Purification of a rat neurotensin receptor expressed in *Escherichia coli*

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A truncated rat neurotensin receptor (NTR), expressed in *Escherichia coli* with the maltose-binding protein fused to its N-terminus and the 13 amino acid Bio tag fused to its C-terminus, was purified to apparent homogeneity in two steps by use of the monomeric avidin system followed by a novel neurotensin column. This purification protocol was developed by engineering a variety of affinity tags on to the C-terminus of NTR. Surprisingly, expression levels varied considerably depending on the C-terminal tag used. Functional expression of NTR was highest (800 receptors/cell) when thioredoxin was placed between the receptor C-terminus and the tag, indicating a stabilizing effect of the thioredoxin moiety. Several affinity chromatography methods were tested for purification. NTR with the *in vivo*-biotinylated Bio tag was purified with the highest efficiency compared with NTR with the Strep tag or a hexa-histidine tail. Co-expression of biotin ligase improved considerably the *in vivo* biotinylation of the Bio tag and, therefore, the overall purification yield. Proteolysis of the NTR fusion protein was prevented by removing a protease-sensitive site discovered at the N-terminus of NTR. The ligand binding properties of the purified receptor were similar to those of the membrane-bound protein and the native receptor. The scale-up of this purification scheme, to provide sufficient protein for biophysical studies, is in progress.

INTRODUCTION

Purification of integral membrane proteins from natural sources can be difficult and time consuming, and the amount of purified protein is often too small for structural studies. Heterologous overexpression in bacteria or eukaryotic cells of the gene or cDNA encoding the desired membrane protein offers an alternative with which to overcome these problems [1]. In addition, DNA cloning techniques make it easy to introduce affinity tags on to the recombinant protein to help its purification [2].

Small affinity tags, such as a hexa-histidine tail (-H), may be preferred over larger tags because they may not interfere with structure or activity in any way and therefore may not need to be removed after purification. The recently developed Strep tag (-S) [3] and Bio tag (-B) [4] are only 9 and 13 amino acid residues long respectively. The Strep tag binds specifically to streptavidin, but not to avidin. The Bio tag is biotinylated *in vivo* in *Escherichia coli* and will therefore bind to both avidin and streptavidin. Affinity chromatography matrices suitable for purifying recombinant proteins with histidine, Bio and Strep tags are commercially available.

Affinity tags can have a profound effect on protein stability. In the past, many unstable soluble target proteins were stabilized by adding flanking affinity tails [5]. By contrast, disruption of the overall conformation of the target protein on the addition of tags has also been reported [6,7]. Since the influence of an affinity tag on a given target protein is difficult to predict, it must be determined experimentally. Furthermore, the successful purification of a recombinant membrane protein by affinity chromatography also depends on other factors such as the choice of the detergent for solubilization and the overall expression levels, which require optimization of the purification procedure for each individual target membrane protein.

We have focused on the heterologous expression in *E. coli* and purification of a rat neurotensin receptor (NTR) [8] following preliminary investigation of several peptide receptors. NTR belongs to the family of seven-helix G-protein-coupled receptors (for a review see [9]) having an extracellular N-terminus and an intracellular C-terminus. In this paper we describe a systematic study of the functional expression of NTR linked at the N-terminus to the *E. coli* maltose-binding protein (MBP) and at the C-terminus to various small affinity tags (histidine tail, Strep tag, Bio tag, antigenic epitopes). Functional expression of NTR was highest when thioredoxin was placed between the receptor C-terminus and the respective tag. We then report a comparison of affinity-purification steps based on the interaction of the biotinylated Bio tag with monomeric avidin, the Strep tag with streptavidin, and a histidine tail with immobilized metal ions. Finally, we describe the purification of functional NTR fusion proteins to apparent homogeneity using a two-step procedure consisting of Bio tag binding to monomeric avidin followed by a neurotensin (NT) column.

EXPERIMENTAL

Expression of NTR in *E. coli*

E. coli strain DH5α (Gibco BRL) [10] was used as the host for recombinant plasmids and was grown in double-strength TY-medium [10] containing ampicillin (100 μg/ml) and 0.2 % (w/v) glucose. For expression of functional NTR fusion proteins, DH5α containing the respective plasmid pRG/II-MBP (or pRG/III-hs-MBP) derivative was grown in 400 ml of medium in a 2-litre flask at 37 °C for 4–6 h. After induction with 0.5 mM isopropyl β-thiogalactoside (IPTG) (and addition of 10 μM biotin in the case of co-expression of biotin ligase) the temperature was decreased to 20 °C. The cells were harvested 40 h later, frozen in liquid nitrogen, and stored at -70 °C.

The cDNA cassette encoding a rat NTR [8] was described previously [11]. A *Bam*HI restriction site was created in-frame

Abbreviations used: -B, Bio tag (13 amino acid residues); BirA, *Escherichia coli* biotin ligase; CHS, cholesteryl hemisuccinate Tris salt (Sigma); -F, Flag octapeptide; -H, hexa-histidine tail; -HMTX, penta-histidine tail followed by the c-Myc epitope; IPTG, isopropyl β-thiogalactoside; LM, dodecyl-β-D-maltoside (Calbiochem); MBP, maltose-binding protein; NT, neurotensin; NTR, rat neurotensin receptor; -S, Strep tag; streptavidin–AP, streptavidin–alkaline phosphatase; TrxA, *E. coli* thioredoxin; $t_{\frac{1}{2}}$, half-life.

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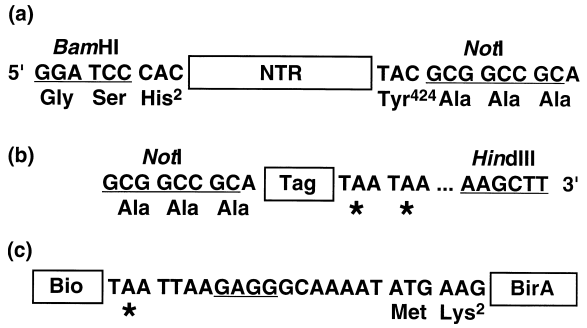

Figure 1 NTR gene cassettes used in this study

The nucleotide sequences of the 5′- and 3′-region of the NTR-gene cassette (**a**) and the tag cassettes (**b**) are given with restriction enzyme sites (underlined) indicated above the sequence. The deduced amino acid residues are shown below the sequence. Numbers refer to amino acid positions in the rat NTR (**a**) and biotin ligase (BirA) (**c**). The amino acid sequences of the various tags are shown in Figure 2. The sequence between the region coding for the Bio tag and the *birA* gene is shown in (**c**). The ribosomal binding site is underlined.

(a)	
Flag	**DYKDDDDK**
Bio	**LGGIFEAMKMEWR**
His	**HHHHHH**
Strep	**AWRHPQFGG**
(b)	
-B	AAA **LGGIFEAMKMEWR**
-H	AAA **HHHHHH**
-S	AAA **SWRHPQFGG**
-HF	AAA **HHHHHH** GT **DYKDDDDK** EF
-HB	AAA **HHHHHH LGGIFEAMKMEWR**
-HS	AAA **HHHHHH AWRHPQFGG**
-HFB	AAA **HHHHHH** GT **DYKDDDDK** EF **LGGIFEAMKMEWR**
-BHF	AAA **LGGIFEAMKMEWR** GG **HHHHHH** GT **DYKDDDDK** EF
-SHF	AAA **WRHPQFGG HHHHHH** GT **DYKDDDDK** EF
-HMTX	AAA **HHHHH** AAA **EQKLISEEDLN**
-TrxA-F	AAA S^{2}[TrxA]A^{109} GT **DYKDDDDK** EF
-TrxA-B	AAA S^{2}[TrxA]A^{109} GT **LGGIFEAMKMEWR**
-TrxA-S	AAA S^{2}[TrxA]A^{109} GT **SWRHPQFGG**
-NTR/SKR-HB	H^{2}[NTR]V^{372} T H^{312}[SKR]Q^{389} AAA **HHHHHH LGGIFEAMKMEWR**

Figure 2 C-terminal tags

Amino acid residues are given in the one-letter code. (**a**) The amino acid sequences of the Flag octa-peptide [68], of the Bio tag, of the hexa-histidine tail, and of the Strep tag are shown. The underlined lysine residue of the Bio tag is biotinylated *in vivo* in *E. coli*. (**b**) The C-terminal tags used in this study are listed. The abbreviated tag names are given on the left-hand side and the corresponding amino acid residues on the right-hand side. The sequence AAA is encoded by the *Not*I restriction enzyme site (Figure 1). TrxA stands for *E. coli* thioredoxin (Ser-2 to Ala-109). -NTR/SKR-HB consists of a C-terminally truncated NTR (His-2 to Val-372) followed by the C-terminus of rat substance K receptor (SKR, His-312 to Gln-389) [69] with the -HB tag. Numbers refer to amino acid positions in thioredoxin, NTR and substance K receptor, respectively. -HMTX stands for the penta-histidine tail followed by the c-Myc epitope [11].

with the codon for His-2 of NTR (Figure 1a) or in-frame with the codon for Thr-43 (T43NTR). A *Hin*dIII restriction enzyme site 3′ of the translation stop codon allowed the insertion of the NTR gene cassettes as *Bam*HI/*Hin*dIII fragments into the expression vectors pRG/II-MBP and pRG/III-hs-MBP (see below). The various C-terminal tag-encoding regions were introduced by PCR and by standard cloning techniques with oligonucleotides. All constructs were confirmed by DNA sequencing (Sequenase, USB). Figure 2 shows the amino acid sequences of the tags used in this study. For co-expression of biotin ligase (BirA), the *birA* gene [12] was placed in an operon-like fashion at the 3′ end of the region encoding the MBP-T43NTR-TrxA-B fusion protein (Figure 1c).

The expression vector pRG/II-MBP was constructed by replacing the *tac* promoter region of plasmid pMal-p2 (New England Biolabs) with the *lac* promoter and double ribosomal binding site of the vector pASK40 [13]. The DNA sequence between the *lac*I^{q} gene and the start codon of the *mal*E gene is identical to pASK40 except that two C-nucleotides precede the ATG codon instead of two A-nucleotides. The codon for Thr-366 of MBP is followed by an in-frame *Bam*HI recognition site and a polylinker including a *Hin*dIII site. Thus, this vector codes for MBP with its N-terminal signal peptide and a multiple cloning site, but lacks the asparagine-linker and the protease factor Xa cleavage site present in pMal-p2.

During large-scale fermentation attempts (but not during expression in shake flasks) pronounced plasmid loss occurred when using DH5α harbouring pRG/II-MBP derivatives as an expression host (Robert Middleton and Richard Hale, unpublished work). Therefore, the expression vector pRG/III-hs-MBP was constructed. The 0.1 kb *Dra*III/*Ava*I DNA fragment (part of the M13 ori) of pRG/II-MBP was replaced with the wild-type *hok*/*sok* gene cassette (see [14–16]) resulting in pRG/II-hs-MBP. Removal of a 75 bp *Nde*I/*Tth*111I DNA fragment containing most of the *nic*/*bom* site [17] gave the parental expression plasmid pRG/III-hs-MBP. Its nucleotide sequence is available upon request. No plasmid loss was observed during fermentation with *E. coli* carrying pRG/III-hs-MBP derivatives; thus, this vector may be generally useful for the expression of membrane proteins in *E. coli*.

Membrane preparation

Spheroplasts were prepared by the method of Witholt et al. [18] and lysed by osmotic shock in ice-cold deionized water [19]. Remaining intact cells were broken open by mild sonication. Protease inhibitors (2 μM leupeptin, 2 μM pepstatin, 0.4 mM PMSF) were present throughout. The membranes were isolated by ultracentrifugation (100000 ***g*** for 1 h at 4 °C), washed with ice-cold buffer A (50 mM Tris/HCl, pH 7.4, 1 mM EDTA, protease inhibitors), and resuspended in ice-cold buffer A containing 10 % (v/v) glycerol. Aliquots of the membrane suspension were flash frozen in liquid nitrogen and stored at −70 °C. EDTA was omitted from buffer A when purification by immobilized metal-affinity chromatography was investigated.

Solubilization of NTR fusion proteins

All steps were carried out at 4 °C. For initial purification trials, NTR fusion proteins were solubilized in buffer B [50 mM Tris/HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, protease inhibitors, 0.6 % (w/v) CHAPS, 0.12 % (w/v) cholesteryl hemisuccinate (Tris salt; CHS), 1 % (w/v) dodecyl-β-D-maltoside (LM)] at a concentration of 5 mg of crude membrane protein per ml. The mixture was gently stirred for 1 h and centrifuged at 100000 ***g*** for 1 h. The supernatant was carefully removed and diluted 1.66-fold with buffer C [50 mM Tris/HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, protease inhibitors, 0.6 % CHAPS, 0.12 % CHS, 50 % (v/v) glycerol] to give a final concentration of 20 % (v/v) glycerol. EDTA was omitted from all buffers for purification of receptors by immobilized metal-affinity chromatography. For the complete two-step purification, the MBP-T43NTR-TrxA-B fusion protein was solubilized in buffer B (pH 7.4) and diluted 2.5-fold with buffer C (pH 7.4) to give a final concentration of 30 % (v/v) glycerol.

Purification of NTR fusion proteins

Initial purification experiments were carried out at 4 °C in buffer D [50 mM Tris/HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, protease inhibitors, 20 % (v/v) glycerol, 0.6 % CHAPS, 0.12 % CHS, 0.1 % LM]. Columns containing 0.5 ml of the appropriate resin were run at a flow rate of 0.15 ml/min. The progress of the purification was monitored by Western blot analysis and ligand binding assay using [^{3}H]NT. Purification of the MBP-NTR-TrxA-S fusion protein was attempted using immobilized streptavidin (Biometra) according to the manufacturer's instructions. Purification of the MBP-(T43)NTR-TrxA-B fusion protein was performed on SoftLink Soft Release Avidin resin (Promega) as described by the manufacturer with the exception that the column was washed with 25 vol. buffer D prior to elution with 5 mM biotin. Purification of the MBP-NTR-HF fusion protein was carried out by immobilized metal-affinity chromatography (Ni^{2+}-NTA agarose, Qiagen), with EDTA omitted from buffer D at all times. The column was washed with 10 vol. of buffer followed by 10 vol. of buffer containing 10 mM imidazole. The fusion protein was eluted with buffer containing 50 mM imidazole.

The complete purification of the MBP-T43NTR-TrxA-B fusion protein was performed via SoftLink monomeric avidin resin followed by a second chromatography step on an NT column. All steps were carried out at 4 °C. Aliquots (45 ml) of solubilized material were loaded batchwise, by gentle rocking for 2 h, on to 2 ml of monomeric avidin resin equilibrated with buffer E [50 mM Tris/HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, protease inhibitors, 30 % (v/v) glycerol, 0.6 % CHAPS, 0.12 % CHS, 0.1 % LM]. The resin was washed five times with 20 ml of buffer E. Receptors were eluted in 2 × 6 ml of buffer E containing 5 mM biotin (2 h incubation followed by a 14 h incubation). Samples (10 ml) of the combined eluate were passed over PD10 gel-filtration columns (Pharmacia) equilibrated with buffer F (buffer E containing 20 mM NaCl). An aliquot (12 ml) of the PD10 column eluate was loaded at a flow rate of 0.125 ml/min on to a 0.5 ml NT column equilibrated with buffer F. The column was washed with 5 vol. of buffer F, followed by 5 vol. of buffer G (buffer E containing 200 mM KCl instead of 200 mM NaCl) and 10 vol. of buffer F. Receptors were eluted in 1.5 ml of buffer H (buffer E containing 1 M NaCl). The NT column was prepared as follows: 2 ml of tetrameric avidin suspension (Sigma A-5150, Lot 42H8290) were succinylated [20] in 50 mM borate buffer, pH 9.0. The resin was washed extensively with buffer I (50 mM Tris/HCl, pH 7.4, 1 mM EDTA) and incubated with 240 nmol (2-fold excess) of biotinylated NT (biotin-βAla-βAla-Gln-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH) in buffer I for 1 h at room temperature. After washing the resin with water, unoccupied biotin binding sites were blocked in buffer I containing 5 mM biotin. The NT resin was washed extensively with buffer I containing 500 mM NaCl, then with water, and stored at 4 °C in buffer I containing 3 mM azide.

NTR binding assay

Saturation binding analysis with [^{3}H]NT (New England Nuclear) on whole *E. coli* cells or membranes was performed as described [11,21]. The data were analysed by non-linear least-squares curve fitting [22,23]. The number of cells resuspended in assay buffer [50 mM Tris/HCl, pH 7.4, 1 mM EDTA, 40 mg of bacitracin per litre, 0.1 % (w/v) BSA] was estimated by measuring A_{600}; 10^9 cells/ml were assumed to correspond to an A_{600} of 1.0. This approach, in combination with ligand binding analysis, led to the calculation of the parameter 'receptors/cell', with some possibility of inherent systematic error. The protein content of crude membranes was assayed as described by Schaffner and Weissmann [24] with BSA as the standard, for the determination of the parameter 'pmol of receptors/mg of crude membrane protein'. Competition experiments with the non-peptide antagonist SR48692 [25] (Sanofi, batch 9200 VI) were carried out in polypropylene tubes, under the same conditions as saturation analysis, in the presence of [^{3}H]NT (0.1–0.2 nM).

The saturation binding of [^{3}H]NT to solubilized receptors was analysed as follows: 20 μl of solubilized receptors were incubated for 1 h on ice in a total volume of 150 μl of assay buffer containing 2 nM [^{3}H]NT and detergent (0.6 % CHAPS, 0.12 % CHS, 0.1 % LM). Non-specific binding was determined in the presence of 2 μM unlabelled NT (Cambridge Research Biochemicals). Separation of bound from free ligand was achieved by gel filtration on 3 ml columns (QS-Q, Advanced Laboratory Techniques, U.K.) of Sephadex G 25 Fine (Pharmacia) (15 %, w/v) equilibrated overnight at 4 °C in buffer (50 mM Tris/HCl, pH 7.4, 1 mM EDTA) containing detergent. A 5 ml aliquot of this slurry was used per column. The columns were precooled to 4 °C and prespun for 3 min at 500 ***g*** (4 °C). Aliquots (100 μl) of the assay mix were loaded on to the columns. Proteins were collected in the void volume at 630 ***g*** (4 °C) and bound ligand was analysed by liquid scintillation counting. Competition experiments with the antagonist SR48692 were carried out in polypropylene tubes, under the same conditions as described above, in the presence of [^{3}H]NT (0.4–0.5 nM). K_i data for membrane-bound and purified NTR were based on the respective K_D values (see Table 2) obtained from [^{3}H]NT saturation analysis.

Western blot analysis

Recombinant NTR fusion proteins synthesized in *E. coli* were detected as appropriate with the mouse anti-(Flag) M2 monoclonal antibody (1:3000) (IBI Kodak), anti-MBP sera (1:3000) (New England Biolabs), or streptavidin–alkaline phosphatase (streptavidin–AP) (1:1000) (Amersham) as described previously [11]. The Western blots were developed by using anti-(mouse IgG) peroxidase conjugate for the M2 antibody or anti-(rabbit IgG) peroxidase conjugate (both Sigma) for anti-MBP sera, employing the substrates diaminobenzidine and hydrogen peroxide. For streptavidin–AP, the substrate system Nitro Blue Tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma) was used as recommended by the supplier. Western blot analysis using 9E10 antibodies directed against the c-Myc epitope was performed as described earlier [11].

N-terminal sequence analysis

Proteins were separated by SDS/PAGE [26] and electroblotted on to polyvinylidene difluoride membranes (Immobilon-P, Millipore) as described [27]. Sequence analysis was performed with an automated gas-phase sequencer (Applied Biosystems 476A/477A).

RESULTS

Increased expression of NTR fusion proteins in *E. coli* using C-terminal tags

We have previously described a fusion protein approach for the functional expression of NTR in bacteria [11] in which MBP is linked to the N-terminus of the receptor. The C-terminus of NTR has now been modified by the addition of various affinity tags (Figure 2) to evaluate immobilized metal-affinity chromatography, immobilized streptavidin, and monomeric avidin resins as first purification steps (see below). Although the only differences between expression plasmids lay at the extreme 3′ end of

Table 1 Expression of NTR fusion proteins in *E. coli*

Expression of NTR fusion proteins was performed in shake flasks with *E. coli* DH5α bearing the respective pRG/II-MBP derivative. Ligand binding analysis on whole cells was performed with [^{3}H]NT. Data are given $\pm$S.E.M. from 3–9 experiments performed in duplicate. Western blot analysis was carried out as described in the Experimental section. Abbreviations: AP, streptavidin-AP; n.a., not applicable. Yes and No are qualitative statements. (Yes) signals obtained with anti-MBP sera and M2 were weaker than expected from signals with streptavidin-AP. [a] Calculated molecular mass of the proteins encoded by the respective expression plasmid. [b] The streptavidin-AP signal obtained with MBP-NTR-TrxA-S was weaker than that with MBP-NTR-TrxA-B, although both fusion proteins were detected with similar intensity by anti-MBP sera.

NTR fusion protein	NT-binding sites per cell	Calculated molecular mass (kDa)[a]	Western blot analysis		
			Anti-MBP	Anti-Flag	AP
MBP-NTR	40 $\pm$ 7	89.9	No	n.a.	n.a.
MBP-NTR-B	22 $\pm$ 11	91.6	No	n.a.	No
MBP-NTR-H	21 $\pm$ 8	90.8	No	n.a.	n.a.
MBP-NTR-S	5 $\pm$ 3	91.1	No	n.a.	No
MBP-NTR-HF	351 $\pm$ 138	92.1	Yes	Yes	n.a.
MBP-NTR-HB	17 $\pm$ 7	92.2	No	n.a.	No
MBP-NTR-HS	0	91.8	No	n.a.	No
MBP-NTR-HFB	36 $\pm$ 16	93.6	(Yes)	(Yes)	Yes
MBP-NTR-BHF	141 $\pm$ 55	93.8	Yes	Yes	Yes
MBP-NTR-SHF	165 $\pm$ 81	93.1	Yes	Yes	No
MBP-NTR-HMTX	341 $\pm$ 80	92.3	Yes	9E10 Yes	n.a.
MBP-NTR-TrxA-F	557 $\pm$ 171	103.4	Yes	Yes	n.a.
MBP-NTR-TrxA-B	695 $\pm$ 188	103.7	Yes	n.a.	Yes[b]
MBP-NTR-TrxA-S	700 $\pm$ 274	103.2	Yes	n.a.	Yes[b]
MBP-NTR/SKR-HB	139 $\pm$ 73	95.2	Yes	n.a.	Yes
MBP-T43NTR-TrxA-B	547 $\pm$ 200	99.1	Yes	n.a.	Yes

the NTR cDNA, surprising differences in expression levels were observed for the various constructs (Table 1). The -HMTX (penta-histidine tail followed by the c-Myc epitope) and -HF tails seem to contribute to higher expression levels compared with the Bio tag, for example, pointing towards a possible stabilization of the fusion protein by these C-terminal tags. NTR fusions with *E. coli* thioredoxin (TrxA) [28] at the C-terminus resulted in the highest expression levels. The stabilizing effect of TrxA may be a result of the remarkable stability of this globular domain [29,30]. All NTR fusion proteins bound [^{3}H]NT with high affinity [11] (see also below).

Solubilization

Careful optimization of the detergent and the buffer composition is critical for obtaining maximum solubilization efficiency and stability of functional receptors in solution. To optimize the solubilization efficiency, several detergents were tested in the presence of 200 mM NaCl. Triton X-100, n-octyl β-D-glucopyranoside, 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine (DHPC), n-octyl tetraoxyethylene (C_8E_4), Zwittergent 3-10 and Zwittergent 3-16 were unsuccessful for solubilization of receptors in functional form due either to inactivation of NTR or to poor solubilization efficiencies. In contrast, CHAPS/CHS and LM allowed solubilization of receptors in functional form. Highest solubilization efficiencies (85 %) were obtained by using a mixture of CHAPS/CHS/LM in the presence of 200 mM NaCl (buffer B). Solubilization with CHAPS/CHS alone resulted in 35 % efficiency, and with LM alone in 30 % efficiency. The buffer composition had a dramatic effect on the half-life ($t_{\frac{1}{2}}$) of receptors in solution. The $t_{\frac{1}{2}}$ of receptors solubilized in LM or CHAPS/CHS was about 15 h or about 25 h at 4 °C, respectively. Using the mixture of CHAPS/CHS/LM increased $t_{\frac{1}{2}}$ to about 50 h. Addition of glycerol (30 %) had a further stabilizing effect up to a $t_{\frac{1}{2}}$ of about 400 h for receptors solubilized in buffer B and diluted with buffer C.

Comparison of affinity purification of various NTR fusion proteins

The current expression levels of NTR fusion proteins (-HF, -TrxA-S, -TrxA-B) (Table 1) are sufficiently high for the development of a purification procedure. However, the first purification step must be powerful because of the relatively low expression level of the receptor fusion protein compared with that of other membrane proteins purified using affinity tags (see for example [31–33]). Several affinity-chromatography methods were considered as possible options for this purpose.

The purification of the MBP-NTR-HF fusion protein by immobilized metal-affinity chromatography was inefficient (10- to 20-fold, determined by ligand binding) (results not shown). The purification factor could be doubled by prolonged washing (50 column vol.) of the loaded Ni^{2+} column. The receptor fusion protein began to elute at a concentration of 20 mM imidazole, preventing stringent washes at higher imidazole concentrations to remove non-specifically bound contaminants. Therefore, another approach was required as a first step in large-scale purification.

To investigate the suitability of the Strep tag for the purification of NTR on immobilized streptavidin, the fusion protein MBP-NTR-TrxA-S was expressed in *E. coli*. Two problems prevented the successful use of this affinity system for receptor purification. First, despite the presence of protease inhibitors, partial proteolysis of the Strep tag occurred during membrane preparation and solubilization as judged by Western blot analysis (results not shown). Secondly, most of the receptor fusion protein did not bind to immobilized recombinant core streptavidin, although a fraction of the fusion protein contained the Strep tag (evidence by Western blot analysis, results not shown). Purification strategies utilizing the Strep tag were not pursued further.

To explore the biotin/avidin system for the purification of NTR, a receptor fusion protein with the C-terminal Bio tag (MBP-NTR-TrxA-B, Table 1 and Figure 2) was expressed in *E. coli*. Initial purification attempts showed promising purification factors of 70 as determined by ligand binding analysis (results

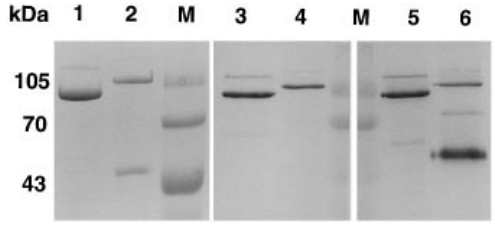

Figure 3 Purification of NTR fusion proteins by the biotin/monomeric avidin system

The proteins MBP-T43NTR-TrxA-B (lanes 1, 3 and 5) and MBP-NTR-TrxA-B (lanes 2, 4 and 6) were partially purified on monomeric avidin resin and analysed by SDS/PAGE (10%). Lanes 1 (2.8 μg of protein) and 2 (1.75 μg of protein), Coomassie Blue staining; lanes 3 (0.56 μg of protein) and 4 (0.35 μg of protein), Western blot analysis with anti-MBP sera; lanes 5 (0.56 μg of protein) and 6 (0.35 μg of protein), Western blot analysis with Strep-AP. Lane M: prestained molecular-mass markers (Gibco-BRL).

Table 2 Pharmacological characterization of NTR fusion proteins

Saturation binding of the agonist [^{3}H]NT and competition of the non-peptide antagonist SR48692 were analysed as described in the Experimental section. Average data are given $\pm$S.E.M. from experiments performed in triplicate.

	Membranes	Purified
[^{3}H]NT saturation (K_D in nM)		
MBP-NTR-TrxA-B	0.12 ± 0.02 ($n = 3$)[a]	
MBP-T43NTR-TrxA-B	0.14 ± 0.04 ($n = 10$)[a]	0.43 ± 0.11 ($n = 6$)
SR48692 competition (K_i in nM)		
MBP-NTR-TrxA-B	0.71 ± 0.11 ($n = 3$)	
MBP-T43NTR-TrxA-B	0.64 ± 0.16 ($n = 4$)	5.06 ± 2.22 ($n = 4$)

[a] An average B_{max} of 10–15 pmol/mg of crude membrane protein was determined.

Table 3 Purification of the NTR fusion protein MBP-T43NTR-TrxA-B

Analysis of the purification of MBP-T43NTR-TrxA-B (average of five experiments) according to the procedures detailed in the Experimental section. Membranes prepared from 1.3 l of an *E. coli* suspension (A_{600} of 3, shake flask expression, pRG/III-hs-MBP derivative, co-expression of biotin ligase) were used for solubilization. Total protein was measured according to the method of Schaffner and Weissmann [24]. The activity was determined by saturation binding of [^{3}H]NT. Recovery data listed in parentheses refer to the NT column.

	Protein (mg)	Activity (pmol/mg)	Purification (-fold)	Recovery (%)
Monomeric avidin (SoftLink)				
Solubilized material loaded	40	27	1	100
Flow through	37	7.3		25
Eluate	0.26	1600	60	38
NT column				
SoftLink eluate loaded	0.23	1600		34 (100)
Flow through	0.13	54		0.7 (2)
KCl wash	0.024	958		2 (6)
Eluate	0.038	6026	223	21 (62)

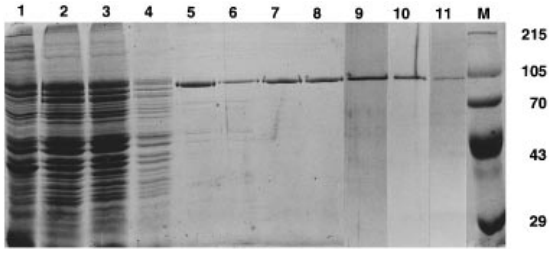

Figure 4 Two-step purification of MBP-T43NTR-TrxA-B to apparent homogeneity

The truncated NTR fusion protein was purified on a monomeric avidin column (lanes 3–5) followed by an NT column (lanes 6–11) as detailed in the Experimental section. The progress of the purification was monitored by SDS/PAGE (lanes 1–8: Coomassie Blue stain, lane 9: silver stain), Western blot analysis (lane 10: Strep-AP, lane 11: anti-MBP sera) and ligand binding (Table 3). Key to lanes: lane 1, membrane preparation (25 μg of protein); lane 2, solubilized material (30 μg); lanes 3–5, SoftLink column flow through (30 μg), wash (10 μg), and eluate after desalting (1.7 μg); lanes 6–8, NT column flow through (0.87 μg), KCl wash (0.68 μg), and eluate (0.64 μg); lanes 9–11, eluate (0.05 μg, 0.14 μg, 0.14 μg); lane M, prestained molecular-mass markers (Gibco-BRL).

not shown). Two problems became evident during purification trials. First, up to 85% of the fusion protein did not bind to the avidin resin and was found in the flow through. 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. Co-expression of *E. coli* biotin ligase led to a remarkable decrease in the amount of receptor in the flow through (now less than 25%), indicating that incomplete biotinylation was a major cause of the initial 'flow through' problem. The second problem during membrane preparation and purification of MBP-NTR-TrxA-B was that a major proteolytic product (presumed to be NTR-TrxA-B) could be detected by Western blot analysis with streptavidin-AP (Figure 3, lane 6), but not with anti-MBP antibodies (Figure 3, lane 4). This fragment co-purified with the full-length receptor fusion protein on the monomeric avidin column, and also on the NT column (see below; results not shown). As a control experiment, a purification was carried out using membranes from *E. coli* harbouring the parental expression plasmid without the receptor cDNA. The membrane fraction, solubilized proteins and the avidin column eluate were analysed by SDS/PAGE and Western blot. No protein was detected in the eluate (Coomassie Blue stain), and no immunogenic signals were observed in any of these three fractions (results not shown). Removal of the N-terminal 42 amino acid residues of NTR (MBP-T43NTR-TrxA-B) abolished the proteolytic fragment, indicating that protease-sensitive sites were located within this region. Indeed, N-terminal sequence analysis of the proteolytic fragment revealed the major sequence Leu-Ser-Leu-Ser-Asn-Gly, and the underlying sequence Leu-Ala-Leu-Ser-Leu-Ser, corresponding to amino acid residues 34–39 and 32–37 of NTR, respectively.

Both fusion proteins (MBP-NTR-TrxA-B and MBP-T43NTR-TrxA-B) bound [^{3}H]NT with the same high affinity (Table 2) [11]. Thus, the use of a monomeric avidin affinity chromatography step on the MBP-T43NTR-TrxA-B fusion protein satisfied all the criteria we required for a high efficiency first step.

Two-step purification of functional MBP-T43NTR-TrxA-B

The truncated NTR fusion protein was purified to apparent homogeneity by using two affinity chromatography steps. Table 3 summarizes the two-step procedure. *In vivo* biotinylation of the Bio tag allowed the pre-purification of the fusion protein by the monomeric avidin system as described above. Isolation of functional receptors was then achieved by ligand affinity chromatography. We exploited the high affinity of biotinylated NT to

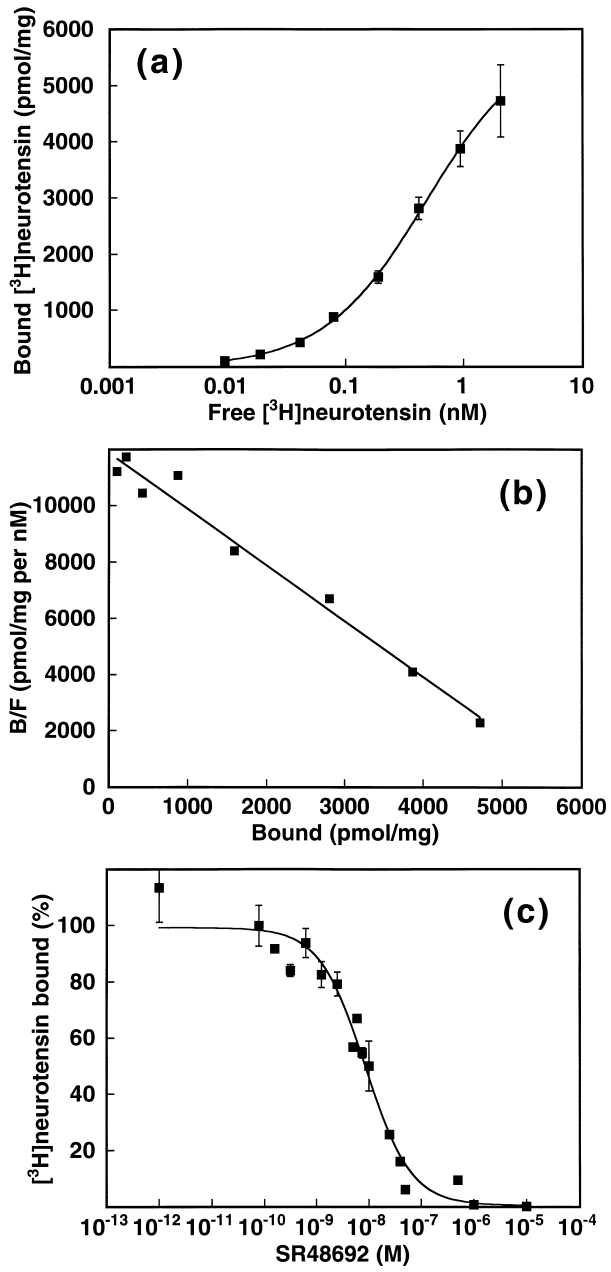

Figure 5 Pharmacological characterization of the purified MBP-T43NTR-TrxA-B protein

(**a**) Saturation binding of the agonist [^{3}H]NT. The specific binding activity amounted to 81.7–93.3% of the total binding activity (for example: 8078 d.p.m. specific binding versus 9888 d.p.m. total binding at a [^{3}H]NT concentration of 2.4 nM). (**b**) Scatchard transformation of the saturation data. Abbreviations: B, bound; F, free. (**c**) Competition of [^{3}H]NT binding by the non-peptide antagonist SR48692. Representative experiments performed in triplicate are shown (see also Table 2). In many cases, the error bars are smaller than the symbols (**a** and **c**).

tetrameric avidin resin for preparation of the NT column. This avoided unwanted leakage of ligand as has been observed when NT was coupled to glutaraldehyde-activated gel [34]. After desalting, the eluate from the monomeric avidin column was passed over the NT column, which retained almost all of the NT binding activity. Non-functional receptors were found in the flow through (Table 3; Figure 4, lane 6). Proteins retained non-specifically on the gel by ionic interaction were eliminated by washing the column with buffer containing 200 mM KCl [35]. A small amount (2%) of functional receptor protein was lost during this step (Table 3) because K^+ (and Na^+) ions decrease the NT binding affinity [36]. Receptor fusion protein, eluted from the NT column with buffer containing 1 M NaCl, was pure as revealed by SDS/PAGE (Figure 4, lane 8). The final yield was 21% of the total activity present in the solubilized material.

Purified receptors bound NT in a reversible and saturable manner and with high affinity (Table 2, Figure 5a). The specific activity was 6134 ($\pm$636) pmol/mg (five experiments in triplicate). A specific activity of 10340 pmol/mg corresponds to a molecular mass of 96.7 kDa for the fusion protein, assuming one ligand binding site per receptor molecule. The selective non-peptide antagonist SR48692 inhibited the binding of [^{3}H]NT to purified receptors (Table 2, Figure 5c).

The $t_{\frac{1}{2}}$ of detergent-solubilized receptors was determined at each stage of the two-step purification by [^{3}H]NT binding assay. The $t_{\frac{1}{2}}$ of solubilized receptors and receptors in the SoftLink column eluate was about 400 h at 4 °C, decreasing to about 320 h for the purified fusion protein. Interestingly, reduction of the NaCl concentration from 1 M to 200 mM decreased the $t_{\frac{1}{2}}$ of purified receptors to about 50 h.

The N-terminal sequence of purified MBP-T43NTR-TrxA-B (and MBP-NTR-TrxA-B) fusion protein was determined by Edman degradation (Lys-Ile-Glu-Glu-Gly-Lys-Leu) and corresponded to the sequence of mature MBP [37]. This established that the purified protein was MBP-T43NTR-TrxA-B and revealed that the MBP signal peptide had been correctly processed.

DISCUSSION

Expression of NTR fusion proteins

Determination of the structure of integral membrane proteins is a challenging task essential to an understanding of the functioning of fundamental biological processes at the atomic level. Unfortunately, the majority of membrane proteins are found naturally in very small quantities, and therefore have to be overexpressed. To date, there are still few examples of highly overproduced membrane proteins, and it is difficult to outline general rules for the successful overexpression of a desired membrane protein [1].

We have previously shown [11] that maximal expression of NTR with a penta-histidine c-Myc tail (HMTX) occurred at low temperature (20 °C) with the *E. coli* cells in the late stationary phase (expression lasts over 40 h). The improvement in expression levels on addition of an N-terminal MBP moiety has also been documented previously. Here we report the influence of C-terminal tails on the expression of NTR fusion proteins. Thus it appears that many factors, rather than one key factor, contribute to higher expression levels of NTR in bacteria.

Two factors, thermodynamic stability and C-terminal sequence, have been reported to determine the susceptibility of model proteins to intracellular degradation [38]. In the case of the N-terminal domain of the λ repressor, non-polar amino acid residues are destabilizing when placed at the extreme, unstructured C-terminus, whereas charged and polar residues show a stabilizing effect. The influence of C-terminal tags on the expression of NTR fusion proteins seems to be more complex and the reason why some tags stabilize NTR is not clear. However, it can be noted that the -HF and -HMTX tails contain clusters of negatively charged residues (Figure 2) which may contribute to increased expression levels compared with fusion proteins with for example -B, -H, or -S tags (Table 1). The

introduction of thioredoxin between the C-terminus of NTR and the affinity tag had the most beneficial effect on expression. Thioredoxin is a very stable molecule and has a compact fold with over 80 % of the polypeptide chain involved in secondary structure [29,30]. This feature may contribute to its stabilizing effect and higher expression levels.

The following data suggest that the NTR fusion protein is correctly oriented in the cytoplasmic membrane of *E. coli*. N-terminal sequence analysis of the purified MBP-T43NTR-TrxA-B fusion protein revealed that the MBP signal peptide had been correctly processed, indicating that the MBP moiety had been translocated across the membrane. This suggests that the N-terminus of NTR is also on the periplasmic side of the membrane, and thus in the same orientation as NTR expressed in brain. Furthermore, ligand binding analysis can be successfully performed with whole *E. coli* cells using [^{3}H]NT, which is a hydrophilic peptide and is highly unlikely to cross the membrane. The ligand binding site must therefore be accessible from the periplasmic side of the membrane, again supporting the above conclusion.

Affinity purification of tagged NTR fusion proteins

Polytopic eukaryotic membrane proteins are difficult to overexpress in their functional form in *E. coli* [1]. The expression levels of NTR are sufficiently high to envisage purification, but are relatively low (10–15 pmol/mg or 0.1 % of membrane protein) in comparison with overexpressed prokaryotic membrane proteins (i.e. melibiose permease, 190 pmol/mg [39]). Therefore, the first purification step has to be very efficient in order to enrich the NTR fusion protein. Affinity tags fused to NTR were considered as a possible way of achieving this goal.

Immobilized metal-affinity chromatography has been used very successfully for the purification of soluble proteins [40,41]. More recently, this technique has also been applied to the purification of membrane proteins in the presence of detergents [31,32,42–48]. This approach, however, was not satisfactory for the MBP-NTR-HF fusion protein, resulting in fairly low purification efficiencies. Stringent washes with buffer containing imidazole to remove non-specifically bound contaminants were not possible due to early elution of the receptors. This may be due to the specific buffer composition necessary to ensure the stability of the solubilized NTR fusion proteins. Interestingly, all the above published purifications were performed in buffers containing non-ionic detergents (e.g. Triton X-100 or dodecyl-maltoside), whereas a mixture of dodecyl-maltoside and the zwitterionic detergent CHAPS was required in the case of MBP-NTR-HF. Loo and Clarke [49] reported that ionic detergents prevented the binding of recombinant P-glycoprotein with a C-terminal histidine tail to an Ni^{2+}-NTA column. Thus CHAPS may have adversely affected the tight binding of MBP-NTR-HF to the Ni^{2+}-NTA resin. However, the presence of CHAPS is absolutely critical for the stability of NTR fusion proteins during purification and it cannot be omitted from buffers (this was studied in detail for the MBP-T43NTR-TrxA-B protein, results not shown).

The Strep tag binds to recombinant core streptavidin [50] and has been used for the purification of soluble proteins [3,50,51]. A novel and elegant approach using engineered Strep-tagged antibody Fv fragments produced in *E. coli* has recently been employed to purify integral membrane protein complexes in a single step from *Paracoccus denitrificans* membranes [52]. Purification of the Strep-tagged NTR fusion protein was unsuccessful mainly due to proteolytic removal of the tag during membrane preparation and solubilization.

Biotinylated proteins are rare in nature [53]; *E. coli* contains only one such protein, the soluble biotin carboxyl carrier protein of acetyl-CoA carboxylase [54]. This makes the biotin/monomeric avidin affinity system an attractive method for purification of recombinant proteins. Recently, *in vivo* biotinylation of overexpressed membrane proteins has been accomplished by fusing them to the biotin acceptor domain (100 amino acids) of the oxaloacetate decarboxylase from *Klebsiella pneumoniae* and purification of the fusion proteins has been readily achieved [33,44,55]. Here we report the use of a much smaller biotinylation domain, the 13-amino-acid-residue Bio tag [4], for the successful pre-purification of NTR fusion proteins with purification factors of 70, as determined by ligand binding analysis. The eluate from the avidin column contained a mixture of functional and non-functional receptors which could be separated subsequently on a NT column (see below). The actual purification factor of the monomeric avidin step is thus probably higher than that estimated from ligand binding analysis.

Co-expression of biotin ligase is crucial for *in vivo* biotinylation of the Bio tag. A high proportion of Bio-tagged NTR did not bind to monomeric avidin resin when the fusion protein alone was expressed in *E. coli*. Co-expression of biotin ligase resulted in a large increase in the amount of receptor binding to the avidin resin, indicating that incomplete biotinylation was the main reason for poor binding.

Two-step purification of functional NTR fusion proteins

The purification of functional NTR to apparent homogeneity required two steps, one based on the C-terminal Bio tag and the other on ligand binding. Several improvements contributed to the successful use of the biotin/monomeric avidin system as the first step in purifying NTR fusion proteins. Co-expression of biotin ligase enhanced considerably the *in vivo* biotinylation of the Bio tag and therefore the overall purification yield. Use of the mutant receptor MBP-T43NTR-TrxA-B improved the homogeneity of the receptor preparation by removing susceptibility to proteolysis.

The SoftLink resin eluate contained functional and non-functional NTR, the latter a result of possible misfolding of receptors during expression, inactivation of receptors during membrane preparation and adverse influences of the detergent on the ligand binding properties of NTR (see also [42]). We used a novel NT column as the second step to separate functional ligand-binding receptors from non-functional receptors. This NT column is based on the coupling of biotinylated NT to tetrameric avidin resin. A similar approach has been successfully used for the purification of other seven-helix peptide receptors [56–60]. The NT column has a high ligand density (theoretical value: 120 μM) and did not give rise to leakage in contrast to that observed when NT was coupled to glutaraldehyde-activated gel [34].

The ligand-binding properties of purified receptor were similar to those of the membrane-bound protein and comparable with NTR expressed in mammalian cells.

Pharmacological characterization of NTR fusion proteins

During the purification of MBP-NTR-TrxA-B, a major proteolytic product, found to be L32/L34NTR-TrxA-B, was detected (Figure 3, lanes 2 and 6). In order to increase the homogeneity of purified receptors and abolish the protease-sensitive sites, a mutant NTR protein (MBP-T43NTR-TrxA-B) lacking the N-terminal 42 amino acid residues of NTR was

expressed in *E. coli*. Mutagenesis studies on substance P receptor suggested that the N-terminal region proximal to transmembrane domain I plays an important role in agonist binding [61]. Similarly, a mutant NTR lacking amino acid residues 45–60 was devoid of NT binding [62]. In our case, both the full-length and the T43-truncated NTR fusion protein had the same affinity for NT (and the antagonist SR48692) (Table 2). This established that the N-terminal 42 amino acid residues of NTR are not involved in agonist binding, which is in agreement with results obtained with a deletion mutant NTR lacking amino acid residues 14–28 [62].

Different molecular forms of high-affinity NTRs have been characterized by purification, photoaffinity labelling and molecular cloning (see ref. [63]). The pharmacological properties of MBP-T43NTR-TrxA-B and MBP-NTR-TrxA-B in membranes or after purification in detergent (Table 2) can be compared with those of NTRs from brain and expressed in mammalian cells. Membrane-bound NTR fusion proteins exhibited the same high affinity for NT (Table 2) as the cloned NTR expressed in COS and LTK$^-$ cells (K_D of 0.16–0.45 nM) [8,64,65]. In addition, the 55 kDa NTR from rat brain, likely to be the glycosylated form of the cloned NTR, bound NT with the same affinity as the NTR fusion proteins; the K_D values for the brain receptor are 0.13 nM (membrane-bound) and 0.45 nM (purified in the presence of digitonin) [66]. The 100 kDa high-affinity NTR from mouse and human brain bound NT with the same affinity (K_D of about 0.3 nM), both when membrane-associated and when solubilized in the presence of CHAPS/CHS [34–36,67]. The affinity for the antagonist SR48692 of membrane-bound NTR fusion proteins (competition of [^{3}H]NT binding by SR48692) was about 9- to 16-fold higher (Table 2) than that of rat NTR expressed in COS cells (K_i of 6 nM) and LTK$^-$ cells (K_i of 11 nM) [25,62]. Saturation experiments with [^{3}H]SR48692 on membranes derived from LTK$^-$ cells resulted in a dissociation constant, K_D, of 3.4 nM [62]. The reason for the slightly higher affinity of the NTR fusion proteins for antagonist is unknown, but may reflect differences in the respective host systems used for expression. The affinity for antagonist of purified MBP-T43NTR-TrxA-B in detergent is about 7-fold lower than that of receptors in membranes (Table 2). A smaller 3-fold decrease was observed for NT affinity. This may reflect some influence of detergent on the ligand binding behaviour of NTR. A similar observation has been reported for the 55 kDa rat brain NTR purified in the presence of digitonin [66].

Conclusions

Determination of the structure of integral membrane proteins requires in most cases a suitable overexpression system and an efficient purification scheme. Our goal is to develop strategies which will allow the biophysical and structural investigation of NTR. To this end, we have achieved increased expression of NTR fusion proteins in *E. coli* by placing the stable thioredoxin moiety between the receptor C-terminus and the affinity tag. The biotin/avidin system provided an excellent pre-purification step suitable for processing solubilized NTR on a large scale. The separation of functional NTR from non-functional polypeptide was achieved by using a novel NT column. The ligand-binding properties of purified receptor were similar to those of the membrane-bound protein and comparable with NTR expressed in mammalian cells. The two-step purification protocol presented here will allow the development of a large-scale purification scheme for NTR fusion proteins. Similar approaches may be useful for a wide variety of other receptors.

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