The peripheral type benzodiazepine receptor (PBR) binds benzodiazepines such as RO5-4864 and isoquinoline carboxamide derivatives such as PK11195. This receptor includes an $M_r$ 18,000 isoquinoline-binding subunit predominantly located in mitochondrial membranes. This protein has been found to copurify with two other mitochondrial proteins, namely the outer membrane voltage-dependent anion channel (VDAC), also known as mitochondrial porin, and the inner membrane adenine nucleotide carrier. In vitro reconstitution experiments suggested that the PBR was a multimeric complex in which the isoquinoline binding site was on the $M_r$ 18,000 subunit, denoted pk18, whereas the benzodiazepine binding site required the association of this subunit with VDAC to be expressed. Untransformed cells of the yeast Saccharomyces cerevisiae are devoid of specific binding sites for isoquinolines and benzodiazepines, whereas yeast cells transformed with a pk18-expressing vector exhibit RO5-4864 and PK11195 binding sites that are pharmacologically identical to those of the PBR. To clarify the role of VDAC and of the adenine nucleotide carrier, if any, in the constitution of the benzodiazepine binding site, yeast host strains were constructed in which the corresponding genes had been knocked out. Mitochondria prepared from pk18-producing cells devoid of either VDAC or adenine nucleotide carrier exhibit both benzodiazepine and isoquinoline carboxamide binding sites with little or no change in the $K_d$ values as compared with the wild-type background. These results rule out the contention that VDAC is dispensable for establishing the benzodiazepine binding site and are in agreement with the hypothesis that the $M_r$ 18,000 subunit carries both the isoquinoline carboxamide and benzodiazepine binding domains.

Benzodiazepine (Bz) binding sites generally fall into two main structurally distinct types. The first is found on the $\gamma$-aminobutyric acid A receptor, the location of which is restricted to the central nervous system. The second is found in peripheral and in central tissues and is widely known as the peripheral-type benzodiazepine receptor (PBR) (1, 2). The PBR, predominantly located in mitochondrial membranes, displays pharmacological characteristics distinct from the central type. Isoquinoline carboxamide (IQ) derivatives like PK11195 that are high affinity ligands of PBR are ineffective in binding to the central type receptor. The subset of Bz molecules that binds to each type of receptor is also markedly different. For instance, RO5-4864 is a Bz that binds to PBR, at least in human and rat (3) and also to the central receptor, but with a much lower affinity. Purification of rat kidney mitochondrial PBR in a form that retained reversible binding properties yielded a protein fraction composed of three polypeptides of $M_r$ 18,000, 30,000, and 32,000. The two latter subunits were identified as the adenine nucleotide carrier (ADC) and the voltage-dependent anion channel (VDAC), respectively (4). Bzs like $[^{3}H]$flunitrazepam and $[^{3}H]$AZN-886 were shown to photolabel the $M_r$ 30,000 and $M_r$ 32,000 polypeptides nonspecifically (5, 6), whereas $[^{3}H]$PK14104, a fluorinated IQ derivative, specifically photolabeled the $M_r$ 18,000 subunit, denoted pk18 (7). It was postulated that the benzodiazepine binding site resided on ADC and/or VDAC, while pk18 carried the IQ binding site (8). Recently, Garnier et al. (9) showed that a purified recombinant construct consisting of pk18 fused to the Escherichia coli maltose-binding protein (MBP) bound the IQ molecule PK11195 but not RO5-4864, a ligand of the Bz family, in an in vitro reconstruction system. RO5-4864 binding sites were restored by adding purified VDAC to the hybrid MBP-pk18 construct, whereas VDAC by itself did not show any Bz or IQ binding. The authors concluded that a functional interaction of pk18 with VDAC was necessary to form the Bz binding site. They suggested that the Bz recognition site was constituted by an association of pk18 and VDAC components or, alternatively, that the site might be situated on either pk18 or VDAC and confirmed with the hypothesis that the $M_r$ 18,000 subunit carries both the isoquinoline carboxamide and benzodiazepine binding domains.
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responding to an M, 30,000 polypeptide which was identified as the yeast VDAC1 gene-encoded mitochondrial porin. By means of a series of deletion and site-directed mutagenesis experiments it was demonstrated that a particular residue of the pk18 sequence, namely Val154, was crucial for the expression of the Bz ligand ROS-4864 binding site (16). A single change M154V mutation was sufficient to confer ROS-4864 binding capability to the bovine version of pk18 which was otherwise unable to bind this ligand (16). These results indicated that recombinant pk18 was not only involved in, but also absolutely required for, Bz binding. In this report, we address the corol-

ary question of whether VDAC, ADC, or both, are necessary for forming the Bz binding site with pk18. This issue can be addressed in S. cerevisiae since, in this microorganism, mutants devoid of functional VDAC or ADC remain able to proliferate under certain conditions. It is therefore possible to knock out the VDAC- and ADC-encoding genes in a pk18-producing strain, and examine the effects, if any, of these genetic alter-

ations on Bz and IQ binding. Two VDAC isoforms exist in yeast. They are synthesized from two distinct genes, namely VDAC1 (17) and VDAC2 (GenBank accession no. P40478). 2 ACD isoforms are encoded by the genes AAC1, AAC2, and AAC3. The AAC2 gene product appears to be the more abundant ADC isoform in cells grown under aerobic conditions (18, 19). In this report, we describe Bz and IQ binding experiments performed on mitochondrial extracts prepared from cells that produce pk18 in the absence of VDAC and ADC.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Yeast Strains, and Media−E. coli strain S17-1 (RP4-2-Tc:Mu-KM: Tn7, pro, hsdR, recA) (20) was used for DNA cloning and trans-kingdom conjugation experiments. E. coli was grown either in Luria Bertani (LB) medium containing 100 μg/ml ampicillin or in 2YT (21).

C3-A5YS86 is a vacular protease-deficient strain of yeast (22). M3 and M22-2 (vac+) are two congenic strains of S. cerevisiae kindly provided by D. M. Forte (17).

S. cerevisiae W30300IV strain (MATa, ade2-1, ura3-1, his3-1, leu2-3, leu2-112, can1-100, cox1-TRP1) is a congenic derivative of W303 that carries a null allele of the cytochrome-oxidase subunit IV-encoding gene COX4. W303 and W303ivox4 are kindly provided by Dr. B. Giardi. W303 derivatives impaired in VDAC were constructed by disrupt-

ing VDAC1 and VDAC2 using URA3 and HIS3, respectively. The general strategy was to express the URA3 or HIS3 gene in a linear plasmid. Two mutants were EL301 (W303 vdac1::URA3), EL302 (W303 vdac2::HIS3) and EL62 (W303 vdac1::URA3, vdac2::HIS3). The disruption of VDAC1 was achieved by replacing a 180-nucleotide internal fragment (BclI-EcoRV) of VDAC1 with URA3. VDAC2 was disrupted by introducing the HIS3 gene within the Mun1 and Stu1 sites. This disruption resulted in the deletion of the entire VDAC2 sequence, except for 280 nucleotides at the 3′ end. Gene dis-

ruptions were confirmed by Southern blotting or polymerase chain reaction amplification analyses (data not shown).

Strain DNY1 (MATa, aac1::LEU2, aac2::HIS3, his3-11, his3-15, trp1-1, ura3-1, can1-100, ade2-1, leu2-3, leu2-112) was a gift from Dr. D. Nelson (23). Strain EL105 derives from DNY1 by disrupting the AAC3 gene. In EL105, the internal BglII-PvuII fragment of AAC3 was replaced by the URA3-encompassing fragment. AAC3 gene disruptions were identified by their poor growth phenotype when placed under anaerobic conditions (18). aac gene disruption was ascertained by genomic DNA analyses using polymerase chain reaction techniques (data not shown).

Yeast strains were grown in complex YP (1% Bacto-yeast extract, 2% Bacto-peptone, DIFCO) media. Carbon and energy sources were generally either 2% glucose or 2% glycerol plus 2% galactose for growth and pk18 biosynthesis induction, respectively. To grow strains impaired in both vdac1 and vdac2 genes, glucose and glycerol were replaced by 2% raffinose. Mutants devoid of ADC were grown in 2% glucose then in 2% galactose for pk18 biosynthesis induction.

Transformation—Yeast mutants were transformed by the lithium acetate method (16) and in vivo DNA transfer by trans-kingdom conjugation between yeast and E. coli cells, a BFR expression cassette including the galactose-responsive TATA box (GRA1) promoter and the PGK terminator (24) and finally URA3 as the selectable marker. pEMR1043 differs from pEMR971 only by the length of the oriT-encompassing fragment which was about 200 bp shorter in the former plasmid. pEMR1130 differs from pEMR1043 only by replacing TRP1 by URA3 as the selectable marker. Plasmid pEMR1179 is a low copy centromeric vector which derives from YEp351 (25). Plasmid pEMR1179 contains the neomycin gene of Tn903 which confers G418 resistance (250–500 μg/ml) and the AAC2 gene expressed under the control of the PGK promoter.

Yeast Transformation—Two methods were used to transform yeast strains. One was by electroporation essentially as described by Becker and Guarente (26) and the other method was by trans-kingdom conjuga-

tion experiments (27) as follows. S17-1 cells were transformed to am-

picillin-resistant LB and harvested in mid-exponen-

tial phase of growth. Cells were then resuspended in 10 mM MgSO4 at a density of 106/ml. Yeast recipient cells were grown overnight in YPG, pelling; 10 ml of 10 mM MgSO4, 1% glucose, 2% raffinose. Mutants were mixed in equivalent amounts, the mix-

ture was then poured onto a filter plate on non-selective YPG-agar medium and left to incubate at 30 °C for 4–16 h. The cells were then resuspended in 10 mM MgSO4 and an aliquot was spread onto selective YNBG medium at 30 °C. Ura− or Trp− exconjugant colonies appeared 3 days later at a frequency of approximately 10−2 per recipient cell.

Receptor Expression and Mitochondria Isolation—Precultures and cultures for PBR expression experiments were done as follows. Precultures were performed in minimal YNBG medium supplemented so as to fulfill the auxotrophic requirements of the strain. Cells were then harvested and resuspended in complex YP medium containing 2% glycerol, 2% galactose, and 1% ethanol. In the cases where mutants did not grow on glycerol-containing medium, these were resuspended by raffinose.

Mitochondria were isolated as already described by Farges et al. (14).

Photoaffinity Labeling of Yeast Mitochondrial Proteins with [3H]PK11195 or [3H]Flunitrazepam—Yeast mitochondrial fractions (100–250 μg of protein) were preincubated with 100 nM [3H]PK11195 (75 Ci/mmol, DuPont NEN) or [3H]Flunitrazepam (80 Ci/mmol, NEN France) in the absence (total binding) or in the presence (nonspecific binding) of 10 μM cold PK11195 or flunitrazepam, respectively. The reaction was performed in the dark in 50 mM Tris/HC1 buffer (pH 7.4) containing 100 mM sucrose in a total volume of 300 μl at 4 °C for 1 h.

The mixtures were placed in 1.7-cm2 cell culture dishes, and samples were exposed to UV light (365 nm) for 15 or 60 min, depending on the photopanning ligand (IQ or Bz, respectively) and analyzed by autoradiographically revealed SDS-polyacrylamide gel electrophoresis. Binding studies of [3H]PK11195 or [3H]Flunitrazepam—Equilibrium binding studies were carried out at 0 °C in 50 mM Tris/HCl buffer (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 10 μM [3H]PK11195 (85 Ci/mmol, DuPont NEN) or [3H]Ro-4865 (84 Ci/mmol, DuPont NEN) in the absence (total binding) and in the presence (nonspecific binding) of 10 μM PK11195 or Ro-4864.

For competition experiments each incubation mixture (0.3 ml) in triplicate contained a 0.1-ml suspension of yeast mitochondria, 20 μM [3H]PK11195 or [3H]Ro-4864, no other addition (total binding) or the unlabeled ligand at the desired concentration or at 10 μM (nonspecific binding). Each sample was incubated for 1 h at 0 °C, and then rapidly filtered through Whatman GF/C filters pretreated with 0.3% polyeth-

ylenimine and washed 3 times with 5 ml of ice-cold Tris/HCl buffer. The filters were then dried and suspended in 5 ml of Beckman Ready Solv. E.P. mixtures for determination of filter bound radioactivity.

RESULTS

Photolabeling of PBR in vac− and aac− Mutants—A photoactivable Bz ligand, namely [3H]Flunitrazepam, was used to label mitochondrial proteinds extracted from wild-type S. cerevisiae cells. Several bands of variable intensities were revealed (Fig. 1). The two major bands apparently corresponded to M, 30,000 and M, 32,000 proteins. The photolabeled M, 30,000 protein was absent from preparations from vac− mutant.
strains and probably corresponded to VDAC. Similarly, the M₄ 32,000 protein was absent from extracts from the adenine nucleotide carrier-defective strains and probably corresponded to the AAC2 gene product. Labeling of both proteins was not inhibited by the addition of excess cold flunitrazepam and could consequently be regarded as nonspecific. Extracts of pEMR1043-transformed cells exhibited an additional band faintly labeled by [³H]flunitrazepam in a reversible manner that apparently corresponded to a M₄ 18,000 polypeptide. This band, likely that of pk18, was also found in pk18-producing vdac cells devoid of the major VDAC, but was hardly detectable in extracts from pk18-producing cells that were devoid of all three AAC gene products. This result confirmed that pk18 was specifically labeled by [³H]flunitrazepam, albeit poorly.

[³H]PK14105 nonspecifically labeled a M₄ 30,000 polypeptide (Fig. 2). This photolabeled protein was not detected in vdac mutant extracts, suggesting that it was yeast VDAC. Gel digestion and sequence analyses of this photolabeled protein confirmed its identity as the VDAC1 gene product (not shown). pk18-Producing cells exhibited a strongly labeled M₄ 18,000 polypeptide. Labeling was inhibited by cold PK14105, as expected. This result confirmed that pk18 was specifically labeled by [³H]PK14105, as already described (5, 13).

Binding of RO5-4864 and PK11195 in Mitochondria-enriched Fractions of VDAC-lacking Cell Extracts—RO5-4864 and PK11-195 binding analyses were performed on mitochondrial extracts from various pk18-expressing yeast strains, some of which were defective in ADC and VDAC (Fig. 3A and B, and Table I). The fact that no VDAC isofrom was present had no influence on the Kᵩ values estimated for both ligands. An approximately two-fold decrease in the maximal binding capacity for both ligands could be observed in the pk18-expressing cells that were devoid of any VDAC gene product, namely EL302 pEMR1130 (Table I), as compared with W303 cells transformed by the same plasmid (Table I). Such a decrease in pk18 expression was confirmed by Western blot experiments (not shown). This might simply reflect a difference in plasmid copy number between the strains, since this parameter can vary from clone to clone in a host cell genotype-independent manner (24, 28).

Binding of RO5-4864 and PK11195 in Mitochondria-enriched Fractions of ADC-lacking Cell Extracts—Strain EL105 pEMR1130 was impaired in the three AAC genes. Scatchard analyses on mitochondria-enriched fractions of extracts from pEMR1130-transformed EL105 cells revealed no dramatic changes in the dissociation constant (Kᵩ) values for [³H]PK11195 and [³H]RO5-4864 (Fig. 3, A and B, and Table I) as compared with an ADC-producing strain. A slight decrease in RO5-4864 affinity could be suspected in cells devoid of the three isoforms, this decrease could, at least partially, be corrected by expressing the AAC2 gene. Thus, even though yeast ADC was clearly not required to form the binding sites of the Bz ligand.
Absence of Cytochrome Oxidase Subunit IV—Similar experiments were performed using a strain that was devoid of any cytochrome oxidase subunit IV, a mitochondrial polypeptide supposedly not linked to PBR. We checked whether the disruption of the COX4 gene that encoded this cytochrome oxidase subunit could affect the affinity of PBR for both types of ligand. As shown on Table I, mitochondria extracted from a pk18-producing cox4 gene disruptant exhibited PK11195 and RO5-4864 binding with normal \( K_D \) values, as expected.

**DISCUSSION**

Photolabeling of PBR using \([\text{H}]\)PK14105 has revealed a polypeptide of \( M_r \) 18,000 in a variety of mammalian cells, as well as in recombinant pk18-expressing yeast cells. Transfection experiments with pk18-encoding cDNA in mammalian cell lines result in increased numbers of both IQ and Bz binding sites. Similarly, pk18-expressing vectors confer both IQ and Bz binding capability to yeast transformants (13). These expression experiments strongly suggest that pk18 by itself contains both the IQ and Bz binding domains. However, there have been several lines of evidence that suggest the association of pk18 with other proteins, including VDAC and ADC. First, mammalian proteins of \( M_r \) similar to VDAC and ADC could be photolabeled with \([\text{H}]\)PK14105 and various photoactivable Bz ligands (1, 5). However, photolabeling appears to be nonspecific. Second, VDAC and ADC were observed to copurify with pk18 (4) and a VDAC-like protein was shown to be associated to the \( \gamma \)-aminobutyric acid A receptor when copurified on a benzodiazepine affinity column (29). These last results suggested a relationship between VDAC and Bz receptors. The fact that VDAC could be labeled by flunitrazepam and that specific reagents that inhibit VDAC function were able to inhibit Bz ligand binding suggested that the Bz binding domain of PBR was on VDAC (8). Further evidence suggesting a role of VDAC in the formation of the benzodiazepine binding site of PBR came from studies using a recombinant chimeric protein consisting of the maltose-binding protein fused to pk18 (MBP-pk18). Purified MBP-pk18 failed to exhibit any RO5-4864 binding sites in a liposome-based reconstitution system, whereas the addition of VDAC restored binding. It is worth noting that purified VDAC by itself did not exhibit any IQ or Bz binding sites, which suggested that Bz binding required the interaction of MBP-pk18 with VDAC in this reconstitution system. In yeast, as already found in mammals, both VDAC and ADC are nonspecifically labeled by irreversible Bzs. However, these proteins are labeled even in wild-type cells which lack pk18 and which do not express any specific IQ and Bz binding sites. It is therefore probable that photolabeling of VDAC and AAC by Bz ligands is irrelevant to PBR.

Recombinant bakers' yeast constitutes an attractive model for elucidating whether VDAC, ADC, or other proteins are indeed required to form the Bz binding site of PBR in addition to pk18. Yeast wild-type cells contain various mitochondrial isoforms of VDAC and ADC but are devoid of pk18 and do not present any specific IQ and Bz binding sites. pk18 can be efficiently produced in yeast cells (13, 15), where it is targeted to mitochondrial membranes (30). pk18 production in yeast results in the formation of IQ and Bz sites that are pharmacologically similar to authentic PBR. In yeast, mitochondrial

### Table I

| Strain/plasmid(s), genotype | Experiment | RO5-4864 | PK11195 |
|-----------------------------|------------|----------|----------|
|                             | \( K_D \)  | \( B_{max} \) | \( K_D \) | \( B_{max} \) |
| W303/pEMR1130               | 1          | 12       | 45       | 2.2       | 80       |
|                             | 2          | 10       | 45       | 2.5       | 80       |
| EL301/pEMR1130, vdc1        | 1          | 26       | 60       |           |          |
|                             | 2          | 13       | 50       | 2.0       | 50       |
|                             | 3          | 15.5     | 60       |           |          |
| EL302/pEMR1130, vdc1 vdc2   | 1          | 13       | 20       |           |          |
|                             | 2          | 12       | 24       | 2.2       | 33       |
|                             | 3          | 16       | 37       | 1.1       | 45       |
|                             | 4          | 14       | 28       |           |          |
| ELW2/pEMR1130, vdc2         | 1          | 20       | 50       |           |          |
|                             | 2          | 9.5      | 21       | 2         | 25       |
| EL105/pEMR1130, aac1, aac2, aac3 | 1   | 45       | 60       |           |          |
|                             | 2          | 12       | 50       | 2         | 60       |
|                             | 3          | 60       | 90       |           |          |
| EL105/pEMR1130 + pEMR1179   | 1          | 16       | 75       | 9         | 53       |
| (AAC2), aac1, aac2, aac3    | 2          | 20       | 20       | 2         | 31       |
|                             | 3          | 20       | 22       |           |          |
| coxIV pEMR1130              | 1          | 10       | 20       | 4         | 44       |
|                             | 2          | 21       | 37       | 2.6       | 47       |

### Table II

| Strain/genotype | RO5-4864 | PK11195 |
|-----------------|----------|---------|
| Wild type       | 43 ± 10  | 8 ± 2   |
| vdc1            | 55 ± 10  | 8 ± 2   |
| aac1, aac2, aac3| 63 ± 10  | 6.5 ± 3 |

### Table III

| Strain/genotype | RO5-4864 | PK11195 |
|-----------------|----------|---------|
| Wild type       | 10 ± 3   | 7 ± 2   |
| vdc1            | 12 ± 1   | 4 ± 2   |
| aac1, aac2, aac3| 13 ± 1   | 16 ± 3  |
functions are dispensable for proliferation, which enables the isolation of mutant strains completely devoid of VDAC or ADC. The fact that such pk18-expressing cells lacking VDAC exhibit R05-4864 binding sites clearly demonstrates that VDAC and ADC are dispensable for the formation of the Bz-specific binding site. Furthermore, a genetic variation affecting a single amino acid residue in the pk18 sequence has been previously shown to be sufficient for restoring or abolishing Bz binding expression in recombinant yeast cells. It is therefore highly probable that both the Bz and IQ binding sites are on the same protein, i.e. pk18. These two binding sites are nevertheless not fully overlapping, as revealed by various chemical, genetic and physiological studies (14, 16, 31–33). Our results with VDAC-lacking cells do not support one of the hypotheses proposed by Garnier et al. (9), namely that the Bz recognition site might be partly on pk18 and partly on VDAC. Garnier et al. based their hypothesis on the observation that in vitro reconstruction experiments the purified VDAC and the purified chimeric construct MBP-pk18 did not exhibit any Bz binding site unless mixed together. That result, in the light of the work we present here, might be interpreted in a different manner. The pk18 domain of the chimeric construct might have adopted a conformation incompatible with Bz-binding, either as a consequence of the fusion with the bacterial protein, or as a result of a partial denaturation event occurring at some step during the purification procedure. The addition of VDAC may have corrected the conformational defect by interacting with the pk18 domain; VDAC would then play a chaperone-like role in this reconstituted system. Quite similarly, a pk19-reconstitution-modulating role could possibly be proposed for yeast ADC in our system, since in the absence of the major ADC isoform, the \( K_D \) and \( K_V \) values estimated for the Bz ligand tend to slightly increase and the flunitrazepam-photolabeled \( M_2 \), 18,000 polypeptide is hardly detectable. Thus, our results do not rule out the alternative hypothesis of Garnier et al. postulating that proteins distinct from pk18 might indirectly modulate the Bz binding site on PBR (i.e. pk18, according to our results) by interacting with this latter polypeptide. The Bz binding domain might correspond to a more restrictive conformational state of the pk18 homopolymer, as compared with the IQ binding domain, this Bz-binding-competent conformation might be stabilized or modulated by other proteins interacting with this complex. Recent results have shown that modifications to the Bz topography are induced upon addition of choriogonadotropin to MA10-Leydig cells, resulting in an increase in both the pk18 cluster size and PBR ligand binding (34). This observation confirms that PBR receptors can exist under distinct morphological states. However, since VDAC, whether from yeast or mammals, is dispensable for the Bz binding site of pk18 produced in yeast cells, the conclusion that the Bz binding site of PBR resides, even partly, on VDAC cannot stand. On the contrary, our results support the assumption that both the IQ and Bz binding sites are fully contained within the pk18 sequence.

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