Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochimica et Biophysica Acta 1610 (2003) 133–140
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
www.bba-direct.com

Expression and purification of truncated, non-glycosylated turkey beta-adrenergic receptors for crystallization

Tony Warne*, Jill Chirnside, Gebhard F.X. Schertler

MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

Received 26 July 2002; received in revised form 17 December 2002; accepted 17 December 2002

Abstract

In order to purify milligram quantities of turkey β-adrenergic receptor (βAR) for structural analysis, we have expressed mutant βARs using the baculovirus system. The initial βAR construct was truncated at both N- and C-termini thus removing an N-glycosylation site. Cys 116 was mutated to leucine and a histidine tag was added at the C-terminus resulting in the βAR construct 20–424/His6. Expression of this construct in Sf9 cells produced 0.5 mg of unpurified receptor per liter of culture which necessitated the use of a fermenter for large-scale production. The yield was improved more than 2-fold to 1.2 mg/l culture by using Tni cells which facilitated the production of receptor on a 4 litre scale in shake cultures. The receptor was purified to homogeneity with 35% recovery giving a yield of 2 mg receptor. A further deletion at the N-terminus (βAR 34–424/His6) eliminated proteolysis which had been observed with the original construct and also increased expression more than 5-fold to 360 pmol/mg solubilized membrane protein. This expression level is one of the highest reported for a G protein-coupled receptor (GPCR) and has enabled us to purify 10 mg βAR for large-scale crystallization experiments.

Keywords: β-adrenergic receptor; Expression; Purification

1. Introduction

The turkey β-adrenergic receptor (βAR) is a member of a large class of cell surface receptors that catalyse hormone- or ligand-stimulated binding of GTP to regulatory proteins (G proteins). These G protein-coupled receptors (GPCRs) are integral membrane proteins with a hydrophobic core of seven transmembrane α-helices. This large and important class of membrane proteins includes many major drug targets [1] but the only crystal structure of a GPCR so far available is that of bovine rhodopsin [2]. The absence of structural information for other GPCRs is partly due to difficulties in their overexpression [3] and these have been reviewed elsewhere [4,5]. The baculovirus expression system has been one of the methods of choice for GPCR overexpression, including the human β2-adrenergic receptor [5,6], with maximal yields of 100 pmol receptor/mg membrane protein being attained in some cases. In order to obtain milligram quantities of the turkey βAR for crystallization experiments, we have used recombinant baculoviruses to overexpress βAR in insect cells. In our optimisation of the overexpression and purification of βAR, we have considered intrinsic expression levels of different insect cell lines, resistance to proteolysis and the presence of post-translational modifications to maximise the yield and homogeneity of the purified receptor. Previous work has indicated the benefits of truncations and mutation in the selection of turkey βAR constructs for overexpression. The turkey βAR has an extracellular N-terminus and an intracellular C-terminus which is extended in comparison to other βARs [7]. The extended C-terminal domain of the turkey βAR (amino acids 425–484) is encoded by an alternatively spliced exon [8] and its function may be to prevent agonist-promoted down-regulation and internalisation, thus maintaining the receptor at the cell surface [9]. The presence of this domain may also impede detergent solubilization [10]. A series of turkey βARs with truncated C-termini have been expressed in insect cells resulting in increased expression and ease of detergent solubilization as well as retention of regulatory activity [10]. Expression was also increased by substituting a leucine for the non-conserved cysteine at position 116 [11].

* Corresponding author. Fax: +44-1223-213-556.
E-mail addresses: ajw2@mrc-lmb.cam.ac.uk (T. Warne), gfx@mrc-lmb.cam.ac.uk (G.F.X. Schertler).

0005-2736/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0005-2736(02)00716-2

The crystallization of bovine rhodopsin [12,13] has shown that glycosylation itself need not interfere with three-dimensional crystallization. However, heterogeneity of glycosylation has been observed for the turkey βAR expressed in Sf9 cells [11], which could interfere with two-dimensional or three-dimensional crystallizations. The turkey βAR has a single N-glycosylation site near the N-terminus (asparagine 14). Removal of the N-glycosylation consensus sequences from the N-terminus of hamster βAR does not affect ligand binding [14,15]. When the turkey βAR is truncated at the C-terminus, it may also be more susceptible to proteolysis at the N-terminus [16]. Both N-glycosylation and N-terminal proteolysis could be prevented by truncation at the N-terminus.

In order to elevate the expression level, reduce proteolysis, avoid N-glycosylation and facilitate purification, we have initially chosen to express a turkey βAR construct encoding residues 20–424 with the mutation Cys 116 Leu and a C-terminal histidine tag (βAR 20–424/His6). This paper describes the expression, purification and characterization of this truncated, non-glycosylated βAR construct and further improvements in the expression and purification to provide material for crystallization.

2. Methods

2.1. Materials

The baculovirus transfer vector pVL1393 was obtained from Invitrogen. The vector pBacPAK8 was from BD Clontech and linearized baculovirus DNA (Baculogold™) was from Pharmingen. Sf9 and Tni (High 5™) cells were obtained from Invitrogen. TNM-FH media was from Sigma and Ex-Cell 405 media from JRH Biosciences. Fetal bovine serum (FBS) was obtained from Hyclone, and Pluronic F-68 from Sigma. Lipid concentrate (100×) was from Gibco. The radiolabelled antagonist ligand [^{3}H](−) dihydroalprenolol was supplied by Amersham. Dodecyl-β-D-maltoside was from Anatrace. Quik-sep disposable columns for ligand binding assays were from Advanced Laboratory Supplies. All other chromatographic materials, columns and instruments were from Amersham Pharmacia. Alprenolol sepharose CL-4B was synthesised as described previously [17]. Calf intestinal alkaline phosphatase (20 U/μl) was from Roche. Shrimp (*Pandalus borealis*) alkaline phosphatase (1 U/μl) from Promega. INDIA™ HisProbe™-HRP (nickel-conjugated peroxidase) was from Pierce. The recombinant baculovirus construct βAR C116L/T424 was kindly donated by Professor Elliott Ross of the University of Texas.

2.2. Preparation of recombinant baculoviruses

βAR constructs were generated by PCR from baculovirus DNA of βAR C116L/T424. This construct encoded residues 1–424 with the mutation of Cys 116 to Leucine with 5′ and 3′ non-coding regions having been removed [9]. The sequence CCCAAAATG was placed at the initiator methionine codon and the constructs were subcloned into the baculovirus transfer vectors pVL1393 (βAR 20–424/His6) and pBacPAK8 (all other constructs) which were used in the co-transfection of Sf9 cells with linearized baculovirus DNA using the manufacturer's recommended procedure after verification by DNA sequencing. Expression was under the control of the polyhedrin promoter. Recombinant baculoviruses were isolated by plaque purification and first passage viruses were screened for βAR expression by solubilization of receptor and ligand binding assays. Recombinant baculoviruses expressing βAR were passaged three times in Sf9 cells to obtain high titre stocks of 2–4 × 10^8 pfu/ml [18] with virus titres being determined by end-point dilution assay [19].

2.3. Cell culture and expression with recombinant baculoviruses

Insect cells were grown in suspension in flasks up to a maximum volume of 500 ml in 2 l roller bottles (Corning) at 27 °C with shaking at 150 rpm. Sf9 cells were grown in TNM-FH medium supplemented with 10% FBS and lipids. Large-scale Sf9 cultures (15–35 l) were grown in a stirred tank fermenter (SGI 50 l) sparged with an air/oxygen mix on demand to maintain at 50% O_2 saturation. Tni cells were grown in Ex-Cell 405 media supplemented with 5% FBS and 0.1% pluronic F-68.

Cells were infected with recombinant virus for maximum expression as follows: when cultures had reached a density of 2–3 × 10^6/ml (Sf9) or 1–2 × 10^6/ml (Tni), virus was added at a multiplicity of infection of 5–10. An equal volume of fresh medium was added immediately afterwards. Cells were harvested by centrifugation 48 h after infection.

2.4. Preparation of insect cell membranes

All membrane preparation and solubilization steps were carried out with ice-cold buffers with the inclusion of the protease inhibitors, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (0.5 mM), leupeptin (2.5 μg/ml) and pepstatin A (3.5 μg/ml).

For small-scale expression testing, cells were pelleted from 1 ml culture, broken by freeze–thaw (two cycles) and resuspended in 1 ml 20 mM Tris–HCl pH 8, 1 mM EDTA. Membranes were first pelleted by centrifugation at 13,000 × *g* for 10 min and, after removal of the supernatant, membranes were solubilized by addition of 1 ml 20 mM Tris–HCl pH8, 1 mM EDTA, 0.35 M NaCl, 0.5% dodecyl-β-D-maltoside (DM) followed immediately by centrifugation at 13,000 × *g* for 10 min to remove unsolubilized material. 5–20 μl volumes of the supernatant were then used in radioligand binding assays.

For large-scale membrane preparation, infected cells from shake cultures (4 l total volume) were harvested by centrifugation at 2500 × *g* for 5 min and resuspended in 100 ml 20 mM Tris–HCl pH 8, 1 mM EDTA. Cells were flash-frozen in liquid nitrogen and stored at − 85 °C. Cells grown in the fermenter were spun down in a continuous flow-through centrifuge (Heraeus Varifuge 20 RS, rotor 8585) at 10,000 × *g*. The pellet was washed once with 20 mM Tris–HCl pH 8, 1 mM EDTA and resuspended in approximately 300 ml of the same buffer before freezing in liquid nitrogen and storage at − 85 °C.

Cells were thawed and resuspended in 20 mM Tris–HCl pH 8, 1 mM EDTA to a final volume of 700 ml (Sf9 cells from 32 l) or 350 ml (Tni cells from 4 l) with use of a DIAX (Heidolph) homogeniser for 2 min at 10,000 rpm to ensure cell-breakage. After centrifugation for 1 h at 150,000 × *g* and 4 °C in a Beckman Ti45 rotor, membrane pellets were resuspended with the homogeniser in the same volume of buffer as before and the centrifugation was repeated. The final pellet was resuspended in buffer with a reduced EDTA concentration (0.2 mM) at 10–20 mg protein/ml and frozen in liquid nitrogen and stored at − 85 °C.

2.5. Solubilization and purification of recombinant βAR from membranes: βAR 20–424/His6

Purification of βAR was performed at 5 °C with a FPLC equipped with valves for column switching, buffer selection and peak collection for automated application to a second column. This allowed the loading and running of the first column (IMAC) and the loading and washing of the second column (alprenolol sepharose) to proceed automatically. For large-scale purifications with a final yield of 2 mg purified βAR 20–424/His6 (determined by protein assay), membranes containing 5–6 mg (100–120 nmol) βAR (determined by ligand binding) in 2 g total protein were thawed and diluted to 10 mg/ml protein in ice-cold 20 mM Tris–HCl pH 8 with the inclusion of 0.35 M NaCl, 3 mM imidazole, 2% DM and protease inhibitors (4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (0.5 mM), leupeptin (2.5 μg/ml) and pepstatin A (3.5 μg/ml)) and centrifuged immediately without prior incubation. After centrifugation for 1 h at 150,000 × *g* in a Ti45 rotor (4 °C), solubilized membrane proteins were directly pumped at 1 ml/min onto a 25 ml (2.6 cm diameter) Ni^{2+} loaded chelating sepharose column pre-equilibrated with IMAC buffer A which comprised 20 mM Tris–HCl pH 8, 0.35 M NaCl, 3 mM imidazole, protease inhibitors and 0.05% DM. IMAC buffer B included in addition 250 mM imidazole.

After sample application the flow rate was increased to 3.5 ml/min and the column was washed with 10 column volumes (cv) buffer A followed by a gradient 0–10% B (2 cv) and a wash with 10% B (10 cv). Fractions containing purified receptor were eluted with a gradient 10–100% B (1 cv) with 100% B maintained for a further 2 cv. When the imidazole concentration applied reached 100 mM, 140 ml of the subsequent eluate was collected.

This fraction was loaded at 0.4 ml/min onto a 5 ml (2.6 cm diameter) alprenolol sepharose column which was equipped with a water jacket. The column was washed at 1 ml/min with buffer A^I (20 mM Tris–HCl pH 8, 0.35 M NaCl, 1 mM EDTA and 0.05% DM) and buffer B^I (as A + 1 M NaC1) as follows: 5 cv buffer A^I; 2 cv buffer B^I; 2 cv buffer A^I; 2 cv buffer B^I and 2 cv buffer A^I. The column was then warmed to 16 °C and the receptor was eluted at 0.4 ml/min with buffer A^I + 0.8 mM (−) alprenolol. The DM concentration was reduced to 0.02% in this elution buffer in anticipation of the ensuing concentration step.

The receptor peak (10 ml) was concentrated to 1 ml in a 50 kDa molecular weight cut-off centricon (Amicon) run at 5000 × *g*. The concentrated fraction was applied to a calibrated superdex 200 16/60 (120 ml) size exclusion column and eluted in 20 mM Tris–HCl pH 8, 0.1 M NaCl, 0.2 mM EDTA, 0.1 μM (−) alprenolol and 0.02% DM at 0.5 ml/min. The receptor peak (5 ml) could be either used directly or further concentrated with a 100 kDa molecular weight cut-off centricon at 1000 × *g* for crystallization experiments. The higher molecular weight cut-off membrane was used at this stage in order to minimize concentration of micellar detergent prior to crystallization.

2.6. Solubilization and purification of recombinant βAR from membranes: βAR 34–424/His6

For the purification of 10 mg βAR 34–424/His6 (determined by protein assay) the above procedure was used with the application of 30 mg solubilized βAR (determined by ligand binding) in 1.8 g membrane protein to the first column. For the purification of βAR 34–424/His6 on a smaller scale (2–5 mg) a 1.6 cm diameter (10 ml) chelating sepharose column was used for the first step. Flow rates, wash, gradient and peak collection volumes for this column were reduced by a factor of 2.5. The purification was then continued as described above. This smaller scale purification was adapted to prepare dephosphorylated βAR 34–424/His6 by placing 100 μl (100 units) shrimp alkaline phosphatase in the receiving vessel for the IMAC peak and including 1 mM $MgCl_2$ in IMAC buffer B.

2.7. SDS-PAGE and N-terminal sequencing

Standard techniques were employed for electrophoresis on 12.5% SDS-PAGE gels [20] except that samples were prepared with a room temperature incubation in sample buffer. For N-terminal sequencing, proteins were electroblotted onto PVDF membranes (Immobilon-P, Millipore) [21]. Sequences were determined with an Edman automated

N-terminal protein sequencer (Procise 494, Applied Biosystems).

2.8. Radioligand binding

Saturation binding assays for solubilized receptor were performed in 50 mM Hepes/KOH pH 8, 15 mM $MgCl_2$, 1 mM EDTA, 0.05% dodecylmaltoside with 20 nM [^{3}H](−) dihydroalprenolol in a final volume of 155 μl which was incubated on ice for 1 h. Bound and free radioligand were then separated by centrifugal gel filtration with Quik-sep columns packed with 2.5 ml sephadex G-25F pre-equilibrated with buffer containing detergent as above. Tritiated antagonist was determined by liquid scintillation counting. Non-specific binding was determined by addition of 3 μM s-propranolol to controls. Purified receptor fractions in gel filtration buffer containing 0.1 μM unlabelled (−) alprenolol were diluted at least 1000-fold in 50 mM Hepes/KOH pH 8, 15 mM $MgCl_2$, 1 mM EDTA, 0.05% dodecylmaltoside and incubated on ice for 30 min to reduce both free unlabelled (−) alprenolol and receptor concentrations before addition of 10 μl (~ 5 ng) receptor to each assay.

Membrane-bound receptors were assayed as described above but with the omission of detergent and with bound and free ligand separated by filtration over GF/B (Whatman) filters on a FH225 filtration manifold (Hoefer).

Ligand binding curves for the solubilized receptor were determined with a range of 0.1–20 nM [^{3}H](−) dihydroalprenolol in the presence of 1.5 ng (0.2 nM concentration) receptor, data were analysed using the program Prism (GraphPad).

2.9. Protein estimation

Protein was assayed by the amido black method [22] with bovine serum albumin as the standard. The application of this method to purified receptor preparations was compared with the results of amino acid analysis which was performed on a Biochrom 20 Amino acid analyser (Amersham Pharmacia) after hydrolysis of the samples in 6 M HCl for 18 h at 110 °C. The results for five 100-μg samples from separate purifications indicated that the amido black method was 99% accurate for this receptor.

2.10. Determination of detergent concentration and specific detergent binding

Detergent concentrations were determined colorimetrically. The method is based on a method for determination of sugars [23], which has been adapted and evaluated for use on glycosidic detergents [24]. For determination of dodecyl-β-D-maltoside, standards were prepared in the range 4–20 μg in 50 μl volume and 250 μl 5% phenol and then 600 μl concentrated sulphuric acid were added. Absorbance values of 0.2–1.4 at 490 nm were obtained for the standards and a linear calibration curve was plotted (R>0.99) from which unknown detergent concentrations could be inferred. Specific detergent binding for purified receptor preparations of known protein concentrations could then be calculated after subtraction of the baseline contribution from the column buffer.

2.11. Dephosphorylation

Receptor samples (0.2 μg/μl) were incubated with calf intestinal alkaline phosphatase (1 unit/μg receptor) for 2 h at 37 °C in the recommended buffer with the inclusion of 0.02% DM. Samples were then diluted in SDS sample buffer and run on 12.5% gels.

3. Results and discussion

3.1. Expression, solubilization, purification and characterization of βAR 20–424/His6

Initially, βAR 20–424/His6 was expressed in Sf9 cells grown in TNM-FH media. The receptor expression level was 50 pmol/mg solubilized membrane protein and the yield in shake cultures was 0.4 μg/ml with cells being infected on reaching a density of 2.5×10^6/ml. In large-scale expression in a 50 litre fermenter, with better aeration than in shake cultures, it was possible to infect cells at a density of 3×10^6/ml, resulting in a final yield of 16 mg from 32 l. This gave sufficient material for development of the purification and initial characterization of the receptor.

When βAR 20–424/His6 was expressed in Tni cells, expression was slightly higher at 60 pmol/mg. When cells were infected at a density of 2×10^6/ml, the yield of receptor was increased 2.5-fold over that obtained from Sf9 cells grown in the fermenter to 1.25 mg/l culture. This increase was sufficient to facilitate the routine purification of receptor with a final yield of ~ 2 mg from 4 l shake cultures.

Solubilization of receptor and membrane proteins from insect cell membrane preparations was determined by ligand binding assays and was found to be 85–90% efficient with a ratio of 2:1 detergent/total membrane protein (w/w) and a minimum of 0.2 M NaCl without any prolonged incubation step. A decrease in turbidity of the membrane suspension was observed immediately on addition of detergent and therefore centrifugation was carried out without delay.

As the predicted molecular weight of this receptor construct is 46.062 kDa, the theoretical maximum specific binding for pure receptor is 21.7 nmol/mg protein, assuming one binding site per receptor. The relatively low level expression of ~ 50 pmol βAR/mg solubilized membrane protein dictates that a purification of at least 400-fold is required to achieve a homogenous preparation. An SDS gel

illustrating the purification stages of the receptor from Sf9 cells is shown in Fig. 1. The purified receptor is clearly visible as the major band in lanes 2–5, having an apparent molecular weight of 40 kDa. It is quite common for hydrophobic membrane proteins to run slightly ahead of their predicted positions. It can be seen in lanes 2–4, but not in lane 5 due to the high loading in this lane, that there is a faint band below the main receptor band. N-terminal sequence analysis of both bands gave the amino acid sequences GATAAPTG for the main upper band and ELLSQQ for the minor lower band. Both sequences correspond to βAR, the upper band which is rather smeared out corresponds to the receptor with only the loss of the initial methionine residue but the lower band indicated some further proteolysis with a further 14 residues having been cleaved off. Western blotting with nickel-conjugated peroxidase indicated the presence of the C-terminal histidine tag in the purified receptor (results not shown). Significant enrichment of βAR was achieved in the IMAC and alprenolol sepharose affinity chromatography steps with the final size exclusion step removing some traces of higher molecular weight contaminants and also serving to exchange the receptor into a buffer with a much-reduced alprenolol concentration for subsequent binding assays. The extremely sharp peak which eluted from the calibrated size exclusion column indicated a homogenous receptor preparation; the elution profile from a 16/60 superdex 200 column is shown in Fig. 2. The elution volume corresponded to a molecular weight for the DM/receptor complex of 120 kDa. No change in elution volume was observed when size exclusion chromatography was performed either without ligand or in the presence of the agonist ligand isoprotenerol (100 μM). It is however likely that significant amounts of (−) alprenolol carried over from the affinity elution could have been present at this stage. A specific detergent-binding ratio of 2.25 (w/w) was consistently determined for receptor fractions from the size exclusion column which had not been subjected to a concentration step. This would imply a molecular weight for the DM/receptor complex of 149 kDa and the size-exclusion data were therefore in accordance with a monomeric receptor preparation. The discrepancy between determined and predicted values might have been due to non-ideal behaviour of the βAR/detergent complex on the size exclusion column.

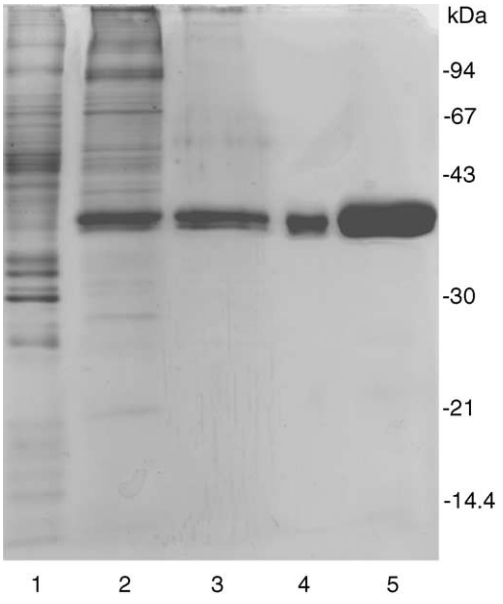

Fig. 1. Silver-stained SDS gel illustrating the purification of βAR 20–424/His6. (1) Solubilized membranes, (2) IMAC, (3) alprenolol sepharose, (4) size exclusion, (5) final concentrated fraction (βAR loadings: lanes 2–4, 2 μg; lane 5, 10 μg).

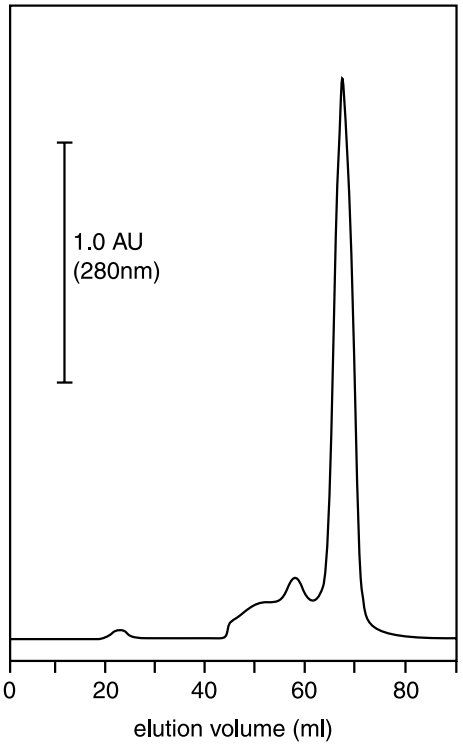

Fig. 2. Elution profile of βAR 20–424/His6 chromatographed on a superdex 200 16/60 size exclusion column.

The receptor could be concentrated up to at least 18 mg/ml with a 100 kDa molecular weight cut-off centricon for crystallization experiments. Even with this higher molecular weight cut-off membrane, some concentration of free micellar detergent was observed. However, if excessive concentration factors were avoided by ensuring that receptor was eluted from the size exclusion column at 0.5–1 mg/ml, the detergent to protein ratio was not significantly elevated as the initial concentration of specifically bound, fully retained detergent exceeded the only partially retained free micellar detergent (0.2 mg/ml) by a factor of 5–10. For a higher receptor concentration factor (50-fold), it was determined that the free micellar detergent was concentrated 10-fold which resulted in a signifi-

cant increase in the detergent to protein ratio. The colorimetric detergent determination method utilised has been validated by comparison with the radiometric method utilising ^{14}C labelled DM and both methods gave similar values [24].

Overall recovery of receptor for the entire purification procedure has been calculated as ~35%. This was based on an initial quantification of receptor in the solubilized membranes by ligand binding which was the only method available for the unpurified receptor. However, quantification of purified receptor was by protein determination as the ligand binding of the highly purified receptor preparation was suboptimal. Ligand binding values of 10–12 nmol/mg were consistently obtained, which corresponded to ~50% of the theoretical maximum. This result is puzzling, as one would expect the receptor to have been active at least up to and including the point of its specific elution by antagonist. It is possible that the purified receptor was only 50% active or that its determination had been only 50% efficient. The dilution of the receptor in the assay mixture was sufficient to reduce the carry-over of non-tritiated antagonist to a maximum of 3.5% of the tritiated antagonist. It is unlikely that there was interference from tightly bound unlabelled antagonist as extended incubations of up to 3 days did not result in any increase in binding sites determined. It is also unlikely that results were affected by poor recovery from the spin columns as 90% recovery of 10 μg quantities of purified receptor was determined by protein assay. It is however possible that the binding may be suboptimal due to delipidation as ligand binding has previously been found to be restored to inactive native turkey βAR preparations by reconstitution [25]. As previously stated, size exclusion and detergent binding data imply, but by no means prove, a monomeric receptor preparation. The affinity of 0.8 nM for (−) alprenolol determined by ligand binding curves performed in the range 0.1–20 nM was slightly lower than the figure of 2 nM previously obtained [10]. Scatchard plots indicated the presence of only one high affinity binding site in the given antagonist ligand concentration range.

3.2. Expression and purification of other βAR constructs

Due to the partial N-terminal proteolysis which was observed on purification of βAR 20–424/His6, it was decided to delete residues 22–33, thus further truncating the construct to produce a more homogenous receptor. The new construct is referred to as βAR 34–424/His6 in order to simplify the nomenclature, although it should be noted that residues 20 and 21 were retained. As this N-terminal deletion resulted in an increase in expression, two further constructs were made to investigate the possibility of purification of an untagged construct (βAR 34–424) and the effect of further N-terminal deletion (βAR 42–424). These constructs also retained residues 20 and 21. The three amino acid sequences of the N-termini of the four constructs are shown with the start of the predicted first membrane span underlined [7].

```
T20   MGATAAPTGSRQVSAELLSQQWEAGMSLL
T34   MG----------AELLSQQWEAGMSLL
T42   MG--------------------AGMSLL
```

Expression of the four constructs in Sf9 and Tni cells on a 20-ml scale is compared in Table 1. In all cases, βAR expression was determined by ligand binding and protein assays on solubilized membrane protein fractions and therefore does not take into account any material which could not be solubilized. Cell densities at the point of infection were 2.5×10^6/ml (Sf9) and 1.5×10^6/ml (Tni).

The deletion of further sections of the N-terminus resulted in increased expression of all the new constructs when compared to βAR 20–424/His6. For βAR 34–424/His6, the increase was 3-fold in Sf9 cells but nearly 6-fold in Tni cells. It has previously been noted that baculovirus-mediated GPCR expression may be enhanced by using Tni cells rather than Sf9 cells [26]. In the case of the βAR constructs we have studied, the difference in expression levels between the two cell-lines was rather variable with the biggest improvement on transfer to Tni cells (3-fold) observed with the best expressed construct (βAR 34–424) and only a minor improvement with the least well-expressed construct (βAR 20–424/His6). Expression was also slightly enhanced when the C-terminal histidine tag was deleted (βAR 34–424). The turkey βAR is a type IIIb membrane protein with an extracellular N-terminus with no leader sequence [27] and the receptor largely follows the 'positive-inside' rule [28], with a large majority of lysine and arginine residues found in the intracellular loops. Significantly, one of four remaining extracellular arginines in βAR 20–424/His6 (Arg 15 was deleted in the original truncation) is at the N-terminus at position 30. This was removed by deletion in the generation of the new constructs and it is

Table 1

βAR construct	Cell line	Expression level [pmol βAR/mg protein] (solubilized)[a]	Total expression [μg βAR/ml culture]	Total expression [receptors/cell] (millions)
20–424/His6	Sf9	48	0.45	4.8
20–424/His6	Tni	64	1.2	21
34–424/His6	Sf9	137	1.2	13
34–424/His6	Tni	362	6.1	110
34–424	Sf9	164	1.25	14
34–424	Tni	470	7.2	130
42–424	Sf9	150	1.2	16
42–424	Tni	147	2.3	67

[a] Determinations of antagonist binding and protein assays were performed on solubilized membrane protein fractions, cell densities at time of infection were 2.5×10^6/ml (Sf9) and 1.5×10^6/ml (Tni).

possible that the removal of this unfavourable residue resulted in the large increase of expression which was observed. In a contrasting approach, the expression of functional human β2-adrenergic receptor has been enhanced by the addition of a cleavable N-terminal signal sequence [6].

The stability of ligand binding was compared for βAR 20–424/His6, 34–424/His6 and 42–424 by following antagonist binding in DM solubilized membranes which had been kept on ice. The first two constructs showed high stability with a half-life of ligand binding in the order of weeks, whereas with 42–424, the half-life was less than 3 days. One of the insights from the crystal structure of bovine rhodopsin has been the interaction of the N-terminus with extracellular loops I and III [2]. If such an interaction were also to occur in the turkey βAR, it could account for the instability of the βAR construct 42–424. No significant difference in stability was observed between βAR 20–424/His6 and 34–424/His6, therefore the N-terminal deletion that had given rise to improved expression of 34–424/His6 appeared not to have compromised the stability of this construct as far as could be determined within the constraints of this assay.

βAR constructs 34–424/His6 and 34–424 were routinely expressed on a 4 l scale in Sf9 and Tni cells for receptor purification and the high expression was maintained, and when infection of Tni cells was carried out at a cell density of 2×10^6/ml, 30 mg unpurified receptor was obtained from 4 litres.

The purification of βAR 34–424 was initially attempted with a combination of alprenolol sepharose and size-exclusion chromatographies. These two steps alone did not yield a homogenous preparation and no further purification step has so far been implemented (results not shown).

When βAR 34–424/His6 was purified, the increased quantity of receptor in the initial solubilizate increased final yields accordingly and we were able to purify 10.5 mg receptor from membranes isolated from 4 l Tni cells. The purified receptor showed a slightly increased electrophoretic mobility and returned the expected N-terminal sequence GAELL, but in other respects, the behaviour and properties of the purified receptor were similar to those of the purified βAR 20–424/His6.

It had previously been observed on SDS-PAGE gels that the main band of βAR 20–424/His6 was rather smeared and fuzzy. In the case of βAR 34–424/His6, the upper part of the main band seemed better resolved into a fainter trailing band. Incubation of both purified constructs with alkaline phosphatase resulted in a more uniform electrophoretic mobility which implied that with both constructs there was some phosphorylation occurring (Fig. 3). The results shown are for Tni cells but it was established that the phosphorylation also occurred in Sf9 cells. In order to improve the preparation, alkaline phosphatases were included in the purification. Shrimp alkaline phosphatase was sufficiently active at 5 °C to produce a receptor preparation

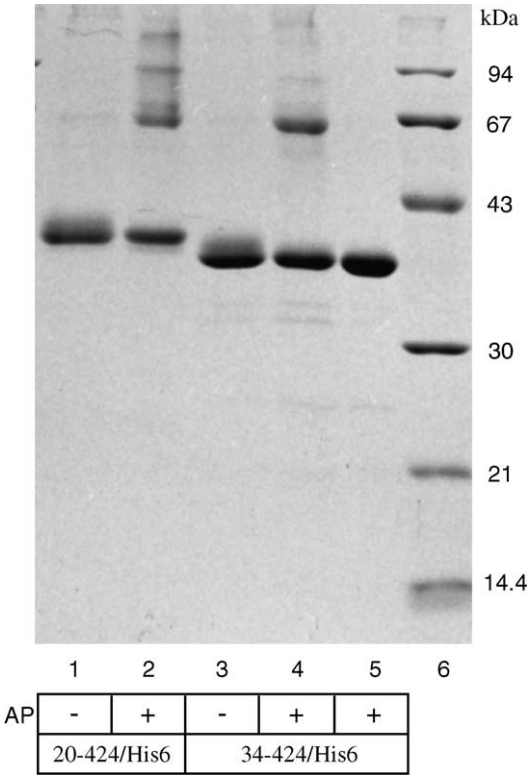

Fig. 3. Dephosphorylation of turkey purified βARs. βAR 20–424/His6: (1) Control, (2) incubated with alkaline phosphatase (AP). βAR 34–424/His6, (3) control, (4) incubated with AP, (5) purified with a shrimp AP incubation step. ~ 1 μg βAR in each lane, (6) molecular weight markers. The gel was Coomassie stained.

which can be judged to be less phosphorylated by its more uniform electrophoretic mobility (see Fig. 3, lane 5). Cys 358 of the turkey βAR is a potential palmitoylation site and the corresponding residue in the human β2AR (Cys 341) is palmitoylated [29], also when expressed in insect cells [30]. In the turkey βAR constructs featured in this study, Cys 358 is followed by 15 serine and threonine residues before the C-terminal truncation at Met 424. It has been shown for the human β2AR that if palmitoylation is prevented, phosphorylation of the receptor is increased [31]. It is possible that the multiplicity of phosphorylation states observed in purified turkey βAR preparations itself results from a heterogeneity in palmitoylation states which has so far not been investigated.

4. Conclusions

In this work, the overexpression and purification of non-glycosylated turkey βARs is described. In addressing a problem with N-terminal proteolysis, we have increased expression levels significantly and we are now able to purify 10 mg quantities of the turkey βAR for large-scale crystallization experiments. The purified receptor has been determined to be partially phosphorylated but the extent of

this remains to be investigated. The phosphorylation of the purified βAR has been significantly reduced by including a dephosphorylation step in the purification. The suboptimal ligand binding properties of the receptor remain a concern and are the subject of continued investigation. Dodecylmaltoside is the only detergent in which we have so far determined the receptor to be stable and the large amount of this detergent specifically bound to the receptor could be a major obstacle to its three-dimensional crystallization. The major focus in our continued crystallization experiments will therefore be on alternative detergents, detergent mixtures and stabilizing conditions.

Acknowledgements

We are grateful to Chris Tate, Reinhard Grisshammer and Olga Perisic at MRC-LMB and Girish Shah and Jim Coote at Glaxo, Stevenage, UK for helpful advice and discussions. We thank Sew Peak-Chew for N-terminal sequence analysis and David Owen for amino acid quantification (both MRC-LMB). We are also grateful to Andrea Urbani at DKFZ, Heidelberg, Germany for advice on detergent quantification. This work was supported by the Medical Research Council, Glaxo SmithKline and Zeneca Pharmaceuticals (UK) through a Link Grant.

References

[1] J. Drews, S. Ryser, Nat. Biotechnol. 15 (13) (1997) 1318–1319.
[2] K. Palczewski, T. Kumasaka, T. Hori, C.A. Behnke, H. Motoshima, B.A. Fox, I. Le Trong, D.C. Teller, T. Okada, R.E. Stenkamp, M. Yamamoto, M. Miyano, Science 289 (2000) 739–745.
[3] C.G. Tate, FEBS Lett. 504 (3) (2001) 94–98.
[4] C.G. Tate, R. Grisshammer, Trends Biotechnol. 14 (11) (1996) 426–430.
[5] M. Bouvier, L. Menard, M. Dennis, S. Marullo, Curr. Opin. Biotechnol. 9 (5) (1998) 522–527.
[6] X.-M. Guan, T.S. Kobilka, B.K. Kobilka, J. Biol. Chem. 267 (31) (1992) 21995–21998.
[7] Y. Yarden, H. Rodriguez, S.K. Wong, D.R. Brandt, D.C. May, J. Burnier, R.N. Harkins, E.Y. Chen, J. Ramachandran, A. Ullrich, E.M. Ross, Proc. Natl. Acad. Sci. U. S. A. 83 (18) (1986) 6795–6799.
[8] J. Wang, E.M. Ross, J. Biol. Chem. 270 (12) (1995) 6488–6495.
[9] C. Hertel, M.H. Nunnally, S.K. Wong, E.A. Murphy, E.M. Ross, J.P. Perkins, J. Biol. Chem. 265 (29) (1990) 17988–17994.
[10] E.M. Parker, E.M. Ross, J. Biol. Chem. 266 (15) (1991) 9987–9996.
[11] E.M. Parker, K. Kameyama, T. Higashijima, E.M. Ross, J. Biol. Chem. 266 (1) (1991) 519–527.
[12] T. Okada, I. Le Trong, B.A. Fox, C.A. Behnke, R.E. Stenkamp, K. Palczewski, J. Struct. Biol. 130 (1) (2000) 73–80.
[13] P. Edwards, J. Li, M. Burghammer, J.H. McDowell, C. Villa, P.A. Hargrave, G.F.X. Schertler, J. Mol. Biol. (submitted for publication).
[14] E. Rands, M.R. Candelore, A.H. Cheung, W.S. Hill, C.D. Strader, R.A. Dixon, J. Biol. Chem. 265 (18) (1990) 10759–10764.
[15] R.A. Dixon, I.S. Sigal, M.R. Candelore, R.B. Register, W. Scattergood, E. Rands, C.D. Strader, EMBO J. 6 (11) (1987) 3269–3275.
[16] A. Luxembourg, M. Hekman, E.M. Ross, FEBS Lett. 283 (1) (1991) 155–158.
[17] M.G. Caron, Y. Srinivasan, J. Pitha, K. Kociolek, R.J. Lefkowitz, J. Biol. Chem. 254 (8) (1979) 2923–2927.
[18] D.R. O'Reilly, L.K. Miller, V.A. Luckow, Baculovirus Expression Vectors: A Laboratory Manual, Freeman, New York, 1992.
[19] L.J. Reed, H. Muench, Am. J. Hyg. 27 (1938) 493–497.
[20] J.E. Coligan, B.M. Dunn, H.L. Ploegh, D.W. Speicher, P.T. Wingfield, Current Protocols in Protein Science, Wiley, 1999.
[21] P. Matsudaira, J. Biol. Chem. 262 (21) (1987) 10035–10038.
[22] W. Schaffner, C. Weissmann, Anal. Biochem. 56 (2) (1973) 502–514.
[23] M. Dubois, K.A. Gilles, J.P. Hamilton, P.A. Rebers, F. Smith, Anal. Chem. 28 (3) (1956) 350–356.
[24] A. Urbani, A. Warne (manuscript in preparation).
[25] J. Kirilovsky, M. Schramm, J. Biol. Chem. 258 (11) (1983) 6841–6849.
[26] D. Massotte, L. Baroche, F. Simonin, L. Yu, B. Kieffer, F. Pattus, J. Biol. Chem. 272 (32) (1997) 19987–19992.
[27] S.J. Singer, Annu. Rev. Cell Biol. 6 (1990) 247–296.
[28] E. Wallin, G. von Heijne, Protein Eng. 8 (1995) 693–698.
[29] B.F. O'Dowd, M. Hnatowich, M.G. Caron, R.J. Lefkowitz, M. Bouvier, J. Biol. Chem. 264 (13) (1989) 7564–7569.
[30] B. Mouillac, M. Caron, H. Bonin, M. Dennis, M. Bouvier, J. Biol. Chem. 267 (30) (1992) 21733–21737.
[31] S. Moffett, G. Rousseau, M. Lagace, M. Bouvier, J. Neurochem. 76 (1) (2001) 269–279.