A Sensory Transducer Homologous to the Mammalian Peripheral-type Benzodiazepine Receptor Regulates Photosynthetic Membrane Complex Formation in Rhodobacter sphaeroides 2.4.1*

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The Rhodobacter sphaeroides 2.4.1 tryptophan-rich sensory protein gene, tspO (formerly crtK, ORF160) encodes a 17-kDa protein which has an unusually high content of aromatic amino acids in general and of L-tryptophan in particular. The TspO protein was localized to the outer membrane of aerobically grown R. sphaeroides 2.4.1 by use of a polyclonal antibody against the purified protein. This protein is present in several-fold higher levels in photosynthetic as opposed to aerobic grown cells. Although tspO lies within the crt gene cluster, null mutations have an intact carotenoid biosynthetic pathway. In the tspO1 mutant there was an increased production of carotenoids and bacteriochlorophylls relative to the wild type, particularly when cells were grown aerobically or semi-aerobically. When present in the tspO gene restored "normal" pigment production to tspO1. The effect of the tspO gene on pigment production was shown to take place at the level of gene expression. Because the tspO gene product of R. sphaeroides 2.4.1 shows significant sequence homology and similarity to the peripheral-type benzodiazepine receptor from mammalian sources, TspO-specific antibodies when probed against liver and kidney mitochondrial protein showed strong cross-reactivity. The role of TspO in R. sphaeroides 2.4.1 and its relation to photosynthesis gene expression are discussed.

Rhodobacter sphaeroides is a member of the proteobacteria, and it is characterized by its metabolic versatility including growth either chemoheterotrophically or phototrophically. A decrease in oxygen level results in the induction of the photosynthetic membrane system designated the intracytoplasmic membrane (ICM). The ICM contains all of the components necessary to convert light energy into chemical energy during phototrophic growth. The ICM is also gratuitously synthesized by R. sphaeroides during anaerobic growth in the dark in the presence of an alternative electron acceptor, such as dimethyl sulfoxide (Me$_2$SO). While ICM synthesis and composition are known to be tightly regulated, the molecular mechanisms which govern the biosynthesis and assembly of the ICM are only beginning to yield to molecular genetic analysis (1).

Carotenoids (Crt) in addition to bacteriochlorophyll (Bchl) are an important structural component of the ICM. They take part in the entrapment and utilization of light energy, and furthermore, have an important antioxidative function during aerobic growth. The crt gene cluster of Rhodobacter capsulatus has been well characterized by (2, 3), and the genes and likely enzymatic activities which they encode have been discussed. In R. sphaeroides, these genes have been recently shown to occupy a similar arrangement as in R. capsulatus (4).

According to the phenotype of a number of Crt mutants in R. capsulatus (3) and in R. sphaeroides NC1B8253 (4) enzymatic activities have been assigned to seven of the eight genes constituting this cluster but not to crtK, herein designated tspO (tryptophan-rich sensory protein). Based on the amino acid sequence deduced from the nucleotide sequence it was suggested that TspO was an integral membrane protein. Most of the enzymes of the carotenoid biosynthetic pathway were also shown to be localized to the cell membrane, and it was proposed that TspO is a site for docking of the enzymes involved in carotenoid biosynthesis (3). However, no evidence either for this or for any other physiological activity associated with this protein has been available, until now.

Comparison of the deduced amino acid sequence of the R. capsulatus TspO with that of adrenal peripheral-type benzodiazepine receptor (PBR) revealed a high degree of homology between the two proteins; of the 129 amino acid residues comprising 75% of each of these proteins, there were approximately 35% identity and a further 15% conservative replacements (5).

The PBR is present in many types of mammalian tissue including kidney, liver, brain, adrenal gland, testes, etc. (6). It has been shown that this 18-kDa protein is localized to the outer mitochondrial membrane and is associated with an outer membrane, voltage-dependent anion channel (VDAC) and adenine nucleotide carrier, which is an inner mitochondrial membrane localized protein. The PBR binds with nanomolar affinity to a variety of benzodiazepines as well as to dicarboxylic porphyrins (7). A number of metabolic activities have been ascribed to the PBR including regulation of steroidogenesis and participation in tetrapyrrole metabolism. However, the precise physiological function of this mitochondrial membrane protein still remains unclear.

Here, we attempt to bring together this disparity of observations by defining a physiologic role for TspO in R. sphaeroides 2.4.1, describing its cellular localization, and further showing that antibody produced against TspO cross reacts quite specifically with a protein(s) of the rat mitochondrion.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, Media, and Growth Conditions—Bacterial strains and plasmids used in this study are described in Table I.**
Cultures of R. sphaeroides 2.4.1 and its derivatives were grown in Siström's minimal medium A containing 0.4% succinate as a carbon source (8) as described previously (9, 10).

Cell growth was monitored turbidometrically with a Klett-Summor serial colorimeter using a no. 66 filter. 1 KU is equivalent to 1 × 10^11 cell/ml. To minimize antibiotic photodegradation (11), liquid cultures of R. sphaeroides grown heterotrophically in the presence of chloramphenicol were placed behind a CS 7–69 filter (620–1100 nm; Corning Glass Works, Corning, NY). Strains of Escherichia coli were grown as described previously (12). When appropriate, antibiotics were added to the following final concentrations: ampicillin (Ap), 100 µg/ml; kanamycin (Km), 25 µg/ml; spectinomycin (Sp), 50 µg/ml; streptomycin (Sm), 50 µg/ml; tetracycline (Tc), 10 µg/ml; and chloramphenicol (Cl), 1 µg/ml for R. sphaeroides. Conjugal matings between E. coli and R. sphaeroides were performed as described by Moore and Kaplan (13). β-Galactosidase and alkaline phosphatase activity in cell extracts—R. sphaeroides cultures were grown to a cell density of approximately 1.8 × 10^9 cells/ml, and chloramphenicol was added to a final concentration of 80 µg/ml to terminate protein synthesis. Assay of β-galactosidase activity in cell extracts was performed as described previously (13). All experiments involving β-galactosidase assay were performed at least twice, with results being reproducible to within ±15%.

In vitro alkaline phosphatase (APase) activity was determined following the hydrolysis of o-nitrophenyl-phosphate as described previously (14).

Cell Fractionation—Subcellular fractions of R. sphaeroides 2.4.1 were prepared by the method of Weiss (15) as modified by Tai and Kaplan (16). The protease inhibitor phenylmethylsulfonyl fluoride was added to cell extracts at a final concentration of 1 mM. Protein concentration was determined using the Pierce reagent (Pierce); bovine serum albumin was the standard.

Carotenoid and bacteriochlorophyll Determination—Pigments were extracted from R. sphaeroides 2.4.1 cells and quantitated according to procedures described (8). HPLC analysis of pigments was done on a Waters HPLC system equipped with a model 411 absorbance detector.

Suitable pigment separation was achieved with a Spherisorb ODS2 (Rainin Instrument, Woburn, MA). The mobile phase was 0.1% phosphoric acid (pH 2.2) in methanol (25 x 0.46 cm) using a gradient elution system described (17).

Spectrophotometric Analysis—R. sphaeroides 2.4.1 cells were harvested by centrifugation (10 min, 10,000 × g, 4 °C) and disrupted by sonication for 5 min (50% duty cycle). Unbroken cells and cell debris were removed by centrifugation (30 min, 30,000 × g, 4 °C). Absorption spectra were analyzed on a SLM DW2000 double-beam UV-VIS-R spectrophotometer (SLM-Amino Instruments, Urbana, IL). Equivalent protein concentrations of the cleared lysate were used when the spectral profiles of different strains of R. sphaeroides were compared. The extinction coefficients reported by Meinhart et al. (18) were used to calculate the levels of individual spectral complexes.

Molecular Techniques—Standard procedures were used for plasmid isolation, restriction endonuclease digestion, isolation of DNA fragments from gels, ligation, and other molecular biological techniques (12, 19). Sequencing was done with an ABI 373A automatic DNA sequencer (Applied Biosystems Inc., Foster City, CA) at the DNA Core Facility of the Department of Microbiology and Molecular Genetics.

DNA Sequence Analysis—Sequence analyses were performed with the computer programs DNA Strider (Institut de Recherche Fondamentale, Commissariat a l’Energie Atomique, Paris, France), Programs FASTA and TFASTA were used to screen EMBL and GenBank data bases for sequence similarities. These programs use algorithms based on that of Lipman and Pearson (20). The program Peptidestructure was used to predict the secondary structure of TspO.

Conjugation Experiments—Plasmid pUI2701 was constructed by introducing 1.1-kb KpnI fragment of pU1124 containing tspO under PrrnB into the KpnI site of the broad host range expression vector pRK415.

Plasmids carrying cta::lacZ and cta::lacZ transcriptional fusions were constructed by the blunt end ligation of 0.13-kb Psil fragment of pU3101, containing promoter region for divergently transcribed genes, cta and ctaI, into the XbaI site of pXY1 vector, SmI/I, in both orientations. The presence and orientation of the insert were verified by sequencing from the 5' end of the lacZ toward the promoter region. Plasmid containing cta::lacZ fusion was designated pU12711, and plasmid carrying cta::lacZ was designated pU12712.

Plasmid pU12715 carrying a translational fusion of tspO and the glutathione S-transferase (GST) gene was constructed by ligation of 0.7-kb BamHI-EcoRI fragment of pU1124, containing tspO, into the BamHI-EcoRI sites of pGEX-2TK vector (Pharmacia).

SDS-PAGE, Western Blot Analysis, and N-terminal Amino Acid Sequencing—SDS-PAGE and Western blotts were performed as described (12). Cultures were grown and cells broken as described (9). Western blotts were probed with antibodies against the major outer membrane protein or against TspO. N-terminal amino acid sequence of the R. sphaeroides 2.4.1 major outer membrane protein was performed at the analytical chemistry center, UT Medical School, on an Applied Biosystems 477 Protein Sequencer using a procedure described (21). Protein was electroblotted onto polyvinylidene difluoride membrane, stained with Coomassie Blue, and sequenced directly.

Expression and Partial Purification of TspO-GST Fusion Protein—TspO-GST fusion protein was expressed in E. coli M109 from pUI2715 after induction with 100 mM β-isopropyl-thiogalactoside for 3 h. Cells were disrupted by French Press and fractionated by centrifugation (10 min, 20,000 × g, 1 h, 150,000 × g). Fractions were subjected to SDS-PAGE, and the TspO-GST fusion protein was found to be localized to the membrane fraction. Membranes solubilized with 1% N-lauroylsarcosine in phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 10.1 mM NaHPO4, 1.8 mM KH2PO4, pH 7.3) and subsequently dialyzed against 0.05% N-lauroylsarcosine in the same buffer. The resulting solution was treated with thrombin (10 units of thrombin/mg protein) at 25 °C overnight. Cleavage products were run at 15% SDS-PAGE, and a strip of gel corresponding to the 17-kDa protein (TspO) was removed and used as an antigen for raising antibodies against TspO.

Antibodies—Antiserum against TspO was obtained from Cocalico Biologicals (Cocalico Biologicals, Inc., Reamstown, PA) using standard procedures. The TspO isolated from SDS-PAGE was used to immunize two white New Zealand rabbits. The immune and preimmune sera were characterized for their reactivity against total E. coli and R. sphaeroides 2.4.1 protein and membrane protein. For immunological detection of transferred proteins, antisera were diluted in TBST (20 mM Tris, pH 7.4, 500 mM NaCl, 0.05% Tween 20) plus 5% bovine serum albumin and incubated with protein transferred to nitrocellulose filters for 1.5 h at room temperature. After extensive washing in TBST and incubation with anti-rabbit secondary antibodies (Biorad, Hercules, CA) for 1 h at room temperature, the blot was developed by using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Antibodies against TspO were affinity purified using the modification of a protocol described (22). A preparative SDS-PAGE was loaded with approximately 0.8 mg of protein extract containing TspO and subjected to electrophoresis. Proteins were electroelutically transferred to a nitrocellulose membrane; the protein band corresponding to TspO was located, removed from the membrane, and blocked 1.5 h in 5%BSA in TBST. After washing three times for 15 min each in TBST, the nitrocellulose strip was incubated overnight at 4 °C in a solution containing 1 ml of serum and 5 ml of TBST. The nitrocellulose strip was washed three times for 15 min each in TBST, and antibodies were eluted with 100 mM glycine, pH 2.2 (five times, 1 ml each). The glycine solution was neutralized with 1 M Tris pH 9.0, and the resulting solution was characterized for antibody reactivity against R. sphaeroides 2.4.1 membrane proteins.

Antibodies against the major outer membrane protein were prepared by C. Dea and S. Kaplan (23).

Photoaffinity Labeling of the R. sphaeroides 2.4.1 Major Outer Membrane Protein—Membrane samples (20 µl) containing 50 µg/ml protein in 50 mM Tris-HCl buffer, pH 7.5, were preincubated with [N-methyl-3H]flunitrazepam (2 nm) for 30 min and then irradiated for 15 min by a Black-Ray ultraviolet lamp (Ultraviolet products, San Gabriel, CA). The samples were then removed from under the lamp and excess of unlabeled flunitrazepam (10 µM) was added in order to displace any remaining [3H]flunitrazepam not covalently bound to protein.

Proteins incorporating the radiolabeled drug were estimated using SDS-PAGE electrophoresis in 12.5% gel (24). The gel was then treated with ENHANCE (DuPont Biotechnology Systems, Boston, MA) according to the procedure recommended by the manufacturer and exposed to Kodak film (Eastman Kodak Co.) for 1.5 mo at ~80 °C.

Materials—Restriction endonucleases and nucleic acid-modifying enzymes were purchased from New England Biolabs, Inc. (Beverly, MA). Antibiotics, ONPG, X-Gal, X-P, and vitamins were obtained from Sigma Chemical Company. Restriction enzyme labeling was performed at research Biochemicals International (Natik, MA). [N-methyl-3H]flunitrazepam was from Amersham (Amersham Corp.). All other chemicals were of reagent grade purity and were used without further purification.
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and R. capsulatus crtK deduced amino acid sequences of the identity was observed between the aligned residues for the Chromosomal tspO—which complemented the mutation(s) in mutant CL1a (25) was designated TSPO1 after Southern hybridization confirmed the insertion of the mutant. The sequences are as follows: R. sphaeroides, R. capsulatus, HsPkbS, R. sphaeroides TspO, MmPkbS, RsTspO, BtPkbS, RsNCIB, RsTspO from wild type strain NCI18253, and RsTspO, R. capsulatus 2.4.1 TspO. Bold are amino acid residues conserved in all seven sequences.

RESULTS

Cloning, DNA Sequence Determination, and Inactivation of the Chromosomal tspO—The 2.1-kb SstI fragment of pAS204 which complemented the mutation(s) in mutant CL1a (25) was cloned into pBS11K+ in both orientations relative to vector sequences. The DNA sequence of both strands was determined using overlapping clones covering the 2.1-kb insert.2 Computer analysis (26) suggested that two open reading frames (ORFs) which were likely to encode proteins were present within the fragment. Comparison of the DNA sequences of these ORFs to computer data base sequence libraries using software described under "Experimental Procedures" indicated significant homology to the DNA sequence of the Crb8 and CrkK genes of R. capsulatus. The ORF, homologous to CrkK, was 480 nucleotides in length (Fig. 1) and shared 60% sequence identity with the R. capsulatus gene. There is 99.7% identity between the nucleotide sequences encoding tspO from R. sphaeroides 2.4.1 and from R. capsulatus NCI18253 (4).

Using the cartridge encoding kanamycin resistance from pUC4K, a tspO disruption in pUI1108 was constructed (Table I). This construction was subsequently used to generate the pSP202-derived vector pUI1110. One Km′Tc′ transconjugant was designated TSP01 after Southern hybridization confirmed the replacement of the wild type tspO by the tspO::Km construction from pUI1110. The absence of vector sequences in the mutant was confirmed when a radioactively labeled pSP202 probe failed to hybridize to genomic DNA from the mutant.2

Analysis of the Deduced Amino Acid Sequence for tspO—48% identity was observed between the aligned residues for the deduced amino acid sequences of the R. sphaeroides 2.4.1 tspO and R. capsulatus crkK gene, with an additional 23% of the aligned residues being similar (Fig. 1). Seven tryptophan residues were conserved in both sequences. Data base searches revealed TspO was 35% identical to the human peripheral-type benzodiazepine receptor; RsCrtK, R. capsulatus CrkK; RsNCIB, R. sphaeroides TspO from wild type strain NCI18253, and RsTspO, R. capsulatus 2.4.1 TspO. In bold are amino acid residues conserved in all seven sequences.

2 M. Wood and S. Kaplan, unpublished results.
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Table I

| Organism and strain or plasmid | Relevant characteristic(s) | Ref. or source |
|-------------------------------|----------------------------|----------------|
| E. coli DH5α                  | DHLαphe;Tn10Dcm            | (30)           |
| HB101                         | lacY1 galK2 supE44 ara14 proA2 rpsL20 recA13 xyl-5 mtl-1 hisD50 mcrB mrr | (33)           |
| S17–1                         | C600-Fp-4-2 (Tc::Mu)(Km::Tn7) thi pro hisD50 hisD50 recA13 | (34)           |
| R. sphaeroides 2.4.1          | Wild type tspO::Kmr         | W. R. Sistrom  |
| Plasmids                      |                            | M. Wood and S. Kaplan |
| pRK415                        | Tc'                        | (35)           |
| pBSIK5+                       | Ap'; with T3 and T7 promoters | Stratagene      |
| pUC4K                         | Source of Km'              | Pharmacia       |
| pSUP202                       | pBR325 derivative, Mob' Ap' Cmr Tc' | (34)           |
| pAS204                        | pRK415 containing the 2.1-kb SstI crtBtspO fragment from pUI8487 Tc' | A. Suwanto      |
| pUI1106                       | pBSIK5- containing the 2.1-kb SstI fragment of pAS204, Ap' | M. Wood and S. Kaplan |
| pUI1107                       | Same as pUI1106 containing 2.1-kb fragment in opposite orientation, Ap' | M. Wood and S. Kaplan |
| pUI1108                       | pBSIK5- containing Km'-encoding HindI fragment of pUC4K inserted at the BalI site of tspO, Ap', Km' | M. Wood and S. Kaplan |
| pUI1110                       | pSUP202 containing 3.7-kb tspO::Kmr fragment of pUI1108 inserted at the SspI site, Tc', Km' | M. Wood and S. Kaplan |
| pUI1124                       | pBSIK5- containing tspO under PrnB Apr | M. Wood and S. Kaplan |
| pUI1180                       | pRK415 containing translational fusion of 32 N-amino-terminal part of tspO and 'PhoA, Tc' | M. Wood and S. Kaplan |
| pUI1182                       | pRK415 containing translational fusion of 89 N-amino-terminal part of tspO and 'PhoA, Tc' | M. Wood and S. Kaplan |
| pUI1830                       | Sm'/Sp'/puf::lacZ           | L. Gong        |
| pCF200Km                      | Sm'/Sp'/Km' puc::lacZ       | (25)           |
| pUI2701                       | Derivative of pRK415 harboring 1.1-kb KpnI fragment of pUI1124 containing tspO under PrnB, Tc' | This study |
| pUI18461                      | Tc' cII fragment from cosmid pUI18641 | (27)           |
| pUI3101                       | Tc' derivative of pRK415 harboring 3.5-kb BamHI fragment of cII gene cluster from cosmid pUI18641 | M. Sabaty      |
| pLX1                          | Sm'/Sp' vector for promoter cloning, lacZYA', xylE | M. Gomelsky    |
| pUI2711                       | Derivative of pLX1, Sm'/Sp' cII::lacZ | This study |
| pUI2712                       | Derivative of pLX1, Sm'/Sp' cII::lacZ | This study |
| pGEX-2TK                      | Ap' vector for overexpression of proteins fused with GST | Pharmacia      |
| pUI2705                       | Derivative of pGEX-2TK containing translational fusion of tspO with GST Ap' | This study |

Absorption spectra of R. sphaeroides 2.4.1 membrane preparations. Cells were grown photoheterotrophically in the light at 10 W/m². Samples of equal protein concentration (1 mg/ml) were examined as described under “Experimental Procedures.”

Characterization of tspO::phoA Gene Fusions—Due to the significant homology between TspO and the membrane-localized mammalian PBRs (Fig. 1) it was anticipated that TspO would be membrane-localized in vivo. The hydrophobicity plot of TspO indicated the possible presence of at least four membrane-spanning domains. Therefore, two translational gene fusions were constructed between the N-terminal portion of tspO and the genetically engineered E. coli alkaline phosphatase (APase) gene phoA which is missing the DNA sequence encoding the signal peptide (13). The fusion containing the 52 N-terminal amino acids of TspO (pUI1180) exhibited APase activity in R. sphaeroides 2.4.1 cells grown both aerobically or photosynthetically (300 and 7500 units of specific activity, respectively). A second fusion following the 89 N-terminal amino acids (pUI1182) was not active under either set of conditions. Thus, these results are consistent with the conclusion that TspO resides within the cell membranes.

Immunochemical Detection of TspO—In order to obtain antibodies against this polypeptide, we cloned tspO under the highly regulated tac promoter into the expression vector pGEX-2TK (Pharmacia) at the BamHI-EcoRI sites, and the construction was designated pUI2715. The protein was then overex-
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**TABLE II**

| Strain       | Condition of growth | B800–850, nM/mg protein | B875, nM/mg protein | Ratio B800–850/B875 |
|--------------|---------------------|--------------------------|---------------------|---------------------|
| 2.4.1        | Dark/MgSO           | 55.8                     | 18.3                | 3.1                 |
| TSPO1        |                      | 56.1                     | 17.1                | 3.3                 |
| 2.4.1        | Photoheterotrophic  | 22.4                     | 18.9                | 1.2                 |
| TSPO1        | 100 W/m²            | 25.2                     | 20.7                | 1.2                 |
| 2.4.1        | Photoheterotrophic  | 49.8                     | 17.6                | 2.8                 |
| TSPO1        | 10 W/m²             | 45.4                     | 23.5                | 1.9                 |
| 2.4.1        | Photoheterotrophic  | 58.7                     | 18.3                | 3.2                 |
| TSPO1        | 3 W/m²              | 63.8                     | 30.8                | 2.1                 |

**TABLE III**

| Strain  | Plasmid | Carotenoids | Bacteriochlorophyll |
|---------|---------|-------------|---------------------|
| 2.4.1   | None    | μg          | μg                  |
| 2.4.1   | pRK415  | 317         | 131                 |
| 2.4.1   | pUC12701| 259         | 83                  |
| TSPO1   | None    | 583         | 143                 |
| TSPO1   | pRK415  | 536         | 141                 |
| TSPO1   | pUC12701| 249         | 38                  |

Fig. 3. Pigment accumulation by R. sphaeroides 2.4.1 and TSPO1 cells. Cells were grown aerobically at 30°C, 60% N₂, 2% CO₂ to an optical density of 20 KU and shifted to semiaerobic conditions (3% O₂, 95% N₂, 2% CO₂) at the time point indicated by the arrow. Pigments were extracted and quantified as described under “Experimental Procedures.”

**TABLE IV**

| Strain | Condition of growth | B800–850, nM/mg protein | B875, nM/mg protein | Ratio B800–850/B875 |
|--------|---------------------|--------------------------|---------------------|---------------------|
| 2.4.1  | Dark/MgSO           | 55.8                     | 18.3                | 3.1                 |
| TSPO1  |                      | 56.1                     | 17.1                | 3.3                 |
| 2.4.1  | Photoheterotrophic  | 22.4                     | 18.9                | 1.2                 |
| TSPO1  | 100 W/m²            | 25.2                     | 20.7                | 1.2                 |
| 2.4.1  | Photoheterotrophic  | 49.8                     | 17.6                | 2.8                 |
| TSPO1  | 10 W/m²             | 45.4                     | 23.5                | 1.9                 |
| 2.4.1  | Photoheterotrophic  | 58.7                     | 18.3                | 3.2                 |
| TSPO1  | 3 W/m²              | 63.8                     | 30.8                | 2.1                 |

To obtain additional insight into the possible target, we used pressed E. coli as a fusion with GST after induction with isopropyl-β-D-thiogalactoside. TspO-GST constituted more than 10–15% of the cellular protein as evidenced by SDS-PAGE. The fusion protein was purified and antibodies against TspO obtained as described under “Experimental Procedures.”

Fig. 4A represents an SDS-PAGE of TspO-GST expressed in E. coli and stained with Coomassie Blue. The band of 44 kDa (lane 2) disappeared following treatment with thrombin and was replaced by two new bands (lane 3) corresponding to GST (27 kDa) and TspO (17 kDa). Using antibodies raised against TspO (Fig. 4B) revealed one immunoactive band in preparations treated with thrombin (lane 2). In the sample of GST-TspO (Fig. 4B, lane 1), both the fusion protein and several bands, apparently corresponding to products of nonspecific cleavage, were detected.

Membrane fractions of R. sphaeroides 2.4.1 and TSPO1 were separated by SDS-PAGE and probed with TspO-specific antibody. In Fig. 5, one band corresponding to a protein of 17 kDa was visible in the outer membrane preparations from 2.4.1 cells grown semiaerobically (lane 1) or photoheterotrophically (lane 3). No immunoactive band could be detected in TSPO1 (lanes 4 and 5). TspO expressed in E. coli from the construct pUC12715 (Fig. 5, lane 6) migrated more slowly in SDS-PAGE than its homologue from the membrane fraction derived from R. sphaeroides 2.4.1. This difference correlated with the presence of an additional 13 amino acid residues in the genetically engineered protein expressed in E. coli.

To gain additional insight into the question of how TspO acts as a sensory transducer, membranes from both aerobic and photosynthetic grown R. sphaeroides 2.4.1 cells were reacted with anti-TspO antibodies following separation on SDS-PAGE (Fig. 5, lanes 1 and 3). Membranes from aerobic grown cells showed low levels of TspO in agreement with the APase results. However, the bulk of the reactive species migrated with an apparent size of ~36 kDa. On the other hand, the level of TspO in photosynthetic grown cells was considerably increased, but the immunoactive species had a size of 17 kDa.

Experiments with Benzodiazepines—R. sphaeroides 2.4.1 was cultured either photoheterotrophically (10 W/m² light intensity) or in dark/MgSO in the presence of varying concentrations of the benzodiazepines flurazepam or flunitrazepam. The growth rate was not affected by either drug in the range of concentrations from 0 to 80 μg/ml. However, when the formation of spectral complexes was examined (Fig. 6A), we observed that the amount of the B800–850 complex was reduced by 60–65% at this concentration of flurazepam.

However, the same effects were observed in experiments with mutant strain TSPO1 (Fig. 6B), thus eliminating the possible involvement of TspO in the benzodiazepine sensitivity of R. sphaeroides 2.4.1.
[N-methyl-\( ^3 \)H]flunitrazepam as a means of following the binding of this drug to cellular proteins. Cells of either R. sphaeroides 2.4.1 or TspO1 grown aerobically or photoheterotrophically were fractionated according to procedures described under "Experimental Procedures." These preparations were preincubated with \( ^3 \)H-labeled flunitrazepam and UV-cross-linked. SDS-PAGE fractionation of proteins and subsequent radioautography revealed only one major radioactive band in the outer membrane preparation corresponding to a protein of approximately 47 kDa (Fig. 7, A and B). No specific binding of \( ^3 \)H]flunitrazepam was detected in either the inner membrane or cytoplasmic proteins. One potential difficulty in detecting benzodiazepine binding to TspO is the very low level of TspO relative to the major outer membrane protein in R. sphaeroides 2.4.1; binding to TspO could be masked, and then special precautions might have to be exercised.

Previous work from this laboratory revealed that the major outer membrane protein or porin from R. sphaeroides 2.4.1 migrates at approximately 47 kDa (23). An antibody raised against this polypeptide was found to react with the same protein binding the labeled drug (Fig. 7C), thus identifying it as the major outer membrane protein. We determined the N-terminal sequence of this polypeptide (EISFSGYAAE) and found it was 44% identical and a further 30% similar to that of the previously reported porin of R. capsulatus (28).

Reaction of Rat Mitochondria with Antibodies to TspO—When we probed preparations of either kidney or liver, rat mitochondria with antibodies raised against TspO, a strong and highly specific cross-reactivity was observed with three protein species from kidney and with two protein species from liver mitochondria (Fig. 8). In both liver and kidney, immunoreactive bands at approximately 30 and 55 kDa were present. In preparation from the kidney mitochondrion additional bands of approximately 35 and 55 kDa were observed; however, these bands were absent from the liver preparation. The relative intensities of immunoreactive bands were much higher in prepa-
sources of activities have been ascribed to the PBRs which are localized in the junction of the two mitochondrial membranes. It was proposed that the PBR in association with two proteins VDAC and adenine nucleotide carrier comprises a porphyrin transport site at the outer mitochondrial membrane, such as the regulation of steroidogenesis and their involvement in porphyrin transport across the mitochondrial membrane. It was proposed that the PBR in association with two proteins VDAC and adenine nucleotide carrier comprises a porphyrin transport site at the junction of the two mitochondrial membranes (6).

We observed that several benzodiazepines, chosen primarily because of their aqueous solubility, and which are known to bind with high affinity to PBRs, affected the biosynthesis of the B800–850 antenna complex in R. sphaeroides 2.4.1. When present in concentrations 0.02–0.1 nm, flurazepam suppressed complex formation by 50–95% in wild type R. sphaeroides 2.4.1 grown phototrophically. However, the same effect was also observed with TSP01. Further studies using a structural analog of flurazepam, flunitrazepam, demonstrated this drug specifically bound to the major outer membrane protein of R. sphaeroides 2.4.1, whether or not TspO is present. It was recently shown that the R. capsulatus major outer membrane protein, or porin, binds with high affinity some of the tetrapyrrole intermediates in Bchl a biosynthesis (28). One possibility is that benzodiazepines when present in micromolar concentrations compete for tetrapyrrole-binding sites. However, why the biosynthesis of the B800–850 complex is inhibited to a greater extent than that of the B875 complex still remains unclear. These results may reflect differences in the assembly pathways for each of these macromolecular complexes.

Comparison of the nucleotide sequence of tspO from R. sphaeroides 2.4.1 with that of R. sphaeroides NCIB8253 (4), revealed 99.7% identity, with three nucleotides absent from the NCIB8253 sequence in the positions between nucleotides 4095 and 4096 (G): 4124 and 4125 (G), and 4146 and 4147 (C), relative to the sequence from 2.4.1. The resulting frameshifts led to a decreased similarity between the two derived polypeptides (91% identity). All nonidentical amino acid residues were found to be localized to the region of the frameshift between positions 62 and 86. The codon usage assessment program indicated five rarely used codons in the DNA sequence from NCIB8253 compared to 2.4.1. However, strains 2.4.1 and NCIB8253 are considered to be similar if not identical (29) on a basis of available sequencing data and Asel DNA digestion patterns.

Disruption of the R. sphaeroides' tspO by insertion of a kanamycin resistance gene did not lead to any significant change in the phenotype of cells grown either aerobically or photosynthetically, except for some small alteration in the ratio between B800–850 and B875 complexes. However, a profound phenotypic difference was observed between the wild type and TSP01 during transition from aerobic to anaerobic growth. TSP01 also produced substantially increased levels of both Crt and Bchl relative to the wild type. Studies showed that there was a 3–5-fold effect of TspO on expression of crtA, crtI and bchF in the mutant when compared to the wild type. Transcription of the puc operon was similarly affected in the mutant strain under semiaerobic conditions. Introduction of tspO in trans into TSP01 mutant or wild type resulted in a severe reduction in pigment accumulation to below the wild type level, which was most evident in cells grown under semiaerobic or aerobic conditions.

Differences in expression of both crt and bch genes between mutant and wild type cells were also detected when cells were grown phototrophically at medium light intensity (10 W/m²) but not at high light intensities (100 W/m²).
R. sphaeroides Tryptophan-rich Sensory Protein TspO

**Fig. 7. Identification of the major outer membrane protein as a [3H]flunitrazepam binding species.** A, Coomassie Brilliant Blue-stained SDS-PAGE. R. sphaeroides 2.4.1 and TSPO1 cells were grown semiaerobically and fractionated as described under “Experimental Procedures.” Lanes 1 and 4, outer membrane preparations; lanes 2 and 5, inner membrane preparations; lanes 3 and 6, cytoplasmic fraction from 2.4.1 and TSPO1 cells, respectively. B, radioautogram of R. sphaeroides 2.4.1 proteins labeled with [3H]flunitrazepam. Lanes are the same as in A, C, immunoblot analysis of major outer membrane protein. Proteins from fractionated cells of R. sphaeroides 2.4.1 were resolved by SDS-PAGE and probed with antibodies raised against major outer membrane protein (porin). Lanes 1 and 4 are the same as in A and B.

**Fig. 8. Immunoblot analysis of mitochondrial proteins.** Preparations of rat kidney and liver mitochondria (gift of Dr. M. McEnery) were solubilized in sample buffer, run on a 12% SDS-PAGE, and blotted onto nitrocellulose membranes. Proteins were probed with antibodies raised against TspO. Lanes 1 and 2, preparations from kidney mitochondria solubilized at 90 and 37°C, respectively. Lanes 3 and 4, preparations from liver mitochondria, solubilized at 90 and 37°C. Lane 5, preparation of overexpressed in E. coli TspO-GST protein treated with thrombin.

We have previously described the existence of an additional oxygen sensing system in R. sphaeroides 2.4.1, the PrrA system which is typical of a two-component regulatory system and where a decrease in oxygen tension results in an activation of photosynthesis gene expression (30). The TspO system appears to involve the negative regulation of photosynthesis gene expression. Whether or not TspO acts through the regulatory protein PpsR remains to be determined (31, 32). However, the possibility exists that a small ligand molecule could be involved in this regulatory pathway as revealed by the effect of benzodiazepines. Thus, R. sphaeroides 2.4.1 appears to have at least two systems able to sense the level of oxygen in the environment. Very recent work also confirms the presence of the Fnr system of aerobic/anaerobic control in R. sphaeroides 2.4.1. In addition, the TspO system also appears to be able to sense differences in light intensity. We do not know the extent of overlap on photosynthesis gene expression between these regulatory systems.

Antibodies raised against TspO specifically recognize this protein in the outer membrane fraction of R. sphaeroides 2.4.1 cells. Membrane localization of this protein was also supported by the results of the TspO-alkaline phosphatase-fusion analysis which showed high level of APase when conjugated to the N-terminal amino acid portion of TspO. It is known that mammalian PBR interacts with VDAC in the outer mitochondrial membrane and adenine nucleotide carrier, localized to the inner mitochondrial membrane. VDAC is a porin-type protein apparently involved in porphyrin transport across the membrane. It is therefore possible to speculate that in R. sphaeroides 2.4.1 TspO interacts with the major outer membrane protein, which is shown to bind porphyrins and benzodiazepines with high affinity, thus affecting biosynthesis and/or assembly of the photosynthetic antenna complexes through an as yet unknown mechanism.

Analysis of the amino acid sequence of TspO does not predict any potential DNA-binding domains in this polypeptide. Therefore, the pathway and mechanism of signal transmission from the membrane-localized TspO to the DNA-bound transcriptional effector is not clear. It would seem reasonable to assume that there is in addition to TspO an inner membrane-localized component of this pathway. The nature of this hypothetical protein is unknown. Nor do we know how this protein might interact with the repressor component of this pathway. We do know, however, that this pathway does not directly affect puf operon expression, but may do so indirectly through Crt and Bchl availability. Therefore, in future studies we plan to determine the mechanism(s) by which TspO senses changes in $O_2$ level, the likely response regulator with which it interacts and to further investigate the functional and structural relationships between TspO and the mammalian PBR.

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4. J. Zielstra-Rylls and S. Kaplan, manuscript submitted for publication.
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