------|-----------------------|---------------|-----------------|
| α1-(309–392) (No. 6)       | 12.1 kDa              | 49 ± 1 kDa    | Size exclusion Chr. |
| S-α1-(1–219) (No. 2)       | 30.7 kDa              | 465 ± 33 kDa  | Size exclusion Chr. |
| HEK-α1-(1–219) (No. 3)     | 25.4 kDa              | 378 kDa       | Sucrose gradient |

[^3H]strychnine binding and, unexpectedly, showed a strong membrane attachment. Secondary structure analysis suggested a significant contribution of α-helices to the N-terminal domain, while no α-helical structure was found in the cytosolic TM3–4 loop.

Following expression in *E. coli*, the large intracellular loop α1-(309–392) was purified under native conditions. Due to inclusion body formation, the extracellular constructs α1-(1–219) and S-α1-(1–219) were refolded after denaturation. While low solubility was a characteristic property of these refolded N-terminal constructs, the additional N-terminal S-tag reduced, but did not prevent, precipitation of the purified protein. This pronounced instability in solution suggested the presence of considerable hydrophobic surface areas, mediating protein aggregation. Thus the isolated N-terminal domain would be expected to show tight membrane association. Indeed, membrane preparation of the construct SP_α1-(1–219) from transfected HEK293 cells, followed by Western blot analysis, confirmed this prediction: the immune signal of the extracellular domain was exclusively co-distributed with the membrane, but not with the soluble fraction. Even at alkaline conditions (pH 11.0) the N-terminal domain SP_α1-(1–219) remained with the membrane fraction. In fact, the entire GlyR and the N-terminal domain showed practically identical behavior under the condition of alkaline extraction, indicating a very tight membrane association of the isolated N-terminal domain. Although this observation may be compatible with an association of the isolated N-terminal domain with the plasma membrane, we could not exclude an association with intracellular membranes or an unspecific precipitation within the cytosol yielding the same result. To further elucidate the subcellular localization of the isolated GlyR N-terminal domain, we performed immunocytochemistry experiments in transfected HEK293 cells. The subcellular distribution of the isolated N-terminal domain was indistinguishable from that of the full-length α1-subunit, confirming its attachment to the plasma membrane. The pronounced tendency toward precipitation as well as a strong membrane attachment of the GlyRα1 N-terminal domain were consistent with the presence of hydrophobic surface areas, as previously proposed from studies of reconstituted GlyRs (19). When the extracellular domain of the α7 nAChR was expressed in *E. coli* as an isolated refolded protein (13), solubility was likewise reduced. Thus, significant hydrophobic interaction stretches within the extracellular domain appear to be a property of many members of the acetylcholine receptor superfamily.

High affinity strychnine binding is an important functional characteristic of the inhibitory GlyR (2). Indeed, we observed identical[^3H]strychnine affinities for the full-length α1-subunit and the isolated N-terminal domains from HEK293 cell preparations. High affinity strychnine binding persisted even in the refolded isolated protein from *E. coli* expression, with an affinity reduced only 3–6-fold compared with the full-length receptor. These observations indicated a proper folding of the extracellular domain into its functional conformation, independent of other extracellular or transmembrane

**Fig. 6.** Determinations of native molecular weight of GlyR constructs. For chromatography, marker proteins were (1) ribonuclease A, 13.7 kDa; (2) chymotrypsigen A, 25 kDa; (3) ovalbumin, 43 kDa; (4) albumin, 67 kDa; (5) γ-globulin, 150 kDa; (6) catalase (232 kDa); (7) ferritin, (440 kDa); (8) thyroglobulin (669 kDa). Mean $K_{av}$ = S.D. of the calibration proteins from triplicate determinations are plotted. A, intracellular loop α1-(309–392), Sephacryl S200 column, MW$_{app}$ = 49 ± 1 kDa. B, extracellular domain α1-(1–219), Sephacryl S400 column, MW$_{app}$ = 465 ± 33 kDa. C, sucrose gradient centrifugation. A 10–40% sucrose gradient was used and GlyR antigen visualized by DORA. Apparent molecular weights are indicated. See Table III for results.
Proteins were buffered in 10 mM potassium phosphate, pH 7.4. Scans baseline corrected and represent the average of 8 individual scans. Intra- and extracellular domains of the glycine receptor. The spectra are in the amide I range are shown.

Absorption bands. Negative bands indicate the peak positions of underlying absorption bands. Gaussian bands obtained from a fit of the amide I and II absorption using the OPUS software. For clarity, only the bands in the amide I range are shown.

Behavior of the full-length GlyR α1-subunit during sucrose gradient centrifugation is compatible with a pentameric association (22, 37). Likewise, molecular weight determination of the GlyR extracellular constructs by size exclusion chromatography revealed a multisubunit assembly. As the hydrodynamic volume of a protein depends on both, its mass and its surface properties, the molecular weight values obtained represent only estimates. Despite these limitations, the apparent molecular weights determined for the cytosolic TM3–4 loop and the truncated extracellular protein would be compatible with tetrameric and 15-meric complexes, respectively. When the N-terminal domain solubilized from HEK293 cells was investigated on sucrose gradients, a bimodal molecular weight distribution suggested multimers of about 5 and 15 subunits. As determinants directing α-subunit assembly are situated within the GlyR N-terminal domain (16), pentameric agglomeration would indeed be expected for the isolated extracellular domain, rather than for the TM3–4 loop. Taken together, our data for the N-terminal domain were in good agreement with the expected pentameric association (4, 22, 37). In contrast, tetrameric association of the isolated TM3–4 loop remains unexplained, but may be attributed to an intracellular interaction site of GlyR subunits.

To investigate secondary structure elements within the isolated extramembranous domains expressed in E. coli, we performed circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopy studies. Spectra taken of the TM3–4 loop α1-(309–392) suggested no α-helical portion at all. While CD and FTIR investigation both predicted significant amounts of β-structure, the distinct CD maximum at 203 nm indicated the formation of a PPII helix. It should be noted that the TM3–4 loop cannot be tested for its functional significance. Spectral signals of a PPII helix, which is also present in the entire reconstituted GlyR protein (41), suggested a correct folding of the prominent secondary structure elements. In contrast, data obtained for construct α1-(1–219) predicted an α-helical content of 24% (CD, 208 nm, Ref. 28) while spectra obtained from construct S_α1-(1–219) pointed to 18% (CD, 208 nm) or 25% (FTIR) α-helices (Table IV). CD and FTIR spectra further indicated significant amounts of β-sheet in the extracellular domain (48% by FTIR investigation), in good agreement with values found for the acetylcholine binding protein (14) and AChR extracellular domains (Table IV). However, low α-helical contents of 8% were found for the AChBP from Lymnaea stagnalis (14), and 12–14% for soluble, monomeric preparations of mouse muscle nAChR N-terminal domains (38, 39). In contrast, preparations of rat α7 and Torpedo N-terminal AChR domains, both forming oligomeric assemblies, showed α-helical contents of 32–44% (13, 36). Even within the AChR family, extracellular domains from...
For the GlyR N-terminal domain, the α-helical content is consistent with the absence of acetylcholine binding protein. As the GlyR extracellular domain structure would be expected for the entire GlyR α1 subunit, the TM3 region contributes a further 5%, a total of at least 17% α-helical structure would be expected for the entire GlyR α1 subunit. This is in qualitative agreement with the prediction of about 20% α-helical content for the reconstituted GlyR α1-subunit (41) or its fragment α1-(165–291) (42). This would suggest that not all transmembrane domains of the GlyR α2β subunit, and is consistent with the absence of α-helices in the TM3–4 loop.

Our results show high affinity strychnine binding for the eukaryotic and refolded N-terminal domains. Interestingly, the isolated extracellular domain showed a strong membrane association when expressed in HEK293 cells, indicating that hallmark features of holoreceptor structure are indeed conserved in the isolated N-terminal domain.

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