FOR THE RECORD

Two β -rich structural domains in GABA_A receptor α_1 subunit with different physical properties: Evidence for multidomain nature of the receptor

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Abstract

The type A γ -aminobutyric acid (GABA_A) receptor is a major inhibitory neurotransmitter-gated ion channel. Previously, we identified a membrane-proximal β -rich (MPBR) domain in fragment C166-L296 of GABA_A receptor α_1 subunit, forming natively pentamers. In the present study, another structural domain, the amino-terminal domain, was shown to exist in the fragment Q28-E165. The secondary structures of both fragments were β -rich as measured using FTIR spectroscopy and estimated from the CD spectra to be 42% and 51% β -strand for Q28-E165 and C166-L296, respectively. The CD spectrum of the combined fragment Q28-L296 was additive of the spectra of the two fragments. In addition, denaturation curves of both fragments were characteristic of cooperative transitions, supporting their domainlike nature. C166-L296 required 6.5 M of guanidine chloride for total denaturation, therefore it is extraordinarily stable, more so than Q28-E165. Moreover, effects of detergent on the molecular masses of Q28-E165 and C166-L296, as monitored using laser-scattering spectroscopy, indicated that intermolecular interactions were much more significant in C166-L296 than in Q28-E165. Effects of pH on their molecular masses suggested that ionic forces were involved in these interactions. Together the results show that the two adjacent fragments form independent folding units, MPBR and amino-terminal domains, different in secondary structure content, denaturation profile, and polymerization status, and suggest that the former may play a more important role in receptor assembly and that the extraordinary stability may underlie its intrinsic tendency to form oligomers. More significantly, the present study has provided direct evidence for the long-postulated multidomain nature of this family of receptors.

Keywords: Recombinant protein; circular dichroism; FTIR; laser scattering; secondary structure; deletion mutagenesis; denaturation profiles

Type A γ -aminobutyric acid (GABA_A) receptors belong to the fast-acting ligand-gated ion channel superfamily that mediates most of the inhibitory synaptic transmissions in

the central nervous system (MacDonald and Olsen 1994; Smith and Olsen 1995; Stephenson 1995). The superfamily of receptors also includes the neuronal nicotinic acetylcholine, glycine, and serotonin receptors. Members of the superfamily share significant sequence similarity and, hence, are believed to have similar structure with a large amino-terminal extracellular region, including a highly conserved extracellular Cys–Cys loop. The remaining part includes four highly homologous transmembrane (TM) regions and

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an intracellular loop of variable length between the putative third and fourth TM regions (Schofield et al. 1987). The pentameric quaternary structure is also believed to be a common feature of the superfamily, with the native receptor forming heteromeric proteins composed of five subunits (Stephenson 1995). The ligand-binding sites were found to be situated at these subunit interfaces (Sigel and Buhr 1997). However, because of the lack of X-ray crystallographic or NMR structures, the prevalent model is largely putative in nature. Little is known about the mechanisms involved in subunit oligomerization and, hence, formation of functional receptors.

Progress on structural studies on GABA_A receptors has been limited by the inadequate supply of the pure protein due to difficulties in expression and purification of large transmembrane proteins. To alleviate these problems, a dissecting–rebuilding strategy (Campbell and Downing 1994) has been adopted: the protein is divided into autonomous folding units (domains) and the structure of the individual domains are characterized before reconstitution of the image of the whole protein. In this regard, our recent success in overexpressing the C166-L296 segment of the GABA_A receptor α_1 subunit (numbering based on the precursor protein) and delineating an integral domain within the segment (Xue et al. 1998, 1999) identifies an efficient system to obtain recombinant protein domains for further characterization. Segment C166-L296 has been demonstrated to form stable β -rich secondary structures and homopentameric assemblies (Xue et al. 2000). It was also shown that it could bind the fluorescent benzodiazepine Bodipy-FL Ro-1986 with affinity in the micromolar range (Hang et al. 2000). Therefore, our system has provided information on the secondary and quaternary structure, and benzodiazepine-binding property of this segment. However, the mechanisms involved in receptor assembly and possible contributions from various parts of the receptor subunit remain to be elucidated.

In the present study, using the previously reported *Escherichia coli* expression system (Xue et al. 1998; Hang et al. 2000), the possible existence of another structural domain present at the amino terminus of the mature α_1 subunit peptide (with the signal peptide constituted of the first 27 residues removed) was explored by observing the effects of deletional mutations on the structural integrity of fragment Q28-E165. Having shown the existence of a structural domain in each of the fragments Q28-E165 and C166-L296, the two fragments were then compared for physical properties such as secondary structure, stability, denaturation profile, and polymerization status. This was facilitated by biophysical means including Fourier transform infrared (FTIR), circular dichroism (CD), fluorescence, and laser-scattering spectroscopy to gain insights into the nature of the two structural domains and their possible contributions to the receptor assembly.

Results and Discussion

Fragment Q28-E165 alone was more resistant to degradation than in fusion with neighboring sequences

The amino-terminal fragment Q28-E165 was overexpressed in *E. coli* and it was found that the fragment was not susceptible to degradation to the extent seen when combined with the neighboring fragment C166-L296, giving Q28-L296 (Fig. 1, lane 2). Carboxy-terminal deletions of the putative transmembrane regions from Q28-L296 decreased protein stability; the resulting fragment Q28-R245 showed severe degradation (Fig. 1, lane 3). Therefore, the relative stability of Q28-E165 provided initial indication on the existence of a structural domain in this region.

Both Q28-E165 and C166-L296 were β -rich, and their secondary structures added up to that of Q28-L296

The far-UV CD spectrum of Q28-E165 has a minimum at 217 nm and a maximum at 194 nm, indicating a β -rich structure and the relatively broad spectrum is indicative of some α -helix component. The percentage of α -helix, β -strand, turn, and random structures are 23.1%, 28.5%, 22.2%, and 25.5%, respectively, as calculated using the CD-Pro package (Sreerama and Woody 2000) compared with 30.9%, 41.9%, 9.2%, and 17.5%, respectively, as estimated using the earlier SELCON program (Sreerama and Woody 1993). In comparison, the spectrum of C166-L296 had a slightly lower α -helix content and was even more β -rich (18.9% α -helix; 31.4% β -strand; 22.3% turn; 26.3% random using CDPro and 23.9%, 51.7%, 7.5%, and 17.1%

Fig. 1. SDS-PAGE analysis of GABA_A receptor α_1 subunit fragments. Expression and purification of receptor segments were as described in Materials and Methods. (A) One day and (B) 100 days after purification. (Lane 1) marker; (lane 2) Q28-L296; (lane 3) Q28-R248; (lane 4) Q28-E165; (lane 5) C166-L296.

using SELCON). Therefore, the two fragments have different secondary structure contents and the changes are similarly reflected in the percentages calculated by the two analyses programs. Interestingly, it can be seen that the spectrum of Q28-L296, the combined fragment, closely resembled the composite spectrum of the two individual spectra for the two smaller fragments (Fig. 2D). This suggests that division of the Q28-L296 fragment into two individual segments has not compromised the protein secondary structure, indicating that the two fragments, Q28-E165 and C166-L296, are sufficient to constitute independent protein folds by their own rights.

The secondary structure contents of the protein fragments determined by far-UV CD were further confirmed using FTIR to avoid possible interferences due to the aggregation status of the fragments. It was found that the FTIR spectra of both Q28-E165 and C166-L296 showed a prominent amide I peak at $\sim 1628\text{ cm}^{-1}$ (Fig. 2C), which with spectra deconvolution, confirmed that the fragments were β -rich. Therefore, polymerization of the fragments did not appear to have significantly altered their CD spectra and interpretation of the CD spectra was not compromised.

Amino- or carboxy-terminal deletion disrupted the structural integrity of Q28-E165

Amino- and carboxy-terminal deletions from the Q28-E165 fragment were performed and changes to the secondary structure and tertiary packing were monitored using CD spectroscopy. The far-UV CD spectrum of Q28-E165 was compared to that of R64-E165 (deletion of the amino-terminal 36 residues containing a predicted α -helix) and Q28-L155 (deletion of the carboxy-terminal 10 residues, a predicted β -strand; Fig. 2A). In comparison to the spectrum of Q28-E165, that of R64-E165 showed lower α -helix content (18.1% α -helix; 34.4% β -strand; 24.3% turn; 23.8% random using CDPPro and 26.5%, 47.2%, 8.6%, and 17.2% using SELCON). The lower α -helix content is consistent with the prediction of an α -helix at the amino terminus (Xue et al. 1999), which had been deleted in this mutant. In addition, the spectrum of Q28-L155 showed an increase in the content of random coil compared to the spectrum of the parent segment Q28-E165 (20.7% α -helix; 29.5% β -strand; 22.1% turn; 28.1% random using CDPPro and 26.8%, 47.4%, 7.8%, and 18.2% using SELCON) suggesting that this pre-

Fig. 2. CD and FTIR spectra of various fragments of GABA_A receptor α_1 subunit. (A,B). Effects of amino- or carboxy-terminal deletions on Q28-E165. Far-UV CD spectra (A) and near-UV CD spectra (B) of Q28-E165 (solid line), R64-E165 (dashed line), and Q28-L155 (dotted line) in 10 mM Tris-HCl at pH 9.5 are compared. (C) The FTIR spectra of Q28-E165 (dashed line) and C166-L296 (solid line) are shown. The prominent amide I region (1600–1700 cm^{-1}) from which the secondary structure contents are calculated is labeled. (D) The far-UV CD spectra of Q28-E165 (dashed line), C166-L296 (dotted line), and Q28-L296 (dash-dot line). The spectrum SIM (solid line) is obtained by averaging the two individual spectra of Q28-E165 and C166-L296.

dicted β -strand is indispensable to the protein structure integrity.

Similarly, changes were observed in the tertiary packing of the segments when deletions were performed (Fig. 2B). The near-UV CD spectrum of Q28-E165, with prominent absorbances in the 280–295 nm region, was characteristic of proteins with ordered tertiary structure. Therefore, the ordered, well-defined tertiary structure of Q28-E165 is clearly shown. The spectrum of the amino-terminal deletion mutant was also indicative of the presence of tertiary packing, but there were intensity differences in the region 260–290 nm, suggesting that the packing around aromatic residues in R64-E165 is different from that in the parent fragment Q28-E165. In particular, the intensity difference at 292 nm is indicative of changes in the environment surrounding Trp residues, as Q28-E165 and R64-E165 have the same number of Trp residues. More severely, the near-UV CD spectrum of Q28-L155 was near zero in the region 270–300 nm, indicating that the aromatic residues are in nonordered states, most likely a result of gross structural changes caused by the deletion of 10 residues from the carboxyl terminus. Therefore, the results indicate that both Q28-E63 (predicted α -helix) and, more so, Y156-E165 (predicted β -strand) contributed to the integrity of the structure domain in Q28-E165.

C166-L296 was more stable than Q28-E165 in denaturation tests

The two fragments were further analyzed by comparing their denaturation characteristics. The denaturation curves of both fragments using urea as denaturant and monitored by both fluorescence and CD spectroscopy are shown in Figure 3A and B, respectively. The sigmoidal shape of the curves for the amino-terminal fragment Q28-E165 indicate that denaturation is a cooperative process with one transition state and strongly supports the suggestion that the fragment forms a regular protein fold. The aggregation status of this fragment has not affected its denaturation, suggesting that the intermolecular interactions involved in aggregation are relatively weak. In addition, the curves from CD and fluorescence spectroscopy are superimposable, suggesting that both changes in secondary structure (far-UV CD; Fig. 3B) and tertiary structure (fluorescence intensities; Fig. 3A) occur concurrently. Interestingly, it can be seen that the denaturation of C166-L296 is not complete even at 10 M of urea, in the presence of 2 mM dithiothreitol (DTT), suggesting that C166-L296 is extremely stable, much more so than most known proteins and more so than Q28-E165.

Total denaturation of C166-L296 can be achieved with ~6.5 M guanidine chloride (GdCl) (Fig. 3C). It can be seen that denaturation for the Q28-E165 fragment was almost complete at 3.5 M of GdCl, at which there was only a small degree of structure change for C166-L296. This structure

Fig. 3. Denaturation curves of the two extracellular domains. (A) The fluorescence emission was scanned from 300 to 400 nm with excitation set at 295 nm. Changes in average emission wavelength with increasing concentrations of urea were monitored. Changes in CD ellipticity at 222 nm with increasing concentrations of urea (B) or GdCl (C) were also monitored. Denaturation of C166-L296 was performed in the presence of 2 mM of dithiothreitol. The denaturation of C166-L296, using GdCl, in the absence of 2 mM DTT is also shown (open triangles in C). The curves were obtained as the line of sigmoidal fit of the data. (squares) Q28-E165; (triangles) C166-L296.

change, also represented in the gradual changes of the urea denaturation curves (Fig. 3A,B), may account for intermolecular interactions. It is notable that a complete one-transition state denaturation curve can be obtained for C166-L296 within the range of 3–6.5 M of GdCl when in the presence of 2 mM of DTT. This suggests that denaturation of the protein under these conditions is a cooperative process, supporting a domainlike structure for the fragment. When denaturation was performed in the absence of the reducing agent DTT, the transition between folded and unfolded protein was less cooperative (Fig. 3C), suggesting that there is a contribution to the protein structure by disulfide bonds.

Polymerization of both fragments were sensitive to SDS and pH, but to different extents

The interactions involved in the assembly of the proteins were further investigated using laser-scattering spectroscopy to monitor the effects of SDS and pH on the polymerization status. At pH 9.6, the average molecular mass of Q28-E165 was measured to be ~3100 kD, suggesting that at

this pH the protein was present as large aggregates. With incremental increase of SDS, it was found that the molecular mass of Q28-E165 rapidly decreased by ~3000 kD on addition of 0.05% SDS (Fig. 4A,C), suggesting that the large aggregates of Q28-E165 were readily disrupted, supporting the suggestion that intermolecular interactions were relatively weak. However, similar addition of 0.05% SDS, at pH 9.6, to C166-L296 only resulted in a decrease from 250 kD to 225 kD (Fig. 4B,C). This suggests that the interactions involved in the polymerization of C166-L296 are much stronger than those for Q28-E165. In addition, it was found that the polymerization status of the fragments was dependent on pH. The molecular mass of C166-L296 was found to change from 1800 kD at pH 7.0 to 130 kD at pH 10.5, whereas that of Q28-E165 changed from 3000 kD at pH 9.0 to 270 kD at pH 12.6 (Fig. 4D). The molecular mass-dependency on pH suggests that surface charges are involved in the polymerization of the fragments.

Conclusion and implication

In conclusion, in addition to the previously identified membrane-proximal domain, MPBR in fragment C166-L296, we

Fig. 4. Effect of SDS and pH on the molecular mass of the two extracellular domains. The molecular mass was calculated using laser-scattering spectroscopy as described by Xue et al. (2001). The elution profile of (A) Q28-E165 and (B) C166-L296, using 10 mM of Gly at pH 9.6 as elution buffer, in the absence (thin line) and presence of 0.05% SDS (thick line) monitored with both UV (dashed line) and laser-scattering (solid line) spectroscopy. The corresponding average molecular mass under the experimental conditions were calculated to be (A) 3100 kD and 58 kD; (B) 250 kD and 225 kD in the absence and presence of 0.05% SDS, respectively. (C) Effect of incremental increase of SDS. The sample and elution buffer was 10 mM Gly at pH 9.6 in the presence of differing concentrations of SDS. (D) Effect of pH. The sample and elution buffer was 10 mM of Gly at different pH. (triangles) Q28-E165; (squares) C166-L296.

now propose the presence of an amino-terminal extracellular domain, formed by residues from fragment Q28-E165 of the GABA_A receptor α_1 subunit. The domainlike nature of this amino-terminal fragment is demonstrated by its denaturation profile and loss of structural integrity with either amino- or carboxy-terminal deletions. The presence of these two domains provides direct evidence for the multidomain nature of this family of receptors, in particular of the GABA_A receptor.

Despite the fact that both fragments are β -rich in secondary structure, there are a number of significant differences in their physical properties. First, the aggregates of Q28-E165 are readily broken using SDS to yield the monomer state whereas the interactions involved in the polymerization of C166-L296 are much stronger as demonstrated by the tolerance to SDS. These strong interactions are likely to contribute to the intrinsic tendency of MPBR to form pentamers similar to that of the native receptor. Second, although the aggregation status of both fragments is dependent on pH, the large aggregates of C166-L296 are more sensitive to high pH than those of Q28-E165. Third, C166-L296 is extremely stable to denaturants, requiring 6.5 M of GdCl for total denaturation, much more than most known proteins and more than Q28-E165. The extraordinary stability may be a factor favoring the stable formation of pentamers. Together, these results strongly suggest that residues in the C166-L296 region may play a more important role than those in Q28-E165 in the quaternary structure of the receptor. This complements the earlier finding that the benzodiazepine-binding affinity of C166-L296 is greater than that of Q28-E165 (Hang et al. 2000). Hence, it is implied that the MPBR domain in fragment C166-L296 of the GABA_A receptor α_1 subunit is both structurally and functionally a highly important domain.

Materials and methods

Materials

The cDNA clone, pCDM8-b α_1 , encoding the bovine α_1 subunit was a gift from Dr. A. N. Bateson of the University of Alberta. The plasmid pTrcHis was purchased from Invitrogen and the fluorescent benzodiazepine ligand Bodipy-FL Ro1986 was from Molecular Probes, Inc. Guanidine and urea are of Sigma Ultrapure grade. All other chemicals were from either Sigma or USB.

Construction of expression plasmids and mutagenesis

All subcloning and mutagenesis were performed with the PCR-based Site-directed Mutagenesis Kit from Stratagene (La Jolla, CA) with slight modifications in that *DpnI* treatment was replaced by gel purification of linear PCR products. A 2.3-kb cDNA (GenBank accession number X05717) encoding the mature peptide (Q28-Q456) of bovine GABA_A receptor α_1 subunit was subcloned from pCDM8-b α_1 to pTrcHis, which has been modified by deletion of the sequence between the mini-cistron and the *EcoRI* site

(Xue et al. 1998). Deletional mutants were made based on this initial subclone. All insert sequences were confirmed by DNA sequencing.

Expression and purification

Fragments of the GABA_A receptor α_1 subunit were expressed and purified as described in Hang et al (2000). The molecular mass and purity of the proteins were estimated using SDS-PAGE.

Laser-scattering spectroscopy

The MiniDAWN LS detecting system from Wyatt Technology Corporation was used for LS measurements. LS spectroscopy was carried out as described in Xue et al. (2001).

CD spectroscopy

All CD measurements were obtained using a JASCO J-720 spectrophotometer at room temperature with a 0.1-cm path length for far-UV CD or 1-cm path for near-UV CD. The protein samples were 0.1 mg/mL in 10 mM Tris-HCl at pH 9.5 for far-UV and 1 mg/mL for near-UV CD. Secondary structure contents were estimated from the far-UV CD spectra using the programs CDPro (Sreerama and Woody 2000) and SELCON (Sreerama and Woody 1993).

FTIR

FTIR spectra were acquired with a Bruker Vector 22 spectrophotometer (Bruker Analytik, Karlsruhe, Germany) equipped with a three-reflection diamond ATR element (DurasamplIR, Sensir Technologies, Danbury, CT). The machine and sample compartment were continually purged with dry nitrogen. Spectra, the average of 250 scans, were acquired at a resolution of 4 cm⁻¹ and twice zero-padded before transformation. Absorption spectra were subsequently analyzed using the Datamax32 software (Galactic Software). The spectra were corrected for evanescent wave sample penetration using a diamond refractive index of 2.42, before buffer subtraction. After buffer subtraction the amide I band was deconvoluted and secondary structure elements were assigned essentially as described by Byler and Susi (1986). Deconvolution was based on a combination of Fourier self-deconvolution and gaussian curve-fitting. Gaussian spectral components were then assigned based on their frequencies.

Fluorescence spectroscopy

All fluorescence measurements were performed at room temperature using a Perkin-Elmer LS50B luminescence spectrometer. The excitation wavelength was set at 295 nm and emission was scanned from 300 to 400 nm. The mean emission wavelength was calculated through integration of the emission spectra at each urea titration point.

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