Topology characterization of a benzodiazepine-binding β -rich domain of the GABA_A receptor α_1 subunit

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Abstract

Structural investigation of GABA_A receptors has been limited by difficulties imposed by its transmembrane-complex nature. In the present study, the topology of a membrane-proximal β -rich (MPB) domain in the C139–L269 segment of the receptor α_1 subunit was probed by mapping the benzodiazepine (BZ)-binding and epitopic sites, as well as fluorescence resonance energy transfer (FRET) analysis. Ala-scanning and semiconservative substitutions within this segment revealed the contribution of the phenyl rings of Y160 and Y210, the hydroxy group of S186 and the positive charge on R187 to BZ-binding. FRET with the bound BZ ligand indicated the proximity of Y160, S186, R187, and S206 to the BZ-binding site. On the other hand, epitope-mapping using the monoclonal antibodies (mAbs) against the MPB domain established a clustering of T172, R173, E174, Q196, and T197. Based on the lack of FRET between Trp substitutionally placed at R173 or V198 and bound BZ, this epitope-mapped cluster is located on a separate end of the folded protein from the BZ-binding site. Mutations of the five conserved Cys and Trp residues in the MPB domain gave rise to synergistic and rescuing effects on protein secondary structures and unfolding stability that point to a CCWCW-pentad, reminiscent to the CWC-triad “pin” of immunoglobulin (Ig)-like domains, important for the structural maintenance. These findings, together with secondary structure and fold predictions suggest an anti-parallel β -strand topology with resemblance to Ig-like fold, having the BZ-binding and the epitopic residues being clustered at two different ends of the fold.

Keywords: Ala-scanning; circular dichroism; fluorescence spectroscopy; fold recognition; epitope mapping; secondary structure; stability; thermodynamic analysis

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

and Olsen 1994; Smith and Olsen 1995; Stephenson 1995). They are clinically important drug targets, and several distinct classes of therapeutic agents including BZ tranquilizers, barbiturates, and general anesthetics may regulate their functions allosterically (Sieghart 1995). A pentameric structure has been suggested for these receptors (Nayeem et al. 1994), with the chloride ion channel formed along the vertical axis of subunit interfaces and several distinct ligand binding sites situated in the large N-terminal extracellular region. In particular, the BZ-binding pocket was attributed to the α/γ subunit interface (Sigel and Buhr 1997). More recently, the homomeric γ_2 receptor was found to be positively modulated by BZs, indicating the presence of BZ-binding sites on the homooligomers (Martinez-Torres and Miledi 2004).

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Abbreviations: AChBP, acetylcholine-binding protein; BFR, Bodipy-FL Ro-1986; BZ, benzodiazepine; CD, circular dichroism; FA, fluorescence anisotropy; FRET, fluorescence resonance energy transfer; GABA_A, type A γ -aminobutyric acid; GdCl, guanidine hydrochloride; Ig, immunoglobulin; LGIC, ligand-gated ion channel; mAb, monoclonal antibody; MPB, membrane-proximal β -rich.

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While experimental determination of GABA_A receptor structure is hindered by the transmembrane nature of the protein, 3D structure prediction by homology modeling is also constrained by the lack of a suitable structural template. Among proteins with known 3D structures, although acetylcholine-binding protein (AChBP) shows the highest sequence similarity to the extracellular domain of LGIC receptors, its identity with the ligand-binding domain of the GABA_A receptor is only 18% (Brejc et al. 2001; Cromer et al. 2002). Nonetheless, fold-recognition algorithms—by which fold types can be predicted even for proteins with little sequence similarities to known structures—have been formulated, and fold recognition may provide useful structural information on the GABA_A receptor protein that can be tested experimentally.

Experimental investigations of GABA_A receptor structures have been facilitated by the overexpression of recombinant receptor domains (Xue et al. 1998; Shi et al. 2002, 2003). On this basis, the N-terminal region of GABA_A receptor α_1 subunit was shown to consist of two β -rich domains formed by residues Q1–E138 and C139–L269, respectively (Shi et al. 2002). Since the C139–L269 segment harboring the MPB domain (Fig. 1A) formed ordered tertiary and rosette-like quaternary structures (Xue et al. 1999, 2000; Hang et al. 2000), and displayed a greater BZ-binding affinity than the neighboring Q1–E138 segment, it was identified to be both structurally and functionally a highly important domain (Shi et al. 2002).

To gain insight into this MPB domain, in the present study protein secondary structure and fold predictions have been combined with experimental approaches based on the characterization of point mutants by means of circular dichroism (CD) spectroscopy, fluorescence anisotropy (FA) measurement, FRET analysis, and mapping of solvent-accessible epitopic sites with mAbs against the MPB domain. The contributions of the five conserved Cys and Trp in this receptor domain to structural stability of a postulated Ig-like fold were also investigated.

Results

Residues important to protein structure or BZ-binding

To identify residues important to protein structure or BZ-binding, the 62 non-Ala residues in P154–L219 segment (Fig. 1A) were Ala-substituted individually, and the resultant mutants were compared to the wild-type MPB domain with respect to their secondary structures and BZ-binding affinity.

Based on the far-UV CD spectra (Fig. 2A) and the CDPro analysis package (Sreerama and Woody 2000),

the wild type protein exhibited 18.5% α -helix, 31.7% β -sheet, 22.3% β -turn, and 27.4% others, including random structures (Table 1). For the mutant proteins, Ala-substitution of each of residues K156, Y160, E166, V167, V179, G185, Q190, L194, D199, Q204, T207, F217, and L219 gave rise to significant changes in secondary structures compared to the wild type (Table 1; Fig. 2A), pointing to the importance of these residues to the secondary structural maintenance. A small structural change encountered earlier upon Ala-substitution of Y210 (Hang et al. 2000) was found not to be significant when the improved computational programs (Sreerama and Woody 2000) of the present study were used (Table 2). It might be noted that within the Ala-scanned P154–L219 segment, secondary structural prediction showed mainly the occurrence of β -strands and loops, with little α -helix (Fig. 1A).

Toward the binding of the fluorescent BZ ligand Bodipy-FL Ro-1986 (BFR), Ala-substitution of each of the residues—G185, S186, R187, and F217—reduced binding affinities to about half that of the wild type (Table 2; Fig. 2B), and comparable to previously reported affinities of the Y160A, T207A, and Y210A mutants (Hang et al. 2000). Of these seven Ala-substitutions, because Y160A, G185A, T207A, and F217A induced protein structural changes, their effects on BZ-binding might be a secondary consequence of such changes. In contrast, the S186A, R187A, and Y210A mutations, insofar that they did not induce any significant secondary structural change (Table 2; Fig. 2A), evidently made a more direct contribution to BZ-binding. These results confirmed the importance of Y210 to BZ-binding first observed in electrophysiological studies (Amin et al. 1997; Buhr et al. 1997), and indicated in addition the involvement of S186 and R187 in BZ-binding.

The Q1–L269 segment, which spans the N-terminal β -rich domain Q1–E138 and MPB domain as delineated previously (Shi et al. 2002), exhibited a K_d of 1.6 μ M toward BFR (Table 2). The higher binding affinity of the larger domain, compared to the K_d of 4.9 μ M exhibited by the MPB domain, likely reflected extra binding contributions made by residues present in Q1–E138 (Fig. 1A). When Ala-substitutions were performed at the three consecutive residues G185–R187, each of the G185A, S186A, and R187A mutants displayed a five- to sixfold decrease in BZ-binding affinity (Fig. 2C). As in the case for the MPB domain, only G185A, but not S186A or R187A, caused significant changes in secondary structures in the longer domain (Table 2). These findings indicate that the structural perturbation induced by G185A, as well as decreased BZ-binding induced by S186A and R187A, were observable with both the longer and the shorter receptor domains. They therefore represented attributes of stable configurations in this portion of the receptor protein that did not greatly vary with the length of the polypeptide.

In contrast to Ala-substitution of S186, which decreased BZ-binding affinity without changing secondary structures, Thr-substitution of this residue did not alter either property (Table 2), demonstrating thereby the important role played by the hydroxy group at residue

Figure 2. Effects exerted by Ala-substitutions of a three-residue cluster. (A) CD spectra of the wild-type MPB domain and its G185A, S186A, and R187A mutants. (B) Saturation anisotropy curves and their Scatchard transformations (*inset*) for BFR binding to the MPB domain, or the longer Q1–L269 domain (C). In both instances: wild type (■); G185A (□); S186A (○); R187A (△).

186 in BZ-binding. When the residue R187 was subjected to substitution by Lys, Trp, or Glu, none of the substitutions brought about any significant structural change, and only R187E yielded a reduced binding affinity comparable to that of R187A (Table 2). Thus, the appearance of a negative charge at this position was not well tolerated with respect to BZ-binding. Unlike G185A, which affected both secondary structures and BZ-binding, the G185P mutation did not significantly alter either parameter (Table 2), suggesting that conformational constraints at residue 185 might be important to protein folding; Gly

and Pro are known to share a propensity to destabilize α -helices and favor β -turns in proteins (Krystek et al. 1995).

Mutating Y160 to Phe, Thr or Trp gave rise to K_d for BFR of 5.7 μ M, 8.0 μ M, or 8.2 μ M, respectively, compared to 4.9 μ M for the wild type (Table 2). Secondary structural change was displayed by Y160T, where the aromatic Tyr was replaced by hydrophilic Thr but not by Y160F or Y160W, where aromaticity was conserved. That Y160W gave rise to a decreased binding affinity unaccompanied by extensive secondary structural change points to a direct contribution by Y160 to BZ-binding, despite the occurrence of structural disruption when Y160 was replaced by Ala. Moreover, the smaller increase in K_d shown by Y160F compared to Y160T or Y160W indicates the importance of the phenyl ring to the contribution by Y160 toward BZ-binding. For residue T207, its semiconservative substitution by Ser, preserving a hydroxy group in the side chain, failed to alter either protein structure or BZ-binding affinity, in contrast to the evident changes in both properties when it was replaced by Ala (Table 2). Therefore a side-chain hydroxy at this position is clearly required for protein structure maintenance and BZ-binding. The Y210F mutant, which lost the hydroxy group but not the aromatic ring in the side chain, showed a K_d value and secondary structural contents close to the wild type. By comparison, when Y210 was mutated to Thr, retaining the hydroxy group but not the phenyl ring, K_d was significantly increased from 4.9 μ M to 9.9 μ M, still unaccompanied by substantial structural changes (Table 2). Therefore the phenyl ring is important at residue 210 for BZ-binding. When Y210 was substituted by Trp, a reduction in binding affinity was accompanied by considerable secondary structural changes. Thus the placement of a bulky indole ring at this position disrupted the protein fold.

The van't Hoff plot of $\ln K_d$ versus $1/T$ for BFR binding to the MPB domain was close to linear (Fig. 3), yielding $\Delta H = -20.55$ kJ mol⁻¹ and $\Delta S = 29.52$ J mol⁻¹ K⁻¹ over the temperature range 12°–35°C. The linearity suggests that the ligand binding mechanisms were largely uniform over these temperatures, likely involving the same set of amino acid side chains on the receptor protein.

FRET and epitope mapping

The relative distances between putative loops and the BZ-binding site could be determined by measuring FRET from Trp probes placed in these loops to the fluorescent BZ ligand BFR. Since the efficiency of FRET is inversely related to the sixth power of the distance R between donor and acceptor (Stryer 1978), significant FRET represents a reliable indicator for proximity, at approximately $R < 2 R_0$ (Robert 1996). Figure 1A shows the positions of Trp-substitutions in

Table 1. *Ala-substitutions leading to alterations in protein structure*

Protein	Secondary structure contents ^{a,b} (%)				BFR–protein dissociation constant ^b K_d (μ M)
	α -Helix	β -Sheet	β -Turn	Others	
MPB domain					
Wild type	18.5 $\pm$ 0.5	31.7 $\pm$ 0.6	22.3 $\pm$ 0.1	27.4 $\pm$ 0.2	4.9 $\pm$ 0.4
Ala-substitutions ^c					
K156A	23.7 $\pm$ 1.5	29.6 $\pm$ 0.6	21.6 $\pm$ 0.6	25.5 $\pm$ 0.5	5.7 $\pm$ 0.5
Y160A	17.5 $\pm$ 0.3	34.2 $\pm$ 0.2	22.8 $\pm$ 0.3	25.4 $\pm$ 0.4	10.3 $\pm$ 2.1
E166A	14.9 $\pm$ 1.2	33.7 $\pm$ 2.0	21.9 $\pm$ 0.4	28.7 $\pm$ 0.5	6.0 $\pm$ 0.6
V167A	20.9 $\pm$ 0.9	32.6 $\pm$ 1.3	23.2 $\pm$ 0.9	22.7 $\pm$ 1.1	6.2 $\pm$ 1.1
V179A	20.1 $\pm$ 0.5	30.5 $\pm$ 0.5	23.5 $\pm$ 0.2	26.5 $\pm$ 0.4	5.9 $\pm$ 0.7
G185A	20.8 $\pm$ 1.0	28.9 $\pm$ 0.8	21.6 $\pm$ 0.1	28.8 $\pm$ 1.3	10.2 $\pm$ 1.1
Q190A	18.1 $\pm$ 1.1	34.1 $\pm$ 0.5	23.8 $\pm$ 0.2	23.6 $\pm$ 1.0	6.1 $\pm$ 1.1
L194A	21.5 $\pm$ 0.4	30.1 $\pm$ 0.8	21.4 $\pm$ 0.2	27.3 $\pm$ 0.6	5.5 $\pm$ 0.5
D199A	16.8 $\pm$ 0.4	35.4 $\pm$ 1.7	23.0 $\pm$ 0.9	25.0 $\pm$ 2.1	4.6 $\pm$ 0.3
Q204A	18.3 $\pm$ 0.1	32.9 $\pm$ 0.3	23.4 $\pm$ 0.7	25.5 $\pm$ 0.2	4.4 $\pm$ 0.9
T207A	22.3 $\pm$ 1.5	27.5 $\pm$ 2.2	22.2 $\pm$ 0.5	28.1 $\pm$ 1.2	10.4 $\pm$ 1.6
F217A	26.2 $\pm$ 0.4	23.7 $\pm$ 1.1	22.5 $\pm$ 1.6	28.1 $\pm$ 2.2	9.6 $\pm$ 0.4
L219A	21.9 $\pm$ 0.8	28.1 $\pm$ 0.1	21.8 $\pm$ 0.5	28.1 $\pm$ 1.4	5.6 $\pm$ 0.7

^a Secondary structure contents were estimated from CD spectra using the three programs in the CDPro package (Sreerama and Woody 2000).

^b “Others” represent unordered/random and unassigned structures.

^c Data were obtained from at least three independent measurements of different batches of protein samples, and shown as mean $\pm$ S.E. Mutant values significantly different ($p < 0.05$) from the corresponding wild-type values in the t test are shown in boldface.

^d Only the Ala-substitutions in the region of P154–L219 that yielded significantly different secondary structure contents ($p < 0.05$) from the wild-type protein are included.

putative loops, at residues where the substitution had been determined not to disturb secondary structures (Table 2). The Forster distances R_0 between these Trp-substituted mutants and BFR, calculated from Equation 6 as given in Materials and Methods, were 23.9–24.0 Å, close to the value of 24.2 Å for the wild-type MPB domain and BFR. This gives an estimate for R to be < 48 Å. Of the six Trp-substituted mutants constructed, Y160W, S186W, R187W, and S206W yielded FRET to BFR that exceeded the wild-type protein with respect to the FRET index INT280/INT340 (Fig. 4), suggesting the proximity of these four residues to the bound BFR. In contrast, the lack of significant FRET enhancement in R173W and V198W point to the greater distances between the BFR and these positions in the polypeptide.

An antibody to a protein antigen may recognize different amino acids from more than one segment of the primary sequence, which in turn furnishes evidence for the physical proximity between these different amino acids (Benjamin and Perdue 1996). In the present study, the mAbs 3E2 and 4F10 raised against the MPB domain specifically recognized the C139–L269 segment of the α_1 subunit, but not the bovine serum albumin or the recombinant Q1–G219 fragment of the GABA_A receptor β_2 subunit (Fig. 5A). When mAb 3E2 was used as primary antibody in the ELISA assay against the MPB domain and its Ala-substituted mutant forms, the assay readings yielded by T172A, R173A, E174A,

Q196A, and T197A were reduced to around 40%, 40%, 20%, 5%, and 40% of wild type, respectively (Fig. 5B). By comparison, the Ala-mutants S159A, Y162A, T163A, R164A, E170A, R177A, E183A, D184A, S186A, R187A, N189A, D192A, S206A, E209A, and Y210A did not depart greatly from the wild type. Since all of the Ala-substitutions compared did not affect the secondary structures (Table 1), these results suggest that T172, R173, E174, Q196, and T197 represented epitopic residues recognized by mAb 3E2. By the same token, E174, Q196, and T197 were identified to be epitopic residues recognized by mAb 4F10 (Fig. 5C). Therefore, each of these two mAbs recognizes simultaneously E174 and Q196–T197. These results indicate that E174 is spatially situated close to Q196–T197, and all three of them are exposed to solvent.

Synergistic and rescuing effects of mutations at conserved Cys and Trp

Inspection of aligned GABA_A receptor sequences (Fig. 1C) reveals that the five Cys and Trp residues in the C139–L269 segment are all conserved between the α and the γ subunits of GABA_A receptor. In the β subunits, four of them are conserved, while one Cys is replaced semi-conservatively by Ser. To define the roles of these well-conserved residues in the MPB domain, Cys was mutagenized to Ala, and Trp to Tyr, in all possible combinations.

Table 2. Mutational effects on protein secondary structure and BZ-binding

Protein	Secondary structure contents ^{a,b} (%)				BFR-protein dissociation constant ^b K_d (μ M)
	α -Helix	β -Sheet	β -Turn	Others	
MPB domain					
Wild type	18.5 $\pm$ 0.5	31.7 $\pm$ 0.6	22.3 $\pm$ 0.1	27.4 $\pm$ 0.2	4.9 $\pm$ 0.4
Ala-substitutions ^c					
Y160A ^d	17.5 $\pm$ 0.3	34.2 $\pm$ 0.2	22.8 $\pm$ 0.3	25.4 $\pm$ 0.4	10.3 $\pm$ 2.1
G185A	20.8 $\pm$ 1.0	28.9 $\pm$ 0.8	21.6 $\pm$ 0.1	28.8 $\pm$ 1.3	10.2 $\pm$ 1.1
S186A	18.8 $\pm$ 0.5	31.2 $\pm$ 0.2	22.0 $\pm$ 0.4	27.9 $\pm$ 0.5	9.9 $\pm$ 0.8
R187A	18.2 $\pm$ 0.7	30.9 $\pm$ 0.7	22.3 $\pm$ 0.2	27.5 $\pm$ 1.1	9.6 $\pm$ 0.8
T207A ^d	22.3 $\pm$ 1.5	27.5 $\pm$ 2.2	22.2 $\pm$ 0.5	28.1 $\pm$ 1.2	10.4 $\pm$ 1.6
Y210A ^d	19.0 $\pm$ 0.4	30.3 $\pm$ 1.3	22.1 $\pm$ 0.3	28.3 $\pm$ 1.1	12.4 $\pm$ 2.0
F217A	26.2 $\pm$ 0.4	23.7 $\pm$ 1.1	22.5 $\pm$ 1.6	28.1 $\pm$ 2.2	9.6 $\pm$ 0.4
Semi-conservative substitutions					
Y160F	17.9 $\pm$ 0.2	32.9 $\pm$ 0.3	21.9 $\pm$ 0.4	26.5 $\pm$ 0.4	5.7 $\pm$ 0.4
Y160T	17.2 $\pm$ 0.3	35.5 $\pm$ 0.7	22.6 $\pm$ 0.1	25.6 $\pm$ 0.6	8.0 $\pm$ 0.4
Y160W	19.0 $\pm$ 1.3	31.2 $\pm$ 1.3	22.7 $\pm$ 0.2	27.0 $\pm$ 0.3	8.2 $\pm$ 0.6
G185P	18.5 $\pm$ 0.3	32.3 $\pm$ 0.5	22.4 $\pm$ 0.3	27.1 $\pm$ 0.6	4.8 $\pm$ 0.9
S186T	18.6 $\pm$ 0.8	33.0 $\pm$ 0.3	22.1 $\pm$ 0.2	26.1 $\pm$ 0.9	5.9 $\pm$ 0.5
R187E	18.3 $\pm$ 0.9	31.5 $\pm$ 0.6	22.4 $\pm$ 0.5	27.5 $\pm$ 1.0	9.1 $\pm$ 0.8
R187K	19.0 $\pm$ 0.7	32.0 $\pm$ 0.8	22.4 $\pm$ 0.3	26.8 $\pm$ 0.2	5.9 $\pm$ 0.7
R187W	18.1 $\pm$ 0.3	32.9 $\pm$ 0.6	22.8 $\pm$ 0.5	26.5 $\pm$ 0.7	4.9 $\pm$ 0.6
T207S	18.8 $\pm$ 1.1	30.5 $\pm$ 1.3	21.8 $\pm$ 0.3	28.6 $\pm$ 1.0	5.8 $\pm$ 0.6
Y210F	18.0 $\pm$ 0.7	32.4 $\pm$ 0.5	22.1 $\pm$ 0.5	27.6 $\pm$ 0.9	4.6 $\pm$ 0.5
Y210T	18.3 $\pm$ 0.6	31.6 $\pm$ 0.4	22.2 $\pm$ 0.2	28.2 $\pm$ 0.3	9.9 $\pm$ 1.5
Y210W	36.4 $\pm$ 1.8	19.2 $\pm$ 1.0	19.1 $\pm$ 0.2	25.3 $\pm$ 0.4	9.7 $\pm$ 0.1
Q1-L269					
Wild type	18.6 $\pm$ 0.7	31.3 $\pm$ 0.6	22.6 $\pm$ 0.1	28.2 $\pm$ 0.3	1.6 $\pm$ 0.4
G185A	22.2 $\pm$ 0.6	27.6 $\pm$ 0.2	22.2 $\pm$ 0.4	27.9 $\pm$ 1.4	9.0 $\pm$ 0.3
S186A	18.3 $\pm$ 0.8	29.9 $\pm$ 1.7	21.8 $\pm$ 0.4	29.7 $\pm$ 0.6	9.1 $\pm$ 0.8
R187A	18.9 $\pm$ 0.7	30.8 $\pm$ 1.0	22.5 $\pm$ 0.5	28.4 $\pm$ 1.4	8.3 $\pm$ 0.3

^{a,b} See footnotes^{a,b} of Table 1.

^c Only Ala-mutants with significantly different ($p < 0.05$) K_d values are included.

^d The importance of Y160, T207, and Y210 to BZ-binding was first observed in electrophysiological studies (Amin et al. 1997; Buhr et al. 1997), and the three Ala-substitutions were previously reported to reduce binding affinity of the MPB domain toward BFR (Hang et al. 2000).

The secondary structure contents of the 31 resultant mutants were estimated based on far-UV CD spectra (Fig. 6A) and the CDPro analysis package (Sreerama and Woody 2000). To facilitate comparison of secondary structural changes between each Cys-Trp mutant and the wild type, the σ index described in Equation 1 in Materials and Methods was used to express the aggregate change in a mutant with respect to α -helix, β -strands, turns, and random structures. On this basis, the five single-substitution mutants, namely C139A, C153A, C234A, W171Y, and W246Y, were found to display relatively minor σ , while the triple mutant C139A/C234A/W171Y yielded the highest σ among the 31 mutants (Table 3; Fig. 6B). Although significant changes in σ were encountered with all the quadruple and quintuple mutants (Table 3), interestingly their σ values were smaller than those of the double mutants C139A/C234A and C139A/W171Y. The far greater σ of these two double mutants as well as C234A/W246Y (Fig. 6B, black columns) than those arising from their constit-

uent single mutations established the occurrence of striking synergism between the constituent point mutations within the double mutants. At the same time, the greater

Figure 3. Thermodynamics of BFR binding to MPB domain. Saturation anisotropy curves were obtained for BFR binding to MPB domain at different temperatures: 285.0 K ($\square$); 291.2 K ($\bullet$); 298.0 K ($\triangle$); 303.4 K ($\blacktriangledown$); 308.3 K ($\diamond$). Inset shows linear van't Hoff plot for the binding.

Figure 4. Spatial proximity of different amino acid residues to the bound BFR as measured by FRET. The variations of the INT280/INT340 index for FRET with protein concentrations are shown for one run of measurements in the main figure: wild type (■); Y160W (□); R173W (○); S186W (△); R187W (▽); V198W (◇); S206W (○). The saturation curves were each obtained by nonlinear fitting of the data to a hyperbolic model. (*Inset*) The bars represent the saturation index values (mean $\pm$ S.E.) averaged from at least three independent measurements of different batches of protein samples. * indicates mutant values statistically different ($p < 0.05$) from the wild type (WT) in the t test.

σ of C139A/C234A and C139A/W171Y compared to triple mutants obtained by a further mutation at either C153 or W246 (Fig. 6B, shaded columns) clearly signaled

the operation of rescue effects within the triple mutants. Similarly a rescue effect was evident when a third mutation, C153A, was added to the double mutation C234A/W246Y (Fig. 6B). Both the synergism and the rescue effects observed in these various instances entailed largely a shift between α -helix and β -strands, with only minor alterations with respect to percent turns and percent random structures.

When BZ-binding by each of the Cys–Trp mutants was compared to the wild type, none of the single mutants showed a significant change in K_d (Table 3). While significant changes in K_d were exhibited by most of the multiple mutants, these same mutants were also characterized by considerable changes in protein secondary structures (Table 3), suggesting that the decreased binding activity observed could be the result of structural perturbations.

With regard to protein stability, since both wild-type and mutant MPB domain were precipitated by low but not high concentrations of guanidine hydrochloride (GdCl), their resistance against this denaturant could be measured at [GdCl] > 2.5 M (Fig. 7). Contributions by disulphide bonds were evident in GdCl-induced denaturation of the MPB domain in the absence of reducing

Figure 5. Effects of mutations on reactivity with mAbs. (A) Specificity of mAbs 3E2 and 4F10 tested by Western blotting performed as described in Materials and Methods using equal amounts of proteins (20 μ g) per lane. (Lane 1) Protein markers; (lane 2) C139-L269 of GABA_A receptor α_1 subunit (MPB domain); (lane 3) Q1-G219 of GABA_A receptor β_2 subunit; (lane 4) bovine serum albumin. *Left* panel shows Coomassie blue stained gel; *middle* and *right* panels show that 3E2 and 4F10 mediated staining of protein band only in lane 2 by sheep-anti-mouse peroxidase-conjugated secondary antibody. (B) Indirect ELISA performed with mAb 3E2 in microtiter wells coated with various proteins: WT, wild-type MPB domain; 159, 162, etc., mutant Ala-substituted at residue-159, 162, etc.; C1, bovine serum albumin; C2, Q1-G219 of GABA_A receptor β_2 subunit. Both C1 and C2 served as negative controls. Results obtained from two separate tests carried out in triplicates were normalized, and shown as percent absorbance relative to WT (mean $\pm$ S.E.). (C) Same as B except that mAb 4F10 was used.

Figure 6. Effects due to mutations of Cys and Trp residues on protein secondary structures. (A) CD spectra of wild-type MPB domain and representative mutants. (B) Synergistic and rescue effects. X-axis shows the various proteins examined, where I–V represent single mutations as indicated. Multiple mutations are shown in terms of their constituent single mutations; e.g., (I, III) represents the double mutations of C139A/C234A. (Top panel) Percent α -helix (percent α , gray) or percent β -strand (percent β , clear) of the indicated wild type or mutant protein; (middle panel) percent turns (percent t, gray) or percent random structures (percent r, clear); (bottom panel) σ , where occurrence of synergistic effects are highlighted in black and rescue effects are highlighted by shading.

agents, resulting in relatively noncooperative unfolding (Shi et al. 2002). Therefore, protein denaturation experiments were performed in the presence of 2 mM DTT to minimize such contributions. The denaturation curves were monitored either by fluorescence or, in cases where fluorescence intensity was greatly diminished by mutational depletion of Trp, by CD measurements.

Among the five single Cys–Trp mutants, the GdCl denaturation curves of C139A, C234A, W171Y, and—to a less extent—W246Y, were left-shifted relative to the wild type and elevated susceptibility to denaturation (Fig. 7A,C). The curve of C153A remained close to that of the wild type. The $\Delta(\Delta G)$, the difference in the free energy of denaturation between a mutant and the

wild type, is a measure of the change in protein stability brought about by the mutation, and could be estimated by means of Equation 8 in Materials and Methods (Pace 1990). The $\Delta(\Delta G)$ s for the single mutants were $-1.03 \text{ kcal mol}^{-1}$ for C153A, $-1.45 \text{ kcal mol}^{-1}$ for C234A, $-1.58 \text{ kcal mol}^{-1}$ for W246Y, $-2.42 \text{ kcal mol}^{-1}$ for C139A, and $-2.73 \text{ kcal mol}^{-1}$ for W171Y. Thus all the single mutants displayed decreased stability, particularly W171Y, which showed the largest change. When measured by fluorescence stopped-flow spectroscopy, W171Y also showed faster unfolding than wild type (Fig. 7D).

Among the multiple substitutions, C139A/C153A yielded a $[D]_{0.5}$ of 4.6 M GdCl (Fig. 7B), and a calculated $\Delta(\Delta G)$ of $-0.83 \text{ kcal mol}^{-1}$. Thus, in reference to C139A ($[D]_{0.5} = 3.7 \text{ M}$ and $\Delta(\Delta G) = -2.42 \text{ kcal mol}^{-1}$), the further Ala-substitution of C153 caused a rescue

Table 3. Mutations of the conserved Cys and Trp

Protein	σ^a	BFR-protein dissociation constant ^a K_d (μM)
Wild type	0.00 ± 0.05	4.9 ± 0.4
C139A	0.07 ± 0.12	5.5 ± 0.6
C153A	0.16 ± 0.10	6.1 ± 0.7
C234A	0.08 ± 0.12	6.2 ± 0.7
W171Y	0.15 ± 0.17	6.1 ± 1.7
W246Y	0.09 ± 0.11	5.9 ± 1.1
C139A/C153A	0.17 ± 0.09	6.4 ± 0.3
C153A/C234A	0.10 ± 0.09	6.7 ± 1.5
C139A/C234A	0.72 ± 0.21	22.1 ± 3.3
C139A/W171Y	0.62 ± 0.23	26.1 ± 3.2
C153A/W171Y	0.20 ± 0.10	6.3 ± 0.9
C234A/W171Y	0.10 ± 0.09	5.7 ± 0.8
C139A/W246Y	0.15 ± 0.09	7.3 ± 1.0
C153A/W246Y	0.12 ± 0.07	5.6 ± 0.7
C234A/W246Y	0.46 ± 0.11	10.1 ± 0.6
W171Y/W246Y	0.11 ± 0.13	6.1 ± 0.4
C139A/C153A/W171Y	0.38 ± 0.13	13.7 ± 1.3
C139A/C234A/W246Y	0.27 ± 0.14	8.9 ± 0.2
C153A/C234A/W171Y	0.36 ± 0.11	6.6 ± 0.4
C139A/C153A/C234A	0.50 ± 0.12	20.4 ± 1.8
C139A/C234A/W171Y	0.82 ± 0.12	20.7 ± 2.0
C153A/W171Y/W246Y	0.40 ± 0.12	6.2 ± 0.9
C139A/C153A/W246Y	0.35 ± 0.12	7.7 ± 0.9
C153A/C234A/W246Y	0.36 ± 0.12	7.3 ± 0.4
C139A/W171Y/W246Y	0.36 ± 0.12	14.9 ± 1.5
C234A/W171Y/W246Y	0.35 ± 0.11	7.2 ± 0.7
C139A/C234A/W171Y/W246Y	0.53 ± 0.13	8.8 ± 0.9
C139A/C153A/C234A/W171Y	0.40 ± 0.11	8.8 ± 0.6
C153A/C234A/W171Y/W246Y	0.40 ± 0.11	7.8 ± 1.6
C139A/C153A/W171Y/W246Y	0.35 ± 0.06	17.4 ± 1.0
C139A/C153A/C234A/W246Y	0.36 ± 0.09	25.0 ± 2.7
3CA2WY	0.38 ± 0.11	14.7 ± 2.5

^a Data were obtained from at least three independent measurements of different batches of protein samples, and shown as mean $\pm$ S.E. Statistical analysis by *t* test was performed and values significantly different ($p < 0.05$) from the wild type are shown in boldface.

Figure 7. Effects of mutations at Cys and Trp residues on protein stability. Denaturation curves were monitored either by fluorescence emission wavelength upon excitation at 295 nm (A,B), or by CD molar ellipticity at 222 nm (C). $\Delta(\Delta G)$ (kcal mol⁻¹), the difference in free energy of denaturation between mutant and wild type, is shown in parentheses. (D) Unfolding rates of W171Y and wild type in the presence of different GdCl concentrations.

effect on protein stability, to result in a much higher $[D]_{0.5}$ and smaller $\Delta(\Delta G)$. Comparably, the introduction of an additional C153A mutation rescued much of the instability of W171Y (Fig. 7C). In contrast, no extensive rescue effect was observed between C139A and C139A/C234A (Fig. 7B). The triple mutant C139A/C153A/C234A displayed a dramatically reduced $[D]_{0.5}$ of 3.0 M GdCl and a $\Delta(\Delta G)$ of -2.96 kcal mol⁻¹ (Fig. 7B). This departed sharply from the σ results, where adding C153A to the double mutant C139A/C234A brought about rescue effects (Fig. 6B). The $\Delta(\Delta G)$ of the quintuple mutant 3CA2WY, where all three Cys were replaced by Ala and both Trp by Tyr, was estimated to be -4.27 kcal mol⁻¹ (Fig. 7C), indicative of extreme instability and establishing clearly the importance of the five conserved Cys and Trp residues to protein stability.

Fold-recognition analysis

Previously, the application of both TOPITS (Rost 1995) and UCLA-DOE (Fischer and Eisenberg 1996) to overlapping fragments of the GABA_A receptor α_1 subunit suggested β -rich structures for the N-terminal two-thirds of the full-length receptor protein (Xue et al. 1999). In Figure 8, the structures yielding higher Z-scores as a measure of goodness of fit were subgrouped into different structural folds based on SCOP classification (Hubbard et al. 1999).

The Z-scores were relatively low (<3 for TOPITS and <3.5 for UCLA-DOE) even for the top structures, indicating that the folding of the MPB domain does not conform well to elucidated structures. Both TOPITS (Fig. 8A) and UCLA-DOE (Fig. 8B), however, suggested that the Ig-like β -sandwich fold would furnish a useful initial approximation for the domain. When the C139–L269 sequence and five homologous sequences of GABA_A receptor α_6 , β_4 , and δ subunits and the glycine receptor α_3 and β subunits were threaded against template structures using the ProFIT fold-recognition program (Domingues et al. 1999), the top-ranking fold was Ig-like β -sandwich (Table 4), in agreement with the TOPITS and UCLA-DOE results.

Discussion

Understanding of the GABA_A receptor has been hindered by the lack of structural information. In the present study, experimental characterizations have been combined with protein structure predictions to gain insight into the MPB domain of the α_1 subunit of this receptor.

Key residues for BZ-binding

Ala-scanning of the P154–L219 region revealed a number of residues, underlined in Figure 1A, that contribute to

Figure 8. Fold recognition. The 20 best-fitting structures identified by TOPITS (A) or the 15 best-fitting structures identified by UCLA-DOE (B) in terms of similarity to overlapping 120-residue fragments of GABA_A receptor α_1 subunit, with a scanning interval of ten residues, are grouped into structural folds based on SCOP classification. Structure folds represented in A include A, Ig-like β -sandwich; B, viral coat and capsid proteins; C, trypsin-like serine proteases; D, supersandwich; E, single-stranded left-handed β -helix; F, segmented RNA-genome viruses' proteins; G, reductase/elongation factor common domain; H, OB-fold; I, near-membrane domain of membrane proteins; J, lipocalins; K, glycosyl-asparaginase; L, galactose-binding domain-like; M, double-stranded β -helix; N, cupredoxins; O, conA-like lectins/glucanases; P, common fold of diphtheria toxin/transcription factors/cytochrome f; Q, colipase binding domain-like; R, C2 domain-like; S, β -trefoil; T, β -prism I; U, β -clip; V, barrel-sandwich hybrid; W, 7-bladed β -propeller; X, 6-bladed β -propeller. Structural folds represented in B include A, Ig-like β -sandwich; B, viral coat and capsid proteins; C, trypsin-like serine proteases; D, streptavidin-like; E, SH3-like barrel; F, segmented RNA-genome viruses' proteins; G, reductase/elongation factor common domain; H, prealbumin-like; I, PH domain-like; J, OB-fold; K, lipocalins; L, glycosyl-asparaginase; M, galactose-binding domain-like; N, conA-like lectins/glucanases; O, common fold of diphtheria toxin/transcription factors/cytochrome f; P, colipase binding domain-like; Q, β -trefoil; R, β -prism II; S, α -amylase; β -sheet domain; T, acid proteases.

the secondary structural integrity of the MPB domain. The structural changes induced by Ala-substitutions at Y160, G185, T207, and F217 were accompanied by significant decreases in BZ-binding affinity (Table 1), possibly from a disruption of the BZ-binding site.

Several methods have been used previously to identify BZ-binding site residues. Covalent labeling with reactive ligands identified the involvement of H102 of α_1 subunit in BZ-binding (Wieland et al. 1992). Assisted by site-directed mutagenesis, radioligand binding assays and electrophysiological studies of recombinant $\alpha_1\beta_2\gamma_2$ GABA_A receptors expressed in cell lines or *Xenopus laevis* oocytes (Smith and Olsen 1995) uncovered the importance of a number of residues to BZ-binding (Sigel and Buhr 1997),

including Y160, T207, and Y210 of α_1 subunit and F77 of γ_2 subunit. Also, the substituted-Cys accessibility method has been utilized to identify A79 and T81 located at the BZ-binding site of γ_2 subunit (Teissere and Czajkowski 2001). However, a limitation common to these methods is that they could not determine whether the observed effect of a particular mutation on ligand binding is directly due to the participation of the mutated residue in the binding process, or an indirect consequence of protein structural alterations caused by the mutation (Cromer et al. 2002).

In the present study, the overexpression of recombinant receptor domains in quantities adequate for both protein structure and BZ-binding studies has made possible a distinction between such direct and indirect

Table 4. Fold recognition for the MPB domain by ProFIT

Final rank	Median rank ^a	Superfamily	Fold	Class
1	5.0	Immunoglobulin	Ig-like β -sandwich	β
2	9.5	Nucleic acid-binding proteins	OB-fold	β
3	11.0	Fibronectin type III	Ig-like β -sandwich	β
4	14.5	Profilin (actin-binding protein)	Profilin-like	$\alpha + \beta$
5	21.5	NAD(P)-binding Rossmann-fold domains	NAD(P)-binding Rossmann-fold domain	α/β
6	26.5	Starch-binding domain	Prealbumin-like	β
7	26.5	DHS-like NAD/FAD-binding domain	DHS-like NAD/FAD-binding domain	α/β
8	27.0	Pyruvate kinase, C-terminal domain	Pyruvate kinase, C-terminal domain	α/β
9	36.0	Barwin-like endoglucanases	Double psi β -barrel	β
10	37.5	Viral-coat-and-capsid-proteins	Viral-coat-and-capsid-proteins	β

^a See Materials and Methods.

Superfamily, fold, and class are as defined in the SCOP database (Hubbard et al. 1999).

effects. Thus the finding that the Y160W, S186A, R187A, and Y210A mutants displayed an increased K_d unaccompanied by significant structural change (Table 2) pointed to the involvement of these four residues in the BZ-binding site. The importance of Y160 and Y210 to BZ-binding was first encountered in electrophysiological studies (Amin et al. 1997; Buhr et al. 1997), and confirmation of their key roles in the present study validates the utility of Ala-scanning for detecting binding site residues. Ala-scanning revealed as well the involvement of S186 and R187, located in a predicted loop region (Fig. 1A) where no BZ-binding residues had been identified previously. The BZ-binding effects of S186A and R187A were even more pronounced (more than fivefold increase in K_d) in the longer Q1–L269 domain than in the MPB domain (Table 2; Fig. 2). Besides being invariant with the length of the polypeptide, the BZ-binding mechanism and its underlying amino acid residues also appeared stable over different temperatures, as indicated by the constancy of the ΔH and ΔS for the ligand binding (Fig. 3). This MPB domain therefore provides a robust structure for delineating the roles of amino acid residues in BZ-binding.

The nature of the side-chain requirements of the different key residues for BZ-binding was defined by semi-conservative substitutions. For R187, the results of four separate mutations (Table 2) suggested that its positive charge contributed to binding, and the retention of function upon Thr-substitution but not Ala-substitution established a key role for the hydroxy side chain of S186 in BZ-binding, possibly through hydrogen bonding. The phenyl ring was shown to be of foremost importance at both Y160 and Y210, likely on account of its capacity for hydrophobic bonding, in keeping with the hydrophobic nature of BZ ligands. Moreover, the heterocyclic nitrogen and keto-oxygen atoms of BZs present potential sites for hydrogen bonding with receptor. In fact, pharmacophore models for GABA_A receptors have been described for hydrophobic interactions and hydrogen bonding with BZs (Zhang et al. 1995) and flavonoids (Huang et al. 2001). In this regard, Y160 and Y210 could furnish hydrophobic sites, and S186, a hydrogen-bonding site.

Thermodynamic analysis showed that the binding of BFR to the wild-type MPB domain fits a linear van't Hoff plot (Fig. 3), yielding constant negative enthalpy change ($\Delta H = -20.55 \text{ kJ mol}^{-1}$) and positive entropy change ($\Delta S = 29.52 \text{ J mol}^{-1} \text{ K}^{-1}$) over the temperature range of 12°–35°C. A negative ΔH is consistent with the presence of van der Waals interactions and/or hydrogen bonding, and a positive ΔS with hydrophobic and/or electrostatic interactions (Ross and Subramanian 1981), which are in accord with the results of site-directed mutagenesis.

The identification of Y160, S186, R187, and Y210 as key residues for BZ-binding is supported by FRET analysis upon placement of Trp as a probe into different positions within the MPB domain. Fluorescence resonance occurred between a Trp residue at positions 160, 186, 187, and 206 and the fluorescent BZ ligand BFR, in contrast to a lack of resonance when Trp was placed at positions 173 and 198. Since the various Trp-substitutions did not elicit significant changes in secondary structures (Table 2), the results clearly pointed to the proximity of Y160, S186, and R187 to the bound BZ ligand, which is a prerequisite for the side chain of these residues making a contribution to BZ-binding. The ligand-proximity of S206 is also in keeping with Y210 as a participant in BZ-binding.

Structural role of CCWCW-pentad

When the five conserved Cys and Trp residues—which may be represented as a CCWCW-pentad—in the MPB domain were mutated, the single mutations did not induce significant changes in protein secondary structure contents (Table 3), but did cause a decrease in protein stability (Fig. 7). With multiple substitutions, striking synergistic and rescue effects with respect to σ as index of secondary structures and $\Delta(\Delta G)$ as index of protein stability, point to interactions among the five residues. The σ and $\Delta(\Delta G)$ rescues were not strictly correlated to one another; for example, adding a further C153A mutation to the double mutant C139A/C234A resulted in rescue effects in σ (Fig. 6B) but no comparable rescue in GdCl denaturation (Fig. 7B). This reflects that whereas σ measured the end-point structural changes, $\Delta(\Delta G)$ measured only the propensity to unfolding. However, when the factors favoring instability are built up to a high level, the propensity to change and the realized change converged. Thus the very large negative $\Delta(\Delta G)$ of $-4.27 \text{ kcal mol}^{-1}$ of the quintuple mutant 3CA2WY signaled the magnitude of the instability induced by the substitution of all five members of the CCWCW-pentad, and correspondingly the high σ for mutants carrying four or five Cys and Trp mutations clearly gave indication of the loss of structural integrity.

Moreover, since single mutations of Cys or Trp did not affect BZ-binding, these residues are not directly involved in the binding. The decreased BZ-binding affinity of the multiply substituted protein (Table 3) was likely an indirect consequence of structural disruption. The crucial importance of the CCWCW-pentad in receptor function is therefore explainable mainly in terms of structure maintenance.

Protein stability is determined by the summation of contributions from many amino acid residues. While additive effects of multiple mutations are not uncommon,

synergistic, and rescuing effects could also arise owing to the fine balance between interacting amino acid residues (Chen et al. 2004). In this light, that the C153A or W246Y mutation could rescue against the σ arising from the double mutations of C139A plus C234A or W171Y (Fig. 6B) suggests that the CCWCW-pentad forms an interacting group that exerts concerted influence on the structure of the MPB domain. The rescue of $\Delta(\Delta G)$ of C139A by C153A toward GdCl denaturation further attested to the opposing structural effects of these two residues. Comparably opposing structural effects were also observed with W171Y and C153A (Fig. 7). The exact rescue mechanisms regarding σ and $\Delta(\Delta G)$ changes, such as the involvement of potential disulfide bonds, remain to be elucidated. Possibly C153 and W246 may stabilize some unfolding intermediates, such that when a synergism between C139A and C234A or W171Y destabilizes the native structure, C153 or W246 could exacerbate the destabilization by favoring the formation of the unfolding intermediates. Under such circumstances, an additional mutation at C153 or W246 may destabilize the intermediates and restore the balance in favor of the native structure. Interestingly, for example, the Ig domain CD2.d1 is known to form partly folded species (Lorch et al. 1999).

Delineation of protein fold topology

In the present study, site-directed mutagenesis revealed that the four residues Y160, S186, R187, and Y210 are important contributors to BZ-binding. FRET analysis also suggests the spatial proximity of Y160, S186, R187, and S206 to the bound BZ ligand (Fig. 4). Moreover, co-interaction of T172, R173, E174, Q196, and T197 with the same mAb (Fig. 5B) established that these five amino acid residues cluster close to one another. Based on the lack of FRET between Trp mutationally placed at R173 or V198 and bound BZ (Fig. 4), this cluster of amino acids are distal to the BZ-binding site.

The secondary structural prediction of the Alas-scanned P154–L219 segment yielded six β -strand segments interspersed with five loop structures (Fig. 1A). Designating each β -strand or loop structure by the position of one of its central amino acid residues, the six β -strands are displayed in Figure 1A as β_{K156} , β_{Y169} , β_{V180} , β_{L193} , β_{V203} , and β_{T215} , and the five loops as L_{T163} , L_{E174} , L_{S186} , L_{V198} , and L_{G208} .

It is noteworthy that the experimentally identified ligand-proximal and ligand-distal residues are located in the predicted loops. Of the ligand-proximal residues, Y160 is located in L_{T163} , S186 and R187 in L_{S186} , and S206 and Y210 in L_{G208} . On the other hand, of the ligand-distal residues, T172, R173, and E174 are located in L_{E174} , and Q196, T197, and V198 in L_{V198} . This indicates that L_{T163} , L_{S186} , and L_{G208} are clustered together

in a ligand-proximal end of the folded protein, while L_{E174} and L_{V198} are clustered together in a ligand-distal end. A simple topology suggested by these results is an anti-parallel arrangement of the six β -strands (Fig. 1B).

Protein fold recognition by threading against template 3D structures using TOPITS (Fig. 8A), UCLA-DOE (Fig. 8B) and ProFIT (Table 4), are suggestive of a resemblance between the MPB domain and Ig-like structures. Of note, the crystal structure of AChBP also conforms to a modified Ig-like topology (Brejc et al. 2001). The anti-parallel β -strand topology depicted in Figure 1B, based on the experimental observations of the present study, is in agreement with the predicted similarity between the MPB domain and Ig-like fold, an anti-parallel β -sandwich.

The 3D structures of members of the Ig-superfamily are more conserved than their sequences (Halaby and Mornon 1998). While structure-based alignment of 52 Ig-like domain sequences revealed low sequence identity (5%–15%) (Halaby et al. 1999), they share a characteristic topology (Bork et al. 1994). A structural motif identified from this group of proteins consists of an invariant Trp packed against a conserved disulphide bridge to form a “pin,” the central core of the protein domain (Lesk and Chothia 1982). This “pin” motif, regarded as a stabilizer of the Ig-fold (Lesk and Chothia 1982; Ioerger et al. 1999), corresponds to the CWC sequence motif with the formula of $CX_{10-17}WX_{26-62}C$ (Smith and Xue 1997). In some Ig-like proteins, an additional Trp residue is located about 10 residues on the C-terminal side of the second Cys of this CWC pin to form a CWCW motif, as exemplified by $CX_{13}WX_{55}CX_{10}W$ in the H-Chain of N9 neuraminidase (Tulip et al. 1992; Protein Data Bank [PDB] code 1NCA) and $CX_{13}WX_{59}CX_{10}W$ in the B-Chain of an anti-idiotypic antibody (Bentley et al. 1990; PDB code 1CIC). In the present study, the CCWCW-pentad in the MPB domain was found to exert a profound and concerted influence on protein stability. Its sequence of $CX_{13}CX_{17}WX_{62}CX_{11}W$ comprises a central $CX_{17}WX_{62}C$ similar to the CWC-triad pin of Ig structures, with an upstream extension of CX_{13} - and a downstream extension of $-X_{11}W$. Thus the similarity between the central portion of the CCWCW-pentad of the MPB domain and the CWC-triad pin of Ig structures extends to both their important structural roles and their sequence formulae.

In conclusion, the present study has identified key residues of the protein BZ-binding site in the MPB domain, and a conserved CCWCW-pentad important to protein stability. The wide array of experimental observations on BZ-binding, FRET, and mAb-recognition gave rise to the suggestion that the alternate interspersing loops between anti-parallel β -strands in the MPB domain are separated into two surface clusters. The BZ-binding amino acid

residues are located in the loops at one end of the protein fold, and the cluster of epitopic residues, at the opposite end. This topology, derived by prediction-assisted experimental characterizations, provides a useful basis for an initial delineation of the structure and function of this BZ-binding domain of the GABA_A receptor α_1 subunit protein.

Materials and methods

Site-directed mutagenesis

Point mutations were incorporated into the cDNA encoding human GABA_A receptor α_1 subunit domains subcloned in the pTrcHis plasmid (Invitrogen) as described (Hang et al. 2000). Mutagenesis was performed with the PCR-based Mutagenesis Kit from Stratagene, and all positive clones were confirmed by DNA sequencing.

Protein expression and purification

The GABA_A receptor recombinant proteins were expressed and purified as described (Hang et al. 2000; Shi et al. 2002, 2003). Molecular weights and purity of the proteins were estimated using SDS-PAGE. The protein concentrations were determined by OD₂₈₀ in the presence of 6 M GdCl₃, with the extinction coefficient ϵ_{280} calculated from the amino acid composition according to Gill and von Hippel (1989): $\epsilon_{280} = 5690 \times W + 1280 \times Y + 120 \times C$, where W is the number of Trp, Y is the number of Tyr, and C is the number of Cys.

CD spectroscopy and secondary structure prediction

Far-UV CD measurements were performed using a JASCO J-720 spectropolarimeter at room temperature with a 0.1-cm path length cuvette. The protein samples were 0.1 mg/mL in 10 mM glycine, 1 mM EDTA (pH 9.6). The spectra were measured at 0.2 nm resolution and at a scan rate of 100 nm/min. All spectra were averages of at least eight scans. The CD spectra were analyzed with the CDPro package (Sreerama and Woody 2000) for estimation of secondary structure contents.

The summation of secondary structural change, σ , between the Cys–Trp mutant and wild-type MPB domain was calculated according to the following equation:

$$\sigma = 1/4 \times \sum_{j=1}^4 \frac{\Delta Z_j^i}{\Delta Z_j^{\max}} \quad (1)$$

where ΔZ_j^i is the absolute value of the percentile change in one of the four j parameters (α -helix, β -strands, turns, and random structures) for mutant i compared to the wild type, and $\Delta Z_j^{\max}$ is the maximum ΔZ_j^i encountered in the entire set of 31 mutants.

Predicted secondary structural elements along the entire polypeptide chain of the GABA_A receptor α_1 subunit were described earlier (Xue et al. 1999). For the Ala-scanned segment P154–L219, these elements, obtained by combined methods of PHD

(Rost and Sander 1993), PROF (Rost et al. 1996), and SOMPA (Geourjon and Deleage 1995) are shown in Figure 1A.

FA titrations and data analysis

Fluorescence measurements were carried out at room temperature using a Perkin-Elmer model LS50B luminescence spectrometer. Steady-state anisotropy data (A) were calculated based on the following equation:

$$A = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (2)$$

where G is the monochromator grating correction factor given by $G = I_{HV}/I_{HH}$, I_{VV} , and I_{VH} , the parallel and perpendicular polarized fluorescence intensities measured with vertically polarized excitation light; and I_{HV} and I_{HH} , the perpendicular and parallel fluorescence intensities measured with horizontally polarized excitation light (Gidwani et al. 2001). The fraction of fluorophore bound (F_B) was determined from anisotropy (A) and quantum yield ratio (Q) using the equation:

$$F_B = \frac{A - A_{\text{free}}}{(A_{\text{bound}} - A)Q + (A - A_{\text{free}})} \quad (3)$$

where A_{free} is the anisotropy of the free fluorophore, and A_{bound} , the anisotropy of the bound fluorophore. Q is given by the ratio of the intensities between bound and free fluorophore. In the saturation experiments, 0.165 μ M BFR (Molecular Probes, Inc.) in 10 mM glycine (pH 9.6) was titrated with protein. The anisotropy was measured at excitation wavelength 490 nm and emission wavelength 511 nm, both with a 5-nm slit. The dissociation constant K_d was estimated by nonlinear least-squares fitting of the saturation curve to a single-site binding model (van den Elsen et al. 1997).

Thermodynamic analysis

The enthalpy change ΔH describes the temperature dependence of the association equilibrium constant, K_a , based on the linear van't Hoff relationship:

$$\ln K_a = -\Delta H/RT + \Delta S/R \quad (4)$$

where R is the gas constant, and T , the absolute temperature (Horn et al. 2001). The heat capacity change ΔC_p was assumed to be insignificant. K_a is the reciprocal of the dissociation constant K_d , obtained from FA measurements at 285.0, 291.2, 298.0, 303.4, and 308.3 K. The binding enthalpy change ΔH and entropy change ΔS were obtained from the slope and the intercept, respectively, of the $\ln K_a$ versus $1/T$ plot (Maksay 1994).

FRET measurements

The efficiency E of FRET, defined as the fraction of donor excitation events that result in the excitation of the acceptor, is a function of the distance R between the donor and acceptor fluorophores, according to:

$$E = \frac{1}{1 + \left(\frac{R}{R_0}\right)^6} \quad (5)$$

Since R_0 , the Forster distance at which E is 0.5, is typically in the nanometer range, FRET provides a reliable indicator for proximity of donor and acceptor over this distance range. The value of R_0 (Å) could be calculated from the equation:

$$R_0 = [8.79 \times 10^{23} \times (n^{-4} \phi_d \kappa^2 J)]^{1/6} \quad (6)$$

where n is the refractive index of the intervening medium, ϕ_d is the fluorescence quantum yield of the donor in the absence of acceptor, κ^2 is the dipole orientation factor, and J (cm³/M) is the spectral overlap integral (Robert 1996). For the estimation of R_0 between the MPB domain and BFR, J was determined from the emission spectrum of the protein in the absence of BFR and the absorption spectrum of BFR. This yielded the J value of 9.41×10^{-15} cm³/M for the wild-type protein, and of 9.43×10^{-15} – 9.50×10^{-15} cm³/M for the Trp-substituted mutants. The quantum yield of the protein ϕ_{MPB} was recorded by a comparative method (Williams et al. 1983) relative to that of free L-Trp in water, with $\phi_{\text{L-Trp}}$ reported to be 0.14 (pH 7.2), 25°C (Kirby and Steiner 1970). ϕ_{MPB} was calculated to be 0.119 for the wild-type protein, and of 0.109–0.113 for the Trp-substituted mutants. The refractive index of the medium was 1.345 measured by a refractometer. κ^2 was assumed to be 2/3, a statistical average for randomly oriented donors and acceptors.

For FRET measurements, protein solutions were mixed with 0.248 μM BFR in the cuvette, and 450–550 nm emission spectra were obtained at both 280 and 340 nm excitation wavelengths with 5-nm slit in a Perkin-Elmer model LS50B luminescence spectrometer. Light at 280 nm excited both BFR and protein intrinsic fluorophores, whereas light at 340 nm excited only BFR. The two sets of emission spectra were recorded in parallel at the two excitation wavelengths. The ratio of maximal fluorescence intensities (INT280/INT340) was used as an index of FRET to correct for any errors in BFR concentration and inner filter effects caused by different protein concentrations (Hang et al. 2000).

ELISA and Western blotting

The mAbs 3E2 and 4F10 against the MPB domain were produced by Berkeley Antibody Company and tested for specificity by Western blot analysis. Bovine serum albumin was purchased from Sigma-Aldrich. Proteins (20 μg) were loaded on 15% SDS-PAGE gel. Following electrophoresis, they were blotted onto PVDF membrane and blocked by 5% skimmed milk in PBS for 1 h before probing with the mAb (1.0 μg/mL) and sheep-anti-mouse peroxidase-conjugated secondary antibody (diluted 1:4000; Amersham Biosciences Ltd.). Protein bands were visualized with enhanced chemiluminescence (Pierce). For epitope mapping, indirect ELISA was performed for the wild type and Ala-substituted MPB domain. For this purpose, microtiter plates were coated with 50 μL proteins, 1 μM in carbonate coating buffer (pH 9.6). The wells were then blocked by 5% skimmed milk. After three washes, 50 μL each of the mAbs (1.0 μg/mL) were added to the wells and incubated at 37°C for 1 h. Following washing and incubation with peroxidase-conjugated secondary antibody for 1 h, the plates were washed and incubated with 3,3',5,5'-tetramethylbenzidine/H₂O₂ (Zymed), a substrate of horseradish peroxidase. Reaction was stopped by the addition of 2 M H₂SO₄, and absorbance was read at 450 nm by an ELISA reader. Assays were carried out in triplicate and repeated twice.

Protein denaturation studies

Proteins were denatured by titration with GdCl₃ in the presence of 2 mM DTT as previously described (Shi et al. 2002). Denaturation was monitored either by means of fluorescence emission wavelength determination upon excitation at 295 nm, or by CD molar ellipticity at 222 nm. For normalization, the fraction of unfolding f_U was calculated using the equation:

$$f_U = (X - X_N)/(X_U - X_N) \quad (7)$$

where X is the measured wavelength or ellipticity variable, and X_N and X_U , its values under the native and fully denatured conditions, respectively.

$\Delta(\Delta G)$, the difference in free energy of denaturation between mutant and wild type, was estimated from the denaturation curves as described (Pace 1990) based on the equation:

$$\Delta(\Delta G) = [D]_{0.5M} \times L_M - [D]_{0.5W} \times L_W \quad (8)$$

where $[D]_{0.5}$ is the GdCl₃ concentration at the midpoint in the denaturation curve; the subscripts M and W denote mutant and wild type, respectively; and L is the slope of plot of ΔG versus $[D]$, according to $\Delta G = \Delta G^0 - L \times [D]$.

Stopped-flow fluorescence spectroscopy

GdCl₃-dependent unfolding rates were measured by fluorescence stopped-flow spectroscopy as described (Lorch et al. 1999). All stopped-flow fluorescence measurements were performed at 25 ± 0.1 °C using an Aviv Model 202 Spectrometer with a stopped-flow attachment (Aviv Instruments). Protein solution was 50 μM, and denaturant GdCl₃ solution was 8 M. The two solutions were mixed at varying ratios to give different final GdCl₃ concentrations. The change in fluorescence intensity upon mixing was monitored at 1-msec intervals. The unfolding rates (k_u) were calculated by fitting the decay curves to the equation:

$$I = I_a \exp(-k_u t) + I_f \quad (9)$$

where I_a is the fluorescence amplitude of the reaction, and I_f , the final intensity.

Protein fold prediction

The 20 best-fitting protein 3D structures were identified by TOPITS (Rost 1995), or the 15 best-fitting ones by UCLA-DOE (Fischer and Eisenberg 1996) in terms of similarity to overlapping 120-residue fragments of the GABA_A receptor α_1 subunit with a scanning interval of 10 residues, as described (Xue et al. 1999), and subgrouped into structure folds according to SCOP classification (Hubbard et al. 1999).

In addition, C139–L269 of the GABA_A receptor α_1 subunit, together with five homologous sequences of the GABA_A receptor α_6 , β_4 , and δ subunits and glycine receptor α_3 and β subunits, were threaded against 3098 structural domains of 2382 protein structures from the PDB, using the fold-recognition program ProFIT from the ProCERYON package (Domingues et al. 1999). The program was set to automati-

cally retrieve information on each structure template from the SCOP database, and rank the different fold types defined by SCOP. The median rank for the top-listed fold types was determined based on the results from the six homologs.

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