Quaternary Structure of the Chloride Channel ClC-2[†]

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ABSTRACT: The chloride channel ClC-2 is thought to be essential for chloride homeostasis in neurons and critical for chloride secretion by the developing respiratory tract. In the present work, we investigated the quaternary structure of ClC-2 required to mediate chloride conduction. We found using chemical cross-linking and a novel PAGE system that tagged ClC-2 expressed in Sf9 cells exists as oligomers. Fusion of membranes from Sf9 cells expressing this protein confers double-barreled channel activity, with each pore exhibiting a unitary conductance of 32 pS. Polyhistidine-tagged ClC-2 from Sf9 cells can be purified as monomers, dimers, and tetramers. Purified, reconstituted ClC-2 monomers do not possess channel function whereas both purified ClC-2 dimers and tetramers do mediate chloride flux. In planar bilayers, reconstitution of dimeric ClC-2 leads to the appearance of a single, anion selective 32 pS pore, and tetrameric ClC-2 confers double-barreled channel activity similar to that observed in Sf9 membranes. These reconstitution studies suggest that a ClC-2 dimer is the minimum functional structure and that ClC-2 tetramers likely mediate double-barreled channel function.

ClC-2 belongs to the large ClC family of chloride-channel forming proteins (*1*), and it has been implicated in important neuronal and epithelial cell functions. For example, ClC-2 expression has been implicated in the modification of $GABA_A$-receptor function in neurons (*2*). Further, it has been suggested that ClC-2 may be important in volume regulation or chloride secretion in epithelial cells (*1*, *3*). Despite the physiological significance of this protein, the structural basis for its function as a chloride channel remains unclear. In fact, the molecular basis for the chloride channel function of ClC channels in general remains the subject of intense debate. Controversy persists regarding such fundamental properties as the number of pores in a ClC channel as well as the number of ClC polypeptides required to form each pore (*4*−*6*).

ClC-0, ClC-1, and ClC-2 share a similar predicted secondary structure (*1*). Furthermore, the macroscopic currents elaborated by these proteins exhibit certain common biophysical features. For example, all of these proteins confer a conductance path which selects amongst anions according to the following sequence: $Cl^- > Br^- > I^-$. Detailed, single-channel studies of ClC-0 and ClC-1 revealed that both channels exhibit a double-barreled permeation path, such that the opening of a single channel permits anion flux through two independent pores (*7*). Therefore, we would expect that a common pore architecture would be conserved among these proteins. However, there is considerable debate regarding the molecular basis for permeation among research groups studying ClC-0 and ClC-1. For example, ClC-0 function is thought to be conferred by a dimer with each polypeptide forming an independent pore (*4*, *5*, *7*, *8*). On the other hand, substituted cysteine accessibility modification experiments by Fahkle et al. suggest that two ClC-1 polypeptides are required to form a single pore (*6*). Hence, it is possible that all ClC family members may not share a common pore structure.

In the present work, we report the first direct studies of the quaternary structure of ClC-2, another member of the ClC family of chloride channels. We found in planar lipid bilayer studies that ClC-2 functions as a double-barreled chloride channel in membranes of Sf9 insect cells. Using chemical cross-linkers and non-dissociative gel electrophoresis, we showed that ClC-2 can exist as a tetramer in these membranes. To directly determine the structure that confers double-barreled chloride channel activity, we developed the methods necessary to purify, reconstitute and separate ClC-2 monomers and oligomers. Planar lipid bilayer studies of each separately reconstituted structure revealed that purified monomers were nonfunctional, dimers conferred a single pore conductance, and double-barreled ClC-2 channel activity was conferred by a purified ClC-2 complex with a larger mass, probably a tetrameric complex. These data support a novel model for a ClC channel pore structure.

RESULTS

ClC-2 Expression in Sf9 Cells Confers Double-Barreled Chloride Channel Activity. The permeation properties of recombinant rat ClC-2 have been described at a macroscopic, but not at a microscopic or single channel level (*9*, *10*). To isolate the single channel activity that is specifically conferred by ClC-2 expression, we compared the channel activities present in Sf9 membranes expressing this channel protein with membranes obtained from untransfected cells. In previous studies (*11*), we showed that the activation, anion selectivity and pharmacological properties of the macroscopic

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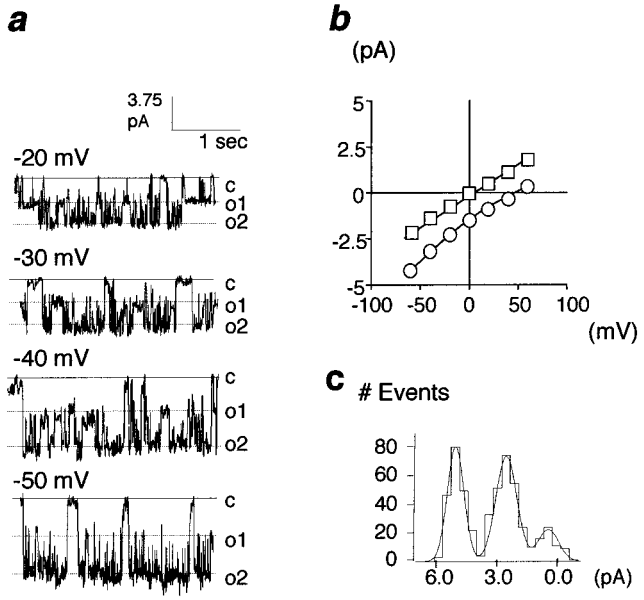

FIGURE 1: ClC-2 expression confers double-barreled channel activity. (a) Recordings obtained from Sf9 membranes expressing ClC-2. Sf9 membranes expressing ClC-2 fused to planar lipid bilayers show three conductance levels within a burst of channel opening. Tracings were obtained with 300 mM KCl in the cis compartment and 50 mM KCl in the trans compartment at holding potentials indicated. The trans compartment is connected to ground. (b) Current−voltage relationships. The current voltage (I−V) relationship of the first conductance level (o1) shows slight inward rectification in the presence of the above solutions. Current reverses at +48 mV under these conditions (open circle). The I−V relationship of the first, 32 pS conductance level is linear in symmetrical solutions, i.e., 50 mM KCl in both bilayer compartments (open squares). (c) Amplitude histograms. The amplitude histogram reveals that the amplitude of the larger conductance; o2 is twice that of the smaller conductance (o1) at the holding potential of −50 mV in the presence of symmetrical solutions, ie. 50 mM KCl in both the cis and the trans compartments. The ratio of the areas under each of the three components of this histogram reflects the relative probability of the channel being in the closed (c), (o1) or (o2) states. The relative probabilities are 0.12, 0.41, and 0.47, respectively. These values are close to those predicted for a binomial distribution: 0.1, 0.43, and 0.46, respectively.

currents associated with ClC-2 expression in Sf9 cells resembled those initially reported by Thiemann et al. in *Xenopus* oocyte expression studies (*10*). In the current studies, the single channel activity of ClC-2 in Sf9 membranes was studied by fusion of membrane vesicles to planar lipid bilayers. Comparison of multiple trials ($n > 20$) from five different membrane preparations from either uninfected Sf9 cells or Sf9 cells infected with recombinant baculovirus containing ClC-2 revealed that a unique conductance was conferred with expression of this channel. As described for the related channel-forming proteins ClC-0 and ClC-1 (*7*, *12*), ClC-2 channels exhibit a chloride-selective "binomial" single channel behavior. The ClC-2 channel opens to two equidistant conductance levels (Figure 1a). Chloride selectivity was confirmed on the basis of reversal potential measurements in the presence of asymmetrical KCl solutions on either side of the planar bilayer (300 mM KCl vs 50 mM KCl (cis vs trans compartments Figure 1b, circles). In symmetrical solutions containing 50 mM KCl, the ClC-2 channel opens to two conductance levels of 32 and 64 pS. As quantitative support for a binomial or double-barreled model for ClC-2 channel activity, we found that amplitude histograms of single channel records were well described by three Gaussian distributions with three equidistant peaks corresponding to one closed and two open states (Figure 1c, squares). Further, the probabilities of the three different conductance levels, corresponding to the areas covered by these Gaussian functions are similar to predictions from a simple binomial distribution.

ClC-2 Exists as Multimeric Complexes in Sf9 Membranes. We employed direct biochemical techniques to determine the quaternary structure that mediates the double-barreled channel activity of ClC-2 in Sf9 membranes: co-precipitation, chemical cross-linking, and non-dissociative polyacrylamide gel electrophoresis. In the first set of studies, we showed that ClC-2 molecules possessing different tags can co-assemble in vivo. ClC-2 molecules possessing a polyhistidine-tag (6×His-ClC-2) were co-expressed with ClC-2 molecules possessing a hemagglutinin (HA-ClC-2) tag in Sf9 insect cells. We had previously determined that insertion of these tags at the amino terminus did not perturb the chloride channel function of ClC-2 (see Materials and Methods). Then, Sf9 membrane proteins were solubilized in dodecylmaltoside (2%) and applied to a nickel resin. Western blot analysis showed that both HA-ClC-2 and 6His-ClC-2 were specifically eluted from the nickel resin (Figure 2a). We know that HA-ClC-2 is binding to the nickel resin through its association with 6×His-ClC-2 because HA-ClC-2 expressed singly in Sf9 cells did not bind (Figure 2a). Conversely, we found that 6×His-ClC-2 protein could be co-immunoprecipated with HA-ClC-2 protein using an antibody directed against the HA tag, confirming the in vivo generation of ClC-2 multimeric complexes.

The size of these multimeric ClC-2 structures in Sf9 membranes was assessed using the bifunctional cross-linking reagent, bis(sulfosuccinimidyl) suberate (BS^3).[1] BS^3 has a relatively short spacer arm (approximately 11 Å in length) and has been used previously to cross-link polypeptides, i.e. Band III protein, assembled in an oligomeric complex (*13*). In the absence of this cross-linker, ClC-2 in Sf9 membranes, stripped of peripheral proteins, was detected predominantly as a single band on SDS−PAGE using an affinity-purified ClC-2 antibody previously described (Figure 2b). The mobility of this band corresponds to the molecular mass of 97 kDa (close to the predicted molecular mass of ClC-2). Weak bands are also apparent at approximately 200−220 kDa and higher. These weaker bands likely correspond to SDS-resistant ClC-2 oligomers as the bands can be competed using the antigenic ClC-2 peptide initially used to raise the antibody (data not shown). Analysis of Sf9 membranes expressing ClC-2 and pretreated with the cross-linker, BS^3 (5 μM) revealed a predominant band migrating as predicted for a ClC-2 tetramer. This result suggests that BS^3 may be protecting a ClC-2 complex, possibly a tetramer, from dissociation by detergent. This effect was due to the efficacy of BS^3 as a cross-linking agent as a different reagent, dimethyl suberimidate−2HCl (DMS), expected to be less effective at the neutral pH of our experimental conditions,

[1] Abbreviations: BS^3, bis(sulfosuccinimidyl) suberate; DMS, dimethylsuberimidate·2HCl; PFO, pentadecafluorooctanoic acid; NPPB, 5-nitro-2-(phenylpropylamine) benzoic acid; DIDS, 4,4′-diisothiocyanato stilbene-2,2′ -disulfonic acid; PE, L-α-phosphatidylethanolamine; PS, L-α- phosphatidylserine; PC, L-α-phosphatidylcholine.

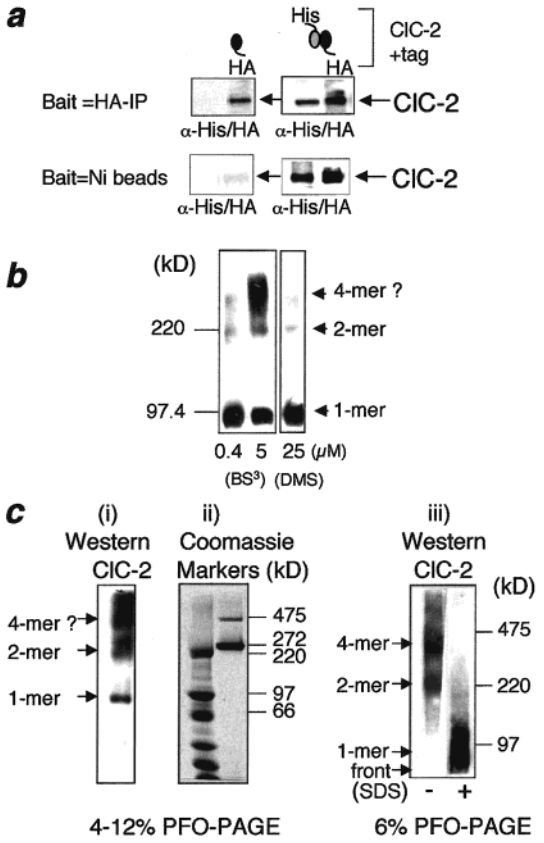

FIGURE 2: Quaternary structure in ClC-2 in vivo. (a) Coassembly of HA-tagged and His-tagged ClC-2 molecules in vivo. Membranes prepared from Sf9 cells either singly infected with recombinant baculovirus containing DNA coding for ClC-2 containing a hemagglutinin (HA-black oblong) tag (left panels) or coinfected with this first baculovirus plus a baculovirus coding for ClC-2 containing a hexahistidine tag (HA-black oblong + His-grey oblong) (right panel) were solubilized using dodecylmaltoside. Capture of HA-ClC-2 molecules by immunoprecipitation using a monoclonal antibody directed against the HA tag (Bait = HA-IP, upper left box) was verified by immunoblotting using this antibody (α-HA). Polyhistidine-tagged ClC-2 molecules were captured via their 6×His tags to a column of Ni-chelated beads (Bait = nickel beads) as confirmed using an antibody against this tag (α-His). Coassembled HA-tagged and His-tagged molecules would be captured using either bait. (b) Cross-linking reagent traps oligomeric forms of ClC-2 in Sf9 membranes. Membranes prepared from ClC-2 transfected Sf9 cells were stripped of peripheral proteins using 6 M guanidine hydrochloride on ice for 30 min at a protein concentration of 4 mg mL^{-1} and analyzed by SDS−PAGE. Western analysis shows that, with low concentrations of the cross-linking reagent BS^3 (0.4 μM), ClC-2 runs primarily as a 97 kDa band, the mass predicted for a monomer. Resuspension of these membranes at a concentration of 0.333 mg mL^{-1} and treatment with the cross-linking reagent, bis-sulfosuccinimidyl suberate (BS^3, 5 μM) for 30 min at room-temperature trapped a higher molecular weight band corresponding to the mass predicted for a ClC-2 tetramer. The cross-linking agent DMS (25 μM) could not trap this higher molecular weight structure. A total of 25 μg of membrane was protein loaded per lane. (c) Nondissociative PFO−PAGE preserves oligomeric ClC-2. Sf9 membranes from ClC-2 transfected cells were stripped of peripheral proteins and then solubilized in PFO−PAGE solubilizer at protein concentration of 1 mg ml^{-1}. (i) Five micrograms of protein was applied to a 4−12% Tris-glycine gel and analyzed by PFO−PAGE. (ii) Coomassie blue stained molecular weight standards; high molecular weight rainbow markers (lane 1), apoferritin (lane 2, 12 mer; 235−240 kDa and 24 mer; 475 kDa) run in tandem with (i) in PFO−PAGE. (iii) Sf9 membranes expressing ClC-2 were solubilized in PFO detergent at a final concentration of 4% (no SDS, −) or solubilized in 4% PFO first and then treated with SDS (+) in a final concentration of 2%.

failed to protect ClC-2 complexes from dissociation in SDS PAGE. The concentration of BS^3 that was effective in cross-linking in the present studies, i.e., 5 μM, is considerably less than the millimolar concentrations used by Jennings and Gosselink in their chemical cross-linking studies of Band 3 protein (*13*). This discrepancy probably relates to the difference in the sample preparation as we used Sf9 membranes and Jennings and Gosselink applied the cross-linking reagent to the extracellular aspect of intact cells (*13*).

Finally, we analyzed ClC-2 expressing Sf9 membranes by pentadecafluorooctanoic acid (PFO)−PAGE, a novel electrophoresis method which partially protects against dissociation of multimeric complexes of both cytosolic and membrane proteins. We have shown previously that this novel electrophoresis method can be used to correctly assess the tetrameric structure of the potassium channel, Kir2.1 and the dimeric and tetrameric structures of Band 3 protein (*14*). Examination of "stripped" Sf9 membranes by PFO−PAGE showed that ClC-2 protein in Sf9 membranes is detected by immunoblotting as one relatively faint band (corresponding to the mass predicted for ClC-2 monomers) and two diffuse bands corresponding to complexes with higher molecular mass (Figure 2c,i). As these bands corresponding to larger molecular weight species are observed in membrane preparations stripped of peripheral proteins, they likely correspond to different oligomeric structures of ClC-2 and not to heteromeric complexes with extrinsic membrane proteins. The molecular mass of these three bands was calibrated using Coomassie blue stained molecular weight standards analyzed in tandem by PFO−PAGE (Figure 2c,ii). The band which corresponds to the approximate molecular mass of a ClC-2 monomer (about 97 kDa) is relatively faint in comparison to the broader bands. The front of the first of the broad bands co-migrates with the 220 kDa rainbow marker and the second band runs slightly faster than the apoferritin 24 mer, that has a molecular mass of 475 kDa. We suggest that these bands likely correspond to ClC-2 dimers and tetramers respectively, and that the dimeric ClC-2 structure may reflect partial dissociation of a native tetrameric complex by the PFO detergent. To confirm that the higher molecular weight proteins do not represent PFO detergent-induced aggregates, we assessed the susceptibility of these complexes to dissociation by SDS treatment. Figure 2c,iii shows that the tetrameric form of ClC-2 seen following PFO membrane solubilization could in fact be disrupted by subsequent addition of the strongly dissociative detergent SDS, supporting our claim that ClC-2 oligomers observed by PFO−PAGE do not represent detergent-resistant aggregates. While these analyses are not definitive, the results of both the chemical cross-linking studies and the PFO−PAGE technique suggest that in membranes, ClC-2 can exist as homomeric complexes, possibly dimers and tetramers.

Purified, Reconstituted ClC-2 Functions as a Channel. As it remains formally possible that the multimeric structures visualized in the preceding experiments are heterogeneous and comprise ClC-2 as well as other integral membranes proteins, we developed methods for the purification and reconstitution of ClC-2 polypeptides. We purified 6×His-ClC-2 from membranes prepared from Sf9 cells expressing this protein using methods previously developed for the purification of the cystic fibrosis transmembrane conductance regulator (CFTR) (*15*). Briefly, 6×His-ClC-2 protein ex-

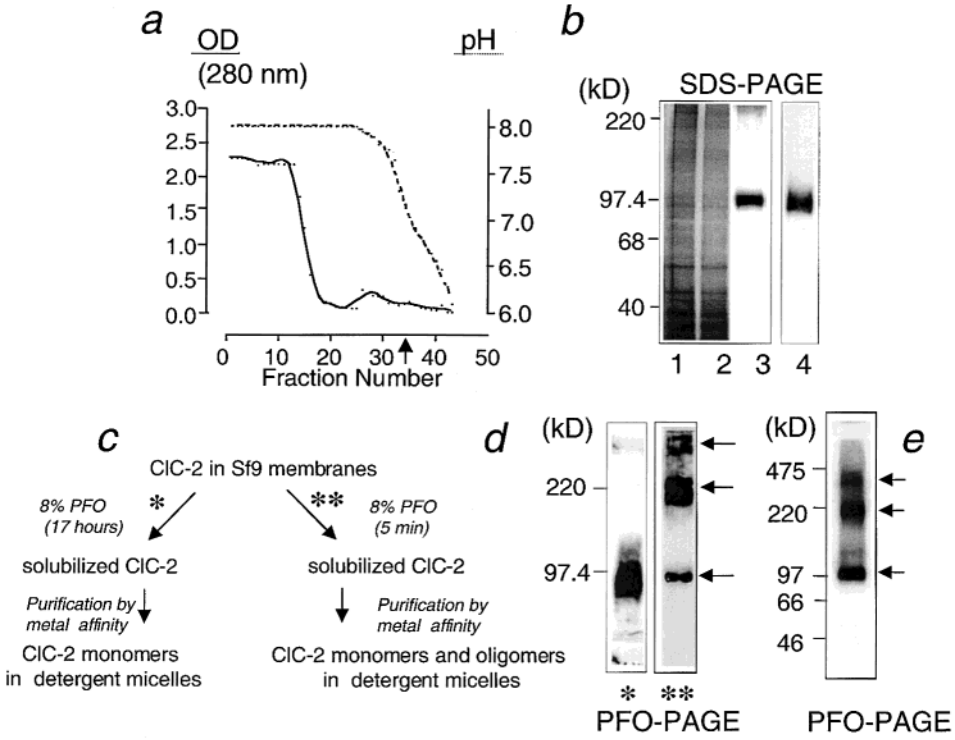

FIGURE 3: Purification and Reconstitution of ClC-2. (a) Elution of His-tagged ClC-2 from a Ni-chelated column using a pH gradient. Elution profile shown. The arrow indicates the fractions in which ClC-2 protein is eluted. (b) Purification of ClC-2 monomers. Lanes 1−3, overloaded silver-stained 8% SDS−PAGE gel; lane 1 = protein applied to column, 25 μg; lane 2 = flow through, 25 μg; lane 3 = purified ClC-2 eluted at around pH 6.8, 2 μg protein. Lane 4 is Western blot of 100 ng of eluted, purified protein. (c) Purification of ClC-2 monomers and oligomers. Flow diagram shows that the length of time during which Sf9 membranes containing ClC-2 are solubilized in detergent determines whether monomers alone (*) or monomers plus oligomers (**) are purified by nickel affinity. (d) Purification of ClC-2 monomers and oligomers. ClC-2 protein purified using method (*) was compared with ClC-2 protein purified using method (**) following PFO−PAGE (4−12% gel) and immunoblotting. Method (*) yields a single band at 97 kDa corresponding to ClC-2 monomer. Method (**) yields three bands corresponding to ClC-2 monomers, dimers (200−220 kDa) and a larger multimeric complex. (e) Reconstitution of purified ClC-2 oligomers. Reconstitution of ClC-2 monomers, purified by method (*), into phospholipid liposomes promotes formation of multimeric complexes. Analysis of reconstituted ClC-2 protein by PFO−PAGE (4−12%) and immunoblotting reveals three bands migrating as 97−100, 200−220, and 410−440 kDa proteins.

pressed in Sf9 membranes was effectively dissociated from interacting proteins during a 17 h treatment with the detergent PFO (8%) and applied to a nickel affinity column. A pH gradient was used to specifically elute ClC-2 protein from the nickel resin (Figure 3). We found that fractions eluted at pH 6.8 contained a 97 kDa protein (visualized by SDS−PAGE and silver staining, Figure 3b, lane 3). The identity of this protein as ClC-2 was confirmed by immunoblotting (Figure 3b, lane 4) using a polyclonal antibody previously described (*11*). The homogeneity of ClC-2 protein eluted at pH 6.8 was greater than 95% pure as determined by densitometry of the 97 kDa protein visualized by SDS−PAGE and silver staining. Analysis of purified ClC-2 by PFO−PAGE followed by immunoblotting similarly revealed a single band corresponding to monomeric ClC-2 with a mass of 97 kDa (Figure 3d, lane *). We estimated the yield from this purification protocol as 1 milligram of ClC-2 protein purified from 1 L of Sf9 cell culture using a modified Lowry assay.

Purified ClC-2 was reconstituted from PFO detergent micelles by dialysis into phospholipid liposomes consisting of PE:PS:PC:ergosterol (5:2:1:1 by weight). In Figure 3e we show by non-dissociative PFO−PAGE and immunoblotting that purified, reconstituted ClC-2 now migrates as three prominent bands, corresponding to the molecular masses predicted for ClC-2 monomers, dimers, and tetramers. This pattern suggests that reconstitution into phospholipid liposomes enhances association of ClC-2 into oligomeric structures as shown diagrammatically in Figure 3c. We predicted that if these oligomeric structures are native to Sf9 membranes expressing this protein and not an artifact of our reconstitution conditions, it should be possible to purify multimers from membranes using less harsh solubilizing conditions. Indeed, we found that we could purify ClC-2 oligomers, in addition to the monomeric protein, from Sf9 membranes solubilized in 8% PFO for only 5 min (Figure 3c, **). Analysis of protein purified following rapid solubilization of Sf9 membranes by PFO−PAGE and immunoblotting revealed three prominent bands (Figure 3d, lane **). Unfortunately, the yield of purified protein from membranes solubilized in this manner was very low (<10 μg/L), possibly due to the fact that the polyhistidine tag in ClC-2 oligomers may be relatively inaccessible for binding to the nickel column and inappropriate for subsequent functional studies. Nevertheless, these results support the claim that purified ClC-2 protein reconstituted into phospholipid liposomes comprises multimeric structures which exist in biological membranes.

We assessed the capacity of purified, reconstituted ClC-2, existing as a mixture of monomers and multimers in proteoliposomes, to mediate accumulation of radiolabeled ^{36}Cl (1.5 mM) in response to an inward electrical driving

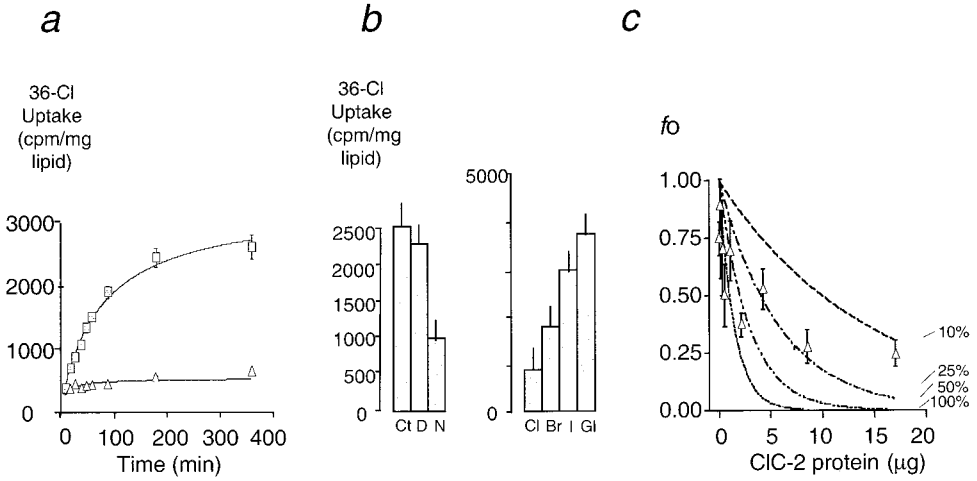

FIGURE 4: Purified, reconstituted ClC-2 can mediate chloride uptake. (a) Time course of electrogenic ^{36}Cl uptake by phospholipid liposomes containing ClC-2. Proteoliposomes contained (0.2 mg of total protein/mg of lipid, filled squares) Empty liposomes did not mediate uptake (open triangles). (b) Pharmacological properties and ion selectivity of purified ClC-2. Left bar graph shows that electrogenic ^{36}Cl uptake mediated by purified reconstituted ClC-2 is inhibited by NPPB (100 μM) but not by DIDS (1 mM). Uptake was measured at 60 min, data points represent mean ± SEM of triplicate measurements. Right bar graph shows that external anions added as 10 mM potassium salts collapse the driving force for ^{36}Cl uptake according to permeability through purified ClC-2, i.e., Cl > Br > I > gluconate. (c) Approximation of percent functional activity of ClC-2. f_o, the fraction of vesicles containing no chloride channels was determined from measurements of ^{36}Cl equilibration relative to the total intravesicular volume (measured from [^{3}H]glutamate distribution) as described by Maduke et al (18). f_o was determined for different vesicle preparations containing varying amounts of purified ClC-2 protein relative to a fixed lipid concentration (3.8 mg of lipids). A family of theoretical curves corresponding to 100, 50, 25, or 10% functional protein were determined according to the relation defined by Maduke et al (*18*): $f_o = \exp(-36\pi N_o \vartheta^2 s m_{ClC-2}/M_{ClC-2} m_L \sigma^3)$. ϑ is 31.38 cm^3/g (±0.7, $n = 10$), M_{ClC-2} is 388 000 g mol^{-1}, $\sigma = 2.3 \times 10$ cm^2 g^{-1}, and $\langle s \rangle$ was 1,0.5, 0.25, and 0.10, respectively, and superimposed on the acquired data (solid triangles ± SEM for triplicate measurements). The software progression used for regression analysis was Prism (Graph Pad, San Diego,CA).

force using a method initially described by Garty et al. (*16*) and modified by Goldberg and Miller (*17*). As expected, empty liposomes failed to mediate the time-dependent cumulative uptake of ^{36}Cl (Figure 4a). On the other hand, proteoliposomes containing purified reconstituted ClC-2 could mediate time-dependent accumulation of ^{36}Cl. These results indicate that ClC-2 protein has been highly purified and that this protein functions as a chloride channel. Further, we determined that flux mediated by purified, reconstituted ClC-2 protein is inhibited by the chloride channel blocker, NPPB, but not by DIDS as expected on the basis of voltage-clamp studies of ClC-2 in biological membranes (*9*−*11*) (Figure 4b). Ion selectivity was also determined using this assay. The relative ability of extraliposomal anions to collapse the driving force for Cl uptake and inhibit cumulative ^{36}Cl uptake reflects the relative selectivity of purified ClC-2 for these anions. As shown in Figure 4b, inhibition of cumulative ^{36}Cl uptake by extraliposome anions exhibits with the following sequence, $Cl^- > Br^- > I^- >$gluconate$^-$, mirroring the anion selectivity sequence previously reported for ClC-2 (*9*, *10*). Hence, the pore properties of ClC-2 have been reconstituted in this assay.

To confirm that a significant fraction of the purified ClC-2 has been functionally reconstituted and is capable of mediating the chloride electrodiffusion described in the preceding paragraphs, we employed the "trapped volume assay" previously described by Miller and his colleagues in the study of EriC, a procaryotic ClC-type chloride channel (*18*). This assay rests on the premise that liposomes loaded with ^{36}Cl and [^{3}H]glutamate and containing one or more active chloride channels will permit the efflux of ^{36}Cl but not the impermeant anion, [^{3}H]glutamate. Further, the value, f_o, the fraction of protein-free liposomes relative to total liposomes (estimated by trapped [^{3}H]glutamate), should decrease exponentially as the amount of reconstituted protein increases. In ideal conditions, where liposomes are unilamellar, spherical, and uniform in size, the percentage of functionally reconstituted protein can be derived quantitatively by fitting the protein concentration dependence of f_o to the equation in the legend of Figure 4c. Visual examination of the experimental data obtained for purified ClC-2 reveals divergence from an exponential fit particularly at the highest concentrations of reconstituted protein. We think that this divergence likely relates to "non-ideal" liposome size and shape. Although we have not directly assessed the structure of ClC-2 proteoliposomes, we know from electron micrographs obtained in our previous studies of the purified cystic fibrosis transmembrane conductance regulator (CFTR) that there is considerable heterogeneity in terms of proteoliposome shape and size (with radii varying from 50 to 200 nm) (*19*). Hence, we elected to describe a range for percent functional protein by superimposing theoretical curves predicted for 100, 50, 25, and 10% functional ClC-2 protein on our empirically derived values for f_o. Conservatively, our data falls within the curves predicted for 10−50% functional protein. Hence, we suggest that a sufficiently large amount of purified ClC-2 has been functionally reconstituted to account for the chloride electrodiffusion described in the present studies.

ClC-2 Tetramers Confer Double-Barreled Channel Activity. In the preceding studies, chloride channel function was measured using preparations of purified, reconstituted ClC-2 protein which were probably comprised of a mixture of monomers and multimers (Figure 3e). To determine which quaternary assembly conferred this activity, we separated each structure in the mixture by gel filtration. Separation by gel filtration first required that the proteoliposomes be disrupted while maintaining the protein complexes intact. This was achieved by adding PFO (4%) to ClC-2 containing

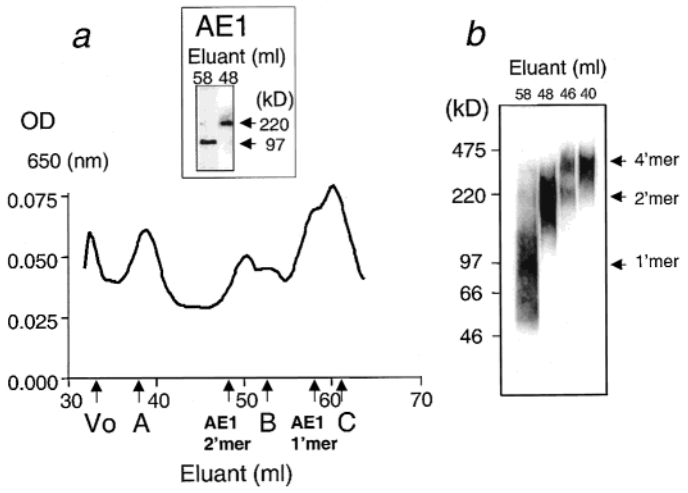

FIGURE 5: Purified, reconstituted ClC-2 separated into monomers, dimers, and tetramers by gel filtration. (a) Separation and size determination of ClC-2 structures, i.e. monomers, dimers, and tetramers by gel filtration. A total of 250 μg of purified, reconstituted ClC-2 was solubilized in buffer A containing 12.5 mM Tris, 0.5 mM DTT, 0.5 mM EDTA, and 4% PFO at pH 7.5 (total volume of 200 μL) and immediately applied to a long, narrow Sepharose CL- 6B column (120 $\times$ 1 cm) previously calibrated with standards in buffer A. ClC-2 protein was eluted with buffer A at 0.5 mL/minute. Each fraction (0.5 mL) was assayed for protein using a modified Lowry assay (*20*). (Insert) Western blot of PFO−PAGE (4−12%) showing band 3 (AE1) monomer (approximately 100 kDa) and dimer (200−220 kDa). AE1 monomers and dimers separated on the same Sepharose column in fractions 58 and 48, respectively, after solubilization of erythrocyte membranes in buffer A. Standards (Vo = blue dextran, A = apoferritin 24 mer is 475 kDa, B = myosin is 202 kDa, C = transferrin monomer 81 kDa). (b) Fractionated ClC-2 monomers, dimers, and tetramers assessed by PFO−PAGE. Effective separation confirmed by Tris-glycine (4−12%) PFO−PAGE analysis of fractions indicated.

proteoliposomes. Then, 250 μg of purified ClC-2 protein in mixed detergent, phospholipid micelles were added to a long narrow Sepharose CL-6B column previously calibrated with standard proteins in the same detergent. This particular column was chosen because it possesses an ideal fractionation range (10^4−10^6 Da). The column length was essential to provide the resolution necessary to effectively separate ClC-2 monomers, dimers, and tetramers. Shorter prepacked columns lack the resolving power to distinguish between ClC-2 dimers and tetramers. The fractions containing ClC-2 protein were assessed for protein quantity by modified Lowry assay (*20*). The three prominent, distinctive peaks were evident from the elution profile. The first peak elutes behind apoferritin 24 mer (475 kDa) and is well separated from the second peak which elutes in the same fractions as dimeric band 3 (AE1, 200 kDa) and the marker protein, myosin (202 kDa). The final peak elutes in the same fractions as monomeric AE1 (100 kDa) and slightly ahead transferrin (81 kDa). The results of this gel filtration experiment are consistent with the interpretation that purified ClC-2 protein in lipid/detergent micelles exists as monomers, dimers, and a higher order structure, probably a tetramer.

To reconstitute each structure separately and with the highest level of fidelity, we rapidly extracted detergent from multiple ClC-2 containing fractions simultaneously by hydrophobic interaction chromatography (*21*) in the presence of excess phospholipid (the same composition as in the previous reconstitution). Detergent extraction and reconstitution into phospholipid was complete within 5−10 min and the reconstituted protein was analyzed immediately by nondissociative PFO−PAGE and simultaneously evaluated for chloride channel function. As shown in Figure 5b, effective separation of ClC-2 monomers, dimers, and tetramers by gel filtration was corroborated by PFO−PAGE. The chloride channel activity of each of the structures was assessed immediately after reconstitution into pure phospholipids using the electrogenic ^{36}Cl flux assay. Excess phospholipid was added to reconstituted tetramers, dimers, and monomers to ensure that the protein to lipid ratio was uniform at approximately 900 ng of protein/mg of lipid. As shown in Figure 6a, ClC-2 monomers cannot support electrogenic chloride flux, while both ClC-2 dimers and tetramers can mediate electrodiffusion. Importantly, these findings support our claim that purified ClC-2 protein rather than a contaminant (of 100 000 kDa or less) confers chloride channel activity. Further, these data show that we have effectively separated ClC-2 monomers from oligomers by gel filtration. Finally, these results show that the formation of a chloride-selective pore or pores by ClC-2 requires at least a dimer and possibly a tetramer.

The single-channel properties of each ClC-2 structure were examined by fusion of proteoliposomes containing either purified ClC-2 monomers, dimers, or tetramers with planar lipid bilayers. As in our previous studies of purified, reconstituted CFTR (*15*) all proteoliposomes were rendered equally fusogenic using nystatin. Fusion events were monitored as nystatin-mediated conductance spikes, as originally described by Woodbury and Miller (*22*). ClC-2 protein was reconstituted such that there were approximately 60 μg of ClC-2 protein in 10 mg of phospholipid. Assuming that the proteoliposomes have a radius of approximately 50−200 nm after sonication and that this size vesicle contains approximately 6−24 $\times$ 10^4 phospholipid molecule (*23*), we estimate that each liposome will contain approximately 3−16 monomers, 2−8 dimers, or 1−4 ClC-2 tetramers. In planar bilayer studies, we found that ClC-2 proteoliposomes containing monomers failed to lead to channel activity in planar bilayers (n = 30, Figure 6b), corroborating results obtained using the flux assay. On the other hand, fusions of proteoliposomes containing ClC-2 dimers (n = 15) led consistently to channel openings to a single conductance level. This pore is anion selective as determined on the basis of current

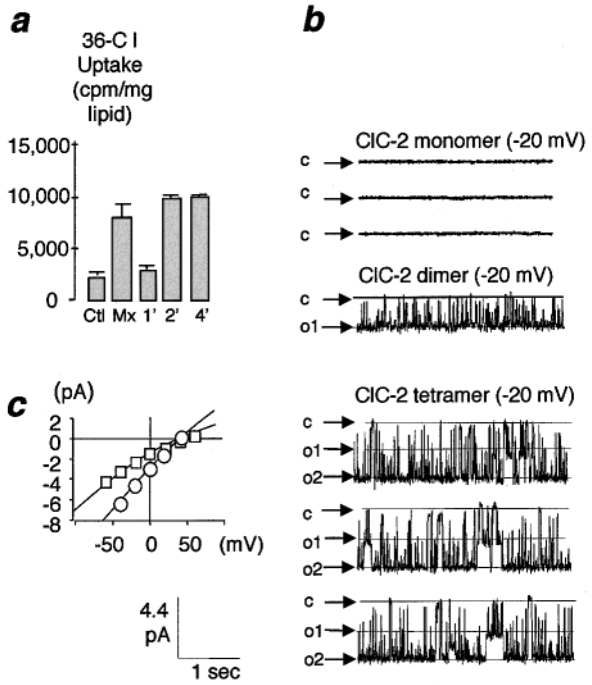

FIGURE 6: Single channel studies of purified ClC-2 monomer, dimers, and tetramers. (a) ClC-2 dimers and tetramers mediate electrogenic chloride flux. Proteoliposomes containing mixed purified ClC-2 structures (Mx) or purified ClC-2 monomers, dimers or tetramers were compared with respect to their ability to mediate electrogenic ^{36}Cl flux. Empty liposomes were controls (Ct) for specific uptake in these studies. In proteoliposomes, protein was normalized at approximately 900 ng mg^{-1} lipid. Two different preparations of purified and size-fractionated ClC-2 protein (fractionated by gel filtration as in Figure 5) were studied, ^{36}Cl uptake at 60 min determined in triplicate and the mean determined for each size-separated structure of each preparation. The bar graphs shows mean and range of uptake by monomeric, dimeric and tetrameric structures separated in the two preparations. (b) ClC-2 dimers and tetramers confer channel activity. Nystatin-doped liposomes containing either ClC-2 monomers, ClC-2 dimers, or tetramers were assessed for single channel activity in planar lipid bilayer studies. The cis compartment contained 300 mM KCl and the trans compartment contained 50 mM KCl. (c) Current−voltage relationships for two open conductance levels of ClC-2 tetramers. The purified, reconstituted ClC-2 tetramer exhibited double-barreled activity similar to that observed in Sf9 membrane studies. The cis compartment contained 300 mM KCl and the trans compartment contained 50 mM KCl. The I−V relationship of the first (open squares) and the second (open circles) conductance levels in the presence of the above bath solutions show current reversal (+48 mV) as predicted for chloride selective pores.

reversal measurements in the presence of asymmetrical ion gradients, i.e., 300 vs 50 mM KCl in the cis and trans bilayer compartments, respectively. In symmetrical 50 mM solutions, the unitary conductance conferred by purified ClC-2 dimers (32 pS) is identical to that of a single conductance level of the double-barreled activity of ClC-2 in Sf9 membranes. These findings suggest that a ClC-2 dimer is required to form a single pore and that, in isolation, this pore can function independently. Each fusion of proteoliposomes bearing ClC-2 tetramers led consistently (18 of 18 fusions) to the appearance of double pore, anion-selective channel activity with two equidistant conductance levels (o1 and o2, Figure 6c). The unitary conductances are 32 and 64 pS, respectively, in symmetrical 50 mM KCl solutions. This activity is similar to the double-barreled activity observed in Sf9 membranes containing ClC-2 with respect to its selectivity for anions and conduction properties (Figure 1). Hence, we have successfully reconstituted the pore properties of ClC-2 exhibited in biological membranes by fusion of purified ClC-2 homotetramers in purified phospholipid bilayers. Further, the coincident appearance of double-barrel channel activity with nystatin-mediated fusion of each proteoliposomes bearing 1−4 ClC-2 tetramers corroborates our previous estimation that 10−50% of purified ClC-2 has been functionally reconstituted. Analysis of rare openings to a subconductance state and the gating kinetics of purified ClC-2 channels will be pursued in our future work.

DISCUSSION

The chloride channel activity of ClC-2 has been implicated in fundamentally important functions, including the regulation of $GABA_A$ receptor function in neurons and chloride secretion by epithelial tissues (*1*−*3*). Hence, it is key that we understand the functional properties of this protein and structural basis for its activity. In this work, we provide the first description of the single channel properties of ClC-2 expressed in Sf9 cells. Like ClC-0 and ClC-1 (*1*, *4*, *8*, *12*), ClC-2 exhibits a double-barrel pore channel activity. Further, on the basis of chemical cross-linking and application of a novel PAGE system to the study of membranes expressing ClC-2, we suggest that ClC-2 may exist as dimers and tetramers. To determine which quaternary structure underlies the double-barrel channel activity in membranes, ClC-2 monomers, dimers, and tetramers were purified from Sf9 membranes and separately reconstituted for channel analysis in planar lipid bilayers. Both ClC-2 dimers and tetramers are capable of forming channels, therefore, dimers form the minimum functional unit of this protein. However, the single-channel activity of ClC-2 dimers resembles a single barrel of the double-barreled channel activity observed in Sf9 membranes and purified, reconstituted ClC-2 tetramers reveal a double-barreled chloride channel function comparable to the activity observed in membranes. Hence, we suggest that the double-barreled chloride channel activity conferred by expression of ClC-2 in Sf9 cells may be mediated by a homotetramer of this protein.

We assessed the quaternary structure of ClC-2 expressed in Sf9 membranes using three experimental approaches; by co-immunoprecipitation of differentially tagged ClC-2 protein, chemical cross-linking in Sf9 membranes, and a novel electrophoretic method [PFO−PAGE (*14*)]. The co-immunoprecipitation studies permit us to conclude that in Sf9 cells, recombinantly expressed ClC molecules form assemblies. The results of the chemical cross-linking and PFO−PAGE studies were consistent with this interpretation and suggest that ClC-2 can form multimeric complexes, probably dimers and tetramers in membranes. However, there are limitations associated with these methods. For example, we cannot definitively rule out the possibility that the multimeric structures detected in baculovirus-infected Sf9 cells following chemical cross-linking with BS^3 are induced by the addition of this cross-linking reagent. Jennings and Gosselink (*13*) concluded from their studies of Band 3 protein that the cross-linker BS^3 induced formation of Band 3 tetramers as these complexes could be converted to dimers upon exposure to high temperature (100 °C). Unfortunately, we could not perform such studies with ClC-2 as ClC-2 is extremely heat

labile. Similarily, we lack proof that the detergent PFO is not inducing oligomerization and the artifactual appearance of ClC-2 tetramers in PFO−PAGE. However, we think that it is unlikely as the oligomeric structures detected by PFO−PAGE analysis could be dissociated to the monomeric form by addition of SDS, suggesting that the multimeric structures we detect are not detergent-induced aggregates. Furthermore, solubilization of ClC-2 containing membranes for short times (approximately 5 min) lead to the detection and purification of tetramers and dimers. On the other hand, solubilization of membranes containing ClC-2 for longer periods (approximately 17 h) yielded mostly monomers. Again, these findings support the notion that the higher molecular weight species are not induced by PFO detergent and ClC-2 dimers and tetramers are likely to exist in Sf9 membranes.

It remains formally possible that two distinct ClC-2 structures may mediate chloride ion flux in biological membranes. While we did not detect "single barrel" activity in planar bilayer studies of Sf9 membranes, it is conceivable that ClC-2 dimers may be functional in membranes of other ClC-2 transfected cell types or in cells in which this protein is expressed endogenously. The results from both the electrogenic flux assay and planar lipid bilayer studies of purified protein suggest that dimers are capable of forming an independent chloride conducting pore. In fact, Malinowska et al. (*24*) have reported that ClC-2G, an isoform of ClC-2 found in the rabbit gastric gland, behaves as a 30 pS channel in microsomal membranes isolated from tissue, a unitary conductance which is very close to that determined for a single pore of purified ClC-2 dimer.

The observations regarding ClC-2 channel structure and function described in this paper support certain hypotheses developed for the pore stoichiometry of the related family members, ClC-0 and ClC-1. Like ClC-0 and ClC-1 (*4*−*6*), the minimal functional unit appears to be a dimer. Further, like ClC-0 and ClC-1 (*4*, *5*, *12*), ClC-2 can mediate double-barrel channel activity. However, our findings also add another layer of complexity to the debate regarding the structural basis for this double-barreled channel function. The research groups of Jentsch and Miller presented compelling evidence to support a structural model for ClC-0 wherein each polypeptide forms a discrete permeation pore (*4*, *5*). In the studies by Middleton et al., two concatenated polypeptides were expressed and chemical modification of a single cysteine introduced close to the permeation path of one ClC-0 polypeptide modified conduction through a single barrel of the double barreled activity (*5*). Hence, each polypeptide of ClC-0 confers a permeation path for chloride ion and a dimer is required for double-barreled channel activity. A comparable experiment was performed in studies of ClC-1. Although single channel data were not presented in this study, Fahlke et al. showed that modification of a cysteine introduced deeply within the putative pore region of ClC-1 caused the same extent of inhibition of whole cell currents regardless of whether one or both polypeptides were modified (*6*). These authors interpreted these data to suggest that two ClC-1 polypeptide comprise a single permeation pore. As suggested by Fahlke (*6*), double-barreled channel activity by ClC-1 may represent binomially distributed subconductance states within a single aqueous pore. Hence, it is not clear whether different oligomeric structures mediate channel function for ClC-0 and ClC-1. The simplest explanation for our findings for ClC-2, namely that each pore of this double-barreled channel is conferred by a ClC-2 dimer, adds more complexity to our understanding of the structural basis of ClC channel activity. Clearly, further studies of the permeation properties of ClC-2 are required to assess the validity of our "simple" interpretation and its potential application to other members of this family of chloride channels.

MATERIALS AND METHODS

Expression Constructs. The open reading frame (ORF) of rat ClC-2 was tagged at the amino terminus with either a hexa-histidine (6×His) tag or a hemagglutinin (HA) tag and then introduced into a high efficiency oocyte expression vector (*25*). The corresponding ClC-2 cRNAs were prepared by in vitro transcription (Ambion,TX), microinjected into Stage VI *Xenopus* oocytes, and the function of the tagged ClC-2 channels compared with that of the wild-type ClC-2 channels using the two electrode voltage clamp method (*10*). The tags did not affect either the activation properties of ClC-2 (hyperpolarization activation) or the selectivity for chloride ion as assessed by reversal potential measurements (data not shown). Subsequently, the tagged ClC-2 constructs were introduced into the pBlueBac4 (Invitrogen, CA) vector for high-level expression in Sf9 insect cells.

Solubilization and Purification of ClC-2. Sf9 insect cells expressing recombinant ClC-2 were harvested from 1 L of medium by centrifugation at 2000*g* at 4 °C, and the cell pellet washed once with PBS. The cells were lysed in a French Press (Spectronic Inst. NY), the nuclei and cell debris pelleted and the supernatant was centrifuged at 100 000*g* for 90 min to pellet a crude membrane preparation. Peripheral membrane proteins were extracted from this membrane pellet using 25 vol of ice-cold 10 mM sodium hydroxide and 0.5 mM EDTA and the stripped membranes pelleted by centrifugation at 100000*g* for 2 h. Integral membrane proteins were solubilized using a detergent solution containing 8% pentadecafluorooctanoic acid (PFO, Oakwood Products Inc., SC) in 25 mM phosphate, pH 8.0, and stirred with a magnetic stirrer overnight at room temperature. The solubilized sample was filtered through a 0.22 μm pore filter prior to applictaion to the Ni-NTA agarose column (Qiagen, CA). Purification of oligomeric ClC-2 followed an identical procedure except that the crude membrane preparation was solubilized for 5 min.

A freshly regenerated 25 mL nickel column was attached to an FPLC column and the ClC-2 containing sample applied at 1 mL/min at room temperature. Then, the column was washed with 100 mL buffer containing 25 mM phosphate, 100 mM NaCl and 4% PFO at pH 8.0 (buffer 1). Buffers containing PFO were prepared by adding the free acid, pentadecafluorooctanoic acid to the buffer and titrating with sodium hydroxide to the required pH. A pH gradient was applied to the column titrating buffer 1 with buffer 2 containing 20 mM phosphate, 4% PFO at pH 6.0 going from 0% buffer 2 to 100% buffer 2 in 100 mL. Three milliliter fractions were collected and analyzed by dot-blot. Immunopositive fractions were analyzed by Western blotting (10 μL of each fraction) and by silver-stained PAGE (50 μL of each fraction). Fractions containing ClC-2 protein eluted at pH 6.8 were pooled and concentrated in an Amicon Centriprep 50 concentrator (no. 4310) to a final volume of 1

mL. This sample was diluted 10 times using a buffer containing 8 mM HEPES, 0.5 mM EGTA, and 0.025% sodium azide at pH 7.2 and reconcentrated to a final volume of 600 μL.

Reconstitution of Purified ClC-2. A suspension containing liposomes (5 mg of lipid in 500 μL of buffer containing 8 mM HEPES was mixed with a sample of nickel column purified ClC-2 (1 mg in 500 μL detergent-containing buffer) (see above). The liposomes were composed of PE:PS:PC: ergosterol (5:2:1:1 by weight). The mixture was dialyzed twice (Spectrapor molecular mass cutoff = 50 kDa, Rancho Dominguez, CA) with 0.025% sodium azide for 18 h against 4 L of 8 mM HEPES and 0.5 mm EGTA at pH 7.2. ClC-2 oligomers fractionated by gel filtration were reconstituted by supplementing 100 μL of each fraction with 1 mg of sonicated lipids and passing this mixture through an Extractigel column (Pierce, IL). Additional lipid was added for subsequent assays of channel function by the "trapped volume" or planar bilayer studies.

Assays of ClC-2 Chloride Channel Activity. (i) Concentrative Tracer Uptake Assay. We used a concentrative tracer uptake assay developed by Garty et al. (*16*) and modified by Goldberg and Miller (*17*) to characterize the chloride conductance properties of reconstituted ClC-2. Proteoliposomes are 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), Tris-glutamate (20 mM) at pH 7.6—to replace external chloride. Uptake was initiated and quantified by addition of 1.0 μCi mL^{-1} of ^{36}Cl . Intravesicular ^{36}Cl was assayed at various time points following separation of liposomes from the external media using a mini anion-exchange column (Dowex 1).

(ii) Approximation of Functional Activity. We used a [^{3}H]-glutamate trapped volume assay as described by Maduke et al. (*18*) to assess the minimum fraction of ClC-2 protein functionally reconstituted as a channel. Briefly, 100 μg of reconstituted ClC-2 protein was mixed with 3.8 mg of *E. coli* total lipids. Then, several aliquots of this preparation containing different masses of ClC-2 protein were obtained and lipid was added to each in order to maintain the lipid mass constant at 3.8 mg. Proteoliposomes were formed by three freeze−thaw cycles followed by 5 s of sonication in buffer containing: 20 mM KCl, 20 mM potassium-glutamate, 20 mM MES−KOH at pH 6.0 with radioisotopes ^{36}Cl and [^{3}H]glutamate added in final concentrations of 2 and 7 μCi, respectively. Passive efflux was initiated by diluting vesicles (100 μL) into 1 mL of buffer lacking radioisotope. After 60 min of equilibration, intraliposomal and extraliposomal radioactivity was assessed. Intraliposomal counts were determined by spinning liposomes through Sephadex G-50 columns.

Planar Bilayer Studies of Purified ClC-2. As in our previous studies, proteoliposome fusion with planar lipid bilayers was facilitated and detected by the introduction of nystatin (120 μg mL^{-1}), a technique originally described by Woodbury and Miller (*22*). 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 μm 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 with a bilayer amplifier (custom-made by M. Shen, Physics Lab., 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.

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