Characterisation of new intracellular membranes in *Escherichia coli* accompanying large scale over-production of the b subunit of F_1F_o ATP synthase

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Abstract Recombinant membrane proteins in *Escherichia coli* are either expressed at relatively low level in the cytoplasmic membrane or they accumulate as inclusion bodies. Here, we report that the abundant over-production of subunit b of *E. coli* F_1F_o ATP synthase in the mutant host strains *E. coli* C41(DE3) and C43(DE3) is accompanied by the proliferation of intracellular membranes without formation of inclusion bodies. Maximal levels of proliferation of intracellular membranes were observed in C43(DE3) cells over-producing subunit b. The new proliferated membranes contained all the over-expressed protein and could be recovered by a single centrifugation step. Recombinant subunit b represented up to 80% of the protein content of the membranes. The lipid:protein ratios and phospholipid compositions of the intracellular membranes differ from those of bacterial cytoplasmic membranes, and they are particularly rich in cardiolipin.

Key words: Over-expression; Membrane protein; Intracytoplasmic membrane; *Escherichia coli*

1. Introduction

One factor impeding the analysis of membrane proteins is the lack of generally applicable systems for their over-expression in quantities sufficient for crystallisation studies [1]. Although *Escherichia coli* is often a successful vehicle for over-expression of both prokaryotic and eukaryotic proteins [2], the expression vectors for most membrane proteins kill the host bacteria [3–5]. In order to try and overcome these difficulties, mutant hosts were selected from *E. coli* BL21(DE3) that allow over-production of some membrane proteins and of globular proteins that could not be expressed in BL21(DE3). These are host strains *E. coli* C41(DE3) and C43(DE3) [5].

Subunit b from the F_o membrane sector of *E. coli* ATP synthase has a hydrophobic segment of about 30 amino acid residues at its N-terminus [6]. The structure of this domain (residues 1–34) in an organic solvent has been solved by NMR [7]. The rest of the protein, which is rich in charged residues, protrudes from the membrane and interacts with the F_1 catalytic domain [8]. Subunit b could not be over-expressed in BL21(DE3), but it was made at high levels in C41(DE3) [5], although the induction of its expression still killed the host cells. This toxicity was removed by use of the host strain C43(DE3), selected from C41(DE3) cells containing an expression plasmid for subunit b [5].

In the work described here, we have found that the over-expression of subunit b in C41(DE3) and C43(DE3) induces proliferation of intracellular membranes. The internal membranes have been purified from cells over-expressing subunit b, and their lipid:protein ratios and phospholipid compositions have been characterised.

2. Materials and methods

2.1. Protein analytical methods

Protein concentrations were estimated by the bicinchoninic acid assay (Pierce Chemicals, Rockford, IL, USA). Proteins were analysed by SDS–PAGE in 12–22% gradient gels. N-terminal sequences were determined with the aid of an Applied Biosystems Procise model 494 protein sequencer. Peptides and proteins were examined by electrospray ionisation mass spectrometry in a Perkin Elmer-Sciex API III$^+$ triple quadrupole instrument.

2.2. Strains and plasmids

The mutants host strains *E. coli* C41(DE3) and C43(DE3) have been described previously [5]. The *unc* E and *unc* F genes, encoding *E. coli* ATP synthase subunits c and b, were amplified by PCR from *E. coli* DNA and cloned separately into the expression plasmid pMW172 [9]. Segments of *unc* F encoding amino acids 1–25, 1–34, 1–48 and 25–156 of subunit b were also cloned into pMW172. Vectors for co-expression of subunits b and c in pMW172 were made with subunit b promoter proximal with a ribosome binding site inserted between the two genes.

2.3. Protein over-expression and isolation of membranes

Bacteria were grown at 37°C in 2×TY medium (16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl, pH 7.4) to an optical density of 0.6 at 600 nm. Then isopropyl-2-D-thio-galactopyranoside (IPTG) was added to a final concentration of 0.7 mM. The cells were grown for a further period at either 37 or 25°C and then centrifuged (2000×*g*, 10 min).

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Abbreviations: IPTG, isopropyl-2-D-thio-galactopyranoside; TLC, thin layer chromatography; HPLC, high performance liquid chromatography; e.s.i.-m.s., electrospray ionisation-mass spectrometry

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Bacteria were resuspended in TEP buffer (10 mM Tris, pH 8.0, 1 mM EDTA and 0.001% (w/v) phenylmethylsulfonyl fluoride) and disrupted by passing the suspension twice through a French pressure cell at 4°C. Intracellular membranes containing subunit b were collected by a 2500×*g* centrifugation, whereas cytoplasmic membranes depleted of subunit b were obtained after ultra-centrifugation at 100 000×*g* of the 2500×*g* supernatant. Intracellular membranes containing subunit b were freed from unbroken cells and debris by resuspension in TEP buffer and centrifugation at 100 000×*g*. Membranes were suspended at a concentration of 2.5 mg of protein/ml. Portions (1 ml) of this suspension were applied to the top of discontinuous sucrose gradients (5–50%, w/v) and centrifuged for 18 h at 155 000×*g*.

2.4. Proteolysis of subunit b inserted in the isolated membranes

Membranes isolated from *E. coli* C43(DE3 were suspended in TEP buffer at a concentration of 2.5 mg of protein/ml. Trypsin (1:20, w/v) was added and the suspension was kept at 30°C. Samples were removed at intervals, and proteolysis was terminated by addition of soybean trypsin inhibitor (fivefold excess by weight). The course of proteolysis was monitored by SDS–PAGE. Peptides were identified by N-terminal sequence analysis. Samples were dissolved in 6 M guanidine hydrochloride and peptides were isolated by reverse-phase chromatography on a C_8 Aquapore RP-300 column equilibrated in 0.1% trifluoroacetic acid and eluted with a linear gradient of acetonitrile. The peptides were analysed by electrospray ionisation-mass spectrometry (e.s.i.-m.s.).

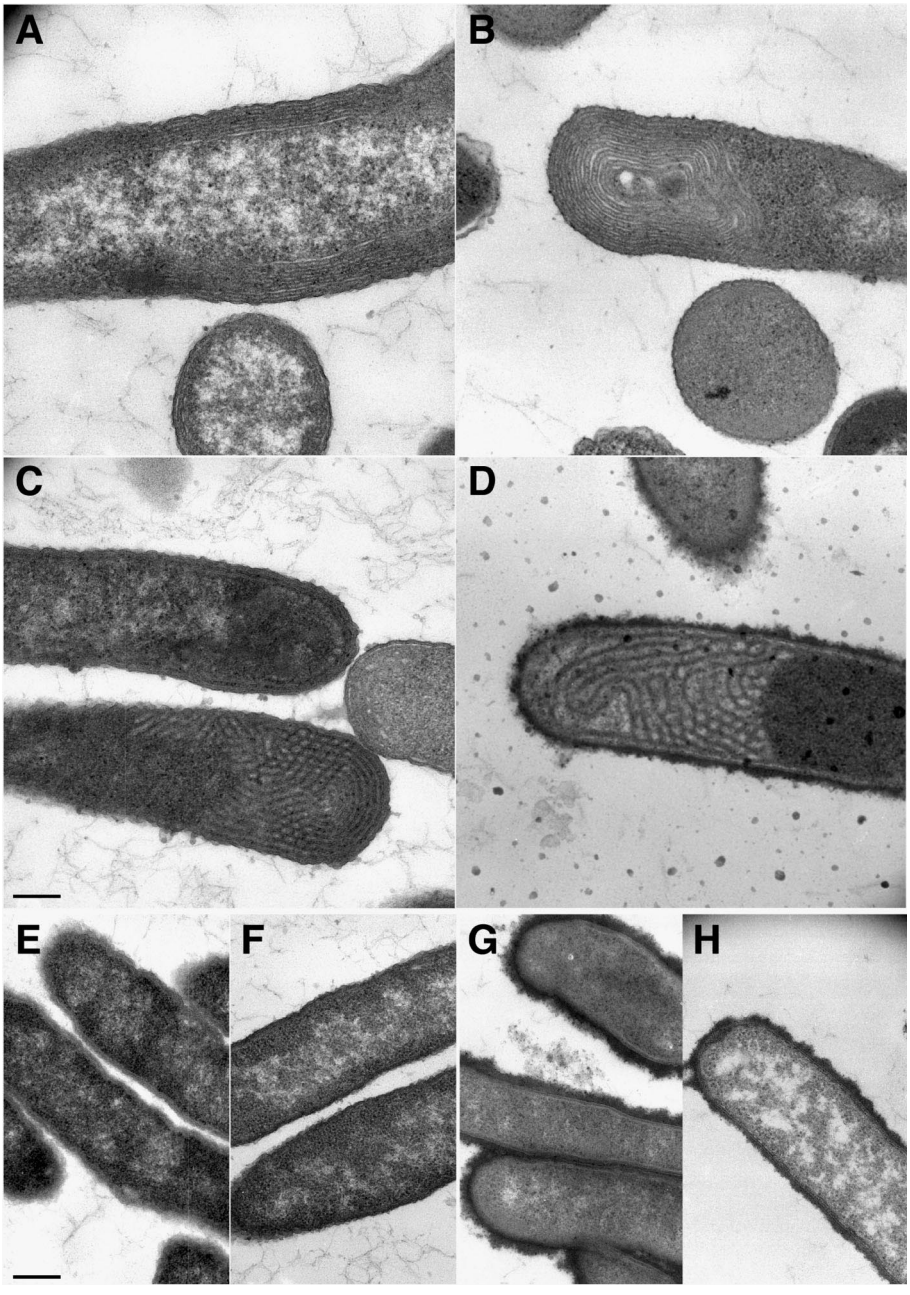

Fig. 1. Electron micrographs of thin sections of *E. coli* cells over-producing subunit b of *E. coli* ATP synthase. A and B: C41(DE3) cells over-producing subunit b grown at 37 or 25°C after induction of expression for 3 or 18 h, respectively. C and D: C43(DE3) cells over-producing subunit b, 3 and 18 h after induction at 37 or 25°C, respectively. E and F: C41(DE3) and C43(DE3) cells containing plasmid without insert, 4 h after addition of IPTG to cultures grown at 37°C. G and H: C41(DE3) and C43(DE3) cells without plasmid grown under the same conditions. The scale bar represents 0.2 or 0.28 μm in A–D and E–H, respectively.

2.5. Phospholipid analysis

Lipids were extracted according to Bligh and Dyer [10]. Phosphorus concentration was determined after acid digestion according to Rouser et al. [11]. The phospholipid composition of the lipid extracts was analysed by thin layer chromatography (TLC) on silica plates using chloroform:methanol:acetic acid, 65:25:10 (by volume) as solvent. Phosphatidic acid (PA) and cardiolipin (CL) were resolved on silica plates using either chloroform:methanol:water:ammonia (68:28:2:2, by volume) for the first dimension, and chloroform:methanol:acetic acid (65:25:10, by volume) for the second dimension, or by pre-treatment with 1.2% boric acid, and chloroform: methanol:water:ammonia (120:75:6:2, by volume) as solvent [12].

2.6. Electron microscopy

Bacteria were fixed with 2% glutaraldehyde, washed with cacodylate buffer (50 mM, pH 7.2), then fixed with 4% osmium tetroxide and washed with Kellenberger buffer [13]. Pellets were embedded in 2% agar, cut and stained in the dark with 0.5% (w/v) uranyl acetate. Samples were dehydrated with alcohol, transferred to propylene oxide/Epon mixtures and finally embedded in Epon 812. Thin sections were cut, adsorbed on electron microscope grids coated with plastic films and stained with 2% uranyl acetate and lead citrate. Membranes isolated from bacteria disrupted either by French press or by EDTA-lysozyme treatment and osmotic shock [14] were adsorbed on to copper grids coated with carbon, and stained with 2% (w/v) uranyl acetate. All samples were examined in a Philips CM12 transmission microscope operated at 120 kV.

3. Results and discussion

3.1. Over-expression of subunit b induces internal membrane proliferation in E. coli

Subunit b was over-produced in C41(DE3) and C43(DE3) bacterial hosts. Maximal level of production of subunit b (30 mg/l of culture) was achieved in C43(DE3) cells 3 h after induction at 37°C or 18 h after induction at 25°C. Over-expression of subunit b was accompanied by proliferation of internal structures that were not observed in controls (Fig. 1). At both 25 and 37°C, the internal membranes in C41(DE3) were vesicle- or cisternae-like structures (Fig. 1A,B), whereas in C43(DE3), a much larger tubular membrane network formed (Fig. 1C,D). In contrast to C41(DE3), C43(DE3) cells over-producing subunit b remained viable even after the formation of the internal networks. Furthermore, 90% of the cells recovered from the culture after induction retained the ability to over-express subunit b.

3.2. Isolation of proliferated membranes

Proliferated membranes from C43(DE3) cells over-expressing subunit b for 18 h at 25°C were easily isolated by centrifugation at 2500×*g* of disrupted cells. Most of the over-expressed subunit b was associated with the 2500×*g* pellet (Fig. 2, lane b). Unbroken cells were removed by a washing step and as shown in Fig. 2 (lane d), the purified intracellular

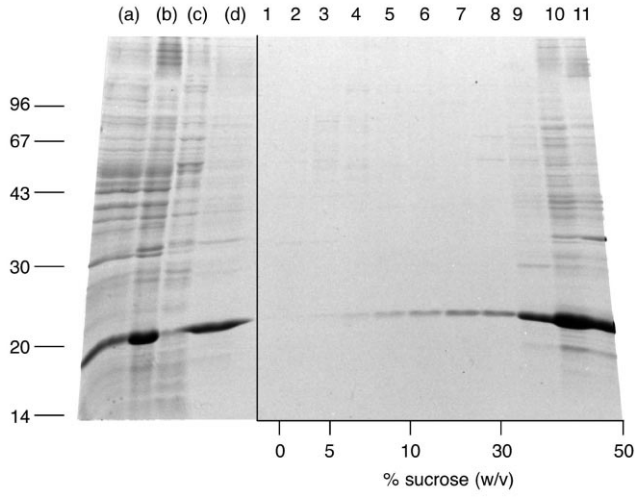

Fig. 2. Protein contents of proliferated membranes isolated from *E. coli* C43(DE3) cells over-producing subunit b. After induction of expression, the cells were kept at 25°C for 18 h and samples were analysed by SDS–PAGE. Lane (a), total cell extract (10 μl); lane (b), low speed pellet (2500×*g*; 30 μg of protein); lane (c), membrane fraction from high speed centrifugation (100 000×*g*) of the supernatant from lane (b) (35 μg of protein); lane (d), high speed pellet obtained by resuspension and washing of material in lane (b) (20 μg of protein). Lanes (1–11), fractions from sucrose step gradient fractionation of material in lane (d) (5 μl samples from 1 ml fractions).

membranes contained almost exclusively subunit b. A second membrane fraction depleted of subunit b was obtained by ultra-centrifugation of the low speed supernatant at 100 000×g (Fig. 2, lane c). Membranes containing subunit b had the appearance in the electron microscope of tubes or ribbons linking large vesicles (Fig. 3). The tubes (or ribbons) and vesicles had buoyant densities on a sucrose gradient of 1.10 and 1.18 g/ml, respectively (Fig. 2, lanes 7 and 10). The vesicles contained more subunit b than the ribbons. Cells disrupted by EDTA–lysozyme treatment and osmotic shock [14] did not swell indicating that the intracytoplasmic network is not an extension of the inner membrane of *E. coli*. Membranes isolated from these cells had also the appearance of tubular structures (Fig. 3B).

Over-expression of the c subunit of the *E. coli* ATP synthase did not induce a massive proliferation of membranes in any of the two strains. Subunit c is a hydrophobic protein consisting of a hairpin of two antiparallel α-helices connected by a short extramembranous loop. Co-expression with subunit b led to the formation of a membrane network similar to the one observed after over-expression of the b subunit alone. Intracellular membranes could be isolated as described above showing that co-expression with subunit b is a potential strategy for targeting and purifying other membrane proteins.

Table 1
Phospholipid contents of proliferated membranes accompanying over-expression of ATP synthase subunit b in *E. coli* C43(DE3)

Membrane sample	Lipid:protein (w/w)	Phospholipid composition		
		CL	PG	PE
low speed pellet	0.701	14.5 ± 0.7	12.5 ± 0.2	72.9 ± 0.8
high speed pellet	0.284	8.2 ± 1.2	13.9 ± 1.2	78.0 ± 0.1
C43(DE3) control	0.376	2.0 ± 0.7	20.3 ± 1.5	77.7 ± 1.6
C41(DE3) control	0.414	4.3 ± 1.2	20.2 ± 0.4	75.5 ± 1.2

The values were obtained from three or four independent determinations. CL, cardiolipin; PG, phosphatidyl glycerol; PE, phosphatidyl ethanolamine.

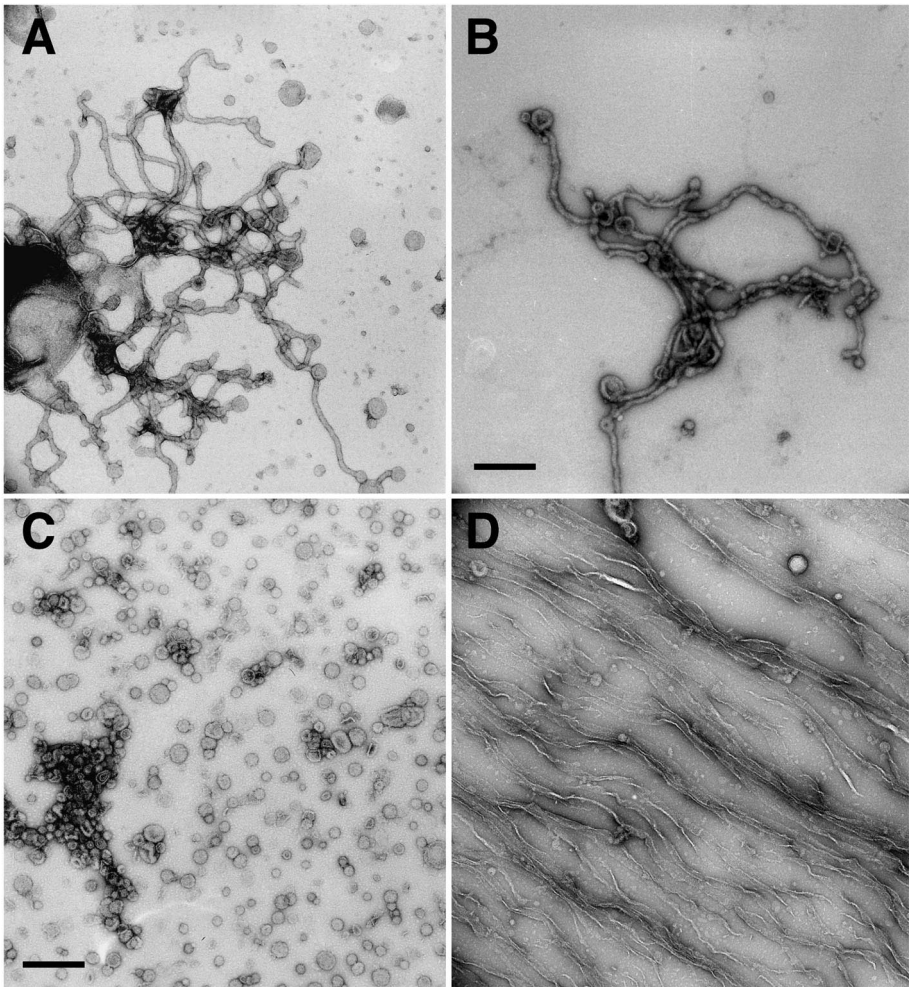

Fig. 3. Electron micrographs in negative stain of membranes obtained by low speed centrifugation from *E. coli* C43(DE3) cells over-expressing subunit b after cell disruption by French press (A), or by osmotic shock (B) (scale bar = 0.2 μm). Membranes were fractionated by sucrose gradient, and analysed by negative stain electron microscopy. C and D: fractions 10 and 7 from the sucrose gradient (see Fig. 2, scale bar = 0.28 μm).

3.3. Phospholipid composition

Under most growth conditions, bacteria maintain a constant of lipid:protein ratio [15], and values of 0.4 are typical of *E. coli* inner membranes [16]. Proliferation of intracellular membranes upon over-expression of some membrane proteins in *E. coli* has been reported previously [17–19] indicating that the cell membrane of bacteria could accommodate large amounts of protein by generation of novel membrane structures. The cells respond to the excess membrane protein biosynthesis by a regulated increase of membrane phospholipid biosynthesis such that the lipid:protein ratio remains nearly constant at 0.4. However, the lipid:protein ratio for the proliferated membranes isolated from C43(DE3) cells over-expressing subunit b (see Table 1) was almost twice as much as the membrane fraction not containing subunit b isolated from the same cells. The combined value of the two membrane fractions was about 0.4 and was similar to the value observed in total membranes from control cells. Therefore, lipids were accumulated in the new membrane structures during large scale over-production of subunit b without changes in the total lipid:protein ratio of the cell. Since the expression of the subunit b occurred without any toxicity, C43(DE3) cells simply adapt their metabolism to compensate for the very high level of expression of the membrane subunit b.

The membranes isolated from C41(DE3) and C43(DE3) cells in which subunit b was not being expressed contain about 2–4% CL. However, in C43(DE3) over-expressing subunit b, the level rises dramatically to about 14% in the proliferated membranes at the expense of its biosynthetic precursor, phosphatidyl glycerol which drops from about 20% in the controls to about 13% (see Table 1). Little is known about the mutations in C41(DE3) and C43(DE3) host strains, but it is unlikely that the increase in CL content accompanying subunit b over-expression is a consequence of a mutation in its biosynthetic pathway, mainly because the levels in control cells are normal. CL levels are known to increase during the stationary phase [20,21]. However, comparison of CL contents with the controls indicates that there is a correlation with over-production of subunit b and higher levels of CL.

3.4. Protein topology and expression of subunit b fragments

The topography of the recombinant subunit b in the isolated membranes was explored by trypsinolysis. Analysis by SDS–PAGE of the peptides in the pellet fraction after tryptic digestion revealed a fragment with an apparent M_r of 7 kDa

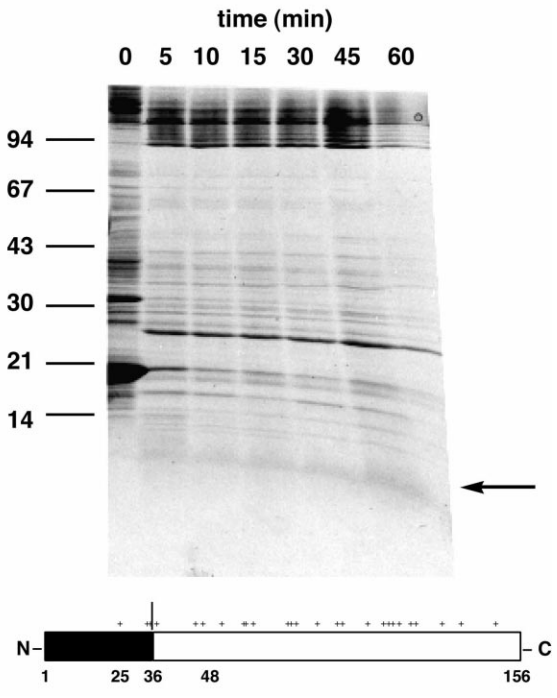

Fig. 4. Trypsinolysis of subunit b incorporated into proliferated membranes. The time course of proteolysis was monitored by SDS–PAGE. The poorly stained band pointed by the arrow shows the peptide identified by NH_2-sequencing, purified by high performance liquid chromatography (HPLC) and analysed by e.s.i.-m.s. The black box represents this fragment (residues 1–36) which is protected by the membrane from tryptic digestion. Relative positions of arginine or lysine residues on the sequence are represented by + signs.

(Fig. 4). Its N-terminal sequence is that of intact subunit b, and its molecular mass measured by e.s.i.-m.s. was 4113.3, corresponding to residues 1–36 of subunit b, and arising by tryptic cleavage after arginine-36. There was no evidence for cleavage after lysine-23, which is consistent with the region around this residue being in a membrane associated α-helix as shown in the NMR structure [7]. These results suggest that the subunit b is correctly inserted into the proliferated membranes.

Based on these observations, truncated forms of subunit b were expressed in C41(DE3) and C43(DE3) under the same conditions used for the full length protein. They corresponded to amino acids 1–25, 1–34, 1–48 and 25–156. None of these over-expressed peptides led to the formation of a large membrane network comparable to the one observed with the full length subunit b. Therefore, both membrane and extramembrane domains are required for intracellular tubular membrane formation. The N-terminus domain of the protein may be required for anchoring the protein into the membrane whereas the soluble domain, which is predicted to have a high content in α-helical coiled-coil structure [22] may be involved in tethering and holding the network of membranes in the cytoplasm.

3.5. Conclusion

Bacterial over-expression of membrane proteins can be improved by optimisation of the host system. Refolding in vitro of membrane proteins from inclusion bodies has been reported previously [23–25]. However, large scale production of membrane proteins for structural studies may require the development of alternative expression systems. As we describe here, high levels of over-expression of subunit b (up to 30 mg/liter of culture) does not lead to the formation of inclusion bodies but it is accompanied by membrane proliferation. Maximal levels of formation of intracellular membrane structures are only achieved by choosing the bacterial host and the growth condition allowing a high level of expression of the recombinant protein without any toxicity associated. It may be possible to over-express other membrane proteins and to arrange for their folding and membrane insertion by expressing them in tandem with subunit b.

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