Expression of an Olfactory Receptor in *Escherichia coli*: Purification, Reconstitution, and Ligand Binding†

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ABSTRACT: An olfactory receptor has been expressed in bacterial cells as a fusion protein with glutathione *S*-transferase (GST). Overexpression of receptor protein yielding about 10% of the cell protein was achieved with mutants lacking the N-terminus and the first transmembrane region or with mutants carrying three positively charged residues in the first intracellular loop. The overexpressed fusion protein accumulated in inclusion bodies and could be solubilized in detergent. It was purified by metal chelation chromatography based on a C-terminal 6-histidine tag, and the GST portion was removed after proteolytic cleavage. The purified receptor was reconstituted into lipid vesicles and specific binding of odor ligands was shown by photoaffinity labeling and tryptophan fluorescence measurements. Thus, for the first time, an odorant receptor/ligand pair becomes available in large amounts for biophysical and screening studies.

The olfactory system of vertebrates recognizes and discriminates thousands of odorous compounds. The enormous capacity of chemical recognition is probably based on a large family of olfactory receptors comprised of several hundreds or a thousand subtypes (Buck & Axel, 1991). These receptors are predicted to contain seven transmembrane segments, consistent with the view that odorant signals are transduced via G-protein-coupled cascades in olfactory sensory neurons (Reed et al., 1992; Breer et al., 1994). Different receptor types are presumed to be tuned to odorous compounds with distinct chemical determinants, and odor coding may be accomplished by expression of one or a few receptor types in individual chemosensory neurons (Lancet et al., 1987; Shepherd, 1994; Mori & Yoshihara, 1995). Soluble ligand-specific odorant binding proteins have also been implicated in the decoding of odor and in signal transduction (Pelosi, 1994; Du & Prestwich, 1995; Prestwich et al., 1995).

Most of the current information on olfactory receptors is based on genes and mRNA studies; the knowledge about the nature of the actual receptor proteins is still very limited. Using the baculovirus/Sf9-system several receptor types have been heterologously expressed (Raming et al., 1993; Gat et al., 1994; Nekrasova et al., 1996). Attempts have been made to determine the ligand specificity of individual receptor types (Raming et al., 1993; Meinken, 1995); however, the functional assays used would be impractical to implement for screening large arrays of odor ligands and receptor types. Several G-protein-coupled receptors (GPCRs)[1] have been expressed in bacterial cells, and when inserted in the inner membrane displayed all the ligand binding properties observed in mammalian cells. However, high-level expression of membrane proteins, and especially of GPCRs, in bacteria appear to be difficult (Schertler, 1992; Grisshammer & Tate, 1995), probably due to the fact that the cells cannot tolerate large amounts of the foreign protein within their membranes. Milligram quantities of purified membrane protein which are required for biophysical studies or attempts to crystallize the protein have only been obtained by overexpressing the protein in *E. coli* as inclusion bodies (IBs), i.e., not integrated in the cell membrane. Proteins derived from IBs are nonnative and have to be folded to the native state. This has been achieved for many soluble, but only for a few membrane proteins (Matsuyama et al., 1992; Fiermonte et al., 1993; Efimov et al., 1994); for an overview, see Grisshammer and Tate (1995).

As far as we are aware, no GPCR has previously been expressed at high level in bacteria. Purification of bacterially expressed GPCRs has only been reported in one case (Tucker & Grisshammer, 1996). In this recent study, microgram amounts of the neurotensin receptor were purified from *E. coli*. Because of the low expression level, the protein was affinity-tagged, and purification was achieved in two highly efficient chromatographic steps.

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[1] Abbreviations: BDCA, 4-benzoyldihydrocinnamaldehyde; DTT, dithiothreitol; GPCR, G protein-coupled receptor; GST, glutathione *S*-transferase; IB, inclusion body; OR, olfactory receptor; PC, phosphatidylcholine; PCC, pyridinium chlorochromate; PMSF, phenylmethylsulfonyl fluoride; sarcosyl, *N*-lauroylsarcosine; TBS, Tris-buffered saline; TM, transmembrane.

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In the present study, an olfactory receptor has been overexpressed as a fusion protein in *E. coli* cells. It was solubilized from inclusion bodies and purified by affinity chromatography. The purified receptor displayed specific interaction with odor ligands.

EXPERIMENTAL PROCEDURES

Materials. Oligodeoxyribonucleotides were synthesized by services at the University Stuttgart−Hohenheim or at the Karolinska Institute, NOVUM, Huddinge. *E. coli* BL21 was from Novagen (ams, Täby, Sweden). The expression vectors pGEX 2a, 2b, and 2c containing an extended multiple cloning site in three frames C-terminal to GST and a thrombin cleavage site were derived from pGEX 2T (Pharmacia). GST antiserum was from Santa Cruz (Santa Cruz, CA, USA). Ni-NTA agarose was from Qiagen (Chatsworth, CA, USA), Extractigel from Pierce (Rockford, IL, USA), digitonin from Calbiochem (La Jolla, CA, USA). A digitonin stock solution was prepared by dissolving 20 mg of digitonin in 1 mL of boiling distilled water, 10 min continued heating to 95 °C, storing at 4 °C over night, centrifuging for 10 min at room temperature (20000*g*) and discarding the insoluble material. The supernatant was used as a "2% digitonin stock solution". All other chemicals were from Sigma (Stockholm, Sweden) or Merck (Darmstadt, Germany) and of the highest purity available.

Construction of Expression Vectors Encoding GST−OR 5 Fusion Proteins. All DNA-related methods were performed according to standard procedures (Sambrook et al., 1989). GST was fused to various parts of OR 5 that are predicted to be extramembraneous, that is, the N-terminus or loops connecting the transmembrane regions. The double-stranded form of the M13 phage containing the OR 5 cDNA was used as a template in PCR and for producing an *Nco*I-*Xba*I fragment. The construct GST−OR 5_{118} was made by isolating an *Nco*I-*Xba*I fragment from the M13−OR 5 vector. (Note: the indexed number is the number of the first OR 5 amino acid residue present in the GST fusion). All other fusions were made by PCR, introducing a *Kpn*I site at the 5′ end of the OR 5 sequence and an *Xba*I site at the 3′ end. The PCR product was ligated in-frame into the multiple cloning site of the pGEX vector after cleaving with the respective restriction enzymes. Constructs containing a 6-histidine tag were obtained by using an extended 3′ oligonucleotide in the PCR reaction that contained the 6 His codons, a stop codon and a *Sma*I site. The mutant GST−OR 5 D52RRRN was made by bidirectional PCR from D52 with overlapping primers. These contained the mutation RRRN including a *Sal*I site (cgt cga cgg aat). The fragments were cleaved with *Sal*I and *Kpn*I or *Xba*I, respectively, and cloned simultaneously into pGEX 2c cut *Kpn*I/*Xba*I. All plasmids were transformed into *E. coli* BL21.

Growth of Cells and Protein Purification. 100 mL of LB-Ampicillin (100 μg/mL) was inoculated with 2 mL of an overnight culture in the same medium and grown at 37 °C until the A_{600} reached 0.8. 100 μM isopropyl β-D-thiogalactoside (IPTG) was added, and growth was continued for 4 h. Cells were harvested by centrifugation at 2000*g* for 10 min and resuspended in 10 mL of 10 mM Tris, 25% sucrose, pH 7.5, and then 1 mM EDTA and 0.25 mg of lysozyme was added and cells were stirred on ice for 1 h. 5 mM dithiothreitol (DTT) and 200 μM phenylmethylsulfonyl fluoride (PMSF) were added, and spheroplasts were lysed by sonication with a tip sonicator (2 min). Insoluble material was collected by centrifugation at 150000*g* for 2 h. The pellet was resuspended in 20 mL of TBS (Tris-buffered saline: 20 mM Tris, pH 7.5, 150 mM NaCl), 1 mM DTT, and centrifuged as above. Finally, the pellet was resuspended in 1 mL of TBS, 1 mM DTT, and stored at −20 °C. Solubilization was achieved by addition of 4 mL TBS containing 1.25% (w:v) *N*-lauroylsarcosine (sarcosyl) and sonication in a bath type sonicator for 1 min at 0 °C. 20 mL of TBS, 0.2% digitonin were added and the sample was centrifuged at 20000*g* for 10 min. Purification was carried out on a Pharmacia FPLC system. The supernatant was loaded onto a Ni-NTA agarose column (4 mL) previously equilibrated with TBS (0.3 mL/min). The column was washed with 20 mL of TBS/0.1% digitonin (1 mL/min), and the protein was eluted in a 20 mL gradient of 0−0.3 M imidazole in the same buffer. The fusion protein eluted as a single peak as detected by monitoring the A_{280}. The corresponding fractions were pooled and dialyzed against 50 mM Tris pH 8.5, 0.2 mM DTT for 4 h. 10 units of thrombin and 0.5% sarcosyl were added, and the sample was incubated for 15 h at 20 °C. After the protein was diluted to 25 mL with TBS, 0.2% digitonin, 0.5% sarcosyl, the sample was rechromatographed on a Ni-NTA column as above. This time, an additional washing step with 10 mL TBS, 1% N-lauroylsarcosine preceded the TBS/digitonin washing.

Reconstitution. 8 mg of egg phosphatidylcholine (PC), 2 mg of phosphatidylglycerol from egg PC, and 20 mg of dodecyl β-D-maltoside were dissolved in chloroform in a glass flask and the solvent was evaporated in an N_2-stream followed by drying at reduced pressure for 1 h. The purified protein (2 mg) was added, and the lipid/detergent film dissolved by gentle shaking at 4 °C. The mixture was passed over an Extractigel column (5 mL) equilibrated with TBS, 1 mM DTT, and the turbid fractions were collected and centrifuged at 150000*g* for 2 h. The pellet was resuspended in 20 mL TBS, 1 mM DTT, and centrifuged as before. Finally, the pellet was resuspended in 1 mL of the above buffer.

Gel Electrophoresis, Immunoblotting, and Protein Determination. Gel electrophoresis was performed on 15% Laemmli gels, Western blotting was done using an ECL kit (Amersham) with anti-GST, anti-Lep, or anti-OmpA serum as the first antibody. The serum dilution was between 1:500 and 1:2000. Protein concentration was determined with a BCA protein kit (Pierce) using bovine serum albumin as a standard.

Pulse−Chase Labeling of the Expressed Proteins. Pulse−chase labeling was done as described (Whitley et al., 1994), except that protein expression was induced by adding 0.5 mM IPTG. The labeled proteins were immunoprecipitated with a mixture of GST and OmpA antiserum and subjected to SDS gel electrophoresis. The radioactive proteins were visualized by fluorography.

Synthesis of a Photoreactive Lilial Analogue. The tritiated photoaffinity label [^{3}H]-4-benzoyldihydrocinnamaldehyde ([^{3}H]BDCA) used in this study was chosen because it shares large parts of its molecular structure with the odorant lilial (4-*tert*-butyl-α-methyldihydrocinnamaldehyde, see Figure 5A) that was shown to activate the odorant receptor OR 5 expressed in Sf9 cells (Raming et al., 1993).

4-Benzoyldihydrocinnamaldehyde. 20 mg (0.079 mmol) of 4-benzoyldihydrocinnamic acid (Olszewski et al., 1995) was dissolved in 6 mL of THF and cooled to 0 °C, 2.0 M borane−dimethyl sulfide complex in THF (18 mg, 0.236 mmol) was added, and the mixture was stirred at room temperature for 4.5 h. Solvents were removed in vacuo, and the residue was dissolved in CH_2Cl_2 (5 mL) and oxidized by stirring for 3 h with 153 mg (0.70 mmol) of PCC (pyridinum chlorochromate) added in two portions. The mixture was diluted with diethyl ether, filtered through Florisil, concentrated in vacuo, and purified on 63−200 μm SiO_2 using 2:1 hexane:ethyl acetate to give a colorless oil in 77% yield.

[^{3}H]BDCA. A 6 mCi sample of [^{3}H]BZDC-NHS ester (Dupont NEN) was dissolved in 1 mL of 1:1 methanol:10% NaOH, stirred 8 h at room temperature, acidified with HCl, and extracted three times with CH_2Cl_2. Combined extracts were dried ($MgSO_4$), concentrated and purified on SiO_2, with monitoring by radio-TLC (2:1:1 hexane:ethylacetate:methylene chloride containing 1% HOAc). The purified [^{3}H]-BDCA acid was reduced with excess borane−dimethyl sulfide complex as described above for the unlabeled material. The mixture was concentrated, oxidized with PCC in CH_2Cl_2 and worked up with ether as described above. The crude [^{3}H]BDCA was purified on SiO_2 with monitoring by radio-TLC. All concentration steps with labeled material were performed using a stream of argon. The product (126 μCi) has an identical R_f on TLC with the unlabeled material. [^{3}H]BDCA was placed in three ampules, and each sample was dissolved in 50 μL of 1:1 heptane:toluene under argon. Ampules were sealed and stored at −20 °C prior to use.

Photolabeling of Receptor Protein. An aliquot of [^{3}H]-BDCA diluted in hexane and an ethanolic dilution of the cold BDCA (ethanol alone was used in the "no competitor reaction") were added to quartz tubes. After removal of the solvent by evaporation under nitrogen gas, the quartz tubes were transferred to an ice water bath and 30 mL of TBS with 1 mM DTT containing purified receptor protein or lipid vesicle solution with reconstituted receptor (1.2 μg of protein each) were added to redissolve the ligand and the competitor. After incubation for 20 min on ice with intermediate vortexing, the reaction mixtures were exposed to ultraviolet light (366 nm) for 20 min; quartz tubes were illuminated from the top and cooled by ice water from the bottom. Following the illumination aliquots of the samples (25 μL) were subjected to SDS polyacrylamide gel electrophoresis. Proteins were visualized by Coomassie-blue staining and the gel was subsequently incubated for 30 min in acetic acid containing 10% 2,5-diphenyloxazole. Drying of the gel was followed by fluorography on Fuji RX film exposed at −70 °C for 1−4 months.

CD Measurement. CD spectra were recorded on a Jasco J-720 spectropolarimeter. Purified receptor was diluted into 50 mM sodium phosphate pH 7.0, 0.1% digitonin to a protein concentration of 3 μg/mL. CD spectra between 195 and 260 nm of the sample and the buffer were recorded separately and the background signal was subtracted.

Measurement of Tryptophan Fluorescence. Purified olfactory receptor in digitonin was concentrated by ultrafiltration to a protein concentration of 10 mg/mL using an Amicon ultrafiltration cell equipped with a PM 10 membrane. 10 μL of the concentrated sample were diluted into 3 mL of 0.02% digitonin in TBS. The tryptophan fluorescence spectrum was recorded on a Perkin Elmer LS 50 spectrofluorimeter (excitation 280 nm, emission 300−360 nm). Lilial was added to a final concentration of 10−1000 μM from 10 or 100 mM stock solutions in DMSO (final DMSO concentration was below 1%), and spectra were obtained as above. Similar experiments were performed on receptor diluted into 0.4% SDS in TBS, on 30 nM tryptophan in TBS/0.02% digitonin and on 40 μg/mL lysozyme in TBS/digitonin. The relative fluorescence intensity measured at the emission maximum (328 nm) was corrected for unspecific quenching by dividing it with the relative intensity measured in the control experiment with tryptophan at the same wavelengths.

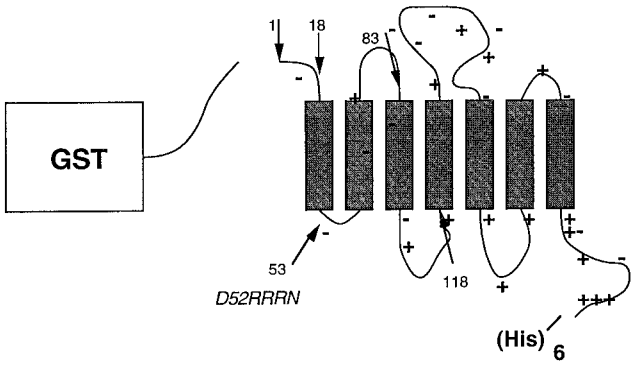

FIGURE 1: Scheme of the constructs used in this paper. The localization of the amino acid residues in OR 5 to which GST was fused are indicated by arrows, as well as the position of the D52RRRN mutation. Charged residues in OR 5 are depicted by "+" and "−".

Data were evaluated assuming that the relative fluorescence intensity of receptor without ligand is 1 and that of the receptor with bound ligand is I_1. It was further assumed that the concentration of unbound ligand [L] is equal to the concentration of total ligand, as the concentration of OR 5 was only around 100 nM. The binding equation can then be written as $K_D = [L](I - I_1)/(1 - I)$, which gives $I = (K_D + I_1[L])/([L] + K_D)$, with K_D being the dissociation constant of the receptor−ligand complex. The data were fitted to the latter equation by a least square fit, leaving the parameters K_D and I_1 free.

RESULTS

Expression of GST−OR 5 Fusion Proteins. The various fusion constructs between GST and OR 5 are schematically shown in Figure 1. Expression of fusion proteins containing either the full receptor sequence (GST−OR 5_1) or the sequence without the first 17 residues (GST−OR 5_{18}) could not be detected on either Coomassie-stained gels or on Western blots (Figure 2A,B). When GST was fused to one of the predicted loops connecting the transmembrane (TM) segments (GST−OR 5_{118}, GST−OR 5_{83}, GST−OR 5_{53}), however, protein expression was high and accounted for about 10% of the cell protein as estimated from scanning of the stained gel. In addition, Western blot analysis using anti GST-antiserum revealed immunoreactive bands at the size expected for the GST−OR 5 fusion proteins (Figure 2B). Cell growth was correlated to protein expression in the sense that cells containing the non-expressed constructs stopped growing immediately after IPTG induction.

Since there is no obvious reason why the first TM segment should prevent translation of the proteins, low-level expres-

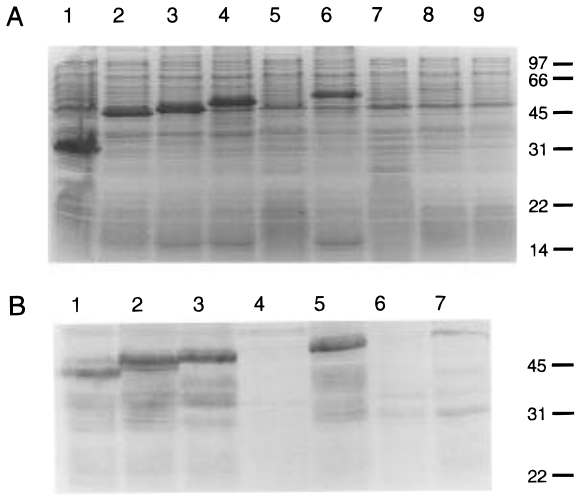

FIGURE 2: Coomassie staining and immunoblot of different GST fusion proteins. Cells were induced with IPTG for 4 h and subjected to SDS−PAGE. (A) Coomassie staining. Lane 1, GST; lane 2, GST−OR 5_{118} (notation: indexed numbers refer to the first amino acid of OR 5 present in the fusion protein); lane 3, GST−OR 5_{83}; lane 4, GST−OR 5_{53}; lane 5, GST−OR 5_{18}; lane 6: GST−OR 5_{18} D52RRRN; lane 7, GST−OR 5_1; lane 8, GST−OR 5_1 D52RRRN-His_6; lane 9: GST−OR 5_1-His_6. In lanes 2, 3, 4, and 6 the fusion protein with an apparent molecular weight of 45−50 kDa is the prominent band. (B) Immunoblot. Lane 1, GST−OR 5_{118}; lane 2, GST−OR 5_{83}; lane 3, GST−OR 5_{53}; lane 4, GST−OR 5_{18}; lane 5, GST−OR 5_{18} D52RRRN; lane 6, GST−OR 5_1-His_6; lane 7, GST−OR 5_1 D52RRRN-His_6. In all lanes except 4 and 6 the fusion protein is detected by anti-GST serum.

sion and block of cell growth may be due to an inappropriate membrane interaction. Bacterial and eucaryotic membrane proteins differ in the distribution of positive charges contained in the loops on the *cis*- and the *trans*-sides of the membrane. While positive charges are enriched on the *cis*-side in proteins from all organisms analysed so far, they occur at a low frequency in periplasmic loops of bacterial proteins but at an average frequency in luminal loops of eucaryotic proteins as concluded from a recent statistical study of GPCRs (Wallin & von Heijne, 1995). This observation suggests that the insertion mechanism may be different for these two groups. From experimental studies it is also known that short loops with several positive charges are difficult if not impossible to translocate to the periplasmic side in bacteria (Nilsson & von Heijne, 1990; Gafvelin & von Heijne, 1994). In the case of OR 5, the loop between TM 1 and 2 is very short (seven residues) and does not contain any positive charges while all other loops are longer and contain at least one positive charge. It is therefore conceivable that the first loop is translocated, resulting in an inverted orientation of TM 1 and TM 2. The rest of the protein will probably not be inserted at all and the translocation machinery might be blocked.

This hypothesis was tested by constructing the mutant GST−OR 5_{18} D52RRRN which contains three positive charges in the loop connecting TM 1 and 2, aimed to block translocation of this loop. Indeed, in contrast to the GST−OR 5_{18} fusion, this mutant protein was expressed at a level similar to the constructs lacking TM 1 (GST−OR 5_{53}, Figure 2A,B). The mutation increased the expression level by at least a factor of 100, as estimated from the lowest detectable amount of fusion protein on Western blots (data not shown). A D52RRRN mutant containing the whole receptor sequence

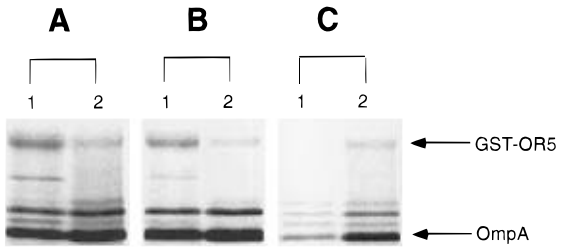

FIGURE 3: Pulse-labeling of constructs containing all seven TM regions. The labeled protein was immunoprecipitated with a mixture of GST and OmpA antibodies. OmpA is produced continuously in growing cells and is therefore an indicator of cell growth. (A) 1 min induction, 1 min labeling. (B) 1 min induction, 1 min labeling, 20 min chase. (C) 40 min induction, 1 min labeling. Lane 1, GST−OR 5_{18}; lane 2, GST−OR 5_{18} D52RRRN. OmpA and GST−OR 5 fusions are depicted by arrows.

(including residues 1−17) was also expressed, though at an approximately 5−10 times lower level (Figure 2B, lane 7).

To investigate why positive charges lead to a much higher expression rate of the protein, a series of pulse labeling and pulse chase experiments were performed using constructs containing TM1 (GST−OR 5_{18}) and either the original D52 or the D52RRRN mutation. Cells were grown on a medium lacking methionine and labeled for 1 min with [^{35}S]-methionine. This was done either 1 min or 40 min after induction with IPTG. In a third experiment, cells were labeled 1 min after induction as described above and chased for 20 min with cold methionine. Labeled protein was immunoprecipitated with a mixture of OmpA and GST antiserum and subjected to SDS−PAGE and fluorography. OmpA is produced continuously in growing cells and was used as an internal control indicative of cell growth.

As shown in Figure 3A, for both constructs expression was detectable in the second minute after IPTG induction. However, 40 min after induction the D52 construct was no longer expressed, and OmpA expression was very low at this time (Figure 3C). In contrast the D52RRRN mutant was still expressed after 40 min. The chase experiment shows that there is no obvious difference in stability between the two constructs (Figure 3B). Similar results were obtained with the full-length receptor (GST−OR 5_1, not shown)

We conclude from these experiments that expression of constructs containing TM 1 and the wild type sequence in the first loop is toxic for the cells and stops protein expression as well as cell growth.

Purification and Reconstitution of OR5$_{18}$ D52RRRN-His$_6$. A Triton X-100 insoluble fraction that contained virtually all of the overexpressed protein was obtained from the broken cells by ultracentrifugation (Figure 4A). Immunoblotting revealed that this fraction also contained some of the Lep and OmpA which are marker proteins for the inner and outer membrane [not shown, see Dalbey (1991) and Freudl et al. (1986)]. These proteins could only be extracted after prolonged incubation (24 h at 4 °C with Triton X-100/EDTA, Figure 4B).

The fusion protein was solubilized in 1% *N*-lauroyl sarcosine (Frangioni & Neel, 1993), and 0.2% digitonin was added. The GST domain should allow for affinity purification of the fusion protein using glutathione sepharose (Smith & Johnson, 1988). However, it was found that only 10% of the solubilized protein bound to the matrix, probably because the GST domain was misfolded. Therefore the hexahistidine tagged fusion protein was expressed and

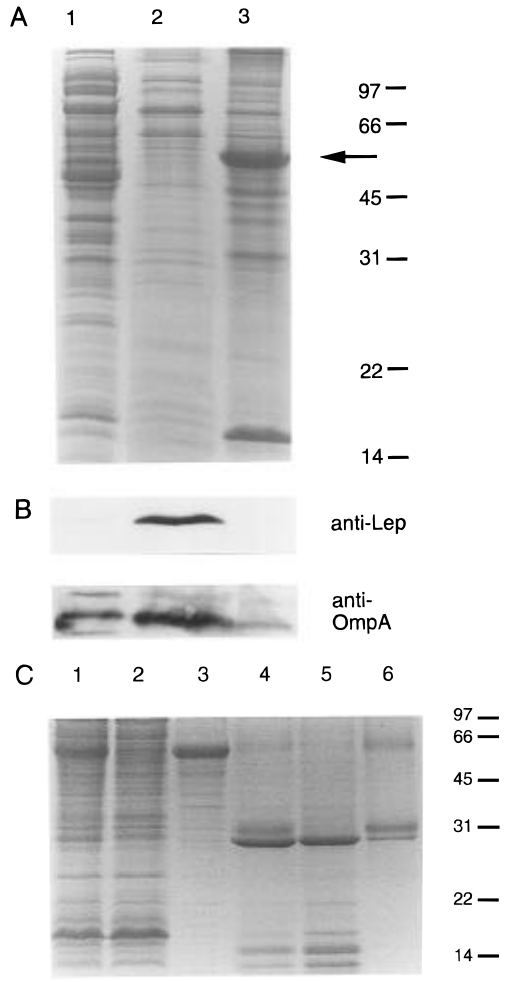

FIGURE 4: Purification of GST−OR 5_{18} D52RRRN-His_6. (A) localization of the overexpressed protein. Lane 1, soluble fraction (supernatant of ultracentrifugation); lane 2, Triton X-100 (1%)/EDTA (0.1 M) extract of the insoluble fraction (24 h extraction at 4 °C); lane 3, remaining insoluble fraction. Each lane contains the protein from 100 μL of cell culture. (B) Western blot with Lep and OmpA antibodies used as markers for the inner and outer membrane of *E. coli*. Lanes as in A. Most of Lep and OmpA is found in the Triton X-100/EDTA extract. (C) Different steps of purification. Lane 1, solubilized protein; lane 2, Ni-NTA agarose flow-through; lane 3, Ni-NTA eluate; lane 4, thrombin-cleaved fusion protein; lane 5, second Ni-NTA flow-through; lane 6, second Ni-NTA agarose eluate. The GST portion of the cleaved protein (26 kDa) runs as a highly stained band at its actual MW, while the receptor (34 kDa) runs as a diffuse and weakly staining band with an apparent molecular mass of 30 kDa. Note that the molar ratio of GST and OR5 after cleavage should be 1 (lane 4). Hence it appears that GST stains much stronger than OR5.

purified on a Ni-NTA agarose column. The GST fusion was maintained as no expressed protein could be detected if the mutant receptor was expressed alone. The protein was eluted in a gradient of 0−0.3 M imidazole. By this approach, most of the contaminants were removed and sarcosyl could be replaced with digitonin, a detergent widely used to stabilize GPCRs (Hulme, 1990).

Following thrombin cleavage the receptor protein was separated from the GST portion as well as from thrombin by a second Ni-NTA affinity purification. Sarcosyl had to be added in this step since otherwise the GST portion strongly associated with the bound receptor and could not be washed off the column. Even under these conditions, some GST could not be removed and was present in the final eluate (Figure 4C). Sarcosyl was replaced with digitonin before eluting the receptor. From 1 L of cell culture, about 20 mg of purified receptor could be obtained.

The purified receptor was reconstituted into lipid vesicles composed of phosphatidycholine:phosphatidylglycerol (4:1) by adding the lipid dissolved in dodecyl maltoside and removing the detergent on an Extractigel column. Again digitonin was chosen as detergent since it has been shown to keep many GPCRs in the native state (Hulme, 1990). Indeed, all other detergents tested (dodecyl maltoside, Triton X-100, cholate, deoxycholate, CHAPS) led to aggregation of the protein. This was reflected in the appearance of oligomers on SDS gels after prolonged incubation that did not occur in the presence of digitonin. The lipid vesicles were washed by ultracentrifugation and the receptor protein was found associated with the lipid as judged from SDS−PAGE (not shown).

Photoaffinity Labeling of Purified Olfactory Receptor. As a first approach to determine the functional integrity of a heterologously expressed receptor protein usually the interaction with specific ligands is assessed. This turns out to be rather difficult for odorant receptors, since binding assays based on centrifugation of reconstituted proteolipid vesicles are not possible because the hydrophobic odor ligands will partition into the lipid fraction. Similarly, in approaches using equilibrium dialysis the ligands will associate with detergent micelles that cannot pass the dialysis membrane. These difficulties have been in part overcome for soluble odorant binding proteins using a novel "reverse phase" binding assay (Du & Prestwich, 1995).

An alternative solution is the use of photoreactive, radioactively labeled ligands which can be covalently coupled to the receptor protein and detected by fluorography of SDS gels. For example, the binding site for an insect pheromone component was recently identified by photoaffinity labeling of a recombinant soluble pheromone binding protein (Du et al., 1994).

In order to analyse if the purified and reconstituted receptor protein is capable to interact with odorous ligands, photoaffinity labeling experiments were performed using tritiated BDCA, a benzophenone containing analogue of the odorant lilial (Figure 5A). Lilial has been shown to activate the receptor OR 5 (Raming et al., 1993; Meinken, 1995). The benzophenone protophore has been used in identification of ligand binding sites in a variety of soluble and membrane associated proteins (Dormán & Prestwich, 1994; Prestwich et al., 1996). The results in Figure 5B demonstrate that the recombinant receptor protein reconstituted in lipid vesicles binds the analogue [^{3}H]BDCA in a concentration dependent manner. The specificity of receptor protein labeling was emphasized in experiments demonstrating that increasing concentrations of unlabeled ligand accomplished a dose-dependent displacement of the radioactive ligand (Figure 5B,C). Importantly, the odorant lilial itself also showed a concentration dependent displacement of the radiolabeled probe (Figure 5D). As can be deduced from Figure 5B, the BDCA concentrations used in these experiments were not saturating. This might be the reason why BDCA is not displaced compeletely by a 1000-fold excess of cold ligand as would be expected under saturating conditions.

CD Measurements. The CD spectrum of purified OR 5_{18}-His_6 is shown in Figure 6A. It shows two minima at 208 and 222 nm typical for α-helical secondary structure.

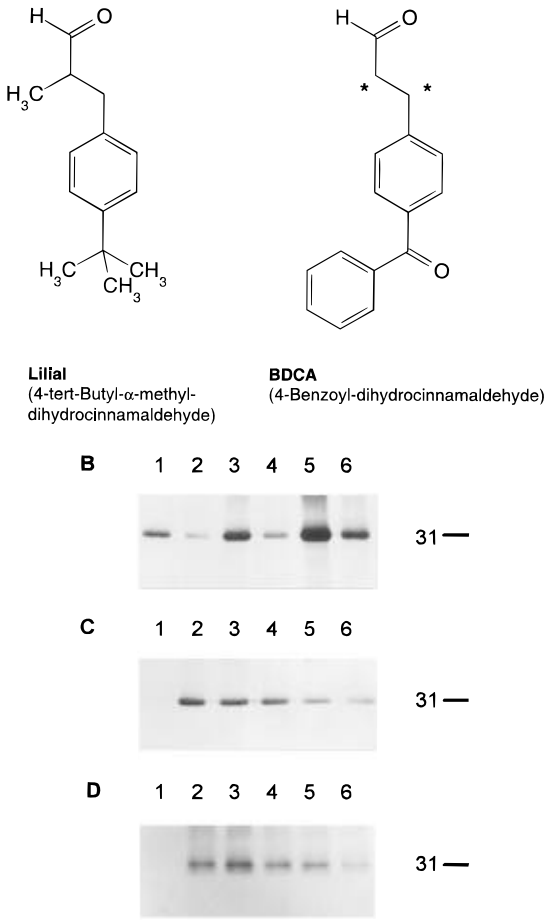

FIGURE 5: Photoaffinity labeling of recombinant olfactory receptor protein using [^{3}H]BDCA. Lipid vesicles containing reconstituted or purified receptor protein were incubated with [^{3}H]BDCA for 20 min in the absence or presence of competitor and subsequently exposed to ultraviolet light. Samples of each incubation mixture were subjected to SDS−PAGE; dried gels were exposed to X-ray films. (A) Structure of BDCA and lilial. The position of the ^{3}H in labeled BDCA is indicated by asterisks (*). (B) Labeling of reconstituted receptor protein using different amounts of [^{3}H]BDCA. Lanes 1 and 2, 200 nM; lanes 3 and 4, 400 nM; lanes 5 and 6, 1 μM of [^{3}H]BDCA. Samples in lanes 2, 4, and 6 were labeled in the presence of a 1000-fold excess of unlabeled BDCA. (C) Labeling of reconstituted receptor protein in the presence of increasing amounts of competitor. The final [^{3}H]BDCA concentration was 200 nM. Lane 1, the sample was not illuminated; lane 2, no competitor was added; lanes 3−6, labeling in the presence of a 25-, 100-, 250-, and 1000-fold excess of unlabeled BDCA. (D) Same labeling experiment as in C but using pure receptor protein and lilial as competitor.

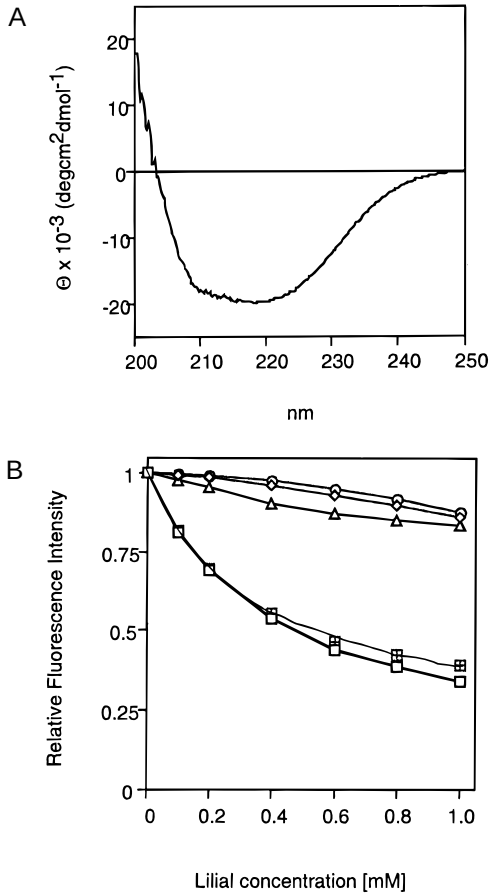

FIGURE 6: (A) CD spectrum of purified receptor solubilized in digitonin. (B) Tryptophan fluorescence upon lilial binding. The relative intensity of the tryptophan fluorescence is plotted versus lilial concentration. (□) OR 5 in TBS/digitonin. (△) OR 5 in TBS/SDS; (○) tryptophan in TBS/digitonin; (◇) lysozyme in TBS/digitonin; (crossed boxes) OR 5 in TBS/digitonin corrected by the value obtained with tryptophan in TBS/digitonin. The fitted binding curve is overlaid these data. From the fit parameters an apparent K_D of 340 μM and an end value of 0.18 for the relative fluorescence intensity at high substrate concentration is calculated.

Secondary structure prediction using the program k2d (Andrade et al., 1993; Merelo et al., 1994) returned 60% α-helix, 13% β-sheet, and 27% random coil content. This is consistent with the view of OR 5 containing about 160 residues (52%) within its seven predicted α-helical transmembrane segments, as suggested from alignment of olfactory receptor sequences (Buck & Axel, 1991; Raming et al., 1993).

Tryptophan Fluorescence Measurements. The sequence of OR 5 contains a single tryptophan in the center of the putative TM 4 region. This position could be closely related to the proposed ligand binding sites in GPCRs (Strader et al., 1994). We therefore investigated if tryptophan fluorescence changed upon ligand binding. Fluorescence spectra of purified OR 5 solubilized in 0.02% digitonin/TBS were recorded at increasing lilial concentrations. It was found that the fluorescence intensity at the emission maximum of 328 nm decreased upon addition of lilial in a concentration dependent manner. In control experiments, lilial was added either to receptor solubilized in 0.4% SDS instead of digitonin, to tryptophan dissolved in the above digitonin buffer, or to a control protein (lysozyme) in digitonin/TBS. In all three cases, a much smaller decrease in fluorescence, probably due to light absorption of lilial, was observed. The data were corrected for absorption and fitted by a least-square fit to the theoretical curve expected in the case of specific binding (Figure 6B). The data suggests that specific binding occurs with an apparent dissociation constant of 340 μM. This appears to be a rather low affinity when compared to other GPCR/ligand pairs. However the ligand probably has to enter the binding site of the receptor from the water phase. Due to its hydrophobicity, lilial will distribute between detergent micelles and water, and the effective ligand concentration in water will be much lower than the total concentration. Because of this artificial situation, the apparent K_D value is not meaningful in terms of the odor detection threshold *in vivo*, when the ligand is transported across the water barrier by odorant binding proteins (Pelosi, 1994) and likely presented in bound form to the GPCR (Prestwich et al., 1995).

Table 1: Effect of Various Odorants on Tryptophan Fluorescence[a]

ligand	relative signal[b] ($c = 10\ \mu$M)	$K_{D,app}$ (μM)
lilial	1.00	340
lyral	0.93	42
citralva	0.01	$>10^5$ [c]
ethylvanillin	0.50	$>10^5$ [c]

[a] The data were obtained as described in Figure 6B and corrected using lysozyme as a control. [b] The relative signal is defined as the decrease in relative fluorescence as compared to lilial $((I - I_0)/I_0)_{ligand}/((I - I_0)/I_0)_{lilial}$. [c] When the data were fitted to the theoretical curve as in Figure 6, negative values for I_1 were obtained, i.e., the fit did not give any meaningful numbers.

However, the fluorescence measurements may give an estimate for the proportion of receptor active in ligand binding. At high ligand concentrations, the fluorescence intensity decreased by 80% indicating that at least 80% of the receptor protein changes fluorescence due to interaction with lilial.

To further investigate the specifity of OR 5 toward various odorants, the same experiments were performed with lyral (an odorant stimulating second messenger response), ethylvanillin, and citralva [the latter odorants are known to be inactive, see Raming et al. (1993)]. Again, lysozyme was used as a control. The corrected values for K_D and the relative decrease in fluorescence at low ligand concentrations (10 μM) are summarized in Table 1. Lyral had a similar effect on OR 5 as lilial, but not on lysozyme. The curves obtained with citralva and ethylvanillin on the other hand could not be fitted to meaningful values with the theoretical function. Both citralva and ethylvanillin had a similar effect on the fluorescence of OR 5 and lysozyme which probably resulted from absorption of light.

DISCUSSION

So far no GPCRs and only a few other eucaryotic membrane proteins have been expressed at high level in *E. coli*. It has been suggested that this may be due to the fact that cells cannot tolerate large amounts of foreign proteins within their membrane (Grisshammer & Tate, 1995). In this study, a mutated olfactory receptor carrying three positive charges in the first intracellular loop has been overexpressed as a fusion protein with GST in bacterial cells. A similar construct with the original receptor sequence was expressed at a much lower level. The degree of protein expression corresponded to the growth rate of the microorganisms; cells carrying a wild type derived construct stopped growing immediately after induction of protein synthesis while cells harboring the mutant construct continued to grow after induction of expression.

The notion that lack of expression of the wild type receptor may be due to partial or improper insertion of the heterologously expressed protein in the bacterial membrane was supported by the finding that the highly expressed mutated protein was targeted into inclusion bodies. All these results suggest that insertion of overexpressed foreign proteins into the bacterial membrane may be toxic to the cells and thus renders low expression levels. Although the heterologously expressed fusion protein was in an aggregated state and certainly misfolded, the specific labeling of reconstituted olfactory receptor protein indicates that detergent exchange and embedding into lipid environment has established at least part of the original tertiary structure; refolding upon reconstitution in liposomes has recently been described for several other membrane proteins (Fiermonte et al., 1993; Efimov et al., 1994). This view is supported and extended by the CD spectrum and the fluorescence quenching studies. In addition, these results suggest that the quenching approach may provide a convenient procedure for a preliminary assessment of receptor/ligand interaction.

The data presented here are to our knowledge the first direct documentation of an olfactory receptor/ligand complex. Lilial, lyral (Raming et al., 1993), and BDCA (R. Schwandner, unpublished data) have previously been shown to activate the second-messenger pathway in Sf9 cells expressing OR5. In this study, we show that binding of lilial and lyral is saturable and in agreement with the predicted binding curve. We also show that binding of BDCA is concentration dependent and specific in the sense that it can be displaced not only by cold BDCA, but also by lilial. As an extension of our studies, we will in future investigate G protein activation by the receptor/ligand complex.

The high level expression of a truncated olfactory receptor construct that upon reconstitution interacts with odor ligands is a novel finding. The easily manipulated bacterial system may allow for large-scale screening programs that may be required to unravel the specific interrelationship between the array of olfactory receptor types and the numerous odor ligands. Expression of G protein-coupled receptors in microbial cells has the drawback that there is no biochemical or cellular response to agonists, due to the lack of appropriate transduction systems. Nevertheless it may be possible to use these host cells for producing high quantities of various olfactory proteins; this could be a first step toward a reconstitution of the olfactory transduction cascade *in vitro*. Future studies will also show whether other eucaryotic membrane proteins can be engineered such that overexpression in bacteria is possible.

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