Cystic fibrosis transmembrane conductance regulator: expression and helicity of a double membrane-spanning segment

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Abstract The gene responsible for cystic fibrosis encodes a membrane protein – the 1480-residue cystic fibrosis transmembrane conductance regulator (CFTR) – in which membrane-based CF-phenotypic mutants alter pore structure and/or impair ion transport. We report the preparation in milligram quantities and conformational characterization of a polypeptide comprised of CFTR transmembrane (TM) segments 3–4, a putative 'helical hairpin' portion of the CFTR TM1–6 domain. The TM segment 3–4 of CFTR was expressed in *E. coli* as a fusion protein linked to the C-terminus of His-tagged thioredoxin. Nickel chelate affinity chromatography, followed by release from the carrier by digestion with thrombin protease, gave free CFTR(TM3–4). Monitoring of the folding properties and conformational state(s) of the TM3–4 polypeptide using circular dichroism spectroscopy indicated a partial α-helical conformation in aqueous buffer, with up to 30% increase in α-helical content observed in membrane-mimetic environments.

Key words: Cystic fibrosis; CFTR; Membrane protein; Protein expression; Circular dichroism; Helical hairpin

1. Introduction

Cystic fibrosis (CF) is an autosomal recessive genetic disease affecting the epithelium, especially the airways, the pancreatic ducts, the sudoriparous glands and bile ducts [1]. The gene responsible for CF has been isolated and predicted to encode a membrane protein – the cystic fibrosis transmembrane conductance regulator (CFTR) [2,3]. CFTR is a member of the ATP-binding cassette protein superfamily and consists of two repeated elements, each comprising six putative membrane-spanning segments and a nucleotide-binding domain. The two repeats are separated by a large polar 'R domain' containing multiple potential phosphorylation sites [4]. While the most well-characterized CF defect in CFTR is localized to the first nucleotide-binding domain residue (ΔPhe^{508}), many mutations in the first transmembrane (TM) domain (TM segments 1–6) are associated with CF disease [5–7]; CF-phenotypic mutants in TM segments 3 and 4 are shown in Fig. 1. Unlike mutant ΔPhe^{508} which is misprocessed and fails to traffic to the apical cell membrane, these TM domain mutants are of particular interest because they are correctly processed and delivered to the cell membrane. However, while certain TM mutations dramatically impair the chloride channel function of CFTR [7], the manner through which various mutants alter the CFTR pore structure or the channel efficiency remains to be characterized on the molecular level.

The cytoplasmic domains of the CFTR have been characterized to some extent [8–12], but comparatively little is known about the transmembrane domains, largely because of the low yields and insoluble aggregation (inclusion body) effects which traditionally accompany the handling of hydrophobic proteins. Solid-phase chemical synthesis is the method of choice for single CFTR segments [13,14], but preparation of multi-spanning hydrophobic segments presents a challenge suited for the techniques of molecular biology. As an initial step in the structural characterization of wild-type and mutant CFTR proteins, we describe here the milligram-level expression in *E. coli* of double transmembrane segments of CFTR

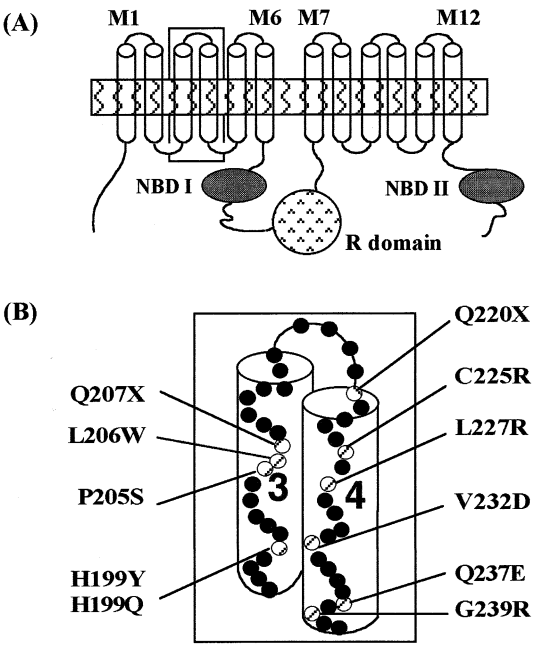

Fig. 1. Top: Schematized domain structure predicted for the cystic fibrosis transmembrane conductance regulator (CFTR) protein. M1–M6, M7–M12, membrane domains; NBDI, II, nucleotide binding domains; R, regulatory domain. The rectangular box delineates the transmembrane segments 3 and 4 (TM3–4) studied in the present work. After Riordan et al. [2]. Bottom: Distribution of mutations phenotypic for cystic fibrosis in CFTR TM segments 3 and 4. X, stop codon. Data taken from the CF Genetic Analysis Consortium, 1995.

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Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; TM, transmembrane segment; Trx, thioredoxin; EK, enterokinase; PAGE, polyacrylamide gel electrophoresis; LPC, lysophosphatidylcholine; LPG, lyso-phosphatidylglycerol; SDS, sodium dodecylsulfate; CD, circular dichroism

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('helical hairpins') as a fusion protein with thioredoxin. Our studies include optimization of protein expression and purification, successful enzymatic cleavage of the CFTR segments from the thioredoxin carrier, and initial structural characterization of CFTR TM3–4 segments.

2. Materials and methods

Chemicals and reagents were purchased from Sigma. Enzymes were from New England Biolabs; enterokinase and thrombin proteinase were from Novagen. SDS mini-gels were obtained from Novex. Bacterial strains were obtained from Novagen: *E. coli* strain DH5a was used for cloning; *E. coli* BL21(DE3) was used in expression studies.

2.1. Construction of expression vectors

Subcloning and transformation steps of *E. coli* were carried out using standard techniques [15]. The polymerase chain reaction (PCR) was used to prepare the cDNA fragments of CFTR TM3–4. A forward primer 5′-GCACCATGGGACTTGCATTGGCAC-3′ (containing an *Nco*I site) and a reverse primer 5′-CGTCT-CGAGTCCCTAGCCCAGCCT-3′ (containing an *Xho*I site) were designed to define and amplify the CFTR coding sequence from base 711 to 858. PCR products of expected size were obtained and cloned into the *Nco*I and *Xho*I sites of the pET32a(+) vector (Novagen) to generate p32CFTM-P3 (Fig. 1). The recombinant plasmid with CFTR(TM3–4) was sequenced to confirm the correct in-frame fusion and the fidelity of the PCR amplification.

2.2. Bacterial expression of TrxCFTR(TM3–4) fusion protein

The *E. coli* strain BL21(DE3) was transformed with the plasmid generated above. Control bacteria were transformed with original pET32a(+). Cultures were grown in Luria Broth medium at 37°C until OD_{600} reached 0.6–0.8, and induced with 0.1 mM IPTG at 25°C. The cultures were incubated for an additional 2–3 h. The cells were harvested by centrifugation at $4000\times g$ for 20 min. The cell pellet was resuspended in 1/10 volume of lysis buffer A (20 mM Tris-HCl, pH 8.0, containing 500 mM NaCl, 5 mM imidazole, 0.1% Triton X-100), or buffer B (same as buffer A but containing 1 M NaCl) and sonicated. The lysate was centrifuged at $16\,000\times g$ for 40 min. The insoluble protein fraction was redissolved in an equal volume of buffer A containing 6 M guanidine hydrochloride. As a control, the vector protein His-tag-thioredoxin-S-gag (but lacking the CFTR(TM3–4) segment) was also prepared in parallel.

2.3. Purification of the TrxCFTR(TM3–4) fusion protein

All steps of purification were performed at room temperature. TrxCFTR(TM3–4) fusion protein was purified from the lysis supernatant by affinity chromatography. *His-bind* resin (Novagen) was packed into a column charged with 50 mM $NiSO_4$ equilibrated with lysis buffer. In typical experiments, crude extract containing the TrxCFTR(TM3–4) fusion protein was mixed with 1/4 volume of prepared resin. After allowing 30 min for equilibration at room temperature, the solution was removed by filtration and the resin beads were eluted with buffer A containing a series of increasing imidazole concentrations. Analysis of the fractions was carried out on Tricine SDS-PAGE and subsequently by Western blotting using an antibody against thioredoxin. Gels were stained with Coomassie Blue R-250 or silver nitrate (the latter for cleaved proteins). Fractions containing fusion proteins were pooled and dialyzed against 50 mM Tris-HCl (pH 7.2) and 100 mM NaCl. Protein concentrations were determined by combining the absorbance at 590 nm (Branford) and the densities on the SDS-PAGE of the protein solutions using a standard curve generated with BSA.

2.4. Proteolytic cleavage and purification of cleaved CFTR(TM3–4)

Purified TrxCFTR(TM3–4) fusion proteins (native) were digested with enterokinase or thrombin proteinase according to the manufacturer's specifications. When the reaction was completed, components of the reaction mixture were separated by SDS-PAGE [16] or affinity chromatography using Thio-resin [17] and S-resin [18]. The separations were carried out at ambient temperature.

2.5. Circular dichroism (CD) spectroscopy

Samples were prepared in aqueous solution containing 50 mM Tris-HCl (pH 8.0) and 150 mM NaCl, with or without addition of 10 mM lyso-phosphatidylcholine (LPC), lyso-phosphatidylglycerol (LPG), or sodium dodecylsulfate (SDS). CD spectra were recorded using an 0.1 cm pathlength demountable cell in a Jasco-J-720A spectropolarimeter at 25°C, and at the physiological temperature of 37°C. Each spectrum was the average of four scans over the wavelength range of 190–250 nm with a step resolution of 0.2 nm.

3. Results and discussion

3.1. High-level expression of CFTR(TM3–4) as a soluble fusion protein with thioredoxin

To express the CFTR(TM3–4) gene product in *E. coli*, the corresponding fragments – which encompass CFTR transmembrane segments 3 and 4 – were inserted into the bacterial expression vector pET32a(+). Fig. 2 presents the design of the fusion construct. Expression of CFTR gene is under the control of a T7 promoter. The C-terminal coding region of the thioredoxin gene was fused to the inserted CFTR(TM3–4) coding sequence (53 aa), linked by an S-tag (15 aa) for enhancing solubility [17] and for detection of the target protein. Between the fusion partner and CFTR(TM3–4), a cleavage recognition sequence for enterokinase (EK), DDDDK, was inserted adjacent to the N-terminus of CFTR(TM3–4). Another potential cleavage site – the protease recognition sequence for thrombin – was inserted such that it would allow release CFTR(TM3–4) from thioredoxin with the CFTR polypeptide containing an N-terminal S-tag sequence. His-tags located both upstream and downstream of CFTR(TM3–4) segments were also incorporated to allow a one-step method for protein purification by Ni-chelate chromatography [19] at all stages of the preparation. Expression of recombinants was carried out after transformation into *E. coli* strain BL21(DE3). Control bacteria were transformed with the original pET32a(+).

Fig. 3 illustrates the expression of TrxCFTR(TM3–4) from its expression plasmid, which was capable of producing the target protein with little difference in levels at 25°C or 37°C. The recombinants of TrxCFTR(TM3–4) from cells migrated

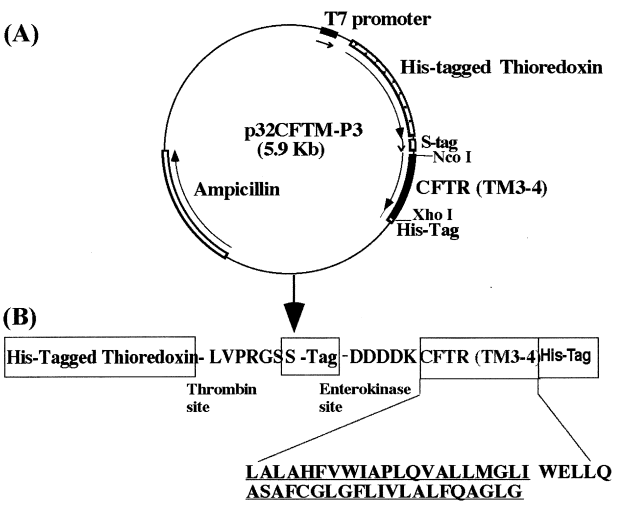

Fig. 2. A: Construct p32CFTM-P3 designed for expression of the TrxCFTR(TM3–4) fusion protein. Trx, His-tagged thioredoxin. The cDNA fragment of CFTR(TM3–4) was synthesized by PCR and ligated into *Nco*I/*Xho*I sites of p32a(+). B: The linearized sequence of the TrxCFTR(TM3–4) fusion protein. The two protease cleavage sites are shown.

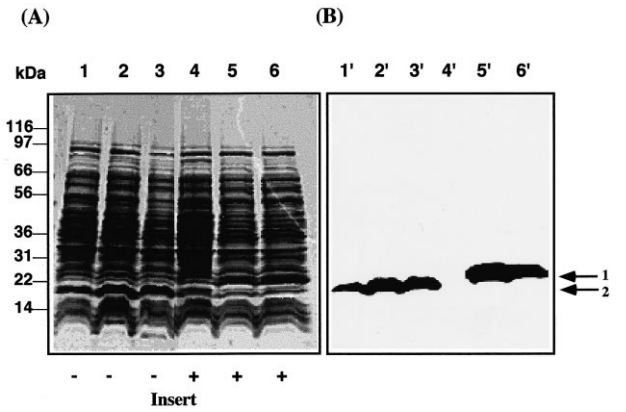

Fig. 3. Accumulation of TrxCFTR(TM3–4) in bacteria. *E. coli* (BL21) strain carrying the p32CFTM-P3 construct was induced for expression. A: Total cell extracts electrophoresed on SDS-PAGE. Proteins were stained with Coomassie Blue R-250. Lanes 1 and 4: uninduced; lanes 2 and 5: induced with IPTG at 37°C; lanes 3 and 6: induced with IPTG at 25°C. B: Western blot using a monoclonal antibody against thioredoxin. Lanes correspond to those given in A.

as a band with an apparent molecular mass of 24 kDa, which was absent in uninduced cells or induced cells without the CFTR(TM3–4) insert (Fig. 3A). An immunologically recognizable band was detected at same molecular mass upon performing a Western blot using anti-thioredoxin (Fig. 3B). It was estimated that the fusion protein accounted for approximately 15–20% of total cell protein.

3.2. Solubility of expressed TrxCFTR(TM3–4)

The production of biologically-active proteins is often hampered by formation of inclusion bodies [20]. Preliminary experiments to optimize the growth conditions and concentration for IPTG induction had shown that a significant fraction of the TrxCFTR(TM3–4) fusion protein is found in inclusion bodies when the protein is induced for 4 h with relatively high concentrations of IPTG (1–5 mM). The positive effect of reduced induction temperature for the production of soluble proteins has been observed, and indeed, we found that TrxCFTR(TM3–4) fusion proteins induced at lower temperature are considerably more soluble; as shown in Fig. 4, greater than 80% of the fusion protein appeared in the insoluble fraction at 37°C while less than 50% was insoluble at 25°C. Using a shorter induction time (2 h), along with a lower level of IPTG (0.1 mM), the fusion proteins produced from the plasmid were almost entirely soluble at 25°C (Fig. 4B). The solubility was enhanced by the presence of detergent (0.1% Triton X-100) and NaCl (up to 0.5 M) in the lysis buffer (data not shown). Increasing salt concentration up to 1 M appeared to have little or no further effect on solubility.

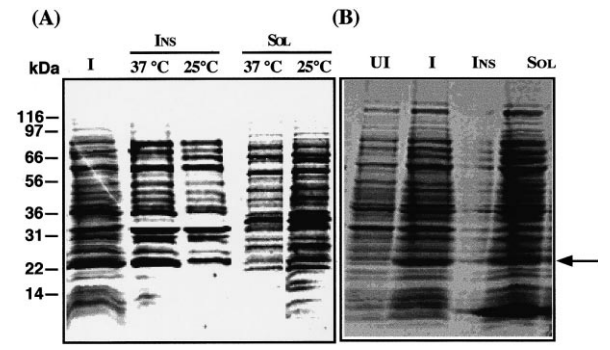

Fig. 4. A: SDS-PAGE showing distribution of expressed TrxCFTR(TM3–4) fusion protein between supernatant (SOL) and inclusion bodies (INS). I, induced (with IPTG) for 3 h, UI, uninduced. Induction temperatures are indicated. B: SDS-PAGE showing optimized solubilization of TrxCFTR(TM3–4), using 2 h induction at 25°C.

3.3. Purification of fusion proteins

Fig. 5 shows a Coomassie Blue stained gel (A) and an immunoblot (B) of the purified TrxCFTR(TM3–4) fusion protein eluted from a nickel affinity column. All of the Triton X-100-solubilized TrxCFTR(TM3–4) protein became bound to the nickel chelate His-bind resin when chromatographed in buffer A (lane 2). Essentially all of the bound fusion protein could be eluted at 400 mM imidazole to give a single band corresponding to the expected molecular mass of approximately 24 kDa on SDS-PAGE. No contaminating protein(s) could be detected by immunoblotting (Fig. 5B). When 0.1% Triton-100 was added into the elution buffer, the resulting proteins were greater than 95% pure, and stable during dialysis against 50 mM Tris-HCl, pH 8.0, 150 mM NaCl. In general, we have been able to produce 15–20 mg of purified fusion protein in soluble fractions from 1 l of LB culture. The purified fusion proteins (with or without dithiothreitol) are very stable and concentrated solutions (1 mg/ml) do not precipitate during storage at 4°C for several weeks.

3.4. Proteolysis of the fusion protein

When the fusion protein was cleaved with 5% (w/w) thrombin at pH 8.0, 37°C, near the optimal cleavage conditions of the protease, 90% of the fusion protein was proteolyzed in less than 3 h. However, more than 80% of the liberated CFTR(TM3–4) appeared to have been degraded. Because of this secondary proteolysis, studies were performed to develop conditions for a more selective cleavage of the linker, but with less internal processing. Lowering both pH and the temperature gave slower, but very selective cleavage. From these results, more suitable conditions for cleavage could be evolved, viz. pH 7.2 and 22°C. Using these parameters, the

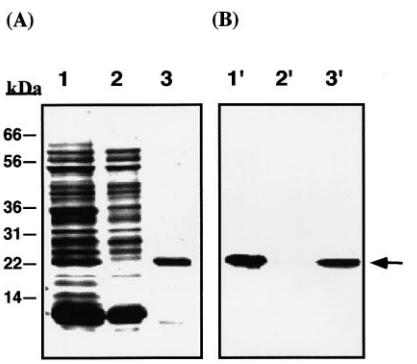

Fig. 5. Purification of TrxCFTR(TM3–4) by affinity chromatography. A: The soluble fraction (shown in Fig. 4B) applied to a nickel chelate affinity column (lane 1). Flow-through at 5 mM imidazole (lane 2). The column was washed with lysis buffer containing increasing concentrations (5 mM to 500 mM) of imidazole; the fusion protein was eluted at 400 mM imidazole (lane 3). B: Western blot using an antibody to thioredoxin. Lane numbers correspond to those given in A.

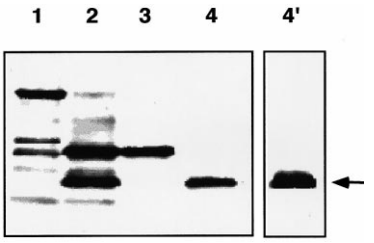

Fig. 6. Thrombin cleavage of TrxCFTR(TM3–4) fusion protein and recovery of CFTR (TM3–4) polypeptide. A: Lane 1, control. Fusion protein incubated with cleavage buffer (3 h) without thrombin. Lane 2, fusion protein treated with thrombin at a protease-to-fusion protein ratio of 1:100 at ambient temperature for 3 h. Lane 3, flow-through of the cleavage mixture applied to S-protein agarose column, showing released thioredoxin. Lane 4, released CFTR(TM3–4), eluted from S-protein agarose column by 4 M urea. Following electrophoresis (16% SDS tricine polyacrylamide gel), the gel was stained with silver nitrate. B: The identity of the CFTR(TM3–4) polypeptide (lane 4 in A) confirmed by Western blotting using an antibody against the S-tag peptide sequence.

fusion protein was completely digested by thrombin in 3 h. Fig. 6 presents the pattern of thrombin cleavage, where the two main fragments were found to correspond to the expected molecular masses of thioredoxin and the CFTR(TM3–4) polypeptide, respectively.

3.5. *Preparative scale recovery of thrombin-cleaved CFTR(TM3–4) segments*

The thrombin cleavage reaction was scaled up to approach the milligram quantities of the CFTR(TM3–4) polypeptide required for detailed structural characterization. When the digestion was completed, as evidenced by the disappearance of the 24 kDa band in the digested samples on the SDS-PAGE, the released CFTR(TM3–4) (containing an S-tag (15 aa); see Fig. 2) could be separated from by-products by various methods. In an initial series of experiments, the thio-resin column unexpectedly failed to retain the thioredoxin fusion partner, with both CFTR(TM3–4) and thioredoxin being removed at the same time; the CFTR(TM3–4) polypeptide was eventually purified by affinity chromatography on S-protein agarose. However, using an S-protein HRP conjugate, a single band was detected as monitored by SDS-PAGE and Western blotting. Following purification by the latter method, the S-resin affinity chromatography yielded approximately 70% recovery (based on molar ratio vs. fusion protein input) of thrombin-released CFTR(TM3–4). In a further protocol, SDS-PAGE gel purification gave the CFTR(TM3–4) polypeptide in ca. 20% yield.

3.6. *Conformational properties of purified proteins*

As established for several membrane proteins, the transmembrane domains of CFTR are expected to be α-helical [21]. To obtain direct experimental evidence of such helical character, CD spectroscopy – which is responsive to the contribution of various protein secondary structural elements – was used to evaluate the overall conformation of purified CFTR-derived proteins. Conformations of the unmodified vector protein (Trx-S-tag), TrxCFTR(TM3–4), and free CFTR(TM3–4) were first examined in aqueous buffer (containing 0.05% Triton X-100) (Fig. 7). Both the vector protein and thrombin-cleaved CFTR(TM3–4) polypeptide display CD spectra in aqueous buffer broadly characteristic of partial

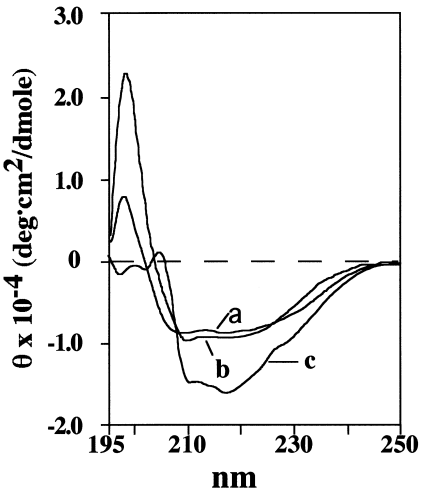

Fig. 7. Circular dichroism spectra of (a) vector (p32) protein (Trx-S-tag); (b) CFTR(TM3–4); and (c) TrxCFTR(TM3–4). Spectra are recorded in 50 mM Tris-HCl, pH 7.2, 100 mM HCl, and 0.05% Triton X-100. Trx, His-tagged thioredoxin, with the His-tag attached to the protein C-terminus. See Section 2 for further details of CD spectroscopic measurements.

α-helical structures (and with coincidentally similar negative ellipticity). The spectrum of TrxCFTR(TM3–4) fusion protein displays considerable α-helical conformation with pronounced double minima near 219 and 210 nm. As the separation of these two minima is relatively narrow ($\Delta\lambda = 9$ nm) vs. typical pure α-helical spectra ($\Delta\lambda = 14$ nm) [22], the spectrum is indicative of the presence of additional ordered structures, presumably the β-sheets which are known to arise in the tertiary structure of thioredoxin [23]. The latter results do attest to the correct folding of (at least) the thioredoxin portion of the fusion protein.

Importantly, the extent of helical structure in the CFTR(TM3–4) polypeptide is enhanced by the addition of 10 mM LPC micelles (Fig. 8b). Since such helix promotion is not detected in the Trx-S-tag vector protein (which lacks CFTR segments) (Fig. 8a), the increase in α-helical content is likely contributed by micelle-protein interactions specific to the putative TM segments of CFTR(TM3–4). Table 1 presents the α-helical content of CFTR(TM3–4) polypeptide in a variety of environments. The largest helical content, calculated as

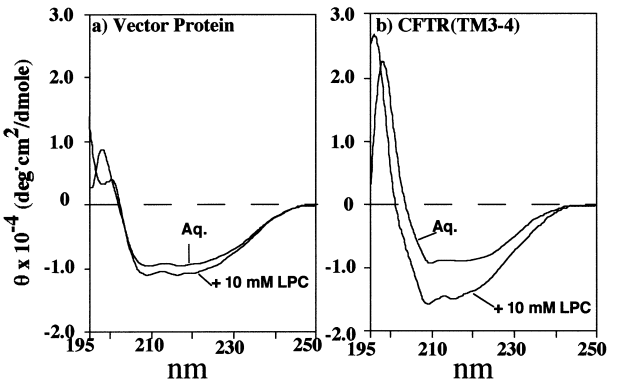

Fig. 8. Circular dichroism spectra showing addition of 10 mM lysophosphatidylcholine micelles (above the critical micelle concentration) to (a) vector (p32) protein; and (b) thrombin-released CFTR(TM3–4) polypeptide. CD spectra were recorded as described under Fig. 7; see also Table 1.

Table 1
Helical content (α%) of thrombin-released CFTR(TM3–4) polypeptide in various environments

Environment	$-[\theta]_{222}$[a]	α %[b]	$-[\theta]_{208}$	α %[c]
Water[d]	11000	29	13000	31
SDS[e]	14800	39	15600	40
LPC	16000	42	17475	46
LPG	18000	47	21740	61

[a]Mean residue ellipticity, expressed in deg cm^2 $dmol^{-1}$.
[b]Calculated by the method of Chen et al. [22].
[c]Calculated by the method of Greenfield and Fasman [24].
[d]Peptide concentration, 4.9 μM; aqueous buffer, 50 mM Tris-HCl, 150 mM NaCl, pH 8.
[e]Micelle concentration, 10 mM.

ca. 60% for the thrombin-released CFTR(TM3–4) polypeptide, is induced by negatively-charged lyso-phosphatidylglycerol (LPG) micelles. Considering the presumed aqueous-based location for the polar S-tag and His-tag residues of CFTR(TM3–4) in the presence of micelles, this helical content of 60% is representative of a nearly full helical conformation for the two effective CFTR TM(3–4) segments.

3.7. Conclusion

We have successfully expressed the Trx-CFTR(TM3–4) fusion protein in high yield in soluble form. Expressed fusion proteins could be readily purified by nickel chelate affinity chromatography. A 1 l culture of BL21(DE3) *E. coli* transformed with the recombinants expressed 15–20 mg of purified soluble fusion protein. Thrombin cleavage was effective in the quantitative removal of the thioredoxin fusion partner from CFTR(TM3–4) polypeptide. Undesirable cleavage of the polypeptide was avoided by optimization of the enzyme digestion conditions. Availability of this expression vector could facilitate production of TM domains of CFTR both for structural/functional, as well as therapeutic, studies.

The gross appearance of CFTR(TM3–4) CD spectra suggests that the polypeptide can readily adopt an α-helical conformation. CFTR(TM3–4) was shown to be incorporated into phospholipid micelles, and, in preliminary results, inserted into phospholipid vesicle bilayers (data not shown). Although the CFTR(TM3–4) polypeptide shows a pronounced tendency to adopt significantly folded structure, it remains to be established whether the two effective TM segments of CFTR(TM3–4) are surrounded by lipids in an essentially linear 'rod-like' form, or whether they have become folded into a 'helical hairpin', where the native-like contacts between TM segments 3–4 – as they occur within the intact CFTR membrane domain – have been restored. No concentration dependence of helicity was observed at polypeptide concentrations between 5 μM to 25 μM – an indication that the α-helical structure formed is intramolecular, rather than intermolecular as has been observed in some model systems (28). Subsequent NMR analysis of CFTR(TM3–4) is expected to provide further insights into the topography of the polypeptide within the membrane environment.

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References

[1] Welsh, M.J. and Smith, A.E. (1995) Sci. Am. 273, 52–59.
[2] Riordan, J.R., Rommens, J.M., Kerem, B.S., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N. and Chou, J.L. et al. (1989) Science 245, 1066–1073.
[3] Rommens, J.M., Iannuzzi, M.C., Kerem, B.S., Drumm, M.L., Melmer, G., Dean, M., Rozmahel, R., Cole, J.L., Kennedy, D. and Hidaka, N. et al. (1989) Science 245, 1059–1065.
[4] Zielenski, J. and Tsui, L.C. (1995) Annu. Rev. Genet. 29, 777–807.
[5] Dean, M., White, M.B. and Amos, J. (1990) Cell 61, 863–870.
[6] Sheppard, D.N., Rich, D.P., Ostedgaard, L.S., Gregory, R.J., Smith, A.E. and Welsh, M.J. (1993) Nature 362, 160–164.
[7] Tabcharani, J.A., Rommens, J.M., Hou, Y.X., Chang, X.B., Tsui, L.C. and Riordan, J.R. (1993) Nature 366, 79–82.
[8] Cheng, S.H. and Gregory, R.J. (1990) Cell 63, 827–834.
[9] Anderson, M.P. and Welsh, M.J. (1992) Science 257, 1701–1704.
[10] Nagel, G. and Hwang, T.C. (1992) Nature 360, 81–84.
[11] Carson, M.R., Travis, S.M. and Welsh, M.J. (1995) J. Biol. Chem. 270, 1711–1717.
[12] Qu, B.H. and Thomas, P.J. (1996) J. Biol. Chem. 271, 7261–7264.
[13] Oblatt-Montal, M., Reddy, G.L., Iwamoto, T., Tomich, J.M. and Montal, M. (1994) Proc. Natl. Acad. Sci. USA 91, 1495–1499.
[14] Goto, N.K., Li, S.-C. and Deber, C.M. (1996) in: P.T.P. Kaumaya and R.S. Hodges (Eds.), Proc. 14th American Peptide Symposium, Mayflower Scientific Ltd., Kingswinford, UK, pp. 271–272.
[15] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[16] Lee, C., Levin, A. and Branton, D. (1987) Anal. Biochem. 166, 308–312.
[17] LaVallie, E.R. and McCoy, J.M. (1995) Curr. Opin. Biotechnol. 6, 501–506.
[18] Kim, J.S. and Raines, R.T. (1993) Protein Sci. 2, 348–356.
[19] Crowe, J., Doable, H., Gentz, R., Hochuli, E., Stuber, D. and Henco, K. (1994) Methods Mol. Biol. 31, 371–387.
[20] Marston, F.A.O. (1987). in: D.M. Glover (Ed.), DNA Cloning III, IRL Press, pp. 59–79.
[21] Akabas, M.H., Kaufmann, C., Cook, T.A. and Archdeacon, P. (1994) J. Biol. Chem. 269, 14865–14868.
[22] Chen, Y.H., Yang, J.T. and Chau, K.H. (1974) Biochemistry 13, 3350–3359.
[23] Holmgren, A. (1985) Annu. Rev. Biochem. 54, 237–271.
[24] Greenfield, N. and Fasman, G.D. (1969) Biochemistry 8, 4108–4116.