# A Monomer Is the Minimum Functional Unit Required for Channel and ATPase Activity of the Cystic Fibrosis Transmembrane Conductance Regulator†

Mohabir Ramjeesingh,‡ Canhui Li,‡ Ilana Kogan,§ Yanchun Wang,‡ Ling-Jun Huan,‡ and Christine E. Bear*,‡,§

*Research Institute, Hospital for Sick Children, and Physiology Department, University of Toronto, Toronto, Ontario M5G 1X8, Canada*

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ABSTRACT: The cystic fibrosis transmembrane conductance regulator (CFTR) normally functions as a phosphorylation-regulated chloride channel on the apical surface of epithelial cells, and lack of this function is the primary cause for the fatal disease cystic fibrosis (CF). Previous studies showed that purified, reconstituted CFTR can function as a chloride channel and, further, that its intrinsic ATPase activity is required to regulate opening and closing of the channel gate. However, these previous studies did not identify the quaternary structure required to mediate conduction and catalysis. Our present studies show that CFTR molecules may self-associate in CHO and Sf9 membranes, as complexes close to the predicted size of CFTR dimers can be captured by chemical cross-linking reagents and detected using nondissociative PAGE. However, CFTR function does not require a multimeric complex for function as we determined that purified, reconstituted CFTR monomers are sufficient to mediate regulated chloride conduction and ATPase activity.

An understanding of the structural basis for the chloride channel and ATPase activities of the cystic fibrosis transmembrane conductance regulator (CFTR)[1] has fundamental basic importance as well as relevance to our understanding of the molecular mechanisms underlying the disease cystic fibrosis. To date, we know that several of the predicted transmembrane helices in CFTR are important in providing a pore for chloride electrodiffusion through CFTR including TM6 and TM12 (*1*−*3*). The two predicted nucleotide binding folds can bind ATP, and at least one, if not both, is capable of hydrolyzing ATP (*4*−*8*). Furthermore, we know that the putative regulatory (R) domain is phosphorylated at multiple sites by protein kinase A and that the structure of the isolated R domain is altered by phosphorylation (*9*−*11*). The amino and carboxy termini of CFTR are involved in interactions with other proteins, and these interactions may be important for the regulation of CFTR itself or its partner proteins (*12*, *13*). However, many important questions remain, and in this paper we address one in particular which has received considerable attention and stimulated a recent debate (*14*): namely, what is the number of molecules of CFTR required to form a regulated chloride channel?

Originally, Marshall et al. (*15*) concluded that CFTR existed primarily as a monomer in membranes, as differentially tagged versions of the protein could not be co-immunoprecipitated. However, more recently, Zerhusen et al. (*14*) showed that concatemers of two tethered CFTR molecules form a single pore with a unitary conductance of 9 pS, the conductance usually attributed to a native single CFTR channel. Cleaving the link between the concatamers with thrombin altered gating kinetics but did not result in the appearance of two channels. Furthermore, a concatemer of a normal protein tethered to a mutant protein known to exhibit altered channel gating led to the appearance of a channel with "hybrid" gating kinetics. These observations prompted the authors to suggest that two CFTR molecules are required to form a single conductance pore.

In the present paper, we report that chemical cross-linking experiments and studies using nondissociative gel electrophoresis show that CFTR exists as monomers and multimers in biological membranes. Further, purified, reconstituted CFTR also exists as monomers and multimers in proteoliposomes. To determine the minimal functional unit of CFTR, purified and reconstituted CFTR monomers and dimers were separated by gel filtration chromatography, and the fractions containing each CFTR structure were reconstituted separately for functional analyses. We found that both monomeric and dimeric CFTR could mediate chloride electrodiffusion and ATPase activity. Hence, our data support the notion that CFTR monomers are fully active. The functional significance of the existence of CFTR dimers in biological membranes remains to be determined.

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* Address correspondence to this author at the Hospital for Sick Children. Telephone: 416-813-5981. Fax: 416-813-5028. E-mail: bear@sickkids.on.ca.

‡ Hospital for Sick Children.

§ University of Toronto.

[1] Abbreviations: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; NBDs, nucleotide binding domains; ABC, ATP binding cassette; PFO, pentadecafluorooctanoic acid; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoylacyl-*sn*-glycero-3-phosphocholine; PKA, protein kinase A; PEI, poly(ethylenimine); sulfo-EGS, ethylene glycol bis(sulfosuccinimidyl succinate); DMS, dimethyl suberimidate hydrochloride; PBS, phosphate-buffered saline; AE1, band 3 protein.

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## EXPERIMENTAL PROCEDURES

*Materials.* Phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), and 1-palmitoyl-2-oleoylacyl-*sn*-glycero-3-phosphocholine (POPC) were obtained from Avanti Polar Lipids (Alabaster, AL). Pentadecafluorooctanoic acid (PFO) manufactured by Fluorochem (U.K.) was obtained from Oakwood Products Inc. (West Colombia, SC). Immobilized Ni−NTA agarose resin was obtained from Qiagen (Mississauga, ON).

*Chemical Cross-Linking of CFTR in Intact Sf9 Cells.* The Sf9 baculovirus expression system was used for large-scale production of wild-type or mutant CFTR proteins as described in our previous publications (*16*). Sf9 cells expressing CFTR were harvested from a 75 $cm^2$ flask, sedimented to form a pellet, and washed using PBS containing the protease inhibitor benzamidine. An aliquot containing $(2-3) \times 10^6$ cells was incubated with 10 mM dimethyl suberimidate hydrochloride (DMS) in PBS at pH 8.5 or 10 mM sulfo-EGS [ethylene glycol bis(sulfosuccinimidyl succinate)] in PBS at pH 8.0. Samples were nutated for 1 h at room temperature, and the cells were pelleted and resuspended with 100 mM Tris, pH 7.5, for 15 min at room temperature before being lysed in 2% SDS.

*Purification of CFTR.* Most of the procedures describing the purification of CFTR-His proteins have been published previously (*17*). Briefly, Sf9 cells harvested from 1 L of suspension culture were lysed in a French press (Spectronic Inst., Rochester, NY), the nuclei and other particles were pelleted, and the supernatant was centrifuged at 100000*g* for 90 min to sediment a crude membrane pellet. Peripheral proteins were extracted from this membrane pellet using 25 volumes of ice-cold 10 mM sodium hydroxide and 0.5 mM EDTA. The "stripped" membranes were sedimented by centrifugation at 100000*g* for 2 h. CFTR was solubilized from this membrane preparation by spinning overnight at room temperature in a solution containing 8% PFO and 25 mM phosphate at pH 8.0. Then, solubilized CFTR protein was purified by virtue of its 10× His tag using nickel affinity chromatography closely following the procedures previously described (*16*). A pH gradient (pH 8.0−6.0) was applied using an FPLC in order to elute CFTR from the column. Fractions possessing CFTR (identified by dot blot) were eluted at approximately pH 6.8 and concentrated at room temperature in Amicon YM100 concentrators (no. 4212, Millipore Corp., Bedford, MA) to a final volume of about 100 $\mu$L. Concentrated samples in 4% PFO (w/v), 25 mM phosphate, and 100 mM NaCl were diluted 1:10 in buffer containing 8 mM Hepes and 0.5 mM EGTA, pH 7.2, and further concentrated to a final volume of 100 $\mu$L to reduce the PFO concentration to 0.4%. Three microliters from each concentrated fraction was subjected to 6% SDS−PAGE gels (Novex, Carlsbad, CA) and analyzed for the quantity and purity of CFTR proteins by Western blot and silver-stained protein gel, as described previously (*18*, *19*). For immunoblotting, the protein was transferred to a nitrocellulose membrane and probed with an anti-CFTR polyclonal antibody generated against a fusion protein corresponding to the predicted NBD2 and C-terminus of CFTR, amino acids N1197−L1480. Immunopositive bands were visualized by enhanced chemiluminescence (Amersham, Oakville, ON).

*CFTR Reconstitution into Liposomes.* Procedures describing liposome preparation are reported elsewhere (*17*). Concentrated fractions containing purified CFTR-His in 0.4% PFO (approximately 100 $\mu$L) were mixed with an excess (1 mg) of a sonicated liposome preparation containing phosphatidylethanolamine (PE):phosphatidylserine (PS):phosphatidylcholine (PC):ergosterol (5:2:1:1 by weight; phospholipids from Avanti Polar Lipids Inc., Birmingham, AL). A lipid control was generated by diluting 1 mg of the liposome mixture containing PE:PS:PC:ergosterol into 100 $\mu$L of a buffer containing 8 mM Hepes and 0.5 mM EGTA, pH 7.2. The concentrated CFTR fractions and lipid control were dialyzed in a Spectra/Por dialysis membrane (Spectrum Laboratories Inc., Rancho Dominguez, CA; molecular mass cutoff 50 kDa) overnight at 4 °C against 4 L of a buffer containing 8 mM Hepes and 0.5 mM EGTA, pH 7.2.

*Separation of CFTR Monomers and Dimers by Gel Filtration Chromatography.* Proteoliposomes containing purified CFTR were collapsed by addition of detergent 4% PFO in a buffer solution also containing 25 mM phosphate, 0.5 mM EDTA, and 1 mM DTT at pH 7.5. A 500 $\mu$L aliquot containing 10 $\mu$g of CFTR protein was applied to a Superose 6 column (120 × 1 cm), and fractions were eluted with 25 mM phosphate, 100 mM NaCl, 4% PFO, 0.5 mM EDTA, and 1 mM DTT at pH 7.5 at flow rate of 0.2 mL/min. Immediately, 100 $\mu$L aliquots of each fraction were supplemented with 0.5 mg of lipid [containing PE:PS:PC:ergosterol (5:2:1:1)], passed through an Extractigel column (Pierce, Rockford, IL), and eluted with a buffer containing 8 mM Hepes at pH 7.2. Larger volume samples were first concentrated in a Microcon concentrator (Millipore Corp., Bedford, MA; 50 kDa cutoff) to about 50 $\mu$L, diluted 10 times with Hepes buffer, and concentrated again to 50 $\mu$L before application to the Extractigel column. The oligomeric status of the CFTR protein in each reconstituted fraction was assessed by PFO−PAGE, as previously described (*20*).

*Assay of the Catalytic Activity of CFTR Protein.* The catalytic activity was measured as the production of [α-$^{32}$P]-ADP from [α-$^{32}$P]ATP by purified, reconstituted, and PKA-phosphorylated CFTR, as described previously (*7*, *17*). Radiolabeled ADP and ATP were separated by poly(ethylenimine) (PEI) chromatography. Correction for spontaneous hydrolysis of ATP in reactions containing proteoliposomes was done by subtracting the [α-$^{32}$P]ADP/[α-$^{32}$P]ATP ratio of control liposomes. The ATPase assay was carried out in a reaction mixture containing 30 $\mu$L of freshly dialyzed proteoliposomes or liposomes, 1 mM nonradioactive ATP, 20 mM Tris, 40 mM NaCl, 4 mM $MgCl_2$, pH 7.5, and 2 $\mu$Ci of [α-$^{32}$P]ATP (10 $\mu$Ci/$\mu$L; Amersham, Oakville, ON).

ATPase reaction mixtures were incubated at 33 °C for 2 h and then stopped by the addition of 14 $\mu$L of 10% SDS and 88% formic acid (v/v). One microliter samples from the ATPase reaction were spotted onto PEI−cellulose plates (VWR, Mississauga, ON) and developed in 1 M formic acid and 0.5 M LiCl, as described previously (*20*). A STORM 840 Molecular Dynamics PhosphorImager was used to visualize ADP production by phosphorylated and nonphosphorylated CFTR samples. The quantity of ATP hydrolyzed was determined using the ImageQuant software package (Molecular Dynamics, Sunnyvale, CA).

*Assays of CFTR Chloride Channel Activity. Concentrative Tracer Uptake Assay.* We used a concentrative tracer uptake

assay developed by Garty et al. (*21*) and modified by Goldberg and Miller (*22*) to characterize the chloride conductance properties of reconstituted, PKA-phosphorylated CFTR. Proteoliposomes were preloaded with 150 mM KCl and centrifuged through Sephadex G-50 columns equilibrated with glutamate-containing salts, potassium glutamate (125 mM), sodium glutamate (25 mM), glutamic acid (10 mM), and Tris−glutamate (20 mM) at pH 7.6, to replace external chloride. Uptake was initiated and quantified by addition of 1.0 $\mu$Ci mL$^{-1}$ or 1.5 mM $^{36}$Cl. Intraliposomal $^{36}$Cl was assayed at various time points following separation of liposomes from the external media using a mini anion-exchange column (AG 1-X8 resin; Bio-Rad, Mississauga, ON).

*Planar Bilayer Studies of Purified CFTR.* As in our previous studies, proteoliposome fusion with planar lipid bilayers was facilitated and detected by the introduction of nystatin (120 $\mu$g mL$^{-1}$), a technique originally described by Woodbury and Miller (*23*). Planar lipid bilayers were formed by painting a 10 mg mL$^{-1}$ solution of phospholipid (PE:PS:POPC in the ratio 4:4:2) in *n*-decane over a 200 $\mu$m aperture in a bilayer chamber. Typically, the cis compartment of the bilayer chamber, defined as that compartment to which liposomes were added, contains 300 mM KCl, and the trans compartment, connected to ground, contains 50 mM KCl. Single channel currents were detected after the addition of the purified catalytic subunit of PKA (220 nM; Promega Corp., Madison, WI) and 1 mM MgATP with a bilayer amplifier (custom made by M. Shen, Physics Laboratory, University of Alabama). Data were recorded and analyzed using pCLAMP 6.0.2 software (Axon Instruments Inc., Foster City, CA). Prior to analysis of dwell times, single channel data were digitally filtered at 100 Hz. Transitions to 50% of the current level (or greater) for a single open pore were considered as a channel opening.

*Statistics.* Statistical analyses of electrogenic flux and ATPase measurements were performed using the unpaired Student's *t* test. Differences between two groups were considered significant with $p$ values $<0.05$.

## RESULTS

*CFTR Exists as a Multimeric Complex in Biological Membranes.* The oligomeric structure of CFTR stably expressed in CHO cells and transiently transfected in Sf9 cells was assessed by nondissociative gel electrophoresis and using chemical cross-linkers. PFO−PAGE is a novel gel electrophoresis method which permits the assessment of the quaternary structure of cytosolic and membrane proteins (*20*). Analysis of CHO membranes expressing CFTR by PFO−PAGE revealed both a major and minor band (Figure 1a, i). The minor band migrated as a 170 kDa protein, consistent with the molecular mass reported for complex glycosylated CFTR protein. The major band migrated as a larger protein, possibly a homodimer or heterodimer containing CFTR (indicated by an asterisk in Figure 1a, i). Similarily, PFO−PAGE analysis of Sf9 membranes revealed major and minor bands consistent with the expected mass of CFTR homodimers and monomers, respectively. To determine the contribution of heterogeneous peripheral proteins to the higher molecular mass complex, we assessed the effect of pretreating CHO membranes bearing CFTR with urea (4 M),

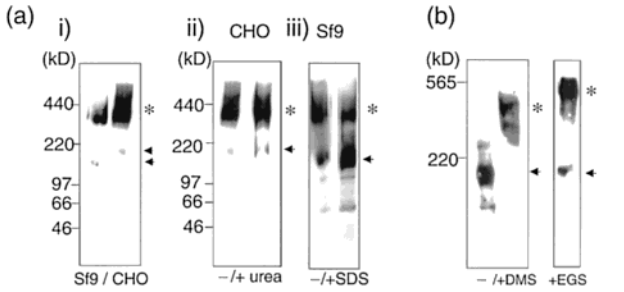


FIGURE 1: CFTR exists as oligomers in vivo. (a, i) Membranes were prepared from Sf9 and CHO cells expressing CFTR, solubilized in 4% PFO, and subjected to PFO−PAGE and Western analysis (polyclonal antibody was used; see Experimental Procedures). The predominant band (*) corresponds to a CFTR multimer. Monomeric CFTR (indicated by arrow) migrates as 170 kDa protein for CHO membrane preparation and as a 140 kDa protein for Sf9 membranes. (a, ii) Treatment of CHO membranes with urea (4 M) does not alter migration of CFTR on PFO−PAGE. (a, iii) Treatment of Sf9 membranes with 2% SDS increases the relative abundance of CFTR in monomeric form (arrow). (b) Intact Sf9 cells untreated (−) or treated (+) with cross-linkers DMS (10 mM) or EGS (10 mM). Membrane preparations were subsequently analyzed by SDS−PAGE.

a treatment which dissociates peripheral proteins (*24*). As this treatment did not affect the migration of the CFTR-containing complex, we suggest that the complex may not represent association of CFTR with accessory cytosolic proteins (Figure 1a, ii). Furthermore, as the higher molecular weight complex could be dissociated by treatment of PFO-solubilized membranes with SDS (2%), we suggest that this higher molecular weight band is unlikely to represent a PFO−detergent-induced protein aggregate (Figure 1a, iii).

*Chemical Cross-Linkers.* DMS, with a spacer arm of 11 Å, and sulfo-EGS, a cross-linker with a spacer arm of 14 Å, were applied to the exofacial surface of intact Sf9 cells to assess the quaternary structure of CFTR. Following cell lysis, analysis by SDS−PAGE, and Western blotting, we found that immunoreactive CFTR isolated from cells treated with either DMS or sulfo-EGS migrated primarily as a major band with a mass greater than that predicted for a monomer (Figure 1b). DMS can permeate cell membranes; hence, it could be capturing interactions of CFTR with itself, with other transmembrane proteins, or with intracellular, peripheral membrane proteins (*25*). The mass of the complex cross-linked by DMS appeared closer to that expected for a dimer than that detected in sulfo-EGS-treated cells. The larger apparent mass of the sulfo-EGS cross-linked complex suggests that the cross-linker with the longer spacer arm may be capturing a larger complex of proteins. Sulfo-EGS cannot permeate the membrane; therefore, this cross-linker must be capturing interactions of CFTR with itself or other membrane spanning proteins.

*Purified CFTR Is Reconstituted as Monomers and Multimers.* To determine directly whether CFTR self-associates to form an oligomeric complex, we assessed the quaternary structure of purified CFTR protein. CFTR expressed in Sf9 membranes was solubilized in pentafluorooctanoic acid (8% PFO) and purified by metal affinity by virtue of its polyhistidine tag (Figure 2a), as previously described (*17*). Analysis by PFO−PAGE revealed that purified, detergent-solubilized CFTR existed primarily as a monomer (Figure 2b, left panel). However, following reconstitution into phospholipid liposomes, a broader band corresponding to

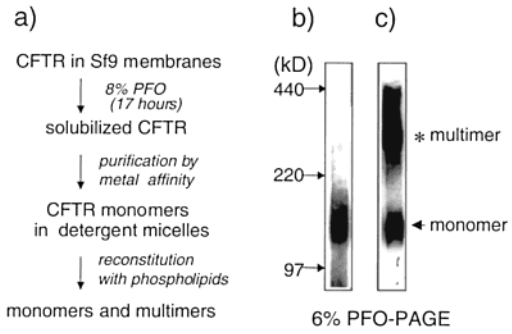


FIGURE 2: Purified, reconstituted CFTR exists as oligomers after reconstitution. (a) Flow chart shows that CFTR monomers are purified in the presence of 8% PFO but reconstitution of purified CFTR in phospholipid liposomes promotes formation of multimers. (b) CFTR purified in PFO exists primarily as a monomer as assessed by PFO−PAGE. (c) Purified CFTR reconstituted in phospholipids exists in oligomeric (probably dimeric) and monomeric forms as assessed by PFO−PAGE.

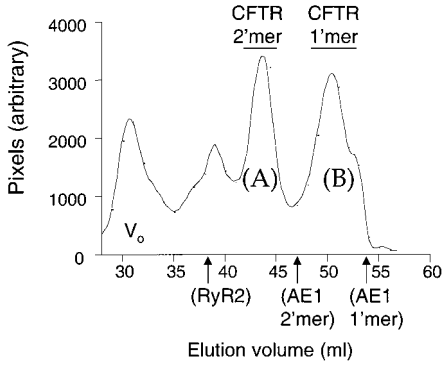


FIGURE 3: Monomers and dimers of CFTR are separated by gel filtration on a Superose 6 column. Proteoliposomes containing purified, reconstituted CFTR were collapsed using detergent (4% PFO) containing buffer as described in Experimental Procedures and applied to a Superose 6 column (120 × 1 cm) calibrated using AE1 monomers and dimers and the ryanodine receptor (RyR2). This elution profile was generated by dot blotting and pixel analysis by NIH Image software. Arrows correspond to volumes in which AE1 monomers (AE1 1′mer, 100 kDa), AE1 dimers (2′mer, 200 kDa), and RyR2 (1′mer, 565 kDa) elute. $V_0$ indicates where the void volume is eluted. Peaks correspond to the molecular mass predicted for CFTR monomers (CFTR 1′mer, B) and CFTR dimers (CFTR 2′mer, A).

multimeric CFTR could be detected in addition to CFTR monomers by PFO−PAGE (Figure 2c). These findings suggest that this higher order structure can be generated during transition from a partially folded to a fully folded protein.

*CFTR Monomers and Dimers Exhibit Chloride Channel Function and ATPase Activity.* We showed in our previous studies that purified CFTR protein mediates PKA phosphorylation-stimulated ATPase activity and phosphorylation-regulated chloride channel activity (*7*, *16*, *26*, *27*). To determine whether CFTR monomers or dimers conferred these activities, we separated each structure in the mixture by gel filtration chromatography. Separation by gel filtration first required that the proteoliposomes be disrupted while preserving the quaternary structure of CFTR. This was achieved by adding PFO (4%) to CFTR-containing proteoliposomes prior to separation. Then, 10 $\mu$g of purified CFTR protein in a 100 $\mu$L volume containing mixed detergent−phospholipid micelles was added to a long narrow Superose 6 column, previously calibrated with the intrinsic membrane protein AE1 (monomers and dimers) and the ryanodine receptor (RyR2) monomer in the same detergent. This particular column was essential to provide the resolution necessary to effectively separate CFTR monomers and dimers. The fractions containing CFTR protein were assessed by dot blot, and pixel intensity was analyzed using NIH Image software to provide an estimate of the relative quantities of CFTR in each fraction. Two prominent and distinctive peaks (A and B) were evident from the elution profile (Figure 3). The protein in peak A elutes between those fractions containing dimeric AE1 protein (200 kDa) and the ryanodine receptor (565 kDa). Hence the mass of the protein eluted in peak A is close to that predicted for a CFTR dimer (CFTR 2′mer, approximately 330 kDa). The protein in peak B is well separated from peak A and elutes in fractions between those containing dimeric (200 kDa) and monomeric (100 kDa) AE1 protein; hence, its mass is consistent with that predicted for monomeric CFTR (CFTR 1′mer, 160−165 kDa). The results of this gel filtration experiment are consistent with the interpretation that purified CFTR protein in lipid−detergent micelles exists as a heterogeneous mixture of monomers and dimers.

To reconstitute each structure separately and with the highest level of fidelity, we rapidly extracted detergent from multiple CFTR-containing fractions simultaneously by hydrophobic interaction chromatography (*28*) in the presence of excess phospholipid (the same composition as in the previous reconstitution). Detergent extraction and reconstitution into phospholipid were completed within 5−10 min. Excess phospholipid was added during reconstitution to ensure an extremely low protein to lipid ratio. For monomeric CFTR, 150 ng of CFTR was reconstituted in 0.5 mg of lipid, and for dimeric CFTR, 163 ng of protein was reconstituted in 0.5 mg of lipid. The reconstituted protein was analyzed immediately by nondissociative PFO−PAGE, and as shown in Figure 4a, CFTR monomers were effectively separated from dimers by gel filtration and these structures were maintained during reconstitution. Reconstituted monomers and dimers remain remarkably stable as evident in Figure 4b. Even after storage of liposomes for approximately 2−3 months at −80 °C and following thawing, PFO−PAGE analysis revealed that monomeric CFTR had not dimerized.

The relative PKA- and MgATP-dependent chloride channel function of CFTR monomers and dimers was determined immediately after reconstitution into phospholipid liposomes by measuring cumulative $^{36}$Cl flux, an assay developed by Garty et al. for the study of populations of ion channel proteins (*21*). We compared the chloride flux mediated by empty liposomes (with no reconstituted CFTR) and liposomes bearing CFTR protein from fraction 50 (monomeric CFTR, as shown in Figure 4a) with flux mediated by liposomes bearing CFTR protein from fraction 44 (dimeric CFTR, Figure 4a). In all cases, liposomes were pretreated with PKA plus MgATP in order to activate channel function. Empty liposomes mediated less $^{36}$Cl uptake than liposomes bearing monomeric CFTR protein ($p = 0.013$). The extent of cumulative flux mediated by protein reconstituted from fraction 50 [monomeric CFTR, 66.1 ± 23.7 (SD) nmol ($\mu$g of protein)$^{-1}$ h$^{-1}$] was not statistically different from that mediated by protein in fraction 44 [dimeric CFTR, 42.4 ± 17.5 (SD) nmol ($\mu$g of protein)$^{-1}$ h$^{-1}$, $p = 0.25$, Figure 4c].

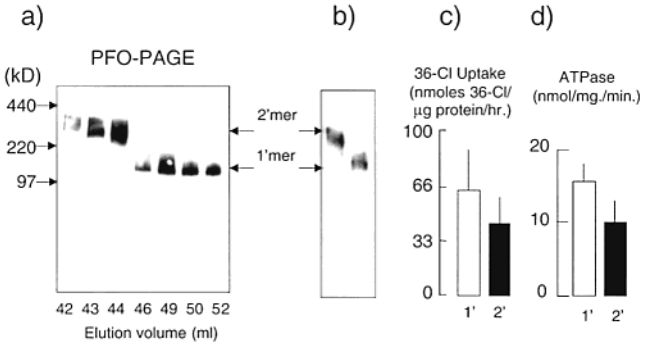


FIGURE 4: CFTR monomers and dimers can mediate chloride electrodiffusion and ATPase activity. (a) CFTR protein in each fraction eluted from the gel filtration column was reconstituted into phospholipid liposomes as described in Experimental Procedures and analyzed by PFO−PAGE and immunoblotting using a CFTR polyclonal antibody. This immunoblot shows that CFTR monomers have been effectively separated from CFTR dimers. (b) Reconstituted monomeric CFTR protein and dimeric CFTR were analyzed by PFO−PAGE 2−3 months after storage at −80 °C and thawing. (c) There is no significant difference between electrogenic $^{36}Cl$ uptake mediated by proteoliposomes bearing monomeric or dimeric CFTR protein; $p = 0.25$. Bars indicate mean ± SD for triplicate measurements. (d) ATPase activity by purified, phosphorylated CFTR protein reconstituted from fraction 49 (monomeric, empty bar; mean of triplicate measurements ± SD) and by phosphorylated CFTR protein from fraction 44 (dimeric, dark bar; mean of triplicate measurements ± SD) are not significantly different; $p = 0.22$.

Hence, both monomeric and dimeric CFTR structures are capable of mediating chloride flux.

The relative ATPase activity of monomeric and dimeric CFTR was also determined after reconstitution and PKA phosphorylation (Figure 4d). Empty liposomes (Ct) exhibit low levels of activity relative to proteoliposomes bearing monomeric CFTR protein ($p = 0.02$). On the other hand, proteoliposomes containing monomeric CFTR [15.3 ± 1.8 (SD) nmol $mg^{-1}$ $min^{-1}$] and proteoliposomes bearing dimeric CFTR [10.8 ± 3.0 (SD) nmol $mg^{-1}$ $min^{-1}$] exhibit comparable ATPase activities (Figure 4d, $p = 0.22$). Two other preparations of purified and reconstituted CFTR were fractionated and similar relative activities measured for fractions containing monomeric and dimeric CFTR.

To study the detailed properties of channel gating by CFTR monomers and dimers, proteoliposomes derived from fraction 50 or 44, respectively, were fused with planar lipid bilayers for single channel analyses. As in our previous studies (*7*, *16*, *26*, *27*), all proteoliposomes were rendered equally fusogenic by the addition of nystatin and ergosterol. We found that fusion of proteoliposomes containing monomeric CFTR (Figure 5a, top panel) conferred single channel activity that was similar to that previously described for CFTR channel function in biological membranes (*29*). As reported for CFTR function in membranes, channel activity by monomeric CFTR required phosphorylation by PKA (200 nM) as well as the presence of MgATP (1 mM). Furthermore, channel activity by monomeric CFTR exhibited selectivity for anions over cations (10:1; data not shown) and a low unitary conductance of approximately 11−12 pS (in the presence of asymmetrical solutions, as described in the legend for Figure 5). These findings suggest that a CFTR monomer is capable of forming a regulated chloride channel with properties similar to those described for the native channel.

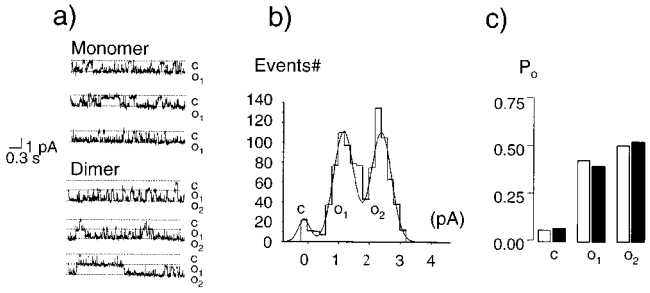


FIGURE 5: CFTR monomers can mediate low-conductance single channel activity. (a) Upper panel: Fusion of proteoliposomes containing monomeric CFTR in the presence of PKA (200 nM) and MgATP (1 mM) with planar lipid bilayers leads to the appearance of channel activity with a unitary conductance of 11−12 pS when bilayer compartments contain asymmetric solutions (300 vs 50 mM KCl; cis vs trans compartments, respectively). Eight bilayer experiments were performed (i.e., fresh liposomes added to a naïve bilayer). Lower panel: Fusion of proteoliposomes bearing dimeric CFTR leads commonly to the appearance of multiple conductance steps, each with a unitary conductance of 11−12 pS channels. (b) Amplitude histogram shows three major conductance levels in channel records of dimeric CFTR channel function, corresponding to the closed conductance level and openings of one or two channels. The area under each peak provides a measure of the relative probability of each conductance level during recordings totaling 3 min in duration. (c) The bar graph in the lower panel compares the relative probability of each conductance level (c = both conductance pores closed, $o_1$ = one conductance pore open, $o_2$ = two conductance pores open) determined from the above amplitude histogram (empty bar; pCLAMP 6.01, Axon Instruments Inc., Foster City, CA) with that predicted if openings of each channel occur independently, i.e., assuming a binomial distribution (dark bar). The following set of equations was used to determine the predicted probabilities of the three current levels (*30*): $P_0 = f_{o1}/2 + f_{o2}$; $f_0 = (1 - P_0)^2$; $f_1 = 2P_0(1 - P_0)$; $f_2 = P_0^2$.

Fusion of proteoliposomes bearing CFTR dimers typically led to the appearance of multiple anion-selective channels similar to those described for monomeric CFTR, with PKA- and MgATP-dependent gating and a unitary conductance of 11−12 pS (Figure 5a, bottom panel). Amplitude histograms of recordings totaling 3 min were fitted by the sum of three Gaussian distributions with equidistant peaks (c, o1, and o2). The relative areas covered by the three Gaussian components yield the probabilities of the closed and of the two open levels, respectively (Figure 5b). The values of the probabilities (empty bars) are similar to the predictions from a simple binomial superposition of two independently gating channels (dark bars, Figure 5c). These data suggest that there is no cooperativity between multiple pores observed following CFTR dimer reconstitution.

## DISCUSSION

Our studies provide direct evidence supporting the hypothesis that the minimal functional unit of CFTR required for regulated channel function and ATPase activity is a monomer. Our findings also suggest that CFTR can exist as a complex, both in biological membranes and in our reconstitution system for purified protein. However, the biological significance for self-assembly of CFTR in membranes remains unclear, as, at least in our reconstitution system of purified protein, coassembly does not appear to regulate intrinsic function.

Our evidence supporting the function of monomeric CFTR rests in large part on our ability to separate purified

monomeric CFTR from purified dimeric CFTR and to study the function of each structure individually after reconstitution into phospholipid liposomes. The fractionation of monomeric CFTR from dimeric CFTR appeared successful on the basis of PFO−PAGE analyses and immunoblotting (Figure 3). However, the possibility exists that each fractionated form could be contaminated by an infinitesimal quantity (i.e., undetectable by immunoblotting) of CFTR protein in a different quaternary structure. It is difficult to rule out the contribution of such contamination in planar lipid bilayer studies of single molecules. However, our macroscopic studies of populations of proteoliposomes containing either monomeric or dimeric CFTR report the cumulative function of all protein in each fraction. As evident in Figure 4, there was no difference in the relative channel and catalytic activity by populations of reconstituted monomers or dimers, providing direct evidence that CFTR monomers are functional and, further, that there is unlikely to be any intrinsic cooperativity between CFTR molecules in a homodimeric complex.

Our findings seemingly contradict a recent report by Zerhusen et al. suggesting that a single conductance pore of CFTR requires assembly of two molecules as a dimeric complex. Their interpretation was based on electrophysiological recordings from concatemers of two CFTR molecules tethered in a head to tail orientation (*14*). In these studies, a single channel pore was observed with hybrid gating when one wild-type (Wt) CFTR molecule was tethered to a mutant CFTR protein known to have altered gating properties (i.e., CFTRΔR). However, as suggested by the authors themselves, although these observations could support a model wherein two CFTR molecules comprise a single pore, alternatively, the appearance of a single pore may reflect an inhibitory effect of the artificial tether on one of the CFTR molecules.

While our data suggest that monomeric CFTR is the minimal channel- and ATPase-forming unit of this protein, we also report evidence that supports the possibility that CFTR can exist in complexes, possibly dimeric complexes, in biological membranes. Specifically, we found that CFTR complexes could be preserved in PFO-solubilized CHO and Sf9 cell membranes and when analyzed by the relatively nondissociative PFO−PAGE method (*20*). Although it is possible that the PFO detergent we employed in these studies may have artificially induced the formation of aggregates, we think that this latter possibility is unlikely as treatment of PFO-solubilized membranes with SDS could effectively dissociate the complex to form monomeric CFTR. In addition, we have shown previously that PFO−PAGE accurately reports the tetrameric structure of inwardly rectifying potassium channels and aquaporin as well as the dimeric structure of AE1 transporters (*17*). Furthermore, two different cross-linking agents applied to the exofacial surface of Sf9 cells could capture CFTR-containing complexes with the approximate mass of a CFTR dimer and higher. Together, our data obtained using membranes and whole cells argue that CFTR associates with itself and possibly other proteins. Our findings, however, contrast with previous studies by Marshall et al. showing that the tagged versions of CFTR could not co-immunoprecipitate with other CFTR molecules with distinctive tags (*15*). These authors suggested that CFTR molecules could not coassemble, at least not through strong, detergent-resistant interactions. Conceivably, the PFO detergent used in the present studies may have the capacity to protect associations between membrane proteins. Further, our findings support the electron microscopic studies of the CFTR quaternary structure in *Xenopus* oocyte membranes which previously revealed a dimeric structure of CFTR in this expression system (*30*).

We also show that purified CFTR protein can assemble as dimers during reconstitution into phospholipid liposomes and that the protein in this structure is functional in assays of ATPase activity and electrogenic chloride flux. Planar lipid bilayer studies revealed that the two channels conferred by fusion of proteoliposomes bearing dimeric CFTR gated independently. Unlike the double barrel channel signature of the ClC family of chloride channels, there is no evidence of a common gate acting to regulate a pair of CFTR conductance pores (*31*, *32*). Hence, in our reconstitution system there is no indication that the formation of a dimeric complex significantly alters the function of individual CFTR pores. On the other hand, in vivo studies point to the possible significance of this dimeric structure in biological membranes. For example, Wang et al. recently published the description of an accessory protein (CAP70), which contains multiple PDZ binding domains, that may tether the carboxy termini of two CFTR molecules (*13*). In fact, these authors suggest that any bifunctional molecule may be capable of tethering two CFTR molecules by their carboxy termini to cause, like CAP70, an increase channel open probability. Hence, dimer formation may lead to changes in function in native membranes because interactions with accessory proteins are enhanced. In our future studies we plan to assess this hypothesis by determining directly whether certain accessory proteins may bind preferentially with purified dimeric rather than monomeric CFTR.

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