FOR THE RECORD

Fragment of GABA_A receptor containing key ligand-binding residues overexpressed in *Escherichia coli*

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Abstract: GABA_A receptor plays a major role in inhibitory synaptic transmission in the central nervous system and is the target of drugs such as the benzodiazepine tranquilizers. The polymeric membrane protein nature of GABA_A receptor has rendered structural elucidation of the receptor a formidable task, greatly hampering structure-based drug design. We report here the first expression in *Escherichia coli* of a fragment of GABA_A receptor. This 131-residue fragment, spanning Cys166 to Leu296 of human GABA_A receptor $\alpha 1$ subunit, contains residues previously suggested to be involved in benzodiazepine binding. The overexpressed non-fusion recombinant protein was purified to near homogeneity and characterized by circular dichroism (CD), which showed that the recombinant protein has well defined secondary structures where β -strands are dominant. The stability of the secondary structures was demonstrated by CD spectra at high pH and elevated temperature. Excluding part of the sequences from the carboxyl terminal of the fragment resulted in dramatic changes in the secondary structures comparable to the effects caused by SDS denaturation. Our results therefore suggest that the 131-residue fragment harbors an integral structural domain of the receptor. The overexpression of the recombinant protein fragment thus opens the way to the biochemical and structural studies of a functionally important region of the receptor, and exemplifies an effective approach of expression and characterization that potentially may be extended to other members of the ligand gated channel receptor superfamily, to which the GABA_A receptor belongs.

Keywords: circular dichroism; domain; extracellular; secondary structure; sodium dodecyl sulfate

GABA_A receptors (Sieghart, 1995; Gupta, 1995; Clement, 1996) are fast-acting ligand-gated chloride ion channels that mediate most of the inhibitory synaptic transmissions in the central nervous system. They play a pivotal role in the regulation of brain excitability, and their functions are allosterically regulated by several distinct classes of therapeutic compounds. These include anxiolytic ben-

zodiazepines, barbiturates, neurosteroids, some volatile anaesthetics and possibly alcohol. The binding of GABA to GABA_A receptors triggers a conformational change that renders the channel transiently and selectively permeable to chloride ion.

GABA_A receptors belong to the ligand-gated ion channel receptor superfamily (Schofield et al., 1987). Sequence data for the superfamily members suggest that they share the same overall tertiary structure. However, in the continuing absence of X-ray crystallographic or NMR structures, the current model for GABA_A receptor structure is largely putative in nature and was formulated based on analogy to a model for nACh receptor (Unwin, 1996), the prototype of the superfamily. The model features a pentameric pseudo-symmetric quaternary structure, a highly conserved extracellular Cys-Cys loop and four highly homologous transmembrane segments.

Progress in structural studies on GABA_A receptors has been limited by the supply of pure protein in any sizable quantity. The native protein is expressed at a very low level as a heterogeneous pool of isoforms. Similarly, expression of recombinant GABA_A receptor in animal (Hadingham et al., 1992; DelRaso et al., 1996) or insect cells (Carter et al., 1992) has been achieved only at levels far too low for structural characterization. Also, endogenous subunits in the animal host cells may contaminate significantly the low-level expressed exogenous receptors (Ueno et al., 1996). Accordingly an overexpression system for large scale production of recombinant GABA_A receptors is urgently needed to allow the purification of sufficient amounts of protein for structural and functional studies. *Escherichia coli* was chosen as the expression host in the present study not only for its high yield of recombinant proteins, but also for the facile biosynthetic labeling it permits of recombinant proteins with stable isotopes for the purpose of multi-dimensional NMR analysis.

Two problems are commonly encountered with cell surface multi-domain proteins such as receptors. First, the hydrophobicity of these membrane-associated proteins presents severe obstacles to structural characterization by current technologies, which have been developed mainly for soluble proteins. These difficulties likely contribute to the hitherto lack of supplies in recombinant GABA_A receptors or any other members of the same superfamily. Secondly, the hinge-like mobility at domain junctions compounds the challenge to their structural resolution. Accordingly, our strategy to address the structural issues concerning GABA_A receptor is to

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produce recombinant protein fragments of potential integral structural domains and resolve individual domain structures prior to an attempt to reconstitute the overall structure. In this regard, the extracellular region of the GABA_A receptor harboring the ligand binding sites is chosen in this study as the first target for analysis on account of its tremendous pharmaceutical interest.

In this study, a 131-residue fragment, Cys166 to Leu296 of human GABA_A receptor $\alpha 1$ subunit, has been successfully expressed in *E. coli* as a non-fusion protein, and purified to near homogeneity (Fig. 1). This fragment spans the region from the conserved Cys-Cys loop to the second putative transmembrane segment. It contains five residues, namely Y161, T162, G200, T206, and V211, previously shown to be associated with benzodiazepine modulation of receptor function (Buhr et al., 1996).

The CD spectrum of the 131-residue fragment, or 166/296, in aqueous solution, has the appearance of a typical spectrum for β -stranded proteins (Fig. 2). The β -strand composition of the fragment measured by CD is reproducible in the neighborhood of 40–50%. Neither increasing the temperature from 20 °C to 60 °C (Fig. 2A), nor elevating pH to 11.5 (Fig. 2B) changed the CD spectra of the fragment, demonstrating clearly the excellent structural stability of the fragment.

Remarkably, the secondary structure of the fragment 166/296 monitored by CD spectra disintegrated when the sequences corresponding to the two putative transmembrane segments were removed from the C-terminal end of the fragment (Fig. 3). For the fragment, 166/276 (Fig. 3A), a 20-residue stretch corresponding to the second putative transmembrane segment, TM2 was removed. Further deletion of TM1, the 28-residue putative first transmembrane segment, gave rise to 166/248 (Fig. 3B) ending at Arg248, the putative boundary between the extracellular and the transmembrane regions. Both mutations drastically transformed the β -strand predominant CD spectra (Fig. 2) to random-coil enriched spectra (Figs. 3A and 3B), featuring a blue-shift in the $\lambda_{\max}$ from 217 nm for 166/296 (Fig. 2) to 208 nm for 166/276 (Fig. 3A) and 203 nm for 166/248 (Fig. 3B), shortening the fragment shortened also the wave length of $\lambda_{\max}$. Interestingly, these effects of deletion on CD spectra could be mimicked by the addition of the denaturing agent sodium dodecyl sulfate (SDS), where increased concentrations of

Fig. 1. Expression and purification of GABA_A receptor fragments. Left lane: protein molecular weight marker; (A) *E. coli* host control; (B) total cell lysates of *E. coli* expressing the 131-residue fragment, 166/296; (C) 8 M urea extracts of *E. coli* expressing fragment 166/296; (D) purified fragment 166/296.

Fig. 2. The CD spectrum of fragment 166/296 remains consistent at (A) increased temperature and (B) pH. (A) solid line = at 20 °C, dashed line = at 30 °C, and other lines are at 40, 50 and 60 °C, in 10 mM Tris-HCl, pH 8.5; (B) solid line = pH 9.0, and dashed line = pH 11.0 of 10 mM sodium borate buffer.

SDS progressively reduced $\lambda_{\max}$ (Fig. 3C). These results suggest that the inclusion of the two putative transmembrane segments facilitates the maintenance of β -strand enriched secondary structure components of 166/296.

Fig. 3. The effects of carboxyl-terminal deletions can be mimicked by SDS addition. (A) The effect of carboxyl-terminal deletion of 20 residues from 166/296; (B) the effect of carboxyl-terminal deletion of 48 residues from 166/296; (C) the effect of SDS: solid line = 0% SDS, dotted line = 0.1% SDS, and dashed line = 0.2% SDS. The buffer used for CD measurement was 10 mM Tris-HCl, pH 8.5.

Recently, Gready et al. (1997) published a consensus three-dimensional model for the extracellular region of ligand-gated ion-channel receptor superfamily based on computer modeling of Gly receptor $\alpha 1$ subunit sequence using *E. coli* biotin repressor structure as a template. This model features a two domain structure consisting of an elaborated SH2 domain at the amino terminal half and a cut-down SH3 domain at the carboxyl terminal half. The two domains are separated by the conserved Cys-Cys loop. Noticeably

in the model, the membrane proximal domain carboxyl terminal to the Cys-Cys loop is essentially an all- β structure. Also, Smith and Olsen (1995) predicted three β -pleated segments in the neighborhood of residues F64 and H101, which are previously suggested to be relevant to ligand binding.

The 131-residue fragment, 166/296, expressed by us is proximately equivalent in position to the carboxyl-terminal extracellular domain in Gready's model, although the fragment has extensions

at both ends compared to the modeled domain. According to Gready's model, the domain in human GABA_A receptor $\alpha 1$ subunit starts at Gln217 and ends at Lys249 in the TM1 segment. Whereas, the expressed 131-residue fragment starts at Cys166 and ends at Leu296 in the TM2 segment. Nevertheless, both Gready et al.'s (1997) model and our CD spectroscopic analysis (Fig. 2) agree on the all- β nature of this part of the receptor.

In conclusion, the present study has accomplished the first over-expression (Fig. 1) in *E. coli* of a fragment of GABA_A receptor, namely Cys166 to Leu296 of human GABA_A receptor $\alpha 1$ subunit, containing key ligand-binding residues (Buhr et al., 1996). The purified recombinant protein shows excellent stability (Fig. 2) and defined secondary structure (Fig. 2). These results indicate that the 166/298 fragment is sufficient for a protein fold. In view of its small size and high stability, this fragment is ideal for further structural scrutiny. Using the same expression system, the present study is being expanded to the whole extracellular domain of the receptor. The benzodiazepine binding capacity of the recombinant protein is currently under investigation. The expression system described herein may make possible the three-dimensional structural elucidation of this functionally important region of GABA_A receptors by X-ray and NMR, and hence structure-based drug-design.

Materials and methods: *Cloning and site-directed mutagenesis:* The prokaryotic expression vector, pTrcHis, purchased from Invitrogen (<http://www.invitrogen.com>) was modified by deletion of the sequence between the mini-cistron and the EcoRI recognition sequence in the multi-cloning-site, so that the amino terminal His-tag is removed and non-fusion proteins can be produced from the inserts. A 0.95 kb cDNA fragment encoding bovine GABA_A receptor $\alpha 1$ subunit sequence¹¹ from the amino terminal upto the end of the second transmembrane segment, TM2, was subcloned into the modified pTrcHis vector between EcoRI and HindIII sites. This part of the coding sequence encodes residues 166 to 296 of GABA_A receptor $\alpha 1$ subunit. In this region, human and bovine share the same sequence. Therefore, the product of this construct is for convenience referred as the fragment 166/296 of human GABA_A receptor $\alpha 1$ subunit. Deletions from carboxyl-terminal of the insert were performed with the PCR-based site-directed mutagenesis kit purchased from Stratagene (<http://www.stratagene.com>). All mutants generated were confirmed by DNA sequencing.

Expression and purification: Expression was achieved by 0.2 mM IPTG induction of LB liquid culture containing Ampicillin 50 μ L/mL, inoculated with *E. coli* harbouring plasmids constructed as described above, and incubated at 37 °C. Recombinant protein products were extracted from the harvested *E. coli* cells with 8 M urea containing 0.1 M β -Mercaptoethanol and purified by gel-exclusion chromatography on a Superdex 200 column (26 $\times$ 60 mm) with a buffer composed of 0.5 M Tris-HCl, pH 9.0, 1 mM EDTA, 0.2 M NaCl, 0.5 M L-Arg, 3 mM L-Cysteine, 0.3 mM L-Cystine, 0.05% deoxycholic acid and 0.05% n-octyl- β -D-glucopyranoside. The sequence and molecular weight of all purified products were con-

firmed by amino terminal protein sequencing and mass spectroscopic analysis, respectively. Protein sequencing service was provided by Protein Service Laboratory, University of British Columbia, and mass-spectrum service provided by Molecular Biology Resource Facility, University of Oklahoma Health Science Center.

CD measurement: Purified recombinant proteins were extensively dialyzed with 10 mM Tris-HCl at pH 8.5. The solutions used for CD measurements were prepared by proper dilution of a stock of about 8 μ M. Protein concentrations were checked by Lowery method. CD measurements were carried out on a JASCO J720 spectropolarimeter. Cells of 0.1 cm path length were used in all the experiments. For the thermal denaturation experiments, the cells were water-jacketed, and temperature control was obtained with a circulating water bath with a thermosensor inserted into the cell. Thermal denaturation curves were acquired at a 4 °C/h speed gradient starting at 20 °C.

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