3 Heterologous expression and purification of the ET_B receptor in *Pichia pastoris*

3.1 Introduction

3.1.1 Main characteristics of *Pichia pastoris*

The methylotrophic yeast *Pichia pastoris* has been developed as an excellent host for the large-scale expression of proteins from different sources (Gellissen, 2000). Typically, the expression of a foreign gene is tightly controlled by the host alcohol oxidase-1 (AOX1) promoter, which is inducible by methanol but repressed when the cells are cultured on other carbon sources such as glucose and glycerol. Therefore, transformed *P. pastoris* cells are first grown to high densities in high concentrations of glycerol or glucose, allowing production of more than 100 g of dry cell weight/l. The production of foreign protein is induced by switching the carbon source to methanol. In the presence of methanol, promoter activity is maximal and AOX1 may then contribute to 30% of the total protein content. Therefore, even very toxic protein can be produced in large scale in this system. Furthermore, heterologous proteins can be secreted at high levels into the culture medium, which facilitates purification of the desired protein. Finally, as unicellular microorganism, *P. pastoris* has major advantages in industrial fermentation. Several GPCRs have been expressed in *P. pastoris*, including ET_A and ET_B receptors (Cid *et al*., 2000; Schiller *et al*., 2000).

As a yeast, *P. pastoris* is an unicellular microorganism that can be cultured and genetically manipulated as *E. coli*. However, it is also an eukaryote and capable of many posttranslational modifications. Yeast-based protein-expression systems are generally regarded as being faster, easier, efficient and economical compared with higher eukaryotic sources, such as insect and mammalian tissue culture cell systems, and usually gives higher expression levels. Yeast grow rapidly and produce proteins using an eukaryotic protein-synthesis pathway, and the costs of the media and equipment to culture yeast are lower than that of mammalian cells. Eukaryotic proteins are produced in an active form and do not need refolding to be active, as is the case for many eukaryotic proteins made in *E. coli*. *Pichia* is especially suitable for expression of proteins that can only be expressed as inclusion bodies in bacteria or that require post-translational modifications that bacteria cannot carry out. A second role played by *Pichia pastoris* in research is its use as a model system to investigate certain areas of

modern cell biology including the organization and function of the secretory pathway in eukaryotes.

3.1.2 Expression construct for heterologous production of the ET_BR in *P. pastoris*

In this work, the expression construct pPIC9KHisFlagΔGPET_BBio (Fig. 3.1) was used for human ET_B receptor production in the methylotrophic yeast *P. pastoris* under transcriptional control of the strong and highly inducible AOX1 gene promoter. In the construct, the receptor-coding region was manipulated as follows: (1) The endogenous cleavable signal sequence of the ET_BR (see Chapter 1), comprising the first 26 amino acids of the polypeptide chain, was removed and instead the coding region of the α-factor prepropeptide of *S. cerevisiae* and an octapeptide FLAG epitope and decahistidine tag were inserted in its place. The α-factor is a yeast secretion signal sequence and therefore should drive the expressed receptor to the cell membrane. The protease-cleavage site is inserted between the α-factor sequence and the receptor, which is recognized by *P. pastoris* Kex-2 protease and should provide efficient α-factor cleavage. (2)The ET_BR coding region was translationally fused to the coding region of the 9.7 kDa biotinylation domain of the *Propionibacterium shermanii* transcarboxylase (Bio-tag). These N- and C-terminal fusions to the ET_BR gene were performed in order to allow immunological detection and purification of the recombinant protein as well as to enhance production level in the recombinant yeast. (3) The putative N-linked glycosylation consensus motif Asn-Ala-Ser at position 59 in the amino acid sequence of the receptor was changed to Gln-Ala-Ser and also the putative metalloprotease sensitive site at amino acid position 64 was removed by codon exchange (R64A) in order to prevent glycosylation and proteolysis. Deletion of the N-glycosylation and the protease-sensitive sites in the construct should not affect the pharmacological properties of the receptor since it has been reported that deletion of the 65 amino acids changed neither ligand binding nor G protein coupling.

Fig. 3.1 Expression construct for the expression of epitope tagged ET_BR in *P. pastoris.* The boxes shown are not drawn to scale. α-factor, coding region for the secretion signal from *Saccharomyces cervisiae* α mating factor prepropeptide; His, coding region for decahistidine; FLAG, coding region for the FLAG tag, DYKDDDDK; ΔGP ET_B, coding region for human ET_BR without N-glycosylation and protease sensitive sites; Bio, coding region for the biotinylation domain of the transcarboxylase from *P. shermanii*.

Also, glycosylation of the receptor was found not to be essential for ligand binding. These modifications within the receptor coding region were performed in an attempt to minimize heterogeneity of the recombinant receptor and therefore were an advantage for crystallization.

3.1.3 Aim of study

Previous attempts to establish a large-scale expression system for ET_BR in baculovirus/Sf9 cells was unsuccessful (see Chapter 2). In order to work towards the goal of structure determination of the receptor-bound ET-1, the receptor is alternatively produced in yeast. The work focused on producing sufficient quantities of receptor-ligand complex for solid-state-NMR using the production system described by Schiller *et al*. (2000).

3.2 Materials and experiments

The materials and sources used are described below.

3.2.1 Growth and media

The protease-deficient *P. pastoris* strain SMD1163 (*his*4, *pep*4, *pbr*) was grown according to the instructions in the Invitrogen Pichia Expression kit manual. Liquid cultures were grown in BMG (buffered minimal glycerol medium, consisting of 1.34% yeast nitrogen base without amino acids, 0.04% biotin, 0.1 M potassium phosphate, pH 6.0 and glycerol at the indicated concentrations), BMM (buffered minimal methanol medium, consisting of BMG without glycerol but with 0.5% methanol) or YPD (1% yeast extract, 2% peptone, 2% dextrose). Routinely yeast cultures were propagated in YPC medium.

3.2.2 Expression

pPIC9KFlagHisET_BBio construct was transformed into the *P. pastoris* strain SMD1163 and transformants with the highest expression according to immunostaining were selected and grown on a large scale. Briefly, 2x 15 ml of BMG medium was inoculated with the clone of interest, and the culture was incubated at 30°C with shaking at 225 rpm for 7 hours. Cells were then diluted 1000-fold into 2x 1 l BMG and grown to the desired A_{600}, usually 5-6 units. The cells were pelleted by centrifugation and induced by resuspending in 12 l of fresh BMM medium to obtain an OD_{600}=1. Cells were grown in baffled flasks at 30°C with shaking at 225

rpm. Additional (0.5% v/v) methanol was added to the culture every 12 h to maintain induction. The cells were harvested by centrifugation after 72-120 h and stored at -80°C.

3.2.3 Preparation of membranes from *P. pastoris*

Cell pellets were thawed on ice and resuspended in ice-cold breaking buffer (50 mM sodium phosphate buffer, pH 7.5, 500 mM NaCl, 100 mM sucrose, 5 mM EDTA and 1g/l BSA plus protease inhibitors (1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin and 10 μg/ml pepstatin A). The cells were passed four passages through a Dynano Mill, Typ KDL A device (Willy A. Bachofen AG, Basel) filled with 0.5 mm glass beads until cells were broken as judged by visual inspection in a light microscope. Lysates were clarified by centrifugation at 3000 rpm for 30 min. Membranes were collected by centrifugation at 200,000×*g* for 120 min at 4°C, washed with Wash-Storage buffer (50 mM Tris/HCl, 1 M NaCl and 10% Glycerol) and resuspended in the same buffer and stored frozen at - 80°C until further use. Between 200 and 300 mg of crude membrane protein per liter of cell culture, or about 18 mg/g of cells, was routinely obtained.

3.2.4 Receptor solubilization and purification

For large-scale purifications with a final yield of 1 mg purified receptor-ligand complex (determined by protein assay), membranes containing 4-7 mg (70-130 nmol) of ET_BR (determined by ligand binding) in 2 g total protein were thawed and diluted to a protein concentration of 3 mg/ml in ice-cold solubilization buffer (50 mM Tris/HCl, pH 7.4, 500 mM NaCl, 10 mM imidazole) and supplemented every 30 min with 1 mM PMSF. Membranes were saturated with a 10-fold excess of ET-1 and incubated for 2 h at 4°C with mild agitation prior to solubilization with 1% (w/v) n-dodecyl-β-D-maltoside (Glycon) by dropwise addition within 30 min. After 2 h at 4°C , unsolubilized material was pelleted at 43,000×*g* for 45 min at 4°C. Solubilized protein was filtered under vacuum through a 0.65 μm cut-off cellulose acetate membrane to remove particulate material. The receptor-ligand complex was purified by batch immobilized metal-affinity chromatography (IMAC). The clarified supernatant was stirred overnight at 4°C with 30 ml of Ni-NTA agarose slurry (Qiagen) preequilibrated with 50 mM Tris/HCl, pH 7.4, 150 mM NaCl (IMAC buffer A) with the inclusion of 50 μM DTT, 0.1% n-dodecyl-β-D-maltoside and 10 mM imidazole. The non-bound protein fraction was removed by centrifugation and the Ni-NTA agarose was packed into a column, washed with IMAC buffer A containing 10 mM imidazole and 0.1% n-dodecyl-β-D-maltoside, followed

by a wash with IMAC buffer A including increasing concentrations of imidazole (30 mM) and decreasing concentration of n-dodecyl-β-D-maltoside (0.05%). The proteins were eluted with buffer A+200 mM imidazole and 0.05% n-dodecyl-β-D-maltoside by mixing for 30 min at 4°C. 10 ml of monomeric avidin gel (Pierce) was added to the collected eluate and batch-bound by end-over-end rotation overnight at 4°C. After the incubation the suspension was poured into a column and washed with several column volumes of wash buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 1 mM DTT, 0.02% n-dodecyl-β-D-maltoside). Protein was eluted with elution buffer (50 mM Tris/HCl, 150 mM NaCl, 0.01% n-dodecyl-β-D-maltoside, 2 mM biotin). The receptor-ligand complex peak was concentrated to 1 ml in a 50 kDa molecular weight cut-off device (Millipore) run at 3000 rpm. Concentrated material was either snap-frozen in aliquots and stored at -70°C or immediately assessed for purity by SDS/PAGE at 4°C or applied to a superose size exclusion column.

3.2.5 Analytical gel filtration

The state of the monomerization and aggregation of the purified receptor-ligand complex was analyzed by gel filtration chromatography on a SMART system using a Superose 6 column (Amersham Pharmacia Biotech). Here, the buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 2 mM biotin) contained 0.01% n-dodecyl-β-D-maltoside.

3.2.6 Gel filtration

Fast protein liquid chromatography (FPLC) gel filtration was performed at 4°C on a Superdex 75 (10/30) column (Pharmacia) run at 0.5 ml/min. The column was equilibrated with one volume of 50 mM Tris/HCl, 150 mM NaCl, 0.01% n-dodecyl-β-D-maltoside at a flow rate of 1 ml/min. The flow through fraction (2 mg/ml) of the monomeric avidin purification containing unbound ET_BR/ET-1 complex was applied to the FPLC column. The content of protein was monitored by measuring the absorbance at 280 nm. Fractions of 4 ml were collected and analyzed by SDS-PAGE.

3.2.7 Eluate concentration

A convenient method for the concentration of dilute protein solutions is the application of ultrafiltration. The eluate fractions were concentrated approximately 10-15fold by means of Vivaspin 20 centrifugal concentrators (Vivascience, Hannover, Germany) according to the manufacturer ´s instructions. The concentrators were equipped with polyethersulfone membranes having a cut-off of 50 kDa. Centrifugation provides the driving force to press

solvent through the membrane thereby resulting in the concentration of the retained macromolecules.

3.2.8 Desoxycholate/TCA precipitation

The Desoxycholate (DOC)/ TCA precipitation (Peterson, 1983) method was used for concentrating dilute protein solutions for SDS-PAGE analysis. The method involves precipitation in the presence of acid and organic solvent, resulting in protein denaturation. The reduced solubility of denatured proteins allows their recovery in a pellet following centrifugation. 25 µl of 1% DOC and 1 ml of 12% TCA were added to 1 ml of test protein sample in a microfuge tube and the contents were mixed by vortexing. The precipitate was recovered by centrifuging the tubes at 14.000 rpm for 20 min at room temperature. The supernatant was carefully decanted, the precipitated protein was dissolved in 20 µl of 2,5% SDS and incubated at 37 °C for 10 min. 5 µl of 4x SDS gel loading buffer to the sample.

3.2.9 Gel electrophoresis

Proteins were separated by 10% SDS-PAGE at 4 °C and visualized by silver staining. Samples prepared in Laemmli sample buffer for ET_BR analysis were loaded on the gel without boiling.

3.2.10 Protein estimation

The protein concentrations were determined using the BCA microplate procedure according to the recommendations of the manufacturer (Pierce) with bovine serum albumin as the standard.

3.2.11 Radioligand binding assays

Saturation binding assays on *P. pastoris* membranes were performed as has been described in detail previously (Schiller *et al.*, 2000). Radioactivity was determined with a γ-counter (1470 Wizard γ-counter, Wallac).

3.2.12 Enrichment of the ET_B receptor/ET-1 complex by ultracentrifugation

The sample (about 10 ml) was divided in four tubes and was centrifuged for 6 h at 8°C and 500 000 g (SW60 Ti, Beckmann). Fractions collected from the top to the bottom of the gradients were analyzed by SDS-PAGE and measured by UV. Then a second ultracentrifugation of the fractions containing receptor was conducted. The gradients were

fractionated for 16 h at 8°C and 400 000 g and divided into fractions collected from the top to the bottom of the gradients. Fractions were analyzed by SDS-PAGE and measured by UV. The obtained pellets were resuspended in 50 mM Tris/HCl, 150 mM NaCl, 0.01% n-dodecyl-β-D-maltoside. The concentrations of purified receptor-ligand complex containing fractions were determined from A_{280} by using a calculated molar absorption coefficient of ε= 75420 $M^{-1}cm^{-1}$.

3.2.13 Gel Staining and immunoblotting

After SDS-PAGE on 10% Laemmli gels, proteins were transferred to PVDF membranes (BioTrace^R PVDF membrane, GelmanSciences) by a semi-dry electroblotting procedure. PVDF membranes were blocked 1h at room temperature in PBS buffer containing 5% low fat milk powder and probed with antibody for 2h at room temperature. Antigens were detected with the monoclonal anti-polyhistidine clone HIS-1 antibody (Sigma) in combination with a secondary anti-mouse Ig horseradish peroxidase linked whole antibody (dilution 1 : 5000). Immunoreactive proteins were detected by chemiluminescence using the ECL-kit (Amersham) following the manufacturer's instructions.
A streptavidin-alkaline phosphatase conjugate (dilution 1 : 5000) (Promega) was used for probing biotinylated protein.

3.3 Results

3.3.1 ET_B receptor expression vector

The expression system and subsequent purification strategy was kindly provided by Prof. H. Michel and Dr. H. Reiländer (MPI for Biophysics, Frankfurt/M.). The ET_B receptor was expressed as a fusion protein (Fig. 3.1), with a Bio-tag linked to its C-terminus. The presence of the α-factor targets the receptor to the membrane. The presence of the Bio-tag at the C-terminus was shown to improve stability of the fusion protein during expression and purification. The receptor to be expressed consists of a single polypeptide chain, which is not glycosylated, with a M_w of 55 kDa.

3.3.2 Receptor expression in shaking culture and membrane preparation

NMR studies of the ET_B receptor will need large quantities of homogeneous protein. Therefore, to obtain enough membranes for subsequent solubilization and receptor

purification, several expressions in shaking cultures on a 12 l scale were done. We have extended this expression system to a 24-L scale on a weekly basis, using shaker culture techniques. The expression of the ET_B receptor was performed as described in Materials and experiments. The details of 12 expressions and 3 membrane preparations are shown in Tables 3.1 and 3.2. Breaking large quantities of yeast cells was performed by using a cell mill-DYNO-mill KDL Typ A. Cell suspension at a wet weight of 30% was optimal for cell breakage. Under these conditions it was possible to break 0.5 kg cells in 30 min by passing a 1.5 l suspension 4 times through the mill. Breaking efficiency, investigated under light microscope was approx. 80%. Bottleneck in membrane preparation lies in the subsequent high spin centrifugation step at 100 000g, which is performed in order to pellet the yeast cell membranes. Large volumes of supernatant obtained after removal of unbroken cells and cell debris had to be centrifuged in a Beckman Ti45 rotor, which has a maximum capacity of 400 ml per run. With 1.5 l -3 l of low-spin supernatant to be centrifuged, membranes to be pelleted and then pelleted again after washing, this remains the most time-consuming step in the course of large scale membrane preparation.

Table 3.1 Yields obtained from the expression in shaking cultures to produce membranes containing the ET_B receptor.

Source	Bio mass [g]	Membrane preparation [g]
Expression no. 1-6 (72 l)	~1200	17
Expression no. 7-9 (36 l)	589	11
Expression no. 10-12 (36 l)	612	10.5

Table 3.2 Production of recombinant ET_B receptor in *P. pastoris*. For measurement of receptor level, membranes were prepared and assayed with [^{125}I]ET-1.

	Membrane preparation 1 (72 l yeast culture)	Membrane preparation 2 (36 l yeast culture)	Membrane preparation 3 (36 l yeast culture)
Membrane protein concentration [mg/ml]	23.6	31.5	25
Total amount of receptor [mg]	31.8	17	20
Amount of receptor per total membrane protein [pmol/mg]	34.6	27	35

From 144 l shaking cultures about 2.4 kg of recombinant yeast cells with production levels in the range of 27-35 pmol/mg were obtained for the Bio-tagged ET_B receptor. Starting from 2.4 kg (wet weight) of yeast cells about 39 g of membrane protein was obtained.

3.3.3 Large-scale solubilization and purification of the ET_B receptor

The purification procedure based on the His-tag and Bio-tag fused N- and C-terminally to the receptor coding region in the construct and also took advantage of the rather essentially irreversible binding of the ET_B receptor by ET-1. Thus, in advance to solubilization and purification, membranes were saturated with an excess of ET-1 in order to protect the receptor during these steps and as a consequence of previous solubilization experiments. Saturation of membranes containing the receptor with ET-1 resulted in higher solubilization rates than without occupation (Schiller *et al*., 2001). The use of both N-and C-terminal tags ensures that only full-length protein will be obtained.

Recombinant ET_B receptor/ligand complex (10 mg) was purified from 144 l of culture in 12 batches. Only small amounts of protein were obtained from one batch, ranging from 0.4 to 1.3 mg, so material had to be pooled from several runs. For each large-scale purification with a final yield of maximal 1 mg of purified ET_B receptor complex (determined by protein determination assay), membranes containing about 6 mg receptor (determined by ligand binding) in about 2 g total membrane protein were thawed. Membrane preparations containing the receptor were incubated with an excess (10-fold K_D molar quantity; K_D= 41.7 pM) of ET-1 in order to stabilize the receptor in its native conformation, and then the receptor was solubilized with 1% n-dodecyl-β-D-maltoside. It was previously shown that the solubilization efficiency of this detergent for the ET_B receptor was >60% (Schiller *et al*., 2001). Although solubilization yield was optimal at lower protein concentrations, large-scale experiments used a concentration of 3 mg/ml in order to reduce the volume to a manageable level (bottleneck was the ultracentrifugation).

To save the labelled ET-1, a new additional step was introduced in the purification procedure. The membrane containing receptor was saturated with labelled ET-1 and after incubation unbound ET-1 was recovered by ultracentrifugation prior to solubilization. The supernatant containing free ET-1 (estimated to be 9/10 of total amount) was stored and reused with additional fresh 1/10 of total ET-1 amount for the next receptor purification cycle.

For purification of the solubilized receptor complex a two-step affinity approach was used with Ni-NTA matrix (His-tag) as a first step and the monomeric avidin (Bio-tag) as a second step. After initial solublization, the sample was clarified by ultracentrifugation, the resulting

supernatant containing solubilized receptor was quite viscous and would not flow through a packed column of Ni-NTA matrix, so adsorption was done in batch. In addition, batch loading of the Ni-NTA resin resulted in much better recoveries compared to column loading. Batch binding of the receptor complex to the Ni-NTA resin was relatively slow, requiring overnight incubation to achieve greater than 80% binding. SDS-PAGE analysis of the eluate from the Ni-column revealed that it contained ET_BR with non-specifically bound major proteins. The Ni-column eluate was further purified by monomeric avidin affinity chromatography.

The receptor fusion protein bound to the monomeric avidin via its *in vivo*-biotinylated Bio-tag. Therefore successful *in vivo* biotinylation in *P. pastoris* was an essential prerequisite for subsequent purification of the recombinant receptor via affinity chromatography. High affinity of biotin for avidin allowed purification of the biotinylated protein under high stringency conditions, thus reducing background binding often observed with other affinity tags that eluted more easily. Purification was carried out in batch. Attempts to purify the receptor on avidin column showed that the majority of the protein was biotinylated in yeast and that a large part fraction of the receptor was in the flow-through and wash fractions. After

Table 3.3. **Purification of the ET_BR.**

Purification no.	Total receptor amount in membrane [mg]	Purified receptor/ligand complex [mg]	Overall recovery of receptor [%]
1	6.6	0.85	12.9
2	6.6	1.13	17.1
3	4.4	0.53	12.1
4	4.4	0.86	19.5
5	4.4	0.37	8.4
6	2.42	0.63	24.8
7	2.38	0.27	11.3
8	4.85	0.8	16.5
9	4.85	1.7	35.1
10	4.85	1.27	26.2
11	4.1	1.17	28.5
12	4.76	0.8	16.8
Total protein [mg]	**54.6**	**10.4**	

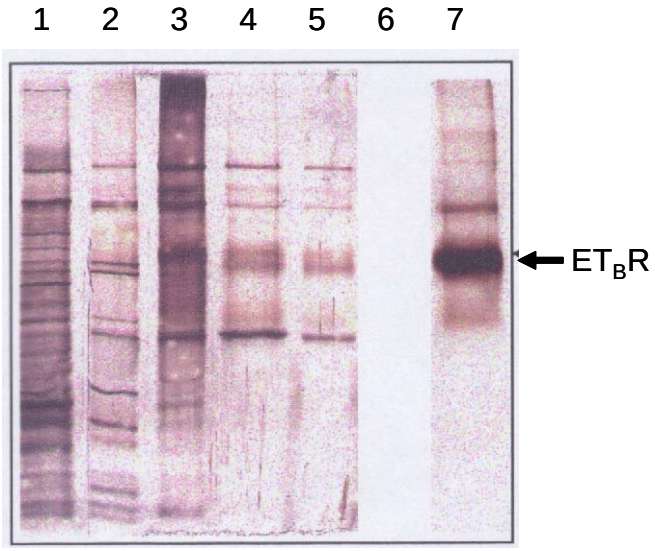

Figure 3.2 10% silver-stained SDS-PAGE of samples obtained from the purification of the ET_B receptor. The purification was performed as described in the Materials and experiments section and is quantitatively documented in Table 3.3. Lanes 1-2: wash fractions of Ni-affinity chromatography, lane 3: eluate of Ni-affinity chromatography, lane 4-5: flow through and wash fractions of avidin affinity chromatography, lane 7: purified and concentrated receptor after avidin affinity chromatography.

this chromatography the receptor was substantially purified. To some extent, the relatively low yield may be due to incomplete biotinylation. Due to suboptimal receptor binding, it was decided to reduce the receptor amount to be purified during each purification procedure. In Fig. 3.2 a silver stained polyacrylamide gel of different purification steps is depicted which confirmed the relative purity of the preparations. In addition to the receptor monomer at an apparent molecular weight of 55 kDa, the Kex2-unprocessed form at 74 kDa was detected.

The details of 12 purifications are shown in Table 3.3, which resulted in a total of 10 mg receptor complex. These results appear to mirror those published with this system (Schiller *et al*., 2001). A maximal overall receptor yield of 35% was calculated based on receptor quantity in the starting membranes and the protein concentration determination in the purified receptor protein preparation. It was in part due to problems with binding of the solubilizate to the Ni-NTA matrix which prevents the maximal experimental yield being obtained. It was often observed that either the solubilizate did not bind well or bound too well to the Ni-NTA matrix, so that the elution fraction contained very low amount of receptor. The purification of the ET_BR in this system proved highly reproducible. Scaling of the purification procedure to increased quantity of receptor in the initial solubilizate would not lead to increased final yield of material to that obtained with this scale (Danka Elez and Eva Molsberger, personal communication). Therefore this scaling was maintained.

3.3.4 Characterization of the ET_B receptor

To assess the stability of the receptor, the elution fraction of the final chromatography step was concentrated with the largest possible cut-off filter (50 kDa) to 1-2 mg/ml. Subsequent analysis of the purified receptor regarding peak shape and retention time, which are informative of oligomerization and aggregation, by Superose 6 gel filtration revealed the presence of one peak which contained almost all of the protein. The symmetrical peak which eluted from the calibrated size exclusion column indicated a homogenous receptor preparation. The elution profile is shown in Fig. 3.3. Based on the calibration of the column the elution volume corresponded to an apparent molecular weight of 87-97 kDa for the dodecyl maltoside/ receptor. However often aggregation of the receptor after purification was detected on the gel filtration column, which could be avoided sometimes by using new monomeric avidin resin for each new purification.

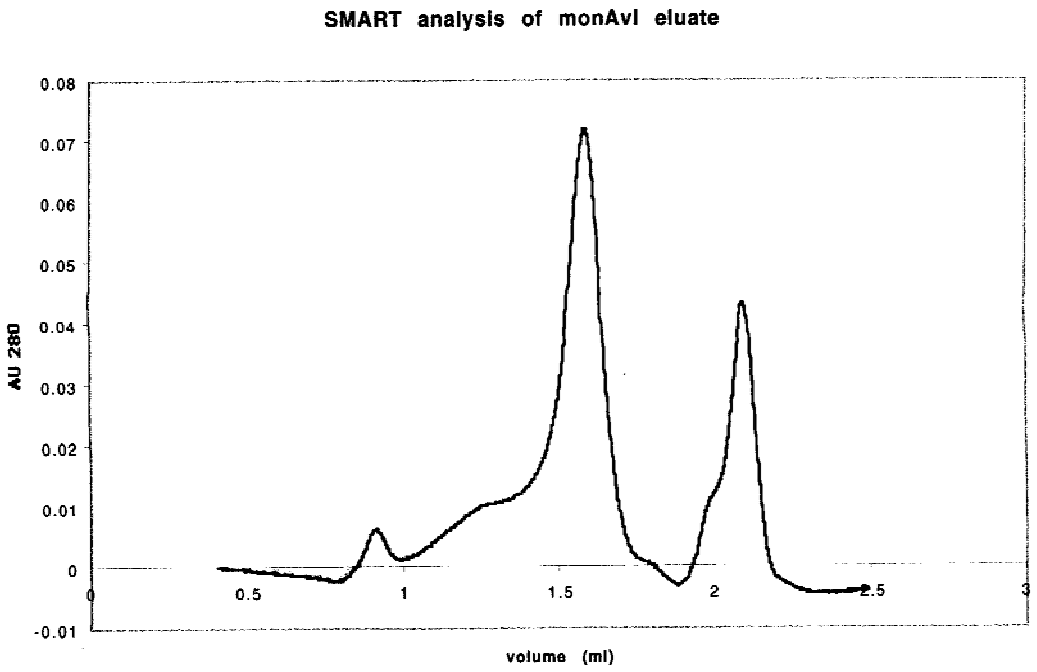

Fig. 3.3. Characterization of the purified ET_BR/ET-1 complex by analytical gel filtration. The state of the monomerization and aggregation of the purified receptor-ligand complex was analyzed by gel filtration chromatography on a SMART system using a Superose 6 column.

3.3.5 Purification of the ET_BR/ET-1 complex from the flow through/wash fractions of the monomeric avidin purification step

Up to 20% of the receptor was estimated not to bind to the avidin resin and was found in the flow through and wash fractions. This could have been due either to a possible effect of detergent on the interaction of biotin with avidin or to the incomplete biotinylation of the Bio-

tag. The flow through was recycled. For the separation of the ET_BR complex from unwanted by-products, which displayed masses of approximately >70 kDa on the SDS-PAGE (Fig. 3.2), the difference in size to the full-length receptor should allow for their efficient elimination in a Superdex 75 gelfiltration run. The elution profile is shown in Fig. 3.4 A. The result was an inefficient separation of the receptor from contaminating by-products as can also be observed on the SDS-PAGE in Fig. 3.4 C. Since the peaks were not well-defined, contamination by other species could not be minimized. In the first peak (fractions 6-7), the main fraction of the ET_BR complex and proteins with high molecular weights were eluted. In the second peak (fractions 9-10), protein content was detected by silver staining with apparent molecular weights of <45 kDa and 66 kDa. In order to analyze the purification profile, the collected fractions were subjected to Western blot analysis with anti-His-tag antibody. As seen in Fig. 3.4 B, two prominent protein bands could be detected in fractions 6-7. The lower one had the apparent M_w of 55 kDa, which correlated well with the calculated mass of the receptor construct itself. The upper one, with an apparent M_w of >66 kDa, represented the unprocessed form of the receptor construct, where the α-factor signal peptide has not been cleaved by *P. pastoris* Kex2-protease. Aggregates were shown at higher M_w. The protein band at <45 kDa was not detected, which was probably a result of receptor degradation.

During another purification trial by loading the flow through/wash fractions onto the Ni-NTA column the 'flow through' problem became evident (data not shown). The receptor did not bind to the resin and was found in the flow through.

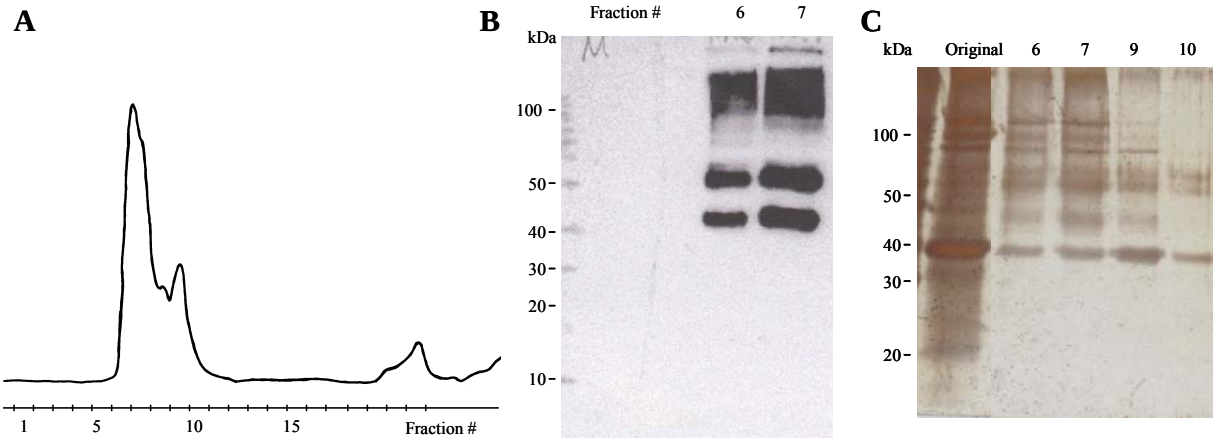

Fig. 3.4. Purification of ET_BR complex obtained from flow through fractions of the monomeric avidin purification step. (A) Profile of a gelfiltration run (Superdex 75) of the ET_BR/ET-1 complex. Shown is the A_{280} trace. (B) Immunological analysis of the purification profile. Collected fractions were loaded on a 10% SDS-PAGE, transferred to a PVDF membrane, and proteins probed with anti-His-tag antibody. (C) Analysis of purity after gelfiltration by 10% SDS-PAGE stained with silver. Lane 1 shows the applied original sample. Rest of the gel shows the elution profile (fractions 6-10).

3.3.6 Enrichment of the ET_B receptor by ultracentrifugation

Solid-state NMR experiments, that can be employed to determine the 3D-conformation of a protein at present, require at least 60 nmol of labelled material, which is challenging to achieve with detergent-solubilized GPCR samples where an increase in concentration of the solubilized receptor leads to an excess of detergent micelles. The resulting high detergent concentration can destabilize the GPCR. The choice of concentration method depended on a number of factors. The usual concentration methods applied to soluble proteins include pressure-based ultrafiltration or buffer removal through dialysis tubing in contact with hygroscopic polymers. Using the ultrafiltration technique, it is usually only the buffer which is removed. While monomeric detergent can pass through ultrafiltration membranes, unless the critical micelle concentration (cmc; the concentration of detergent below which detergent is monomeric in solution and beyond which all additional added detergent forms micelles) is high relative to the total detergent concentration, the amount which is removed during concentration procedures will typically be negligible. Dialysis works well for high cmc detergents, but for low cmc detergents possessing large micelles (such as dodecyl maltoside), dialysis fails. Another consideration is that large protein/lipid vesicles can generally be concentrated by pelleting using ultracentrifugation.

To enable us to a achieve a greater enrichment of the receptor complex in the NMR rotor (ca. 10 mg in 50 µl) compared to usual methods (typically 10mg/ml), an alternative method using ultracentrifugation was assessed for this purpose. We used a simple ultracentrifugation method based on differential sedimentation velocity of protein, free and micellar detergents where the ET_BR-micelles were separated from the mixture of free and micellar detergents devoid of receptor. To enrich the receptor complex by ultracentrifugation a method was adopted which was successfully applied for detergent-solubilized bovine rhodopsin previously (ca. 2 mg/50 µl; Dr. L. Krabben, personal communication). After the first ultracentrifugation run, fractions obtained were next measured spectrophotometrically (Table 3.4 A), subjected to SDS-PAGE and analyzed for the presence of receptor. UV-measurements indicated high concentrations of ET_B receptors in the lowest fraction (4 mg/ml). Fractions at the bottom were rich in receptor, while top fractions contained no receptor. Additionally, unlike bovine rhodopsin, upon ultracentrifugation the receptor became insoluble and precipitated in a pellet. A second ultracentrifugation run was made to prepare a fraction richer in receptor, wherein, fractions containing the receptor were pooled and refractionated by ultracentrifugation. After the second fractionation (Table 3.4 A), the distribution of receptor closely resembled the first ultracentrifugation, with the receptor accumulating in the soluble high-density fractions at the

bottom (18%). Under these conditions however, most of the receptor was recovered in an insoluble fraction (82%) pelleted during high-speed centrifugation at any centrifugation step. The protein composition is visualized in Fig. 3.5. The receptor could be concentrated up to 11.7 mg/ml for solid-state NMR experiments. With this procedure the receptor density achieved was 12 fold enriched over the unconcentrated sample. However, the recovery of the ET_BR/ET-1 complex was very low (less than 1.8 mg from 10 mg of purified receptor complex, see Table 3.4 B).

Table 3.4 A. Protein concentrations of fractions obtained after the first ultracentrifugation run.

Number of tubes	Fraction	Volume [µl]	Dilution	A_{280}	Protein amount per tube [µg]	Total Protein amount [µg]
4	Top	550		n. d.		
4	Middle	550		n. d.		
4	Bottom	550	/	0.01	4	16
4	Protein fraction 1	150	1:6	0.03	18	72
4	ET_BR/ET-1	250	1:200	0.038	1000	4000
1	Pellet wash I	800	/	0.9	470	470
1	Pellet wash II	800	/	0.489	260	260
1	Pellet wash III	800	/	0.3	160	160
1	Pellet wash IV	800	/	0.2	104	104
1	Pellet wash overnight	1200	/	0.843	980	980

Following ultracentrifugation, the distribution of ET_B receptors, in fractions collected from the top of the gradient, was determined by monitoring absorbance at 280 nm. Pellets were redissolved with buffer containing detergent and thereafter were called Pellet wash fractions.

Table 3.4 B. Protein concentrations of fractions obtained after the second ultracentrifugation run.

Fraction	Volume [µl]	Dilution	A_{280}	Total Protein amount [µg]
Top	1000	/	0.07	46
Middle	400	1:1.5	0.18	71
Bottom	100	1:6	0.34	1000
ET_BR/ET-1	150	1:300	0.178	1750

The distribution of ET_B receptors was determined as in Table 3.4 A.

3.3.7 Localization of the truncation site of the ET_BR

In Fig. 3.5, the pellet fractions, which were redissolved with buffer containing detergent and thereafter called wash fractions, showed two predominant distinct bands on SDS-PAGE and represented 45- and 60-kDa polypeptide molecules. It is noticed that the ratio of the high to low M_w species was an one to one stoichiometry in wash I, while only with succeeding washes (II-IV) to redissolve the pellet, the ratio varied and the smaller fragment appeared to be a minor component. This suggested that the 55 kDa ET_B receptor may contain a sensitive proteolytic site which produced the 45 kDa polypeptide. Immunological analysis with anti-His-tag antibody and streptavidin-alkaline phospatase conjugate (Fig. 3.6) indicated that a 10 kDa portion from the 55 kDa receptor construct was missing at the C-terminus, giving rise to the loss of the Bio-tag. Therefore the 45 kDa ET_BR is a C-terminally truncated product of the full-length form (55 kDa) and should be still active in binding to ET-1. We found that during the ultracentrifugation proteolysis of the receptor occurred.

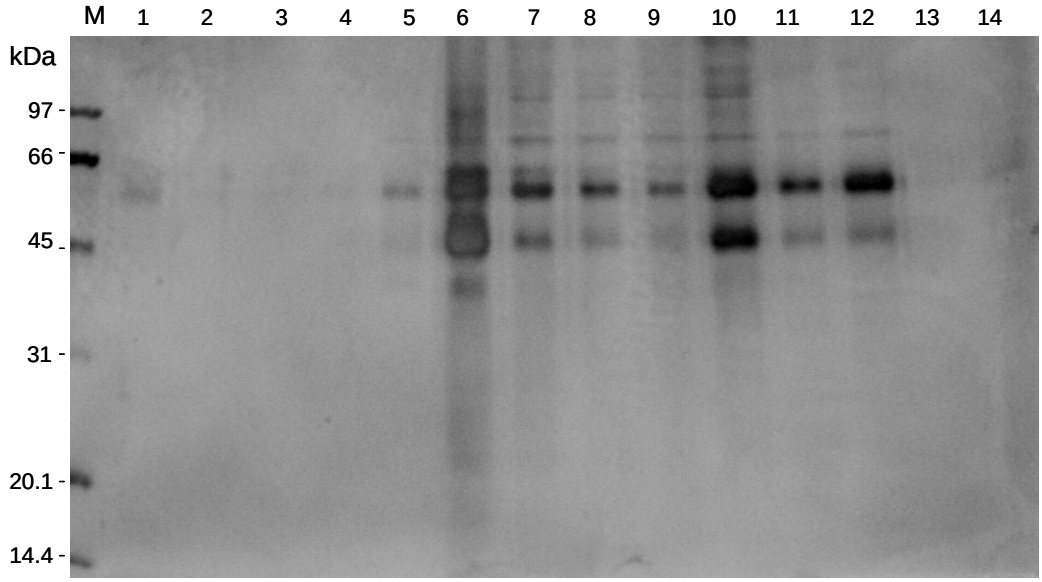

Fig. 3.5. SDS-PAGE analysis of enriched ET_BR/ET-1 by ultracentrifugation. The enrichment was performed as described in Materials and methods and is quantitatively documented in Tables 3.4 A, B. The following fractions were analyzed by 10% SDS-PAGE and silver staining. Lane M: molecular weight standard; lane 1-9: fractions from the first ultracentrifugation run (in the order: bottom, middle, top, protein fraction (1:6 diluted), ET_BR/ET-1 (1:100), wash I, wash II, wash III, wash IV); lane 10-14: fractions from the second ultracentrifugation run (in the order: pellet wash, ET_BR/ET-1, bottom, middle, top).

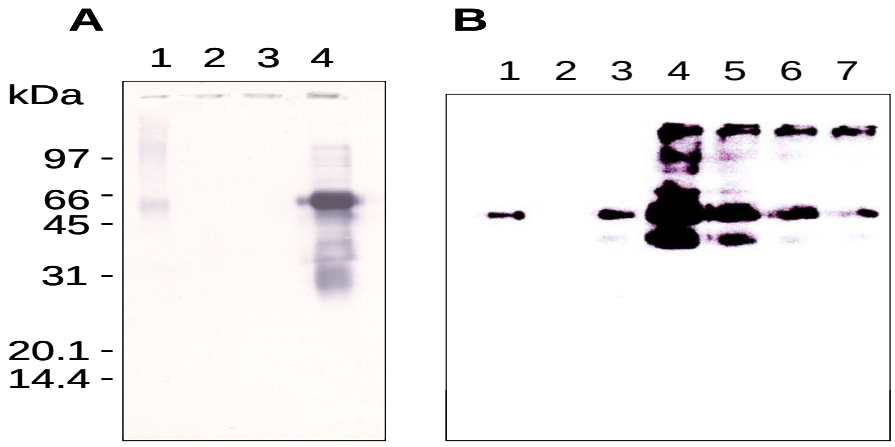

Fig. 3.6. Immunological analysis of collected fractions of ET_BR obtained after ultracentrifuation. (A) Proteins probed with streptavidin-alkaline phospatase conjugate. Lane 1: wash I fraction containing ET_BR/ET-1, lane 2-3: negative controls, lane 4: positive control. (B) Proteins probed with anti-His-tag antibody. Lane 1: bottom fraction, lane 2: protein fraction (1:6), lane 3: ET_BR/ET-1 (1:100), lane 4-7: wash I, II, III and IV.

3.4 Discussion

3.4.1 Expression and purification of the ET_BR

Initially, the ET_B receptor was expressed in Sf9 cells (Chapter 2). In this study, the ET_B receptor was produced in the yeast *P. pastoris* because the highest receptor yield was obtained using yeast as host. The best expressing clone of HisFlagΔGPET_BBio, with an expression of 40 pmol/mg of membrane protein in shaking culture (Schiller *et al*., 2000) was used. Using the expression system and purification protocols outlined above we were able to obtain about 10 mg of pure ET_BR/ET-1 complex from membranes isolated from ca. 144 l *P. pastoris* cells. Some additional modifications were made to the given purification protocol in order to save the labelled ET-1 and to offer improvement in yield. The amount of ET-1 required for receptor purification (practically, 1.2 mg of ET-1 was required for 3 mg of expressed receptor to give a final yield of 1 mg ET_BR/ET-1) could be significantly reduced by including an ultracentrifugation step in the purification procedure. Our inability to high large scale purification due to aggregation required many purification batches to obtain sufficient material. The overall yield from this two step purification was ~40%. The method produced protein of sufficient purity.

Compared to receptor production in yeast, the receptor yield from the insect cells is lower (Chapter 2) and the system handling is more time consuming. The amount of active receptor obtained can be dependent on the vector construct. Schiller *et al.* (2000) reported the

influence of the nature of C-terminal tags on the expression of ET_BR fusion proteins. The highest yield of the ET_BR was obtained with clones containing the Bio-tag. These findings revealed a 6-fold larger yield of the active receptor in the case of the clone containing the C-terminally fused Bio-tag compared to the one without it. The assumption is that this relatively long Bio-tag stabilizes the receptor.

The receptor was purified in complex with ET-1 since binding of the ligand renders the receptor more stable and less prone to aggregation (Doi *et al*., 1997, Schiller *et al*., 2001). However the disadvantages of this approach are that separation of functional ligand-binding receptors from non-functional receptors and binding measurement of active receptor quantities after purification are not possible. Non-functional ET_BR can result of possible misfolding of receptors during expression and inactivation during membrane preparation. It is argued that the receptor purified from *P. pastoris* membranes was active, since it has been proven previously that receptor purified in the ligand-bound state retained full biological activity as revealed by G protein activation experiments (Doi *et al*., 1997). However, scepticism still persists as to whether the isolated protein was active. This scepticism can only be at rest when the isolated receptor can be successfully reconstituted in phospholipids vesicles with purified G_q and shown to be functionally active. This experiment validates the biological activity of the isolated receptor-ligand complex in the guanine-nucleotide-exchange reaction by G_q.

3.4.2 Receptor enrichment by ultracentrifugation and receptor stability

Stability problems were encountered during the enrichment of the solubilized receptor/ligand complex using ultracentrifugation. After ultracentrifugation most of the receptor protein (80%) precipitated in pellets whose SDS-PAGE analysis revealed a major double band with an appropriate molecular weight of about 60 kDa and one band of 45 kDa. The band at 60 kDa represents the full-length receptor, whereas the presence of the 45 kDa band indicated C-terminal proteolysis by protease. Previous studies have reported, that in various tissues, ET_BR exists not only as a 52 kDa intact species but also as a 34 kDa species that is generated through cleavage in the N-terminal extracellular domain by a metalloprotease(s) (Kozuka *et al*., 1991; Saito *et al*., 1991; Takasuka *et al*., 1994). These two species have been shown to be able to bind ligands with high affinity. Although the protease cleavage site (R64↓S65) in the receptor sequence was removed by mutating Arg64 to Ala, it is possible that the receptor still undergoes N-terminal degradation. This was reported by Grantcharova *et al.* (2004) that this modification does not prevent receptor proteolysis in HEK293 cells and that the cleavage

appeared to be independent of the receptor sequence. The physiological role of N-terminal receptor cleavage is unclear. In this work, immunological analysis of the fractionation with both streptavidin conjugate and anti-His-tag antibody indicated that the receptor was truncated at the C-terminus, which was also observed with the ET_BR production in *P. pastoris* by Schiller *et al*. (2000). It seemed that proteolytic conversion from high to low M_r species took place during concentration of the receptor sample. It is noteworthy that ET_BR-ET-1 complexes are more susceptible than ET_AR-ET-1 complexes to proteolysis (Takasuka *et al*., 1992).

The receptor was solublized in 1% dodecyl maltoside in which it was determined to be stable (Schiller *et al*., 2001) but the large amount of this detergent specifically bound to the receptor could be a major obstacle to its three-dimensional crystallization. So enrichment of the receptor was achieved in the chromatography steps also serving to exchange the receptor into a buffer with a much-reduced detergent concentration. By this approach most of the detergent was removed and the cmc was reached (ca. 0.01%, 0.17 mM). Extensive concentration of the purified ET_BR/ET-1 complex was required for structural studies. Concentration by centrifugal filtration or stirred-cell will increase the detergent concentration to a level that is not acceptable for protein stability and sample preparation. Also, a small desalting column can remove some excess detergent but the protein is diluted in this step. Therefore, the method we used to remove/decrease the detergent during the course of concentration was ultracentrifugation. After ultracentrifugation, the heterologously expressed receptor was in a precipitated and certainly misfolded state. Here, only speculative conclusions can be drawn regarding causes for precipitation.

This ultracentrifugation method usually used to pellet proteoliposomes was successfully applied to solubilized bovine rhodopsin/retinal complex in 0.1% dodecyl maltoside from Sf9 membranes (data not shown). The recovery level of intact ET_BR/ET-1 protein was relative to that of rhodopsin quite low. The extremely low level of intact ET_BR/ET-1 complex indicated that the stability of ET_BR was not the same as that of rhodopsin. Nevertheless, the secondary structures resembled each other. The differences dictating the efficient enrichment of rhodopsin might reside in the detergent concentration (0.1% vs. 0.01%) and in the lipid composition. The detergent-ET_BR/ET-1 complex was at a concentration equal to the cmc range (~0.01%, 0.17 mM), which will vary according to the composition of the solvent. At a concentration equal to the cmc, the detergent spontaneously aggregates into a micelle. At concentrations above the cmc, there is an equilibrium between monomers (present at a concentration approximately equal to the cmc) and an increasing concentration of micelles.

During the ultracentrifugation run, detergent reduction might occur through progressive sedimentation of the detergent-ET_BR/ET-1 micelles in equilibrium with the constantly replenished monomeric detergent molecules. However, the detergent layer, wrapped around the protein, might dissolve at free monomer concentrations below its cmc, which entailed the precipitation of the receptor.

It is to question if a higher dodecyl maltoside concentration than 0.01% (e.g. 0.1%), would have led to aggregation of the receptor complex using the ultracentrifugation method. Answering a question with a question is if the ET_BR/ET-1 complex produced in *Pichia* would also have aggregated by extensive concentration using centrifugal filtration. The original receptor sample consisted of about 1 mg/ml. Considering a membrane protein of 55 kDa (0.18 mM) at a typical concentration of 10 mg/ml, concentrating would have led to a 10-fold higher detergent concentration (0.1%, 1.7 mM).

A second question arises what detergent concentration should be used. For a purified membrane protein, the ratio of micelles to membrane protein molecules should be fairly small, as low as 1.5-2 (Helènius *et al*., 1979). A 1.5-fold excess of micelles to protein would require a micelle concentration of 0.27 mM. The number of detergent molecules in a micelle is given by its aggregation number (for dodecyl maltoside, it is about 85). Therefore, a dodecyl maltoside solution at a total concentration that is above the cmc is about 32 mM. A typical concentration used is 1-10 mM or even higher.

Receptor aggregation showed that this detergent membrane system did not mimic the structural properties of native bilayers in terms of maintaining the ET_B receptor in a native and functional state. The detergent and lipid concentration of the purified protein is not characterized and this may be a cause for the failure in sample preparation. Thus, characterization of the sample solution consists of determination of types of lipids present since these arise from solubilization of the ET_BR from membranes of the *Pichia* system, determination of detergent and lipid concentrations and characterization of the composition of solubilized receptor complex itself. A rapid Fourier-transformed infrared (FTIR) spectroscopic method for characterizing the levels of lipid and detergent in detergent-solubilized receptor samples would be advantageous. The FTIR data on lipid- and detergent-receptor ratios is correlated with solubility (as assessed by dynamic light scattering) and structural stability (as assessed by FTIR) to aid in the development of protocols for preparing solubilized receptor. In general, the resemblance to native membranes decreases in the order: vesicles, bicelles, mixed micelles and micelles.

Detergent exchange and embedding into lipid environment before ultracentrifugation may have stabilized the tertiary structure. Refolding upon reconstitution in liposomes has been described for several other membrane proteins (Efimov *et al*., 1994). Alternatively, it should be investigated if dodecyl maltoside can be replaced during purification with digitonin, a detergent widely used to stabilize GPCRs (Hulme, 1990).

The Bio-tagged ET_BR has been targeted for crystallization trials and was, therefore, expressed in a deglycosylated state to avoid heterogeneity of glycosylation. Deglycosylation does not hinder ET-1 binding. The structural consequences of deglycosylation may be insignificant compared to the effects of simple changes in membrane lipid composition although oligosaccharides have various biological functions, ranging from the protection from proteolysis to folding and location.

Since binding of ET-1 to ET_BR does not readily dissociate, we determined whether ET_BR could be saturated with reused ligand and then solubilized as a receptor-ligand complex. Membranes were incubated both with reused and unused ET-1 and washed by centrifugation in order to remove the excess of unbound ligand. Washing, presumably, does not induce dissociation of ligand, validating the use of this method for the cost-saving preparation of ET_BR complexes. Pre-incubation of membranes with a low concentration of unused ET-1 (1/10 of total amount) together with reused ET-1 (9/10 of total amount) could have reduced subsequent binding to a greater extent than when the same concentration of unused ET-1 was incubated with membranes. The receptor precipitation may be a result of progressive proteolysis of the receptor.

3.4.3 Conclusions

The enrichment of the receptor remains a concern and should be the subject of continued investigation. In addition, it should be investigated how factors, such as glycosylation, lipid-protein ratios, and detergent-protein ratios, influence solubilized receptor stability.