Topological Analysis of the Peripheral Benzodiazepine Receptor in Yeast Mitochondrial Membranes Supports a Five-transmembrane Structure*

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The peripheral benzodiazepine receptor, implicated in the transport of cholesterol from the outer to the inner mitochondrial membrane, is predicted by hydrophathy analysis to feature five membrane-spanning domains, with the amino terminus within the mitochondrial periplasm and the carboxyl terminus in the external cytoplasm. We have tested these structural predictions directly by immunodetection of c-Myc-tagged peripheral benzodiazepine receptor on intact yeast mitochondria and by specific labeling in yeast membranes of cysteine residues introduced by site-directed mutagenesis. The combined results support the model originally proposed with some minor but important modifications. The theoretical model predicted relatively short α-helical domains, only long enough to span a phospholipid monolayer, whereas the results presented here would support a model with extended α-helices sufficiently long to span an entire membrane bilayer, with concomitant shorter loop and tail regions.

The peripheral (or mitochondrial) benzodiazepine receptor (PBR)† possesses a benzodiazepine binding site that is clearly distinct from the modulator site of the neurotransmitter γ-amino-nobutyric acid receptor. PBR is present in most, if not all, tissues and is particularly abundant in the outer membrane of mitochondria. PBR has been suggested to be required for the transport of cholesterol from the outer to the inner mitochondrial membrane where steroid biosynthesis takes place (1). The receptor also appears to play a key role in modulating mitochondrial electrophysiology, which suggests its implication in other published investigations, we propose a model in which the cytochrome c terminal oxidase complex of Escherichia coli (14). A three-dimensional model for PBR was proposed and studied using molecular dynamics simulations (12). It was concluded from this model that the α-helices were too short to cross an entire bilayer membrane but corresponded approximately to one phospholipid layer. The work described here was aimed at determining whether the PBR model was correct or needed to be refined.

In view of the paucity of crystallographic data for membrane proteins several techniques have been developed to investigate their topology. Each of these techniques relies on the localization of the hydrophilic loop and tail regions by proteolytic degradation, by antibiotic selection, or by various labeling strategies. In the present report, we describe two quite distinct labeling approaches we have adopted to investigate the PBR model. The first made use of c-Myc epitope insertions with subsequent immunodetection; in the various loop regions the epitope was flanked by pentaglycine to improve antibody accessibility, an innovation used successfully for topological studies of bacteriorhodopsin (15). In the second method, designed to obtain a more refined topological analysis, we mutated the PBR to introduce cysteine residues into various regions that were subsequently visualized by sulfhydryl labeling techniques (16). From the results obtained we were indeed able to confirm the pentahelical PBR structure; but by taking into consideration other published investigations, we propose a model in which the transmembrane helices are longer than those previously thought to exist.

EXPERIMENTAL PROCEDURES

Construction of the Expression Plasmids in S. cerevisiae—Expression plasmids in yeast were all derived from PEM971 (7). The yeast strains were C13 ABYS86 (17 Mat a, leu2-3, leu2-112, ura3-Δ5, his3, prb1-1, prbl-1, prcl-1, cps1-1, Cir). EL301 (W303 vdac1::URA3) and EL105 (MATa, aux1::LEU2, aux2::HIS3, aux3::URA3, his3-Δ3, his3-15, trp1-1, ura3-1, can1-100, ade2-1, leu2-3, leu2-112) are, respectively, vdc and aux mutants described in Ref. 24. Yeast cultures, transformation, and mitochondrial preparation were as described previously (7, 18).

Construction of c-Myc-tagged PBR—Fusions of the human PBR with the c-Myc epitope were carried out by the overlap extension polymerase chain reaction method (19) using the hot-start procedure. Taking c-Myc c1 as a typical example, a sense primer situated on the expression
vector pEMR971 upstream from the PBR initiation codon, 5'-AAAA-TAGCTCCAGTACCAG (400 ng) and antisense primer 5'-CCTTC-TCCGAGACGCTCTCACTGCAGGCTGCGGCG (400 ng) (the underlined nucleotides are part of the c-myc epitope, loweercase nucleotides are those of PBR) in 50 mM of Tris-HCl, pH 8.3, containing 0.2% NP-40, 0.067% SDS, 5% glycerol, 0.1% β-mercaptoethanol, and 0.2 mM of dNTP (1 μl of 5 mM, 50 μM final), were capped with an AmpliWax bead (Perkin-Elmer) and heated at 70 °C for 5 min followed by 5 min at 20 °C. A solution (50 μl) containing pEMR971 (100 ng), 10 mM Pfu buffer (Stratagene, 25 μM) was added, and the mixture was heated at 95 °C for 2 min and then cooled to 50 °C for 1 min. Amplification was carried out for 10 cycles of 20 s at 94 °C, 20 s at 60 °C, and 2 min at 72 °C using a Tecne 1 thermocycler. Exactly the same procedure was carried out with an antisense primer downstream from the PBR stop codon in pEMR971 5'-TACGTCAGGC- GATTCCTGGT (400 ng) and 5'-AATGCTACCACTCCAGGACGTCC- GGCGGCGTGTGAGGGGCTGCCGATGG (400 ng) (underlined and lowercase nucleotides as described above). The two amplified products were purified on 2% agarose, extracted from the gel using a Sephaglass Bandprep purification kit (Pharmacia Biotech Inc.), and approximately equal quantities of amplicons were fused using the same hot-start procedure with Pfu polymerase and five cycles of the same cycle conditions. Finally, the sense and antisense primers described above (400 ng of each) were added, and 15 cycles using the above cycle conditions were carried out. The c-myc-PBR-My-c fusion digested with NotI and BamHI and cloned into the corresponding site in pEMR971. The amino- and carboxyl-terminal c-Myc fusions lacking the pentaglycine flanks were obtained by the insertion of double-stranded synthetic oligonucleotides into appropriate restriction sites. The sequences of these and subsequent constructs were verified by the dideoxynucleotide chain termination method.

**Binding Studies with [3H]PK11195 and [3H]Ro5-4864**—Equilibrium binding studies were carried out at 0 °C in 50 mM Tris-HCl, pH 7.4. Saturation experiments were done as follows. Each incubation mixture (0.3 ml) in triplicate contained a 0.1-ml suspension of yeast mitochondria and the appropriate amounts of [3H]PK11195 (85 Ci/mmol, NEN Life Science Products) or [3H]Ro5-4864 (84 Ci/mmol, NEN Life Science Products) in the absence (total binding) and in the presence (nonspecific binding) of 100 mM 5FM (Interchim) and were incubated at 4 °C for 20 h. The supernatants containing the unbound antibodies were titrated by ELISA. Microtiter plates (ICN) were coated with sonicated mitochondria (Bioblock 72442, power 40, duty cycle 50%, 2 min) and incubated with anti-c-Myc 9C10 monoclonal mouse antibodies using Western immunoblotting and by binding experiments (Fig. 2). The topological model of the PBR, as proposed previously by Bernassau et al. (12) on the basis of the amino acid sequence, is shown in Fig. 1. To study the model we first adopted a c-Myc epitope-scanning procedure. At the cDNA level, the 10 amino acid c-Myc epitope was inserted into the various loops of PBR between hydrophobic amino acids (Fig. 1) to give four constructions denoted c-Myc c1, c2, m1, and m2 (c and m denoting a cytoplasmic and mitochondrial polypeptide, without the flanking glycines). After transfection into yeast we first ensured that expression had occurred and that the PBR had translocated into the yeast mitochondrial membranes in a correctly folded form. This was done by testing the ability of the membrane to be recognized by anti-c-Myc antibodies using Western immunoblotting and by binding experiments.

**RESULTS**

The c-Myc Epitope Scanning Procedure—The topological model of the PBR, as proposed previously by Bernassau et al. (12) on the basis of the amino acid sequence, is shown in Fig. 1. To study the model we first adopted a c-Myc epitope-scanning procedure. At the cDNA level, the 10 amino acid c-Myc epitope was inserted into the various loops of PBR between hydrophobic amino acids (Fig. 1) to give four constructions denoted c-Myc c1, c2, m1, and m2 (c and m denoting a cytoplasmic and mitochondrial location, respectively). To make the epitope more accessible to antibodies it was flanked at each end by 5 glycine residues (15). We also placed the c-Myc epitope either at the amino terminus or carboxyl terminus of the polypeptide, without the flanking glycines. After transfection into yeast we first ensured that expression had occurred and that the PBR had translocated into the yeast mitochondrial membranes in a correctly folded form. This was done by testing the ability of the membrane to be recognized by anti-c-Myc antibodies using Western immunoblotting and by binding experiments. All of the constructs but one could be detected (Fig. 2). The c-Myc m2 fusion could not be detected, and subsequent Western analysis of the cytoplasmic fraction (not shown) revealed a total absence of expression of this construct. The different intensities observed in Fig. 2 reflect construct expression levels because an anti-PBR antibody (5) gave similar results (data not shown). Nearly all of the tagged receptors bound [3H]PK11195 with an affinity similar to that of the wild type PBR, c-Myc cl binding being a little attenuated. In contrast, the [3H]Ro5-4864 binding varied, the lowest affinity being as-
associated with the amino-terminal-tagged receptor. The addition of an alternative, hemagglutinin-derived epitope at the amino terminus or inserted into the m2 region gave results similar to those with the c-Myc fusions (data not shown).

Having ensured that the modified PBR was essentially similar to the wild type PBR, we next looked at the location of the epitope using the reverse ELISA technique. Either predominantly intact or fully lysed mitochondria were mixed at various concentrations with a known amount of anti-c-Myc antibodies. Mitochondria-bound antibodies were pelleted, and free antibodies were assayed in the supernatants by ELISA. As a control we used antibodies against an integral inner membrane protein, F1-ATPase. The results presented in Fig. 3 clearly show that intact mitochondria-dependent antibody depletion was obtained with c-Myc c1, c-Myc c2, and c-Myc at the carboxyl terminus, but not with c-Myc at the amino terminus or c-Myc m1. Antibodies raised against F1-ATPase were more depleted with lysed than with intact mitochondria, as would be expected; the incomplete depletion may be attributable to a lack of efficiency of the antibodies. In line with initial experiments, the depletion of antibodies from the supernatant was less efficient with the amino-terminal c-Myc construct than with the others. However, the experiments tended to support the model schematized in Fig. 1. Unfortunately, the lack of expression of the c-Myc m2 construct meant that the putative m2 loop could not be verified by this procedure. Furthermore, the c-Myc insertion technique could obviously not be used to verify amino acids thought to form transmembrane regions. Therefore, in subsequent experiments we adopted a chemical labeling method.

Localization of Cysteine Residues in Mutated bPBR and bPBR-hPBR Sandwich Hybrids Using 5FM and BM—The fluorophore 5FM (16) and BM (23) react with Cys residues in polar and nonpolar environments, respectively. 5FM will also react with Cys in the presence of detergent, which allows it to penetrate into lipophilic regions (16). Subsequent detection of fluorescence indicates whether the Cys is situated in a polar loop region or within a lipidic membrane region. We used the bPBR and a bPBR-hPBR-bPBR sandwich hybrid for these experiments because in previous work we found that the recombinant bPBR was expressed more than the hPBR and would, therefore, be easier to detect by fluorescence in yeast mitochondria (6, 18). Because the hPBR contains 2 Cys residues at positions 19 and 153 and bPBR contains a unique Cys residue situated in a putative intramitochondrial region at position 135 in m2, a sandwich hybrid PBR containing no Cys was constructed by replacing a bPPR fragment by the corresponding hPBR fragment, the points of fusion being indicated in Fig. 1.

**FIG. 1.** Schematic structure of the original PBR model (12). The primary sequence shown is that of a hybrid bPBR-hPBR comprising bPBR (amino acids 1–41), hPBR (amino acids 42–147), and bPBR (amino acids 148–169) with Val-154 (gray circle) to ensure a Ro5-4864 binding site. The lines indicate the points of fusion. c1 and c2 refer to extramitochondrial cytoplasmic loops, m1 and m2 to intramitochondrial loops, Ct and Nt to the carboxyl- and amino-terminal domains, respectively. The positions of the c-Myc epitope (ep) insertions are shown; G5 means 5 glycine residues. Also noted are positions of the cysteine insertion (C3) and replacements (filled circles). Double circles correspond to acidic amino acids and bold circles to basic amino acids.

**FIG. 2.** Detection and binding affinities of the c-Myc-tagged PBRs. Mitochondrial proteins (30 μg) were separated by 15% SDS-polyacrylamide gel electrophoresis. After electrophoresis the proteins were transferred to nitrocellulose, and the c-Myc-tagged proteins were detected by immunoblotting as described under "Experimental Procedures." The c-Myc positions above the immunoblot refer to those in Fig. 1. 0 corresponds to the wild type PBR without c-Myc. ND is not detected. Binding assays with PK11195 and Ro5-4864 were done as described under "Experimental Procedures." *K*<sub>d</sub> is expressed in nM and B<sub>max</sub> in pmol/mg of protein.
This was used as a template for introducing Cys into various regions of the receptor (Fig. 1 and Table I). In addition, all of the receptors contained a valine at position 154 (bPBR(Val-154)) to ensure Ro5-4864 binding (6). Each of the constructs described, except the Cys-61 mutant, bound both radiolabeled PK11195 and Ro5-4864 to the same extent as the wild type PBR (data not shown), indicating that they were inserted correctly into the mitochondrial membrane.

Using intact or lysed mitochondria, the bPBR(Val-154) and the various mutants were mixed with 5FM and allowed to react for 4 min at 0 °C as described (16). Labeling was monitored by measuring the fluorescence associated with 18-kDa protein bands separated by SDS-polyacrylamide gel electrophoresis (Fig. 4). As expected, no 18-kDa band from intact mitochondria bearing PBR devoid of Cys residues was labeled (no Cys in Fig. 4, B and G). In contrast, the Cys-41 bPBR mutant (Fig. 4, A, B, and G) could be detected in intact mitochondria as early as 1 min after the addition of the reagent (Fig. 4A). These results indicated the specificity of the reagent for the PBR cysteines, at the same time showing the ready accessibility of Cys-41. Panel A also shows that the Cys-3 insertion mutant was not labeled within 4 min, but it was labeled rapidly in broken mitochondria after 4 min (Fig. 4C). In intact mitochondria, however, some labeling of this mutant took place after 10 min (data not shown). These results indicate that the reagent could penetrate through intact mitochondria after a certain time and also that the Cys-3 insertion mutant was clearly in an intracellular hydrophilic environment. Other mitochondrial proteins were also labeled, in particular abundant proteins of about 30 kDa. Among the latter were the voltage-dependent anion channel and the adenine nucleotide carrier because these bands disappeared from the yeast mutants aac<sup>-</sup> and vdac<sup>-</sup> (24) lacking the genes encoding these proteins (Fig. 4O). Interestingly, the VDAC protein was labeled in intact mitochondria (i), and the AAC protein could only be labeled in broken mitochondria (b and bt), which reflects the fact that AAC is an inner membrane protein.

Importantly, bPBR(Val-154) with its unique Cys at position 135 failed to react with 5FM in either intact or broken mitochondria under the conditions used for labeling the other Cys and could only be labeled when the mitochondria were broken in the presence of Triton X-100 (Fig. 4L). These results show that Cys-135 in the bPBR is apparently situated in a transmembrane region rather than in m2, but the bPBR may be an exception since the PBR of other species have an Arg at this position (Fig. 1). The Cys-135 mutant was also labeled by BM in intact mitochondria (data not shown). The neighboring, fully conserved Ser in position 130 is clearly in an accessible internal loop because Cys-130 could be labeled with 5FM in broken mitochondria (Fig. 4K).

Subsequent experiments aimed at firmly establishing transmembrane and loop domains used the bovine-human-bovine sandwich receptor into which a single Cys was substituted. The results are shown in Figs. 4 and 5 and summarized in Table I for the 5FM labeling. The Cys residues that were labeled rap-
idly by 5FM in intact mitochondria were at positions 26, 41, 102, 106, and 168 (Fig. 4, F, G, J, and N), indicating that their cytoplasmic location was compatible with the model. In contrast, Cys-75 and Cys-130 were only labeled in broken mitochondria (Fig. 4, I and K), indicating a polar, intramitochondrial location.

The result with the Cys-3 insert led us to make two further mutants near the amino terminus. Cys-12 and Cys-19 were also partly labeled with BM in intact mitochondria (Fig. 5), suggesting a lipophilic environment. In the first set of experiments we inserted c-Myc epitopes into the putative outer and inner loop regions of PBR. Antibodies raised against c-Myc were used as membrane-impenetrable reagents that will chemically modify only exposed residues at specific sites in the protein and, conversely, lipophilic reagents that only react with residues in a membrane environment. In the first set of experiments we inserted c-Myc epitopes into the putative outer and inner loop regions of PBR. Antibodies raised against c-Myc were used as membrane-impenetrable labeling reagents. Various c-Myc-tagged PBR were translocated into the mitochondria of recombinant yeast cells, in a correctly folded form according to binding experiments with an antibody raised against a synthetic peptide from this region of the PBR (25). Although we previously presented PBR models with five transmembrane regions (6, 12), we did not exclude other possible structures, notably one in which both the amino- and carboxyl-terminal regions were located in the cytoplasm (6). The results presented here support the former model.

To establish membrane protein topology various techniques are employed, notably the production of chimeras incorporating a foreign reporter in putative loop regions to establish their location with respect to the membrane and the use of membrane-impenetrable reagents that will chemically modify only exposed residues at specific sites in the protein and, conversely, lipophilic reagents that only react with residues in a membrane environment. In the first set of experiments we inserted c-Myc epitopes into the putative outer and inner loop regions of PBR. Antibodies raised against c-Myc were used as membrane-impenetrable labeling reagents. Various c-Myc-tagged PBR were translocated into the mitochondria of recombinant yeast cells, in a correctly folded form according to binding experiments with PK11195 and Ro5-4864. After the localization of the epitope in intact or permeabilized mitochondria, it was clear that the m2 loop could not be detected by this method, and Western blot analysis indicated the absence of this fused receptor in the mitochondria.

We subsequently employed cysteine labeling techniques with the polar 5FM and the apolar BM reagents, techniques that can only be used if the target protein is produced at a high level. For this reason, we turned to bPBR and a bPBR-hPBR hybrid, haps attributable to a distortion of the binding sites. The Cys-153 bPBR mutant was also in a lipophilic environment since it was labeled with 5FM only in broken mitochondria in the presence of Triton (Fig. 4M) and in intact mitochondria with BM (data not shown). This last result is interesting inasmuch as the mutation adjoins the valine at position 154 previously shown (6) to be directly implicated in Ro5-4864 binding. Val-154 would appear to be at the interface of the fifth transmembrane region and cytoplasm.

**DISCUSSION**

Hydropathic analysis of the 169 amino acids that constitute the rat (8), bovine (10), human (18), and mouse (9) PBR reveals the presence of five hydrophobic regions in each of the receptors. Alignment of the sequences (6) clearly shows either identical amino acids in these putative transmembrane regions or conservative replacements. The recently described outer membrane sensory protein of the proteobacterium *R. sphaeroides* has also been shown recently (3) to have a close structural and functional relationship with the PBR. Therefore, it is a reasonable assumption that the topology of these receptors is identical. There are now several lines of evidence that support the general model proposed by Bernassau et al. (12), according to which the amino terminus of the PBR points toward the interior of the mitochondrial outer membrane, and five transmembrane regions lead to a highly charged carboxyl terminus exposed to the cell cytoplasm. From extensive site-directed mutagenesis modifications of hPBR and bPBR followed by expression in yeast and binding experiments in intact yeast mitochondria, we recently showed (6) that Glu-29, Arg-32, and Lys-39 are in a loop in the cell cytoplasm, c1 (Fig. 1) and that they somehow affect Ro5-4864 binding. Furthermore, we postulated that the carboxyl-terminal region was also in the cytoplasm, perhaps close to c1 (6). More direct evidence for the cytoplasmic location of the carboxyl terminus has come from experiments with an antibody raised against a synthetic peptide from this region of the PBR (25). Although we previously presented PBR models with five transmembrane regions (6, 12), we did not exclude other possible structures, notably one in which both the amino- and carboxyl-terminal regions were located in the cytoplasm (6). The results presented here support the former model.

We subsequently employed cysteine labeling techniques with the polar 5FM and the apolar BM reagents, techniques that can only be used if the target protein is produced at a high level. For this reason, we turned to bPBR and a bPBR-hPBR hybrid,
receptors that can be produced in high amounts in yeast mitochondria. After ensuring that a PBR variant lacking Cys in its sequence could not be modified with 5FM or BM, we used this technique to ascertain whether various Cys residues were located in aqueous or apolar environments. Because it has been reported (26) that hydrophilic reagents are capable of crossing lipid membranes, care must be taken in using them to draw conclusions about receptor topography, unless kinetic aspects are considered. We found that 5FM treatment of intact mitochondria labeled all of the Cys residues predicted to be in three of the domains, c1, c2, and carboxyl terminal, within 4 min, clearly establishing an extramitochondrial, cytoplasmic localization of these regions. This period was therefore chosen for the 5FM experiments because a longer treatment could label intramitochondrial residues. However, the latter residues also reacted within 4 min after we lysed the mitochondria. Significantly, the Cys-135 residue predicted to be in the m2 loop was labeled by 5FM only in the presence of Triton X-100. This result showed this residue to be localized not in an accessible loop region but inside the fifth transmembrane helix. However, the other species contain an Arg at this position, suggesting that the Cys-135 residue lies near the hydrophilic face of the phospholipid and also indicating some flexibility regarding the extent to which the hydrophobic domains are embedded in the membrane. In contrast, residue 130 is clearly situated in the m2 loop region.

In the original model (Fig. 1) it was proposed that around 18 amino acids formed each of the five transmembrane regions and that the PBR reached only halfway between the outer and inner surfaces of the outer mitochondrial membrane (12). The Cys labeling results with 5FM would indicate, however, that the intramitochondrial residues are in a hydrophilic environment and, therefore, that the transmembrane regions are longer than hydrophobicity analysis would suggest. The exact
beginning and end of transmembrane regions are notoriously difficult to establish, as was pointed out recently in an electron-cryoscopic refinement of bacteriorhodopsin at 3.5-Å resolution (27). However, several theoretical methods to predict such regions exist. It has been postulated (11) that Arg/Lys patches occur at the cytoplasmic ends of receptors present in plasma membranes, their positive charges serving to anchor the receptor to the negatively charged phospholipids. No Arg/Lys patches occur in the PBR of the four species known at present, and it is uncertain whether such anchors exist. However, an alignment of the sequences (6) reveals several perfectly conserved Arg or Lys at positions 32, 39, 69, 103, and 166. In addition, by accepting His in the analysis, positions 27, 46, 46, and 162 can be added. All but two of these positive charges (69 and 76/77) are found in or near the putative extramitochondrial regions or in the cytoplasm (Fig. 1). Our results show that residue 26, just before the positively charged residue 27, is clearly in the cytoplasm. Arg-103 in c2 also appears to be far from the membrane, the nearby positions 102 and 106 being shown here to be in the cytoplasm. Finally, we found Cys-153 to be located in the fifth transmembrane region of the hPBR, but the bPBR has an Arg at this position which, as in the case of Cys-135 discussed earlier, would again suggest that the residue is at the membrane surface. The adjacent Val-154, important for Ro5-4864 binding, is also near the membrane-cytoplasm interface.

It is interesting to note that all four PBR sequences presently available exhibit fully conserved glycines, a residue often involved in structures that terminate α-helices (28), near the proposed carboxyl ends of each of the first three putative transmembrane regions; but it must be clearly stated that no evidence exists to show that the transmembrane regions have an α-helical structure. Finally, several of the structural features discussed here are to be seen in the R. sphaeroides protein described by Yeliseev et al. (3).

In conclusion, we have obtained experimental data that strongly support the theoretical topographical PBR model shown in Fig. 1 (6, 12), but the data lead us to suggest that some refinements should be made to the model. In particular, because the putative intramitochondrial loops are accessible to hydrophilic reagents, the transmembrane domains may be longer by 3–4 residues thereby completely traversing the outer mitochondrial membrane.

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