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Purification of the membrane binding domain of cytochrome b_5 by immobilised nickel chelate chromatography

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Abstract

The purification of a eukaryotic membrane protein has been achieved using a prokaryotic expression system. Bovine cytochrome b_5 is an integral membrane protein (M_r~16 500). It comprises of a globular haem containing catalytic domain positioned at the N-terminus of the protein and a hydrophobic membrane binding segment at the C-terminus. The membrane binding domain (MBD) is resistant to purification using conventional strategies that have proved successful in isolating the soluble haem containing fragment. We report here a versatile purification method for the isolation of the MBD involving a gene fusion system. The fusion protein incorporates thioredoxin at the amino terminus and six histidines as the metal affinity binding site followed by cytochrome b_5 in a pET expression system. This supports high level expression of cytochrome b_5 in *E. coli* C43(DE3) cells. The fusion protein is effectively solubilised from lysed cells with Triton X-100. A step gradient elution with imidazole under non-denaturing conditions on a His-Bind nickel chelate affinity column, saturated with proteins as a crude cell extract, purified the protein in a single step. Proteolytic digestion of pure fusion protein, with trypsin, yielded the MBD. This fragment was further purified by RP-HPLC to a final yield of ~10 mg/l.

Keywords: Purification; Membrane binding domain; Cytochrome b_5; Nickel chelates; Thioredoxin

1. Introduction

Cytochrome b_5 is an amphipathic electron transfer protein. The polypeptide consists of two distinct domains linked by a trypsin sensitive region: a short hydrophobic C-terminal membrane binding domain comprising of approximately 40 amino acid residues which anchors the protein to the endoplasmic reticulum membranes and a larger globular segment containing the haem catalytic domain that projects into the cytosol [1]. In liver microsomes cytochrome b_5 is an integral part of a system responsible for oxidative conversion of stearyl-CoA to oleoyl-CoA [2] and has been implicated as a participant in the cytochrome P-450 dependent hydroxylation reactions [3]. A similar protein lacking the C-terminal region is found in erythrocytes and catalyses the reduction of methaemoglobin [4]. Due to its high solubility and its ease of isolation the haem binding domain has been the subject of most structural and functional studies [5] including detailed crystallography [6] and nuclear magnetic resonance (NMR) spectroscopy [7]. By contrast, little definitive structural data is available for the membrane binding domain. Progress in this area has been hindered due to difficulties encountered during the isolation of full length cyto-

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chrome b_5 where the hydrophobic membrane binding domain (MBD) promotes aggregation [8].

In part, these difficulties can be overcome by fusing the protein of interest to a second protein that is soluble and one that is expressed efficiently and to high levels in prokaryotes. Recently this approach has proved to be effective in expressing ''problematic'' proteins. Some of the original fusion systems, such as those employing *E. coli* genes *lac*Z [9], *mal*E [10] or *trp*E [11] as the highly expressed partners, can resolve translation initiation difficulties, but they do not always solve the intrinsic solubility problems of recombinant proteins. Other systems, such as those employing *Staphylococcus* protein A [12] and *Schistosoma* glutathione S-transferase (GST) [13] as fusion partners, provide a biochemical property that can be exploited as an affinity tag for the effective purification of proteins. In particular these systems may prove useful for the expression of membrane proteins that generally show strong tendencies to aggregate when individually expressed in *E. coli*.

In this laboratory the MBD of cytochrome b_5 has been expressed independently of the soluble catalytic domain, as a C-terminal fusion to GST (unpublished results). Protein expression resulted in the formation of inclusion bodies that required detergent solubilisation in order to purify the fusion product. Although the production of inclusion bodies may facilitate protein purification, the recovery of a soluble and active molecule after treatment with strong denaturing agents is often low [14] and this was indeed the case with the GST-MBD fusion system.

Here we report the use of a fusion expression system based on *E. coli* thioredoxin (*trx*A) [15]. Whole cytochrome b_5 is expressed as a fusion protein with a metal affinity binding site that includes a stretch of six histidine residues integrated into the recombinant protein to facilitate purification by binding to a transition metal ion [16]. The system alleviates the tendency of the fusion protein to form inclusion bodies. In view of the difficulty in directly expressing membrane proteins in heterologous systems, the expression of hydrophobic proteins or membrane proteins to high levels as thioredoxin fusions followed by selective cleavage may provide a generally useful expression and purification strategy. The adopted procedure is reproducible and well suited to large scale purification utilising two simple chromatographic steps.

2. Experimental

2.1. Equipment

Reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on a Gilson HPLC 306 operating system consisting of two pump modules, a 811C mixing chamber accompanied with a manometric 805 module equipped with a variable-wavelength 115 UV detector and a Rheodyne injection port assembled with a 1-ml sample loop. The system was controlled using Gilson software. Column eluates were routinely measured using the absorbance at 280 nm. Samples were loaded onto the column in 1-ml aliquots.

Chromatographic columns, fraction collectors and PhastSystem electrophoresis apparatus were products of Amersham Pharmacia Biotech (UK). Electroporation was carried out using a Gene Pulser (Bio-Rad, UK).

2.2. Chemicals and reagents

Unless otherwise stated, all laboratory reagents were of analytical grade or better. Water used in the preparation of solutions was deionised and obtained from an ''Ultra Pure Milli-Q system'' (Millipore). Acetonitrile (HPLC grade) and globin low-molecular-mass protein markers ranging from 16 000–2500 were obtained from Merck (UK). Trifluoroacetic acid, trypsin, L-1(tosylamido)-2-phenylethyl-chloromethyl ketone (TPCK), EDTA (disodium salt, dihydrate), nickel sulphate, imidazole, potassium dihydrogenphosphate (KH_2PO_4), dipotassium hydrogenphosphate (K_2HPO_4), molecular mass markers, Triton X-100 (TX-100), phenylmethyl sulphonyl fluoride (PMSF), tris(hydroxymethyl)aminomethane (Tris), sodium chloride (NaCl), lysozyme, DNaseI and bovine serum albumin (BSA) were all purchased from Sigma (UK). Agarose was obtained from Gibco (Paisley, UK), glycerol from Fisher (UK) and IPTG

and X-Gal were obtained from Melford Labs. (Ipswich, UK).

Restriction enzymes and DNA ligases were purchased from Promega or Kramel Biotechnology (Northumberland, UK), whilst all DNA sequencing reagents were obtained from Flowgen (Staffordshire, UK), α ^{35}S-dATP radioisotope (10 mCi/ml) from Amersham Pharmacia Biotech and a DNA Cyclist (Exo$^-$) Kit was purchased from Strategene (Cambridge, UK). A polymerase chain reaction (PCR) ''ready-to-use'' Master Mix of dNTPs and buffer was obtained from Advanced Biotechnologies (Surrey, UK).

All bacterial growth media were obtained from Difco (UK), Oxoid (UK) and Sigma. The His-Bind resin was a product of Calbiochem (UK).

2.3. Culture media

Bacterial cultures were routinely grown in LB media supplemented with ampicillin at a final concentration of 50 μg/ml at 30°C under aerobic conditions. Growth on phosphate rich media (TYP media) required 12 g/ml Bacto-Tryptone, 24 g/l yeast extract, 2.3 g KH_2PO_4, 12.5 g K_2HPO_4 and 4 ml/l glycerol.

2.4. Bacterial strains and plasmids

E. coli strain JM109 was used for plasmid mediated transformation during the cloning of the DNA encoding for cytochrome b_5 (Ala1–Asn133) and for the propagation and isolation of plasmid DNA. In the case of protein expression, the *E. coli* strain C43(DE3) a derivative of BL21(DE3) [17] was used. The cloning vector pGEM-T was obtained from Promega, whilst the expression vector pET-32b(+) was purchased from Calbiochem.

2.5. Recombinant DNA methods and reagents

DNA treatments with restriction enzymes, ligation reactions, agarose gel electrophoresis, growth of bacterial cells, selection of recombinants and plasmid preparations were carried out following standard procedures as described by Maniatis et al. [18].

2.6. Cloning of cytochrome b_5

Using the coding sequence of full length cytochrome b_5 present in the plasmid pKK223-3, the DNA was amplified using the following oligonucleotide primers in a PCR. PCR primers for sense 5′-GCG**CCATGG**CTGAAGAAAGC-3′ and antisense 5′ - CCTG **AAGCTT** ATT TAG TTC TCC GAA GT-GTA-3′ strands were used and incorporated a *Nco*I and a *Hind*III site (as indicated in bold) at the 5′ and 3′ ends to facilitate cloning into an expression vector. Reactions were carried out using 44 μl ''ready-to-use'' Master Mix containing 1.25 units of Taq polymerase, 75 m*M* Tris–HCl (pH 8.8 at 25°C), 20 m*M* $(NH_4)_2SO_4$, 1.5 m*M* $MgCl_2$, 0.01% (v/v) Tween 20, 0.2 m*M* of each dNTP, 2.5 μl of each primer (15 μ*M*) to which 1 μl of template DNA (~10 ng) was added. The reaction mixture was overlaid with mineral oil to prevent evaporation and 29 cycles were performed with denaturation at 94°C for 60 s, annealing at 51°C for 60 s and extension at 72°C for 60 s in a MiniCycler (MJ Research). A single DNA product was observed on a 0.8% agarose gel.

The amplified DNA product (~4000 base pairs), purified from an agarose gel was conveniently ligated via the 3′-dA overhangs (a feature of Taq polymerase activity) into the vector pGEM-T containing complementary 3′ terminal thymidine at both ends of the double stranded DNA. The ligation reaction was used to transform JM109 by electrochemical methods and positive clones were selected using the blue–white β-galactosidase assay. The plasmid DNA of white colonies was isolated and restriction digest analysis was used to identify the target DNA. Subsequently the sequence of this DNA was confirmed by PCR sequencing using the DNA Cyclist (Exo$^-$) kit.

2.7. Construction of an expression vector

The recombinant pGEM-T vector (designated pGEM-b5w) was digested with *Nco*I and *Hind*III and the resulting cytochrome b_5 fragment was inserted directly into the corresponding sites of the expression vector pET-32b(+). Ligation products were transformed into *E. coli* C43(DE3) by electroporation.

The sequence integrity of the final construct designated pET-b5w was confirmed by DNA sequencing using commercially available primers complementary to sequences either side of the target DNA.

2.8. Expression of cytochrome b_5–thioredoxin fusion

Growth and protein production conditions were optimised to give the highest level of cytochrome b_5 expression. In a typical incubation, a single colony of *E. coli* C43(DE3)/pET-b5w was grown overnight (LB media supplemented with 50 μg/ml ampicillin) to the stationary phase. A 5-ml aliquot of this sample was used to inocculate 1 l of TYP media supplemented with ampicillin and was grown with vigorous shaking at 30°C until the cell density measured from the absorbance at 600 nm, reached a value between 0.4 and 0.6. At this point IPTG was added to a final concentration of 1 m*M* and the cells were grown for a further 20 h. At this stage the cells acquired a distinctive colouration indicative of haem protein expression. Cells were harvested by centrifugation at 10 000 *g* at 4°C for 15 min.

To assess the solubility of the over expressed fusion protein cells were initially lysed and osmotically sensitive spheroplasts were prepared with the aid of lysozyme and EDTA treatment [19].

2.9. Cell lysis–lysozyme EDTA treatment

Cell pellets harbouring the fusion protein were washed with 100 m*M* Tris–HCl, pH 7.4 and all subsequent steps were performed on ice unless otherwise stated. Cells were resuspended in 33 m*M* Tris–HCl, 20% sucrose, pH 8.1 (four volumes/gram of pellet). EDTA added at a final concentration of 1 m*M* was followed by lysozyme at 5 mg/ml of cell suspension. The suspension was incubated at room temperature with gentle stirring for 20 min. Centrifugation of this suspension at 10 000 *g* for 30 min at 4°C, resulted in a straw coloured supernatant which was discarded. To the resulting spheroplasts ice cold water was added, to promote cell lysis and PMSF was added at a final concentration of 1 m*M*. Crystals of DNaseI were added and the mixture stirred for a further 10 min. The cytoplasmic fraction was separated from the membranous material by centrifugation at 10 000 *g* for 30 min.

2.10. Solubilisation of cytochrome b_5–thioredoxin fusion protein

Detergent solubilisation of *E. coli* membranes was achieved by the dropwise addition of 20% Triton X-100 (v/v) resuspended in 50 m*M* Tris–HCl, 1 m*M* EDTA, pH 8.1 to yield Triton X-100 at a final concentration of 2%. The suspension was supplemented with PMSF and thoroughly mixed with a Potter–Elvehjem homogeniser. This was followed by sonication on ice (3×20 s bursts at full power). The membranes were then routinely incubated for ~16 h with constant stirring at 4°C in the presence of detergent.

The resulting cloudy suspension was clarified by centrifugation at 10 000 *g* for 20 min at 4°C to remove insoluble material and cell debris. This yielded a clear orange coloured supernatant (120 ml) indicative of the solubilisation of the cytochrome b_5–thioredoxin fusion protein (b5w–trx). At this stage of the purification procedure, the solubilised b5w–trx fusion protein was a mixture of both apo and holo forms of cytochrome b_5.

2.11. Reconstitution of b5w–trx with hemin

Apo-cytochrome b_5 was reconstituted by the addition of hemin (prepared in 0.2 *M* NaOH). Small aliquots (10 μl) of hemin solution were added to the orange coloured detergent extract on ice and stirred. The uptake of haem was monitored by an increase in absorbance at 412 nm at regular intervals on a Hewlett-Packard 8452 diode array spectrophotometer. The success of haem reconstitution was visually obvious by the gradual development of an intense red coloured solution.

2.12. His-Bind nickel chelate chromatography

Prior to application of the detergent extract to a His-Bind nickel column, the sample was dialysed extensively against 5 m*M* imidazole, 0.5 *M* NaCl, 20 m*M* Tris–HCl, pH 7.9 to remove EDTA which

would otherwise chelate Ni^{2+} and thereby strip the column of its active affinity group. All chromatography steps were carried out at 4°C. The dialysed sample was centrifuged at 10 000 *g* for 15 min to remove particulate debris and the solution was applied to the His-Bind resin (11.5×1.6 cm XK16 Pharmacia Biotech column) at a constant flow-rate of 3.8 ml/min. Modifications to the basic protocol [20] included the omission of the second ''wash buffer'' step and with all column buffers containing 2% (v/v) Triton X-100. The fusion protein was eluted with a gradient of 0–1 *M* imidazole in 20 m*M* Tris–HCl, 0.5 *M* NaCl, pH 7.9. Homogeneity of the eluted fractions was assessed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE).

2.13. SDS–PAGE

This was performed at each stage of the purification process according to the procedure of Laemmli [21]. A Pharmacia PhastSystem was used for the detection of low concentrations of protein (<μg quantities). The system utilises pre-formed Hi-Density gels (40×40×0.5 mm) obtained from Pharmacia Biotech with a polyacrylamide concentration of 25%. Protein samples were solubilised with 10% (w/v) SDS and 2 μl of the sample was placed in individual pre-formed wells. Approximately ~1 μl of this sample was applied to the surface of the gel. The temperature of the apparatus was kept at 15°C and following the completion of electrophoresis gels were silver stained using the procedure of Rabilloud et al. [22].

2.14. Cleavage of the fusion protein

Purified cytochrome b_5–thioredoxin fusion protein was treated with trypsin at room temperature with gentle stirring in the presence of TPCK in 10 m*M* Tris–HCl, 10 m*M* $CaCl_2$, pH 8.1 for 1 h. The solution contained equimolar concentrations of trypsin and TPCK and a ratio of 10:1 (molar equivalent) of b5w–trx fusion protein to trypsin.

2.15. Purification of MBD by RP-HPLC

The MBD was separated from the trypsin digest material by RP-HPLC (Table 1). Separation was achieved by applying a gradient ranging from 39.9–99.9% acetonitrile (HPLC grade) against water (Milli-Q filtered through a 0.45-μm nitrocellulose membrane). Both mobile phases included 0.1% trifluoroacetic acid. The RP-HPLC column was a semi-preparative 300 Å Rainin Dynamax C_8 alkyl bonded silica column (250 mm×10 mm) in conjunction with a C_8 guard column. The columns were connected to a Gilson HPLC system and linked to a UV detector. Gradient runs lasted 120 min at a flow-rate of 0.8 ml/min. Prior to injecting digested material onto the column the samples were centrifuged at 12 000 *g* for 5 min and loaded onto the 1-ml sample loop. The collected peaks, monitored by absorbance measure-

Table 1
Typical purification of the MBD of cytochrome b_5 from *E. coli* cells C43(DE3) transformed with pet-b5w from 1 l of cell culture[a]

Step	Volume (ml)	Total protein[b] (mg/ml)	b5w–trx content[c] (mg/ml)	MBD content (mg/ml)
Cells	120	11.92	2.38	0.45
Spheroplasts	184	4.60	2.38	0.45
Membrane	104	7.0	2.10	0.35
TX-100	120	6.13	1.23	
Pure b5w–trx (I)	40	2.5	2.50	
Pure MBD (II)	10	1.5		

[a] Purification of trx–b5w by His-Bind nickel chelate chromatography (I) and separation of MBD from trypsin treated b5w–trx by RP-HPLC (II).
[b] The total protein represented is determined from the Lowry assay as described in Section 2.16.
[c] Estimated from SDS–PAGE gel assuming trx–b5w is present at 20% of total protein in the sample.

ments at 280 nm, were concentrated by rotary evaporation.

2.16. Protein concentration determination

Total protein concentration was determined by the Lowry reaction [23] using bovine serum albumin as a standard.

3. Results and discussion

3.1. Over expression of the fusion protein in E.coli

The DNA encoding cytochrome b_5 was inserted downstream from the thioredoxin gene (*trx*A) and a stretch of six histidine residues in pET-32b(+) (Fig. 1). This results in the expression of target DNA from the T7 lac promoter and consists of a cytochrome b_5 fusion to thioredoxin (trx), a water soluble protein with a M_r of 11 665. The constructed plasmid pET-b5w transformed into *E. coli* C43(DE3) and grown in the presence of IPTG (1 m*M* final concentration) produces an intense, over expressed, band at ~33 000, corresponding to the cytochrome b_5–thioredoxin (b5w–trx) fusion protein, when analysed using SDS–PAGE (Fig. 2). It was apparent that growth at 37°C resulted in the majority of the fusion protein being expressed in the apo form as observed by a faint hint of red colour in the cell pellet. When the growth temperature was lowered to 30°C the cell culture was a visibly more intense colour suggesting a greater incorporation of haem by cytochrome b_5. The accumulation of apoprotein in high levels in expression systems such a pET system, has been reported previously and may arise from protein synthesis being seven times greater than in the *E. coli* host [24]. Consequently a mixture of apo and holo protein is produced since haem synthesis and incorporation cannot be maintained at comparable levels to protein expression.

SDS–polyacrylamide gels of the total protein content of these cells identified a protein migrating with an apparent molecular mass of 33 000. This band represents the major protein expressed by these cells. The molecular mass is consistent with the combined masses of thioredoxin and cytochrome b_5 together with the additional residues between these

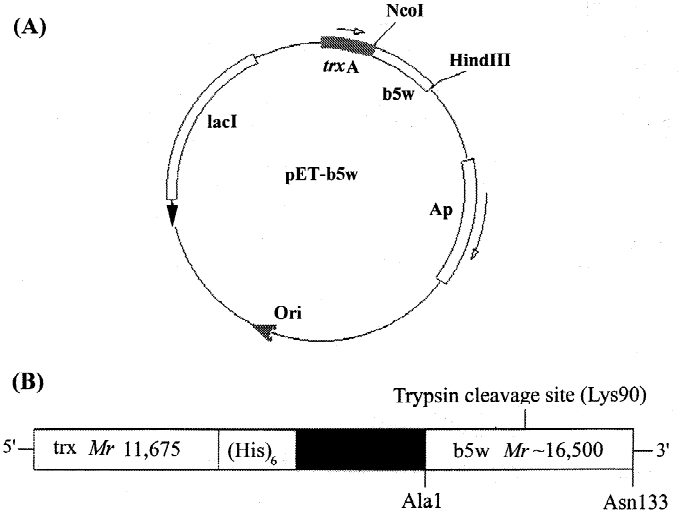

Fig. 1. (A) Plasmid map of the expression vector, pET-b5w showing the position of the fusion protein in relation to the thioredoxin gene (*trx*A). Abbreviations used are: b5w, the coding sequence of cytochrome b_5 showing the first (Ala1) and last residue (Asn133); *trx*A, the *E. coli* thioredoxin gene; Ap, gene coding for ampicilin resistance; Ori, origin of replication; *lac*I, the lac repressor gene. (B) A schematic representation of the cytochrome b_5–trx fusion protein expressed in *E. coli* C43(DE3) cells. The area indicated in black highlights additional residues between trx and b5w.

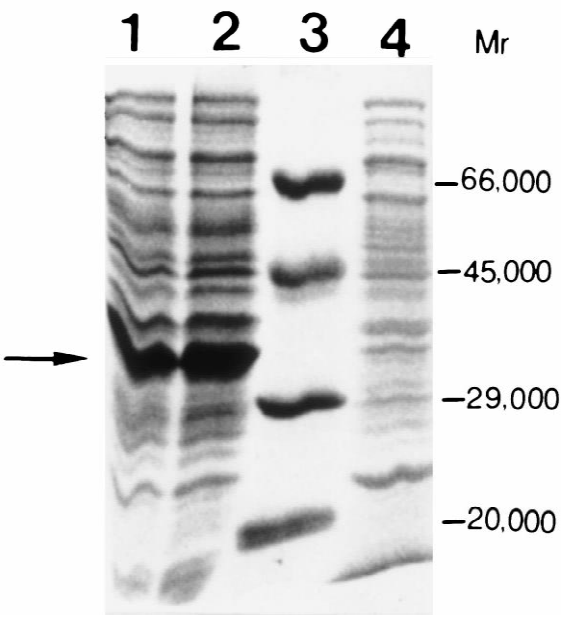

Fig. 2. Coomassie Blue stained SDS–PAGE (12.5%) of proteins of *E. coli* C43(DE3) cells containing the expression plasmid pET-b5w at two temperatures. The arrow indicates the expressed b5w–trx fusion protein migrating at ~33 000. Lane 1, overnight growth of cells induced with IPTG (1 m*M* final concentration) grown at 37°C; lane 2, IPTG-induced cells grown overnight at 30°C; lane 3, M_r protein standards; lane 4, cells grown in the absence of plasmid.

proteins. In the absence of plasmid, there is no expressed band demonstrating that this fusion protein is not an endogenous product of the bacterial cell but a product of the plasmid pET-b5w.

3.2. Subcellular location of b5w–trx in E. coli

Using *E. coli* C43(DE3) transformed with the plasmid pET-b5w the location of the expressed product was investigated by preparing periplasmic, cytoplasmic and membrane fractions from bacterial cells. Spheroplasts were prepared and subsequently ruptured by osmotic shock using ice cold water. Centrifugation leads to the release of the cytoplasmic contents into a supernatant fraction. All of the colour arising from the expression of holo-cytochrome b_5 is found in the membrane fraction whilst the supernatant remains colourless. These fractions were analysed by SDS–PAGE (see Fig. 3a) to confirm that the fusion protein of ~33 000 is associated with the membranes of *E. coli* C43(DE3) cells. Despite its comparatively small size the MBD is able to direct the insertion of the fusion protein to the host membrane.

3.3. Detergent extraction of b5w–trx

Bovine cytochrome b_5 (Ala1–Asn133) can be solubilised from *E. coli* membranes by treatment with Triton X-100 [25,26]. Similarly, the addition of 2% Triton X-100 resulted in solubilisation of over 95% of the fusion protein, as assessed by SDS–PAGE shown in Fig. 3b. The membrane suspension was clarified by centrifugation and almost all of the coloured haem was found in the supernatant fraction whilst the residual membranous material did not retain any significant amounts of colour confirming the effectiveness of Triton X-100 as a solubilising detergent. Detergent concentrations lower than 2% were not as effective resulting in aggregation of the fusion protein over prolonged time periods.

Previous attempts at intracellular expression of the MBD as a C-terminal GST fusion led to the formation of insoluble aggregates even when the membranes were solubilised with Triton X-100 (unpublished results) and other detergents. Electron microscopy of intact cells expressing the MBD as a GST fusion were identified as discrete spheroidal bodies. Treatment of the inclusion bodies with reducing agents in combination with detergent resulted in their partial solubilisation but also led to the loss of binding to the glutathione affinity column. This was presumably due to unfolding of GST around the ligand binding site. Although, in many respects inclusion body formation is a consequence of recombinant protein expressed at high levels, it has been documented that formation can also occur after cellular disruption and thus proteins which are potentially soluble may also aggregate [27]. Formation of inclusion bodies may arise from inappropriate aggregation of partially or incorrectly folded proteins [28] expressed at high levels. From Fig. 3b it is clear that linking cytochrome b_5 to a stable and highly soluble fusion partner such as thioredoxin can prevent the formation of aggregates. This result has been observed in other systems [29].

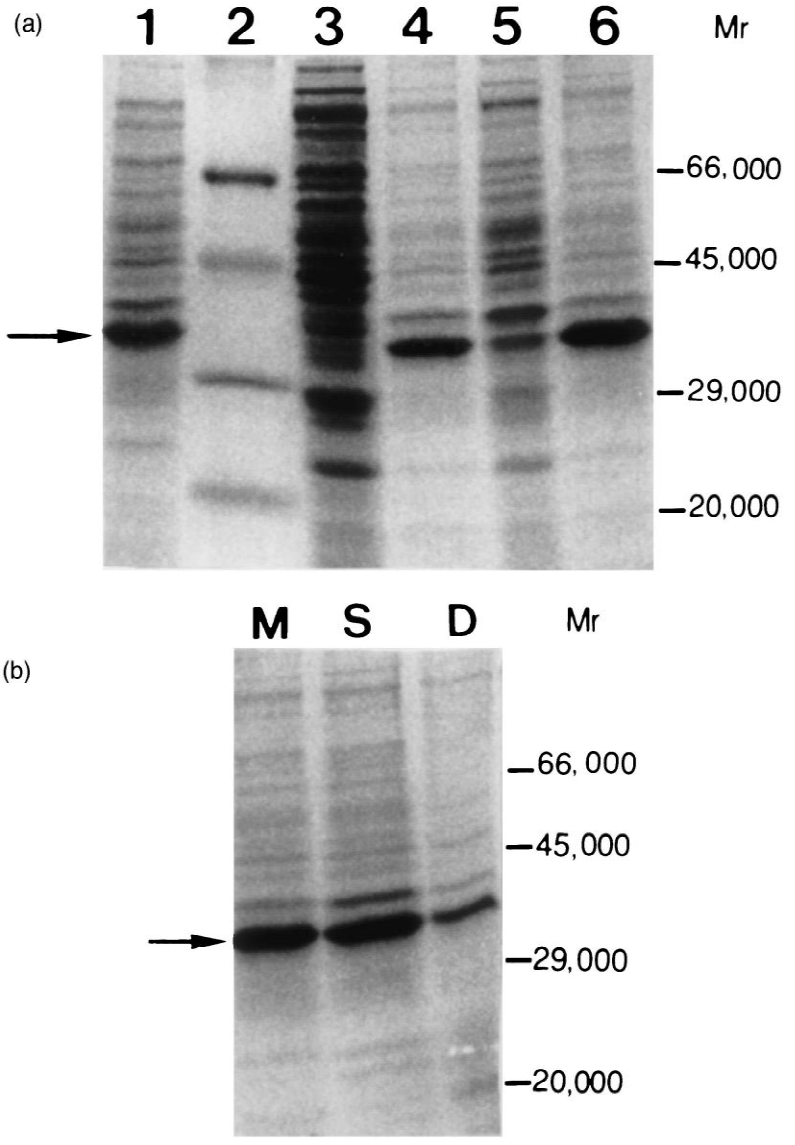

Fig. 3. SDS–PAGE (12.5%) of cells expressing the fusion protein. The cells are stained with Coomassie Blue and show the position of the thioredoxin fusion protein (indicated by an arrow). (a) The subcellular location of b5w–trx. Approximately 15 μg of total protein was loaded into each lane. Lane 1, whole cells; lane 2, M_r protein standards; lane 3, soluble periplasmic fraction; lane 4; spheroplasts; lane 5; crude cytoplasmic fraction; lane 6, isolated membranes. (b) Detergent solubilisation of b5w–trx with 2% (v/v) TX-100. Isolated membranes designated M, supernatant fraction from TX-100-treated membranes designated S and the resulting membranous cell debris designated D, respectively.

3.4. Reconstitution of apo-cytochrome b_5-thioredoxin

Analysis of the expression of the fusion protein suggests a substantial proportion of apo-cytochrome b_5. This was confirmed by the addition of exogenous hemin. This resulted in an intense absorbance at 412 nm (see Fig. 4) characteristic of the oxidised state of cytochrome b_5. It is clear that thioredoxin does not prevent the formation of the haem binding region of

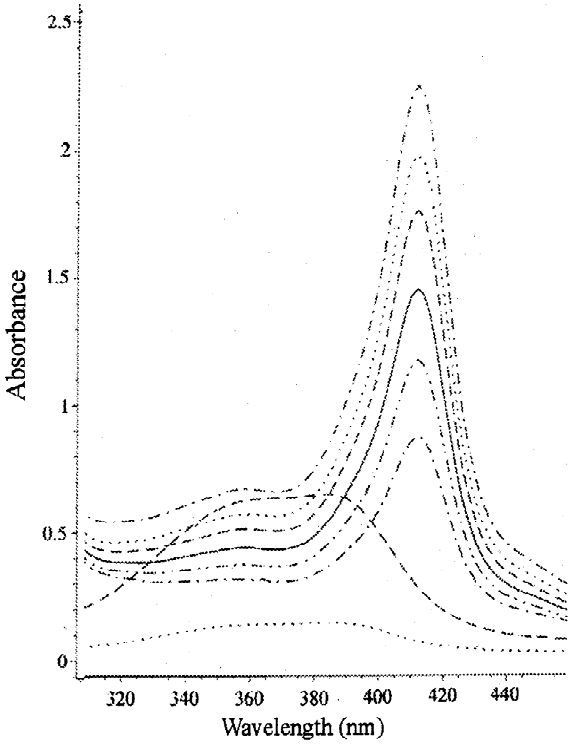

Fig. 4. The increase in absorbance at 412 nm following the addition of hemin to solubilised b5w–trx fusion protein. The profiles indicate the ability of the fusion protein to bind exogenous haem. Free hemin is indicated with a broad absorption at 385 nm with traces shown at two different concentrations. In comparison, cytochrome b_5 containing haem has a narrower absorption spectrum.

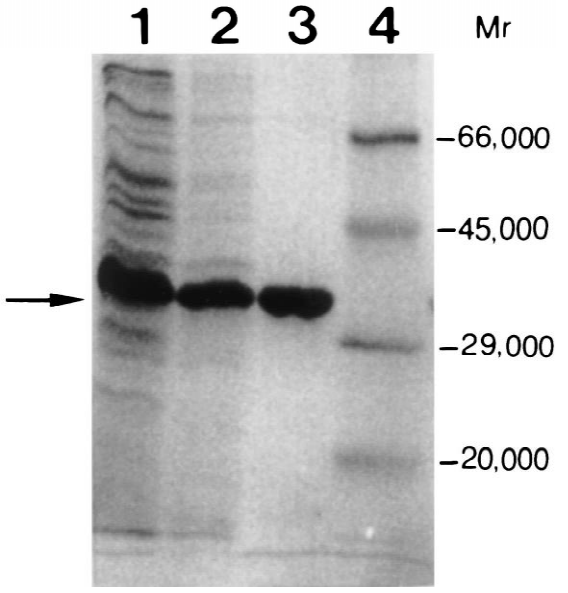

Fig. 5. SDS–PAGE demonstrating expression, solubilisation of membranes and one-step purification of the fusion protein. The gel contains a constant concentration of acrylamide (12.5%) and is stained with Coomassie Blue. Purification of the His-tagged b5w–trx from *E. coli* strain C43(DE3) was carried out as described in experimental methods (Section 2.12) and was achieved in a single column step. A 15-μg amount of protein was loaded in each well. Lane 1, whole cells; lane 2, starting material applied to a His-Bind nickel column, and lane 3, purified b5w–trx. An arrow indicates the migration of b5w–trx.

cytochrome b_5 that extends from residues 1 to 90 nor does it stop haem insertion.

3.5. Purification of b5w–trx by His-Bind nickel affinity chromatography

Reconstituted fusion protein was dialysed extensively against imidazole ''binding buffer'' to remove excess hemin as well as EDTA. The solubilised extract was then loaded onto a His-Bind nickel affinity column and migration of the protein was observed by the presence of a red band. All coloured fractions with an absorbance at 412 nm were collected and pooled. SDS–PAGE (Fig. 5) confirmed that the fusion protein had been purified to homogeneity in a single step using the procedure described in Section 2.12. The results highlight the selectivity of the column in favouring binding of those polypeptides containing the $(His)_6$ tag. In addition, the His-Bind metal affinity step holds a number of advantages over other bioaffinity chromatographic methods. The small metal chelates used in this procedure are stable in the presence of a wide range of solvents and over a large temperature range [30]. In addition resins can be recycled and regenerated by replenishing the supply of the metal ion (Ni^{2+}). Using this procedure approximately 90 mg of the pure fusion protein could be routinely obtained from 1 l of cell culture.

3.6. Trypsin cleavage of b5w–trx

From the purified fusion protein resulting from the His-Bind nickel affinity column aliquots containing approximately 2–3 mg of the pure fusion protein were subjected to trypsin digestion. Experiments

showed that cleavage was optimal in the presence of low concentrations of SDS (7 m*M*, ~0.2% w/v) and that the reactions were essentially complete after 1 h. The digest material was then further purified by RP-HPLC.

3.7. Isolation of MBD by RP-HPLC

The MBD of cytochrome b_5 was effectively separated from other components of the trypsin digest sample using a C_8 silica based column. The elution profiles measured from the absorbance at 280 nm showed four well resolved peaks eluting at ~24, ~52, ~58 and ~95 min (Fig. 6). Each of these peaks were collected and concentrated by rotary evaporation and were further analysed by SDS–PAGE. Analysis of the fourth peak, eluting at 70% acetonitrile, showed a single homogenous band on an SDS–PAGE gel (Fig. 7). A single band indicated the high purity of this fragment isolated from a single column step on a C_8 RP-HPLC column. The hydrophobic peptide corresponding to an apparent M_r of 5000 was in close agreement to that predicted from its respective amino acid sequence. The M_r of this peptide was further confirmed by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS).

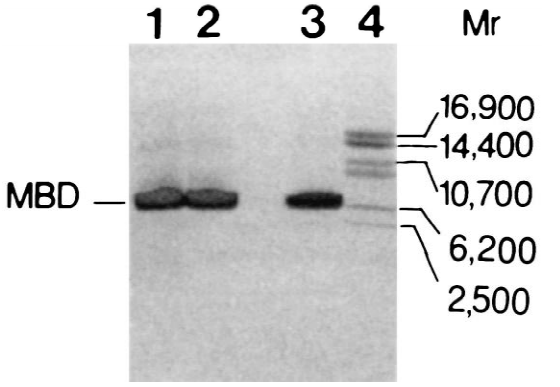

Fig. 7. SDS–PAGE gel of the final product as observed on a 25% acrylamide gel stained with silver, run on a Pharmacia PhastSystem. The gel shows pooled material from the fourth hydrophobic peak after fractionation on a C_8 RP-HPLC column. Lanes 1, 2 and 3, purified MBD; lane 4, globin M_r protein standards, 16 900, 14 400, 10 700, 8100, 6200 and 2500. The purified MBD exhibits an apparent M_r of ~5000.

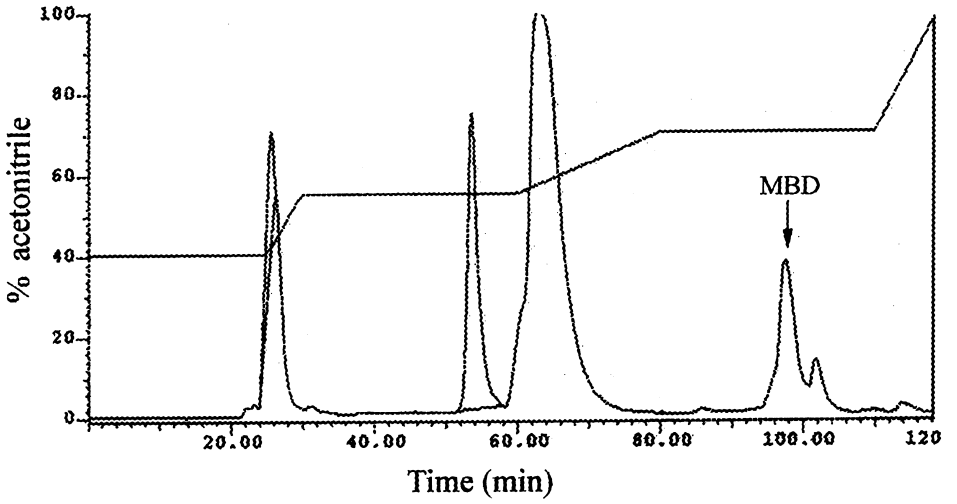

Fig. 6. The elution profile of the trypsin treated fusion protein monitored from the absorbance at 280 nm. A gradient of 39.9–99.9% acetonitrile is indicated by the thick line whilst the lighter line indicates the absorbance profile at 280 nm. The first peak (24–29 min) was identified as trypsin, whilst the second (52–56 min) was due to the haem binding fragment of cytochrome b_5. The third peak (59–70 min) corresponds to the thioredoxin fragment and the fourth peak (95–100 min) eluting at 70% acetonitrile was identified as the MBD.

4. Conclusion

The high levels of expression achieved with the b5w–trx fusion protein coupled with its useful affinity for nickel ions, makes the pET-32b(+) plasmid a powerful system for the over production of heterologous products. Its use can now be extended to the expression of membrane proteins.

Although the MBD has been cloned and expressed the over production of this fragment of cytochrome b_5 has not been universally successful. The formation of insoluble inclusion bodies (unpublished results) with the GST fusion system achieved limited success whilst the expression of full length cytochrome b_5 results in much lower yields [31]. The difficulties arising during isolation of hydrophobic or membrane bound proteins has hampered progress in their structural and functional characterisation.

The MBD of cytochrome b_5 has not been purified in similar yields and purity using recombinant methods. In some cases expression of proteins via inclusion bodies can offer advantages in purification [32]. This arises because inclusion bodies are almost exclusively composed of the expressed protein [33,34]. However, devising an appropriate solubilisation and refolding procedure can remain a significant hurdle. The expression plasmid pET-32b(+) used in this study encodes the fusion partner thioredoxin [35] which dramatically increases the solubility of many proteins normally produced in an insoluble form in *E. coli*. Its usefulness is supported by the observation that a variety of mammalian cytokines and growth factors previously produced in *E. coli* as insoluble aggregates [36] were rendered soluble and retained biological activity when expressed as thioredoxin fusions.

In summary, we report for the first time the over expression and purification of the MBD of cytochrome b_5 via a thioredoxin fusion protein. Although, expressed as a membrane bound protein it is readily made soluble by extraction with detergent. Pure fusion protein could be obtained from a crude membranous cell extract in a single step by His-Bind immobilised nickel chelate chromatography using an imidazole gradient elution. Trypsin cleavage of the b5w–trx yielded the MBD of the correct size and in high yield (~10 mg/l). With such high expression levels in *E. coli*, the two-step column purification described in this study presents an efficient route for preparative scale amounts of the hydrophobic MBD. The high yield highlights the effectiveness and simplicity of this procedure and the qualities of thioredoxin as a fusion partner. The expression system used here should allow detailed biophysical studies of the isolated MBD. This purification route may also prove suitable to proteins particularly membrane proteins, that are prone to inclusion body formation or low levels of expression.

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