HHS Public Access

Author manuscript

Protein Expr Purif. Author manuscript; available in PMC 2016 December 01.

Published in final edited form as:

Protein Expr Purif. 2015 December ; 116: 159–166. doi:10.1016/j.pep.2015.09.018.

Thermal stability of purified and reconstituted CFTR in a locked open channel conformation

Luba A. Aleksandrov, **Timothy J. Jensen**, **Liying Cui**, **Joseph N. Kousouros**, **Lihua He**, **Andrei A. Aleksandrov**, and **John R. Riordan***

Department of Biochemistry and Biophysics and Cystic Fibrosis Treatment and Research Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

Abstract

CFTR is unique among ABC transporters as the only one functioning as an ion channel and from a human health perspective because mutations in its gene cause cystic fibrosis. Although considerable advances have been made towards understanding CFTR's mechanism of action and the impact of mutations, the lack of a high-resolution 3D structure has hindered progress. The large multi-domain membrane glycoprotein is normally present at low copy number and when over expressed at high levels it aggregates strongly, limiting the production of stable mono-disperse preparations. While the reasons for the strong self-association are not fully understood, its relatively low thermal stability seems likely to be one. The major CF causing mutation, ΔF508, renders the protein very thermally unstable and therefore a great deal of attention has been paid to this property of CFTR. Multiple second site mutations of CFTR in NBD1 where F508 normally resides and small molecule binders of the domain increase the thermal stability of the mutant. These manipulations also stabilize the wild-type protein. Here we have applied ΔF508-stabilizing changes and other modifications to generate wild-type constructs that express at much higher levels in scaled-up suspension cultures of mammalian cells. After purification and reconstitution into liposomes these proteins are active in a locked-open conformation at temperatures as high as 50°C and remain monodisperse at 4°C in detergent or lipid for at least a week. The availability of adequate amounts of these and related stable active preparations of homogeneous CFTR will enable stalled structural and ligand binding studies to proceed.

Keywords

membrane protein; ion channel; CFTR; ABC protein; protein thermal stability; cystic fibrosis

*Corresponding author. jack_riordan@med.unc.edu.

Conflict of Interest

None

Author contributions

LAA purified and reconstituted the stabilized CFTR; TJ developed suspension cell cultures and isolated CFTR containing membranes from them; LC designed, constructed and transfected cDNA plasmids; JNK maintained and harvested large scale CFTR expressing cell cultures; LH contributed to design and construction of mutant constructs; AAA performed all CFTR ion channel assays and measured their thermal stability; JRR wrote the manuscript

Introduction

The CFTR protein product of the gene mutated in patients with cystic fibrosis is unique among members of the large ABC protein family, acting as an ion channel rather than a transporter[1]. While the common fold of the CFTR channel and ABC exporters is apparent, the detailed structural differences that distinguish them functionally are not. This uncertainty is primarily due to the fact that although 3D structures of several exporters have been determined[2–9], CFTR has remained refractory to structure determination at high resolution because of difficulties in obtaining homogeneous active protein for analyses. The channel is normally expressed at low levels in epithelial cells and there is no rich natural source. When heterologously over expressed the protein self-associates strongly and has limited detergent solubility[10]. This behavior is exacerbated as levels of expression are increased. Although some proportion of the very large number of channel proteins that can be expressed in bacculovirus infected insect cells exhibit activity[11], the bulk of the protein is soluble only in strong denaturing detergents[12, 13]. This poor solubility, in mild crystallization-compatible detergents, is shared by the protein expressed in Saccharomyces cerevisiae[14] in which the protein is retained in the endoplasmic reticulum[15]. In mammalian cell systems, CFTR expression and functional characterization in membranes has been performed using CHO[16, 17], BHK[18, 19], and HEK-293 cells[20]. CFTR could be readily solubilized from membranes of these cells by mild detergents including dodecyl-maltoside, neo-pentylmaltosides, and others enabling purification of small amounts suitable for low resolution structural analysis by electron microscopy[19–21].

The goal of the current work was to generate milligram amounts of homogeneous human CFTR protein that is active, thermally stable and in a restricted conformational state. This objective was achieved employing a strategy in which the thermal stability of modified constructs was first evaluated functionally in membranes from rapidly generated small cultures prior to scaling-up for expression in large mammalian cell suspension cultures for purification and reconstitution.

Materials and Methods

Mammalian cell expression of CFTR

Wild-type and mutated CFTR cDNA constructs were generated in the pNUT selectable expression plasmid and transfected into BHK-21 cells as previously described[22]. Individual colonies surviving methotrexate selection were expanded on the surfaces of standard cell culture flasks in DMEM/F12 medium supplemented with 5% FBS and containing 500 μM methotrexate. These adherent cells were adapted to growth in suspension by first gradually weaning them from this medium to SFM II 293s suspension medium (Invitrogen) supplemented with 2% FBS, 2x glutamine, 250μM methotrexate. Once cells were adapted to the suspension medium, they were switched from normal tissue culture treated plastic dishes to non-tissue culture treated ones and passaged until the majority of cells in the culture grew as free floating clumps rather than loosely attached to the substrate. Once cells were actively growing in suspension, they were switched to standard spinner cultures for expansion. Cells were further expanded until cell numbers reached ~$2x10^6$/ml.

Cells were maintained in humidified incubators at 37°C with 5% CO_2 during the adaptation process. When the spinner cell culture volume reached 500 ml, the cultures were expanded to 700 ml and transferred to 2L square PETG plastic medium bottles (Fisher). At this point the cultures were transferred to a New Brunswick Scientific model I2500 floor shaker incubator and maintained at 37°C without CO_2 at a rotation rate of 120 rpm. Cultures were maintained at a minimum cell density of $2x10^6$ cells/ml with the caps slightly loosened to allow for air exchange. Cultures could be maintained at cell densities as high as $5x10^6$ cells/ml, however culture health was optimal at $3–4x10^6$ cells/ml. The levels of CFTR expression at each stage were monitored by Western blotting of whole cell lysates and small-scale membrane preparations using methods described earlier[23]. The relative amounts of mature (diffuse major, more slowly migrating band containing complex N-linked oligosaccharide chains and immature (sharp minor, more rapidly migrating band with core N-linked chains) CFTR forms were measured with an Odyssey Imaging system (Li-Cor Bioscience, Lincoln, Nebraska). The immature endoplasmic reticulum located biosynthetic intermediate is removed during the differential detergent solubilization (see below) and is not purified.

Isolation of microsomal membranes

Typically lots of 8–10 bottles, each containing700 ml cultures at ~$3.5 – 4x10^6$ cells per ml were harvested by centrifugation at 2500 rpm using a GS3 rotor and washed with cold PBS yielding approximately 80 ml of packed cells. After the PBS wash, cells were resuspended with 10 times the cell pellet volume in ice cold lysis buffer (50 mMTris-HCl pH 7.4, 1 mM EDTA) containing 3X concentrated protease inhibitors: (Leupeptin (RPI) 3.0 μg/ml, Aprotinin (RPI) 6.0 μg/ml, Pefabloc (RPI) 150 μg/ml, Benzamidine (Fisher) 36 μg/ml, E64 (AG Scientific) 10.5 μg/ml. The resuspended cells were incubated on ice for 10 minutes then disrupted with 5 strokes of a 100 ml Dounce homogenizer. Sucrose buffer (25 ml of 1.5 M sucrose, 50 mM Tris pH 7.2) was added to the homogenate and mixed with 2 more strokes. Unbroken cells and cell debris were removed by centrifugation at 5000 rpm in a GS3 rotor. The crude membranes were collected by centrifugation of the GS3 supernatant in a SW28 rotor at 26,500 rpm for 30 minutes at 4°C. The membrane pellets were collected and resuspended in buffer A (see below) containing 2X protease inhibitors). Membranes were stored at −80°C prior to solubilization. Protein concentrations were measured using bicinchoninic acid assay (BCA reagent from Pierce Thermo Scientific).

CFTR purification

Buffer constituents: Buffer A; 40 mMTris-HCl, pH 7.4; 0.5 M NaCl; 0.1M arginine; 20% glycerol. Buffer B; 50 mM Na-phosphate, pH 7.6; 0.5 M NaCl; 0.1 M arginine; 15% glycerol. Buffer C; 40 mMTris-HCl, pH 7.4; 0.15 M NaCl; 10% glycerol. All buffers contained a protease inhibitor cocktail (benzamidine at 120 μg/ml, E-64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanido)butane] at 3.5 μg/ml, aprotinin at 2 μg/ml, leupeptin at 1 μg/ml and Pefabloc at 50 μg/ml) and 5mM Mg-ATP.

Membranes containing CFTR were resuspended in buffer A containing 0.01% DMNG (Decyl Maltose Neopentyl Glycol; Anatrace), incubated for 15 min at 4°C and centrifuged at 26,000 rpm in an SW-28 Rotor for 35 min. The supernatant was discarded and the pellet was resuspended and solubilized in buffer B containing 1% DMNG. After a 30 minute

incubation at 4°C the suspension was centrifuged at 26,000 rpm for 35 minutes. The detergent extract was mixed with Co-Talon resin (Clontech) and gently rotated for 3h at 4°C. The resin was washed with buffer B containing 25 mM imidazole and 0.2% DMNG followed by 50 mM imidazole with 0.1% DMNG. CFTR was eluted with 180mM imidazole in buffer C containing 0.01% DMNG and dialyzed against the same buffer lacking imidazole overnight at4°C. The CFTR eluates were concentrated up to the desired protein concentration using centrifugal filter units with a 150kDa cut off (Pierce Concentrators; Thermo Scientific).

Liposome preparation and CFTR reconstitution

Lipid stock solutions in chloroform were mixed in the desired proportions (DOPE, DOPC, DOPS, Cholesterol; 48:22:12:18) and dried in a rotary evaporator. All manipulations with the lipids were conducted under nitrogen gas. The dried lipids were suspended in buffer C to a final concentration of 20 mg/ml. The lipid suspensions were sonicated with 3 cycles of 5 min each in a water bath sonicator (Avanti) to generate small unilamellar (SUVs) vesicles. The SUV suspension was flash frozen in liquid nitrogen followed by slow thawing at room temperature to fuse the SUVs into large multilamellar vesicles (LMVs) that were kept frozen at −80°C until use.

In preparation for incorporation of CFTR, LMVs (10mg/ml) were extruded through 1000 nm and then 400nm polycarbonate membranes (15 times each) to form Large Uni Lamellar Vesicles (LULVs). These were diluted to 5 mg/ml of lipids in buffer C containing 10 mM Mg-ATP and 20% glycerol to yield final concentrations of MgATP and glycerol of 5 mM and 10%, respectively in the liposome suspension. Liposome destabilization at room temperature by incremental addition of Triton X-100 at 2 min intervals was monitored by measuring the optical density of the suspension at 540 nm. After addition of DTT to a final concentration of 5 mM and protein kinase A to 25 U/ml and incubation for 1 h at 4°C, the purified CFTR protein was mixed with the destabilized liposomes at a 1:50 (w/w) protein to lipid ratio and incubated for 30 min at 4°C with gentle agitation. To remove the detergent and form the proteoliposomes, 3 additions of Bio-Beads SM2 were made at 4 h intervals. Proteoliposomes were separated from the beads by centrifugation at 2000rpm and kept at 4°C until functional analysis.

Single channel analysis

Planar lipid bilayers were formed with a 3:1 mixture of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (Avanti Polar Lipids) as detailed previously[24]. CFTR ion channels were transferred into the preformed lipid bilayer by spontaneous fusion of membrane vesicles containing naturally folded CFTR constructs or proteoliposomes containing purified and reconstituted protein. To maintain uniform orientation and functional activity of CFTR channels transferred into the bilayer, 5 mM MgATP, 50 nM PKA and membrane vesicles or proteoliposomes were added in the cis compartment only. All measurements were done in symmetrical salt solution (300mM Tris–HCl, pH 7.2, 3mM $MgCl_2$ and 1mM EGTA) under voltage-clamp conditions using an Axopatch 200B amplifier. The membrane voltage potential of −75 mV is the difference

between cis and trans (ground) compartments. Data analysis was performed as described earlier[25].

Heating and temperature control were established by a temperature control system TC2BIP (Cell Micro Controls, Norfolk, VA, USA) with heating element covering the “trans” compartment outer side surfaces and bottom surface. Because of the bulky “trans” compartment it takes approximately 2–3 minutes to achieve a uniform temperature distribution across all compartments after the first indication of the expected temperature by the local temperature sensor in the chamber. Over the temperature range from +20°C to +40°C the wild type CFTR single channel has a well-resolved open state with linear dependence between open state conductance at −75 mV and the holding temperature as found previously[26]. We use this linear relationship as an intrinsic thermometer to define the real temperature in the vicinity of the channel during the non-stationary temperature RAMP experiments where temperature distribution across the experimental chamber is not uniform. Therefore in all experiments under non-stationary temperature conditions the actual temperature was indicated by the single channel conductance rather than by the local thermometer reading. We consider the thermal stability of wild type CFTR function in terms of its ability to support single channel function with stable open state conductance for the next 5 minutes after initial 5 minute incubation at the temperature of interest. The inactivation temperature (T_i) was defined as the temperature 1°C above the highest temperature with a stable open state during the total 10 min period.

Results

Thermal sensitivity of wild-type CFTR channels

The low thermal stability of the mutant form of CFTR (ΔF508) that causes most cystic fibrosis has been extensively studied[27–33] because of the obvious clinical relevance. Extensive biophysical studies of the isolated domain (NBD1) in which F508 normally resides showed that its thermal melting temperature (Tm) was reduced ~6°C compared to that of the wild-type domain[30]. Functionally the full-length ΔF508 protein becomes inactive at temperatures in the 30°C range and above[28, 29]. While the wild-type protein is of course active at physiological temperature and to at least 40°C[26], higher temperatures have not been investigated. To pursue the hypothesis that limited thermal stability may be a major factor contributing to difficulties in generating well-behaved protein for structural studies we assessed the influence of higher temperatures on channel function. After prolonged uniform gating at 40°C (Fig.1a, upper tracing) temperature was ramped up to 45°C at a rate of 1°C /min (middle tracing). Gating appeared to become somewhat less uniform soon above 40°C followed by a progressive increase in variability and the appearance of sub-conductance states. After approximately 1 min at 45°C there was an abrupt loss of near-full conductance gating. This real time transition is shown in expanded scale in the lower tracing. Not surprisingly, neither the precise transition temperature nor the time at that temperature before the transition occurred is identical in repeated single channel tracings. However, there was a high degree of overall reproducibility and the inactivation temperature (T_i) varied from 43°C to 45°C depending on the rate of temperature increase during the ramps. Having established the critical temperature range, we followed single

channel activity at constant temperatures around this range (Fig. 1b). Each tracing represents gating over a period of five minutes after a previous five minute equilibration of the chamber to each of the temperatures indicated. Stable open states were maintained over the entire period at temperatures up to 43°C. At 44°C normal gating continued for only approximately one minute after the pre-equilibration and did not appear at all at 45°C. Thus, defined as the temperature 1°C above the highest temperature with a stable open state, we conclude that the T_i of wild-type CFTR under these conditions is 44°C. This provides a reference point for comparison of the thermal sensitivity of stabilized CFTR variants.

Expression and function of stabilized CFTR constructs

Guided by modifications that we previously found to greatly improve the stability, maturation and function of ΔF508 CFTR[24, 32, 33], we made the same mutations in the wild-type and assessed their influence on expression and channel function in BHK cells. As seen in Fig. 2a the "2PT" variant with NBD1 mutations S492P, A534P and I539T and the ΔRI variant, from which the Regulatory Insertion (residues 404 – 435) was deleted both increased expression levels substantially compared to the wild type. In Fig. 2b it can be seen that RI removal had little influence on open probability (second tracing), consistent with an earlier study[33]. Channel open probability was substantially reduced in 2PT due to the introduction of the two prolines into the mobile Q loop (S492P) and SDR (A534P) regions of NBD1 (third tracing). Substitution of residue H1402 in NBD2 alone, predicted to participate in and be the "linch-pin" in the NBD2 ATP binding site[34], had little effect on expression levels but increased channel P_o to a very high level (fourth tracing). When the two types of NBD1 modification were combined, expression and maturation were greatly increased as observed in the Western blots (Fig. 2a) while channel open probability (fifth tracing) was similar to that of the 2PT variant. When the H1402S mutations was added to the combined NBD1 modifications, the amount of CFTR expressed by the cells (and present in isolated membranes) was increased 3 to 5 fold compared to the wild-type and the mature/immature band intensity ratio as an index of maturation also was elevated (not shown). The channel P_o was increased to nearly 1 indicating that the channel was virtually locked open at 37°C.

The thermal stability of the ΔRI/2PT/H1402S channel in isolated microsomal membranes was evaluated and it was found to retain full activity at 45°C for at least 1h (Fig 3, top tracing) and remain active during a temperature ramp from 45°C to 50°C albeit with open state subconductances most evident in the extended lower tracing at 50°C. Thus, the increased amounts of protein expressed and greater thermal stability encouraged us to proceed with purification and characterization of this variant.

Scale-up and purification

We had previously purified small quantities of CFTR expressed in BHK cells grown on the surfaces of tissue culture plates[19]. For production of larger amounts necessary for crystallization trials and other biophysical studies, the cells were adapted to high density suspension culture on a reasonably large-scale, enabling the generation of lots of ~ 5 x 10^{10} cells as starting material for purification. This number of cells yielded ~ 1g of microsomal membranes from which ~ 2 mg of wild-type and ~ 10 mg of the ΔRI/2PT/H1402S variant

could be purified. The purity of these preparations as assessed by SDS-PAGE and their homogeneity on gel filtration in DMNG is shown in Fig. 4. The purified ΔRI/2PT/H1402S protein was highly homogeneous as judged by heavily loaded SDS-PAGE gels (Fig. 4a) and appeared monodisperse in the low concentration of the DMNG (at 3 x CMC) detergent in which it was purified (Fig. 4b). The molecular mass of the major peak was estimated at 320 kDa from multi-angle light scattering analysis (MALS), substantially less than that predicted for a dimer but greater than that of a monomer. This may be due to the detergent micelles binding to monomeric CFTR. The gel filtration profile of the purified ΔRI/2PT/H1402S protein was unaltered after storage at 4°C for at least two weeks and little changed when kept at 16°C for this period of time (data not shown).

Reconstitution and thermal stability of purified CFTR

Efficient reconstitution into liposomes was achieved by fairly standard procedures detailed in Methods (Fig. 4c). In the case of the wild-type protein, when the proteoliposomes were fused with planar bilayers, only very rare channel openings were detected even at the low temperature of 25°C in contrast to the robust gating observed when microsomal vesicles of the cells from which the protein was purified were fused with the bilayer (Fig. 5, left panels). At 30°C with the reconstituted wild-type protein, no full conductance openings, but only the so-called fast flickering mode (ffm), characteristic of disrupted CFTR[24, 33], were detected, whereas with the microsomal vesicles, the P_o of wild-type CFTR channels was approximately doubled when the temperature was increased from 25°C to 30°C as expected (Fig. 5, right panels).

In marked contrast to the minimal CFTR channel activity observed with the purified wild-type protein, the purified ΔRI/2PT/H1402S protein was fully active at temperatures of 25, 30 and 37°C with very high open probability in all cases (Fig. 6, left 3 tracings). Indeed this behavior persisted for at least 1 hour even at 45°C (Fig. 6, top right tracing). When temperature was continuously increased further, robust gating continued until 50°C, although with sub-conductance states appearing over this range (Fig. 6, middle right tracing). Nevertheless, stable function in the “locked-open” mode continued for at least ½ hour at 50°C (Fig. 6, lower right tracing). Thus, this highly expressed construct appears to fulfill several criteria required for structural analyses including greatly increased life-time and thermal stability as well as a preferred conformational state in which the channel is locked open. The virtually identical single channel properties of the protein before and after purification and reconstitution are emphasized in Fig. 7. Fusion of proteoliposomes containing the homogeneous protein with the planar bilayer shown in panel (a) resulted in the very rapid appearance of multiple channels (panel b), reflecting the high frequency of active molecules in the population. Individual members of this purified and reconstituted population had the same conductance and open probability as those in the membranes of the cells from which they were obtained (panel c).

Discussion

Limited thermal stability of proteins after removal from their cellular environments often prevents them from remaining soluble and mono-disperse for extended periods so that their

inherent properties including three-dimensional structures can be determined. Indeed, low structural stability may make even obtaining these proteins in pure form very difficult because the quality control systems of the cells in which they are expressed recognize them as incompletely or incorrectly folded and degrade them[35]. The proportion of molecules avoiding this fate still may not have achieved a complete fully-folded state and have a strong tendency to associate with self (aggregation) and other proteins during purification steps. These latter problems are further exacerbated in the case of membrane proteins where hydrophobic segments become exposed when extracted from the bilayer with detergents. The presence of inherently disordered regions in proteins also may diminish their overall thermal stability and proteins that must be highly dynamic in the performance of their physiological function require a fine balance between stability and mobility.

The early recognition that wild-type CFTR folds and assembles very inefficiently in cells, with only about 25 –30 % of the nascent polypeptide maturing[36], may reflect the fact that it has difficulty achieving this balance. Attention was further focused on this issue when it was found that the mutation causing most cystic fibrosis, ΔF508, made the protein much more thermally unstable so that virtually none of the mutant polypeptide matured at mammalian physiological temperature[27]. Deletion of F508 from isolated NBD1 reduces its T_m by ~ 6°C[30] and this destabilization is reflected in the function of the full-length mutant channel[30, 32].

The relationship of the functional T_i values we have determined to the gross conformational perturbation of the protein as may be reflected in a cooperative melting transition measured by calorimetry or spectroscopy is not yet known. These values may or may not correspond. The T_i might reflect a more subtle predenaturational change in a structural element that is rate limiting in channel gating, possibly in the pore itself or in a key regulatory site. As discussed extensively by Privalov and colleagues[37, 38] multiple such local unfolding events are likely to occur in large multimeric proteins, possibly gradually, in overlapping stages. Although different CFTR channel sub-conductance states appeared when temperature was continuously increased, the complete loss of activity occurred rather abruptly. Interestingly, the T_i of the wild type channel activity (~44°C) is in the same range as the Tm of isolated NBD1[30]. Although this could be entirely coincidental, the fact that our variants with increased T_i have greater stability and longer lifetime in cells and after purification is consistent with a more global stabilization of the protein. It will now be important to determine Tm values of the purified full-length protein by biophysical methods both before and after reconstitution.

Many second site mutations in NBD1 of ΔF508 CFTR and at least one small molecule promote its conformational maturation[32]. We have found that these effects are not specific to the ΔF508 mutant, however, but also improve the maturation of the wild-type protein. When this set of NBD1 stabilizing mutations were combined with the H1402S substitution in NBD2 the level of mature protein expression in BHK cells increased several fold, enabling purification of milligram quantities of homogeneous protein that remained monodisperse at concentrations > 3 mg/ml in a low concentration of DMNG (3X CMC).

Importantly, this pure protein could be quantitatively incorporated into liposomes in a fully functional state in which it remained for periods longer than two weeks. Thus, the preparation appears to be quantitatively and qualitatively suitable for crystallization trials in detergent or lipid and the structural information that may be obtained will reflect that of an active folded state. With this construct as a platform it may be possible to introduce further modifications that drive the channel to different functional states and then determine the structural differences between them.

Acknowledgments

Support of this work was by grants from the NIH, RO1-DK051870 and PO1-HL10873 and the Cystic Fibrosis Foundation. We thank Christie Broulliette for providing purified P-glycoprotein.

Abbreviations used

CFTR	cystic fibrosis transmembrane conductance regulator
ABC	ATP-binding cassette
NBD1	N-terminal nucleotide-binding domain
CF	cystic fibrosis
CHO	Chinese hamster ovary
HEK	human embryonic kidney
BHK	baby hamster kidney
T_m	melting temperature
T_i	inactivation temperature
RI	Regulatory Insertion (residues 404 – 435)
2PT	variant with NBD1 mutations S492P, A534P and I539T
Q loop	residues contacting the gamma-phosphate of ATP
SDR	structurally divers region
DMNG	Decyl Maltose Neopentyl Glycol
MALS	multi-angle light scattering analysis
DOPE	1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine
DOPC	1,2-Dioleoyl-sn-glycero-3-phosphocholine
DOPS	1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine
SUV	small unilamellar vesicles
LMV	large multilamellar vesicles
LULV	large unilamellar vesicles
PKA	protein kinase A
RIPA	radioimmunoprecipitation assay

ER	endoplasmic reticulum
RAMP	gradual increase with constant slope

References

1. Hunt JF, Wang C, Ford RC. Cystic fibrosis transmembrane conductance regulator (ABCC7) structure. Cold Spring Harb Perspect Med. 2013; 3:a009514. [PubMed: 23378596]
2. Dawson RJ, Locher KP. Structure of a bacterial multidrug ABC transporter. Nature. 2006; 443:180–185. [PubMed: 16943773]
3. Ward A, Reyes CL, Yu J, Roth CB, Chang G. Flexibility in the ABC transporter MsbA: Alternating access with a twist. Proc Natl Acad Sci U S A. 2007; 104:19005–19010. [PubMed: 18024585]
4. Aller SG, Yu J, Ward A, Weng Y, Chittaboina S, Zhuo R, Harrell PM, Trinh YT, Zhang Q, Urbatsch IL, Chang G. Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. Science (New York, NY). 2009; 323:1718–1722.
5. Jin MS, Oldham ML, Zhang Q, Chen J. Crystal structure of the multidrug transporter Pglycoprotein from Caenorhabditis elegans. Nature. 2012; 490:566–569. [PubMed: 23000902]
6. Shintre CA, Pike AC, Li Q, Kim JI, Barr AJ, Goubin S, Shrestha L, Yang J, Berridge G, Ross J, Stansfeld PJ, Sansom MS, Edwards AM, Bountra C, Marsden BD, von Delft F, Bullock AN, Gileadi O, Burgess-Brown NA, Carpenter EP. Structures of ABCB10, a human ATP-binding cassette transporter in apo- and nucleotide-bound states. Proc Natl Acad Sci U S A. 2013; 110:9710–9715. [PubMed: 23716676]
7. Lee JY, Yang JG, Zhitnitsky D, Lewinson O, Rees DC. Structural basis for heavy metal detoxification by an Atm1-type ABC exporter. Science (New York, NY). 2014; 343:1133–1136.
8. Kodan A, Yamaguchi T, Nakatsu T, Sakiyama K, Hipolito CJ, Fujioka A, Hirokane R, Ikeguchi K, Watanabe B, Hiratake J, Kimura Y, Suga H, Ueda K, Kato H. Structural basis for gating mechanisms of a eukaryotic P-glycoprotein homolog. Proc Natl Acad Sci U S A. 2014; 111:4049–4054. [PubMed: 24591620]
9. Srinivasan V, Pierik AJ, Lill R. Crystal structures of nucleotide-free and glutathionebound mitochondrial ABC transporter Atm1. Science (New York, NY). 2014; 343:1137–1140.
10. Chang XB, Kartner N, Seibert FS, Aleksandrov AA, Kloser AW, Kiser GL, Riordan JR. Heterologous expression systems for study of cystic fibrosis transmembrane conductance regulator. Methods in enzymology. 1998; 292:616–629. [PubMed: 9711587]
11. Bear CE, Li C, Kartner N, Bridges RJ, Jensen TJ, Ramjeesingh M, Riordan JR. Purification and functional reconstitution of the cystic fibrosis transmembrane conductance regulator (CFTR). Cell. 1992; 68:809–818. [PubMed: 1371239]
12. Li C, Ramjeesingh M, Wang W, Garami E, Hewryk M, Lee D, Rommens JM, Galley K, Bear CE. ATPase activity of the cystic fibrosis transmembrane conductance regulator. J Biol Chem. 1996; 271:28463–28468. [PubMed: 8910473]
13. Ramjeesingh M, Li C, Garami E, Huan LJ, Hewryk M, Wang Y, Galley K, Bear CE. A novel procedure for the efficient purification of the cystic fibrosis transmembrane conductance regulator (CFTR). The Biochemical journal. 1997; 327(Pt 1):17–21. [PubMed: 9355728]
14. NC; VK; TR; LA; JRR; RCF; Al-Zahrani, Ateeq. Structure of the cystic fibrosis transmembrane conductance regulator in the inward-facing conformation revealed by single particle electron microscopy. AIMS Biophysics. 2015; 2:131–152.
15. Kiser GL, Gentzsch M, Kloser AK, Balzi E, Wolf DH, Goffeau A, Riordan JR. Expression and degradation of the cystic fibrosis transmembrane conductance regulator in Saccharomyces cerevisiae. Arch Biochem Biophys. 2001; 390:195–205. [PubMed: 11396922]
16. Tabcharani JA, Chang XB, Riordan JR, Hanrahan JW. Phosphorylation-regulated Clchannel in CHO cells stably expressing the cystic fibrosis gene. Nature. 1991; 352:628–631. [PubMed: 1714039]
17. O'Riordan CR, Erickson A, Bear C, Li C, Manavalan P, Wang KX, Marshall J, Scheule RK, McPherson JM, Cheng SH, et al. Purification and characterization of recombinant cystic fibrosis

transmembrane conductance regulator from Chinese hamster ovary and insect cells. J Biol Chem. 1995; 270:17033–17043. [PubMed: 7542655]

18. Lukacs GL, Mohamed A, Kartner N, Chang XB, Riordan JR, Grinstein S. Conformational maturation of CFTR but not its mutant counterpart (DF508) occurs in the endoplasmic reticulum and requires ATP. EMBO J. 1994; 13:6076–6086. [PubMed: 7529176]
19. Rosenberg MF, Kamis AB, Aleksandrov LA, Ford RC, Riordan JR. Purification and Crystallization of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR). J Biol Chem. 2004; 279:39051–39057. [PubMed: 15247233]
20. Hildebrandt E, Ding H, Mulky A, Dai Q, Aleksandrov AA, Bajrami B, Diego PA, Wu X, Ray M, Naren AP, Riordan JR, Yao X, DeLucas LJ, Urbatsch IL, Kappes JC. A stable human-cell system overexpressing cystic fibrosis transmembrane conductance regulator recombinant protein at the cell surface. Molecular biotechnology. 2015; 57:391–405. [PubMed: 25577540]
21. Zhang L, Aleksandrov LA, Zhao Z, Birtley JR, Riordan JR, Ford RC. Architecture of the cystic fibrosis transmembrane conductance regulator protein and structural changes associated with phosphorylation and nucleotide binding. Journal of structural biology. 2009; 167:242–251. [PubMed: 19524678]
22. Chang XB, Tabcharani JA, Hou YX, Jensen TJ, Kartner N, Alon N, Hanrahan JW, Riordan JR. Protein kinase A (PKA) still activates CFTR chloride channel after mutagenesis of all ten PKA consensus phosphorylation sites. J Biol Chem. 1993; 268:11304–11311. [PubMed: 7684377]
23. Kartner N, Augustinas O, Jensen TJ, Naismith AL, Riordan JR. Mislocalization of delta F508 CFTR in cystic fibrosis sweat gland. Nature Genetics. 1992; 1:321–327. [PubMed: 1284548]
24. Aleksandrov AA, Kota P, Cui L, Jensen T, Alekseev AE, Reyes S, He L, Gentzsch M, Aleksandrov LA, Dokholyan NV, Riordan JR. Allosteric modulation balances thermodynamic stability and restores function of DeltaF508 CFTR. J Mol Biol. 2012; 419:41–60. [PubMed: 22406676]
25. Aleksandrov AA, Cui L, Riordan JR. Relationship between nucleotide binding and ion channel gating in cystic fibrosis transmembrane conductance regulator. J Physiol. 2009; 587:2875–2886. [PubMed: 19403599]
26. Aleksandrov AA, Riordan JR. Regulation of CFTR ion channel gating by MgATP. FEBS letters. 1998; 431:97–101. [PubMed: 9684873]
27. Denning GM, Anderson MP, Amara JF, Marshall J, Smith AE, Welsh MJ. Processing of mutant cystic fibrosis transmembrane conductance regulator is temperature-sensitive. Nature. 1992; 358:761–764. [PubMed: 1380673]
28. Hegedus T, Aleksandrov A, Cui L, Gentzsch M, Chang XB, Riordan JR. F508del CFTR with two altered RXR motifs escapes from ER quality control but its channel activity is thermally sensitive. Biochimica et biophysica acta. 2006; 1758:565–572. [PubMed: 16624253]
29. Liu X, O'Donnell N, Landstrom A, Skach WR, Dawson DC. Thermal instability of DeltaF508 cystic fibrosis transmembrane conductance regulator (CFTR) channel function: protection by single suppressor mutations and inhibiting channel activity. Biochemistry. 2012; 51:5113–5124. [PubMed: 22680785]
30. Protasevich I, Yang Z, Wang C, Atwell S, Zhao X, Emtage S, Wetmore D, Hunt JF, Brouillette CG. Thermal unfolding studies show the disease causing F508del mutation in CFTR thermodynamically destabilizes nucleotide-binding domain 1. Protein Sci. 2010; 19:1917–1931. [PubMed: 20687133]
31. Wang C, Protasevich I, Yang Z, Seehausen D, Skalak T, Zhao X, Atwell S, Spencer Emtage J, Wetmore DR, Brouillette CG, Hunt JF. Integrated biophysical studies implicate partial unfolding of NBD1 of CFTR in the molecular pathogenesis of F508del cystic fibrosis. Protein Sci. 2010; 19:1932–1947. [PubMed: 20687163]
32. He L, Aleksandrov AA, An J, Cui L, Yang Z, Brouillette CG, Riordan JR. Restoration of NBD1 Thermal Stability Is Necessary and Sufficient to Correct ΔF508 CFTR Folding and Assembly. J Mol Biol. 2015; 427:106–120. [PubMed: 25083918]
33. Aleksandrov AA, Kota P, Aleksandrov LA, He L, Jensen T, Cui L, Gentzsch M, Dokholyan NV, Riordan JR. Regulatory insertion removal restores maturation, stability and function of DeltaF508 CFTR. J Mol Biol. 2010; 401:194–210. [PubMed: 20561529]

34. Zaitseva J, Jenewein S, Jumpertz T, Holland IB, Schmitt L. H662 is the linchpin of ATP hydrolysis in the nucleotide-binding domain of the ABC transporter HlyB. The EMBO journal. 2005; 24:1901–1910. [PubMed: 15889153]

35. Wolff S, Weissman JS, Dillin A. Differential scales of protein quality control. Cell. 2014; 157:52–64. [PubMed: 24679526]

36. Kopito RR. Biosynthesis and degradation of CFTR. Physiol Rev. 1999; 79:S167–173. [PubMed: 9922380]

37. Privalov PL, Dragan AI. Microcalorimetry of biological macromolecules. Biophysical chemistry. 2007; 126:16–24. [PubMed: 16781052]

38. Privalov PL. Microcalorimetry of proteins and their complexes. Methods in molecular biology (Clifton, NJ). 2009; 490:1–39.

Highlights

- Full understanding of the unique ABC ion channel, CFTR awaits high resolution structure determination
- Because of its marginal thermodynamic stability, expression and purification in a mono-disperse active form is extremely challenging.
- Here we have employed functional assays of channel activity to assess the effectiveness of different modifications on thermal stability and then expressed and purified the most stable constructs.
- Expression of these constructs in large-scale mammalian cell suspension cultures provided multi-milligram amounts of a stable locked open channel conformation that was fully active with unaltered channel parameters after reconstitution into proteoliposomes.
- The availability of these and related stable preparations enable the initiation of studies of 3D structure and the binding of modulating small molecules.

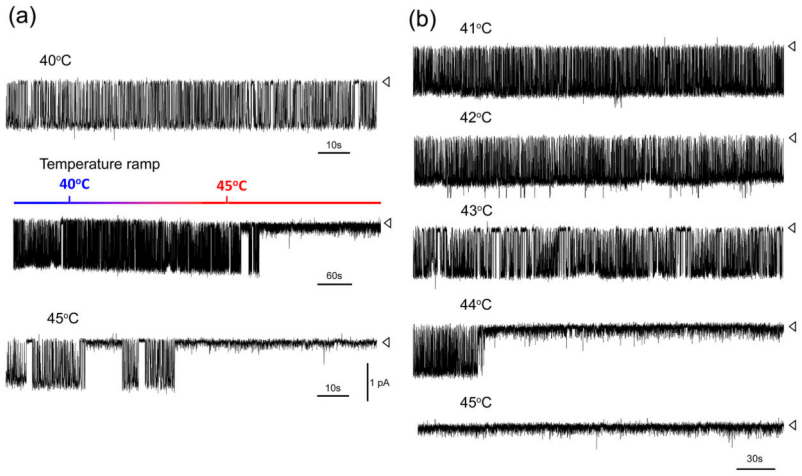

Figure 1.

Temperature sensitivity of wild-type CFTR channels transferred from microsomal membrane vesicles to planar lipid bilayers (see Methods). (a) The upper tracing is a 2 min recording at 40°C. This uniform full conductance gating persists for at least 1 h. The middle tracing shows the response to a temperature ramp (1°C/min) from 40°C to 45°C and the lower tracing, extension of the recording with the temperature held at 45°C. Time scale bars are below each tracing. The current scale bar opposite the lower tracing refers to all tracings. Closed channel state is indicated by arrow-heads. (b) Channel gating during 5 min at fixed temperatures between 40°C and 45°C after a previous 5 min pre-equilibration at each temperature.

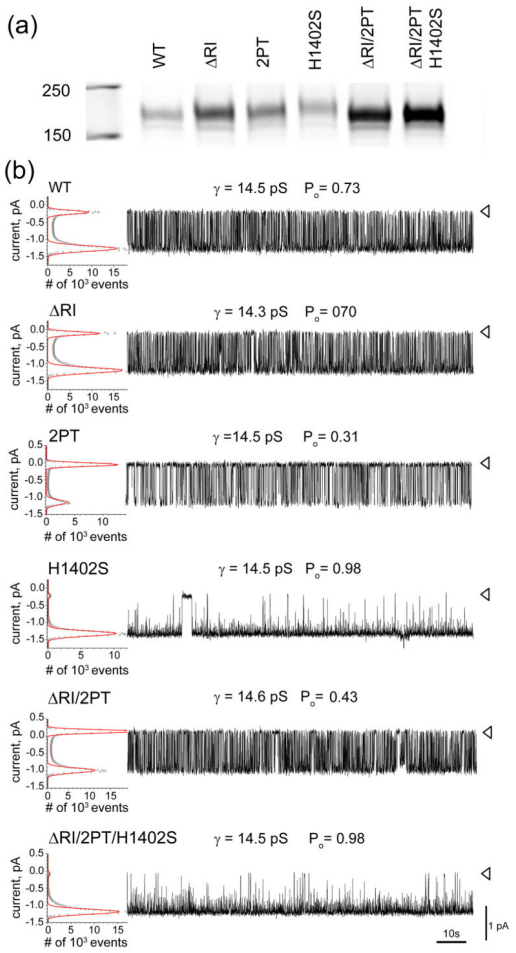

Figure 2.

Influence of different stabilizing mutations on CFTR expression and channel activity. (a) Western blot illustrating relative amounts of CFTR in lysates of BHK cells expressing the variants indicated above each lane. 25 μg of total cell protein was loaded and blot probed with mAb 596. (b) Single channel recordings of 2 min duration when microsomal membrane vesicles from the same cultures were fused with planar bilayers. Unitary conductance and open state probability calculated from the all-points histograms to the left are indicated above each tracing.

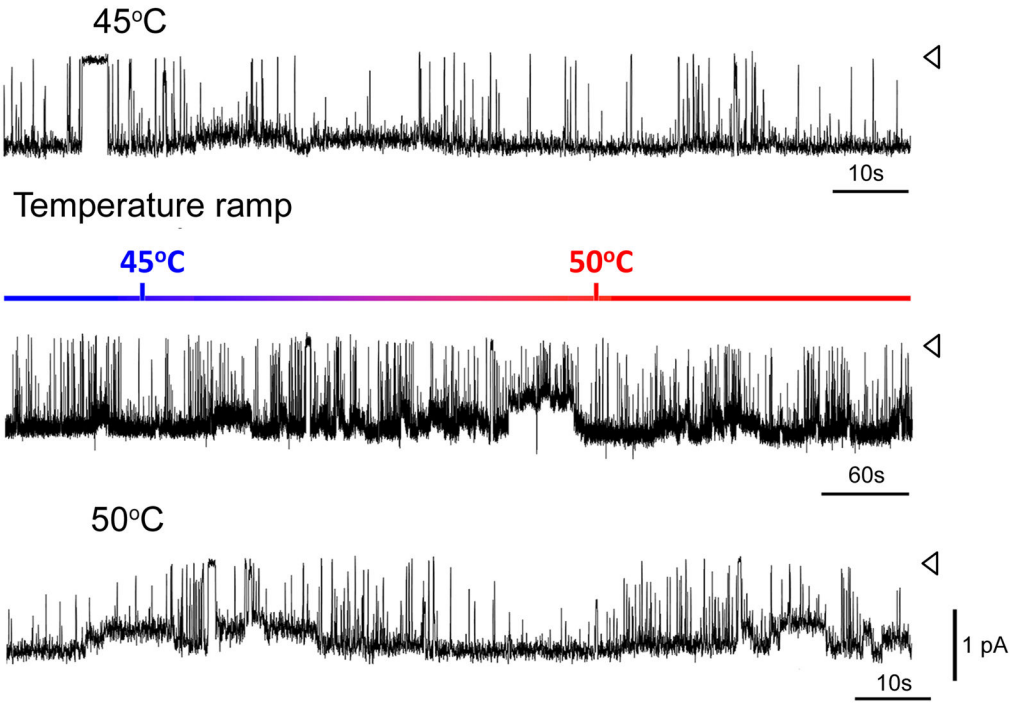

Figure 3.

Thermal sensitivity of NBD1 and NDB2 modified variant, ΔRI/2PT/H1402S. The experimental protocol was similar to that with the wild-type channel in Fig 1, except that the upper tracing was at 45°C, the temperature ramp (1°C/min) in the middle tracing from 45°C to 50°C and the lower tracing at 50°C. Note that the channel inhabits a very high P_o state even at these elevated temperatures. Although the variant visits transient subconductance states at the higher temperatures, the channel remains active in a predominantly open state at 50°C that is near the upper limit of bilayer stability.

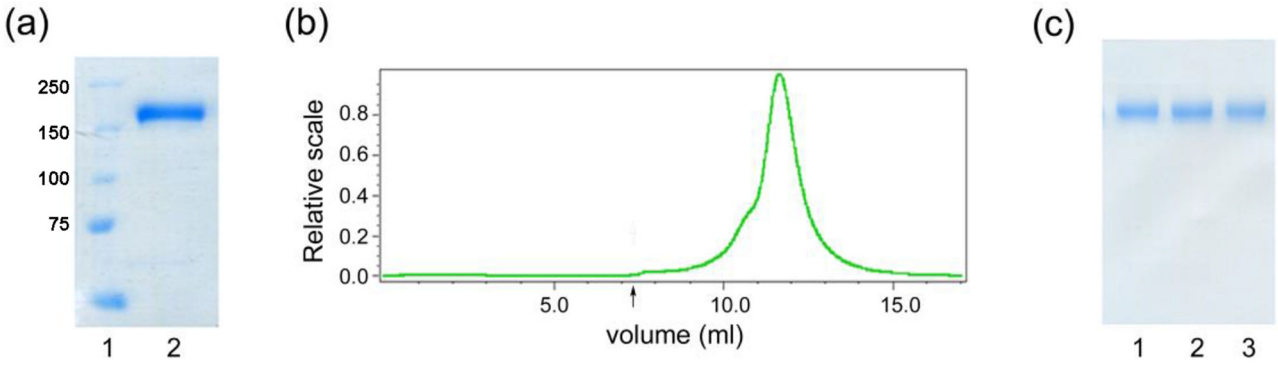

Figure 4.

Purified ΔRI/2PT/H1402S CFTR protein before and after reconstitution. (a) Coomassie blue stained SDS-PAGE (7.5% acrylamide) of molecular weight standards 250, 150, 100, 75, 50 kDa in lane 1 and 2μg of purified protein in lane 2. (b) Gel filtration chromatogram (Shodex KW-804) showing elution profile monitored by tryptophan fluorescence (excitation 290 nm, emission 340 nm). Void volume is indicated by the arrow. (c) SDS-PAGE showing the purified protein in detergent (DMNG) (lane 1) and following reconstitution into liposomes before (lane 2) and after (lane 3) detergent removal with Biobeads.

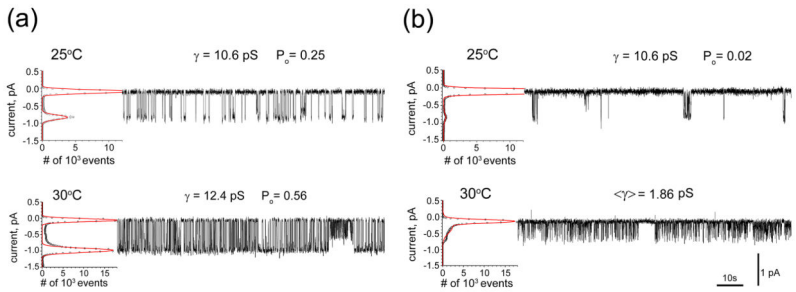

Figure 5.

Wild type CFTR channels activity before and after purification and reconstitution. (a) Single channel recording at 25°C (upper) and 30°C (lower) on fusion with bilayer of microsomal vesicles from BHK cells expressing wild-type CFTR. (b) Recordings at same temperatures on fusion of purified and reconstituted wild type CFTR with bilayer. The transport capacity of the structural unit <γ> for the mutants with no well-resolved peaks on the all points histogram was defined as mean current divided by applied potential difference that is exact analog of γP_o used for the channels with well defined open state [24].

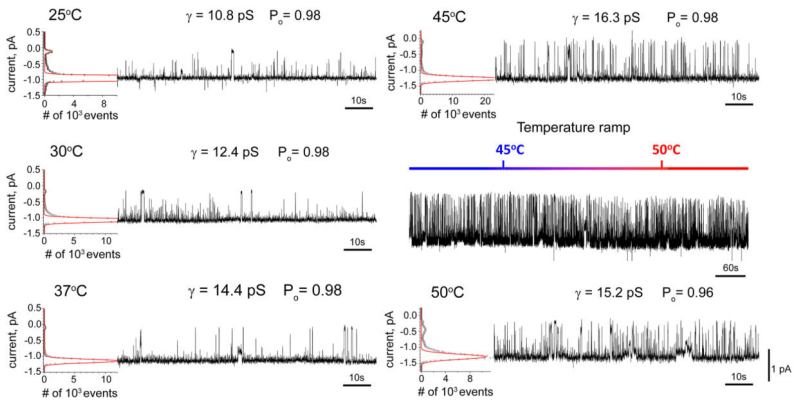

Figure 6.

Thermal stability of purified and reconstituted ΔRI/2PT/H1402S CFTR. The first 3 tracings in the left column and the first in the right column and their accompanying all-points histograms indicate that the stabilized channel is essentially locked-open at temperatures ranging from 25°C to 45°C. This behavior persists as the temperature is ramped from 45°C to 50°C (compressed middle right tracing) and when held at 50°C (lower right tracing).

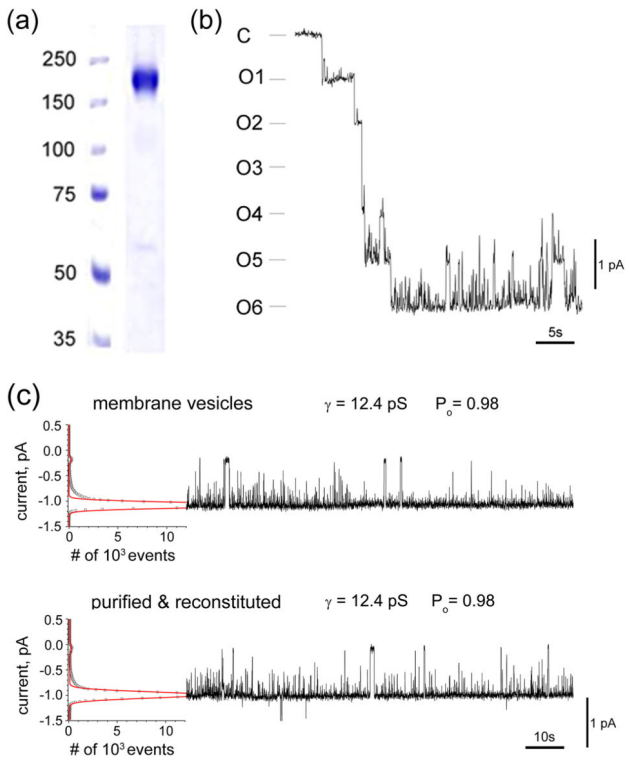

Figure 7.

Stabilized ΔRI/2PT/H1402S CFTR channels are identical before and after purification. (a) Heavily over-loaded Coomassie blue stained SDS-PAGE of protein reconstituted into proteoliposomes. (b) Multiple channels appeared within seconds of proteoliposome addition to the bilayer chamber. (c) Single channel tracings and parameters on fusion with bilayer of microsomal vesicles containing this construct (upper tracing) and the reconstituted proteoliposomes (lower tracing). The total amount of proteoliposomes used for single channel recordings is 10 times less than that used for multiple channel recording shown in (b).