From these experiments, we deduce two molecular features of the ClC-0 channel. First, the sidedness of MTS modification demonstrates that residue 519 faces the internal solution. The electrostatic influence of this residue suggests that it provides mechanistically relevant surface charge near the cytoplasmic entryway of the Cl^- conduction pathway. Second, these results provide strong evidence that the ClC-0 channel is a two-pore homodimer, as previously proposed on the basis of velocity-sedimentation behaviour in nondenaturing detergent[10]. Residue 519 exerts its graded electrostatic influence on permeation exactly twice in the channel complex, once separately in each conduction pathway. Moreover, the frequencies of the hybrid C519–K519 and homomeric C519 channels in our preparations are close to the 2 : 1 prediction for a random mixture of equally expressed subunits in a dimer.

The ClC-0 channel is thus constructed by a 2-pore–2-polypeptide architecture unprecedented among eukaryotic ion channels. This structural design is distinct from that of the familiar channels gated by voltage, cyclic nucleotides, or neurotransmitters, which are built around single pores formed on symmetry axes at the junction of 4 or 5 similar subunits[22]. In ClC-0, the twofold axis, assumed to exist in the homodimer, cannot be coincident with either pore. Instead (Fig. 4*a*), the two identical pores must be formed off-axis[10], as in the 3-pore–3-polypeptide construction of bacterial porins[23,24]. Consequently, we expect that many parts of the polypeptide contribute pore-determinants, in contrast to the localized pore-forming sequences of voltage-gated cation-conducting channels[25]. ClC-0 is the only member of the ClC family studied at the single-channel level[14], and so it is not known whether its double-barrelled structure is a unique feature of this homologue or a general property of the entire family. Because we view a membrane protein's quaternary architecture as a fundamental characteristic rather than a modulatable molecular embellishment, we speculate that the two-pore homodimeric character of ClC-0 applies to all members of this large family of Cl^- channels.

Note added in proof: Similar results were obtained by Ludewig *et al.* (*Nature*, this issue pp. 340–343). □

Methods

Purification, expression and reconstitution of ClC-0 channels. Functional ClC-0 channels were purified from *Torpedo californica* electroplax plasma membranes or from HEK-293 cells transiently transfected with a cDNA coding for ClC-0 from *Torpedo marmorata*[1]. CHAPS-solubilized channels were bound to an immunoaffinity resin based on the RM2 epitope (EGQQREGLEAVKVQTEDP, near residue 650), and eluted with the RM2 peptide as described[10]. Purified ClC-0 (5–10 μg) was reconstituted by gel filtration into proteoliposomes (2 mg ml^{-1}), which were subsequently fused into planar lipid bilayers, to give single-channel behaviour identical to that seen without CHAPS treatment[10]. The ClC-0 clone[1] (generously supplied by T. J. Jentsch) was altered by removal of the flanking untranslated sequences and by incorporation of convenient restriction sites, and transferred to the mammalian expression vector pCDNA3 (Invitrogen). HEK-293 cells (50 100-mm plates) were transfected overnight with 0.5 mg CaP_i-precipitated DNA, collected after 40 h, and extracted with CHAPS for subsequent purification of ClC-0. Hybrid channels were purified similarly after transfection of HEK-293 cells with equal amounts of ClC-0-C519 and of ClC-0-K519 cDNA in which the RM2 epitope had been replaced by the HA epitope.

Single ClC-0 channels were recorded under asymmetrical ionic conditions, with 5 mM Tris-Cl, pH 7.4, and 600 mM NaCl (internal), 200 mM NaCl (external). Records were filtered at 200 Hz and analysed as described[13]. We confined our analysis to properly oriented single channels displaying stationary gating among well defined conductance levels for enough time to collect data at a minimum of 5 voltages; these data-selection criteria applied to over 50% of all the single-channel incorporation events we observed.

Modification of single channels by MTS reagents. MTS derivatives[21] carried the following functional groups: ethylamino (MTSEA), ethyltrimethylamino (MTSET), ethylsulphonate (MTSES). After incorporation of a single C519 channel in a bilayer, a control *I–V* curve using at least 10 voltages was recorded. The desired methanethiosulphonate reagent (1–10 μM) was then added to the internal solution, with a 15-s burst of vigorous stirring, and data were continuously collected over the next few minutes at a constant holding voltage in the range −80 to −120 mV. Individual 'hits' by MTS reagents were observed as abrupt changes in single-channel current, as in similar experiments on diphtheria toxin channels[26].

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Two physically distinct pores in the dimeric ClC-0 chloride channel

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THE *Torpedo* chloride channel ClC-0 (ref. 1) is the prototype of a large family of chloride channels that have roles in transepithelial transport[2] and in regulating electrical excitability[3–6] and cell volume[7]. ClC-0 opens in bursts with two identical conductance levels of ~8 pS (refs 8–10). Hyperpolarization slowly increases the probability of bursts ('slow gating'), and depolarization increases channel opening within bursts ('fast gating'). Replacing serine 123 by threonine changes rectification, ion selectivity and gating, but retains the typical bursting behaviour with two identical independent albeit reduced, conductance states (~1.5 pS). Coexpression with wild-type ClC-0, either as covalently linked concatamers or as independent proteins, leads to bursting channels with two different pores. Our experiments strongly suggest that conductance, ion selectivity and 'fast' gating are determined only by the single subunit forming a single pore, independent from the attached pore; in contrast, 'slow' gating is a function of both subunits. Thus ClC-0 is a homodimer with two largely independent pores.

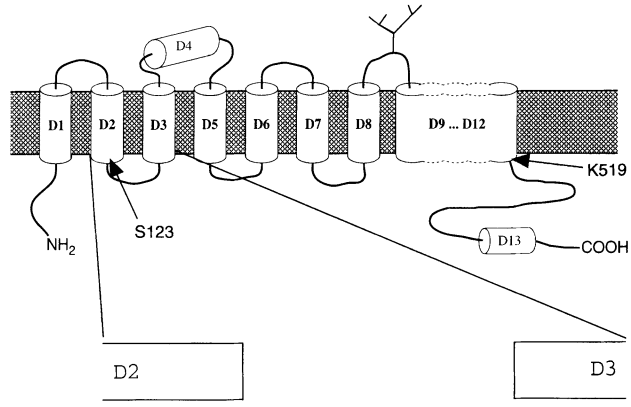
A region between transmembrane domains D2 and D3 is highly conserved across all known ClC proteins[11], including those from yeast[12] and bacteria[13] (Fig. 1). In ClC-0, a conservative substitution of serine 123 by threonine (S123T) led to fast gating being slowed (Fig. 2*a*) and a slight outward rectification of open channels and a relative loss of ion selectivity (Fig. 2*b*). Wild-type ClC-0 activates slowly at negative voltages owing to the voltage-dependent slow gate, but no such effect was observed with S123T up to −150 mV (Fig. 2*c*).

The dominant negative effects of ClC-1 mutations found in myotonia congenita indicate that ClC-1 is a homomultimeric channel[5,6]. We therefore investigated whether wild-type (WT) and S123T ClC-0 proteins can form heteromultimeric channels, and constructed head-to-tail concatameric channels (S123T–WT and WT–S123T). Expression of control WT–WT concatamers (Fig. 2) yielded currents indistinguishable from wild-type ClC-0. Macroscopic current measurements indicate that rectification, ion selectivity and fast gating of the S123T–WT concatamer have properties very similar to those of the wild type (Fig. 2*a*, *b*). However, there was no slow activation by hyperpolarization up to −150 mV (Fig. 2*c*). Similar results were obtained with a concatamer of reverse order (WT–S123T). These results cannot be explained by a superposition of independent wild-type and S123T currents, and indicate the formation of heteromultimeric channels.

Similar to the wild type, S123T channels show two identical subconductance levels (Figs 3*c* and 4*a*) within bursts. In contrast to the ~8 pS conductance of wild-type ClC-0 and WT–WT concatamers (Fig. 3*a*), S123T single-channel conductance was only ~1.5 pS. Consistent with the slower macroscopic current relaxations (Fig. 2*a*), single-channel gating time constants were increased by about threefold.

S123T–WT concatamers show ~1.5, ~8 and ~9.5 pS conductance levels (Figs 3*b*, *d* and 4*a*). Similar results were obtained for WT–S123T concatamers (Fig. 3*e*) and with coexpression of non-linked S123T and wild-type ClC-0 proteins (data not shown). As expected, we also observed homomeric wild-type or S123T channels in the latter case. Importantly, the heteromultimer also displays long time stretches of missing channel activity (Fig. 3*d*). Hence these channels (and S123T homomers) retain a slow gate operating on all three conductance levels, although it has lost its voltage dependence.

On the basis of single-channel analysis of the native *Torpedo* channel, and on effects of the inhibitor 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS), a 'double barrelled' channel with two identical pores was proposed[8,9]. In this model, WT–S123T channels would have two different pores, one WT pore of ~8 pS and one mutant pore of ~1.5 pS. We show that this model is correct, and that the permeation and gating properties of an individual pore are independent of the second attached pore. Replacement of intracellular chloride by bromide reduced the conductance of the large pore of WT–S123T channels by ~50%, although the less selective small pore was reduced by only ~20% (Fig. 3*e*, *f*). This reduction is independent of the state (open or closed) of the second pore, and does not depend on the identity of the attached pore, as similar ion selectivities are found for homomeric wild-type or S123T channels, respectively (data not shown). The same independence holds for open probabilities of both pores (Fig. 4*b*, *c*). Further, gating kinetics of the 8-pS pore are independent of whether it is associated with a wild-type or mutant pore, and the (difficult to measure) mutant S123T pore roughly retained its slower gating kinetics within the heteromer (data not shown). Slow gating, in contrast, which acts on both pores simultaneously, depends on both pores, as the apparent voltage independence of slow gating of the S123T mutant was imposed on the heteromeric S123T–WT channel (Fig. 2*c*).

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                  123
                  T
mutant
 ClC-0    IVSPQAVGSGIPELKTIIRGAVL.HEYLTLRTFVAKTVGL
 hClC-1   LISPQAVGSGIPEMKTILRGVVL.KEYLTMKAFVAKVVAL
 hClC-5   VFAPYACGSGIPEIKTILSGFII.RGYLGKWTLVIKTITL
 hClC-7   FIEPVAAGSGIPQIKCFLNGVKI.PHVVRLKTLVIKVSGV
scClC     YVAPMATGSGISEIKVWVSGFEYNKEFLGLLTLVIKSVAL
ecClC     KYAPEAGGSGIPEIEGALED...QRPVRWWRVLPVKFFGG
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FIG. 1 Topology model[14] of the ClC-0 chloride channel and location of mutations. S123 is located in a highly conserved stretch at the proposed cytoplasmic end of transmembrane domain D2, and K519 is located at the end of D12. Strongly divergent ClC members are given for comparison: hClC-1, human muscle Cl^- channel mutated in myotonia[4–6]; hClC-5, human kidney Cl^- channel mutated in kidney-stone diseases[2]; hClC-7, a broadly expressed human ClC member[11]; scClC, *Saccharomyces cerevisiae* ClC[12]; and ecClC, a ClC from *Escherichia coli*[13]. Bold type indicates conserved sequences.

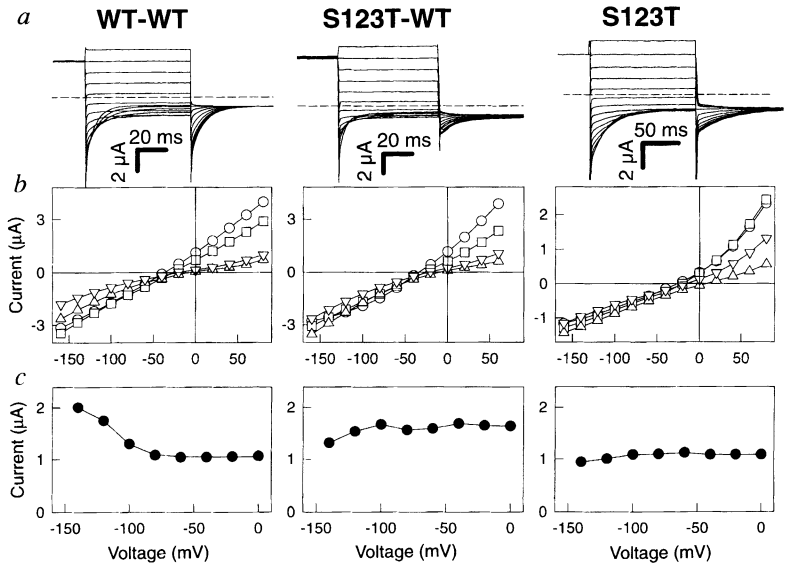

FIG. 2 Macroscopic currents of ClC-0 mutants and concatamers as determined by two-electrode voltage clamping. Results are shown for WT–WT concatamers (left), S123T–WT concatamers (middle) and S123T ClC-0 mutant monomers (right). WT–WT concatamers yield currents indistinguishable from wild-type channels[10,14], and WT–S123T concatamers yield currents similar to S123T–WT concatamers (data not shown). *a*, Typical traces at 104 mM chloride (ND96). After activating the slow gate by hyperpolarization and a prepulse to +60 mV to open maximally the fast gate, oocytes were clamped sequentially to voltages between +80 and −160 mV for 75 ms (WT–WT and S123T–WT) or 150 ms (S123T), followed by a constant pulse to −100 mV. Hyperpolarization closes the fast gate. The broken line indicates zero current. *b*, Instantaneous (open pore) currents measured at the beginning of the test pulse with partial replacement of external chloride (circles) by bromide (squares), iodide (triangles), and nitrate (inverted triangles). Note the slight outward rectification and relative loss of ion selectivity with the S123T mutant. *c*, Currents at +60 mV as a function of hyperpolarizing prepulses of 5 s duration. This voltage activation (by the 'slow gate') is lost both in the S123T mutant and in the S123T–WT concatamer. Because wild-type conductance is about fivefold larger than S123T conductance (Fig. 3), macroscopic currents of heteromers resemble wild-type currents except for the absence of slow gating.

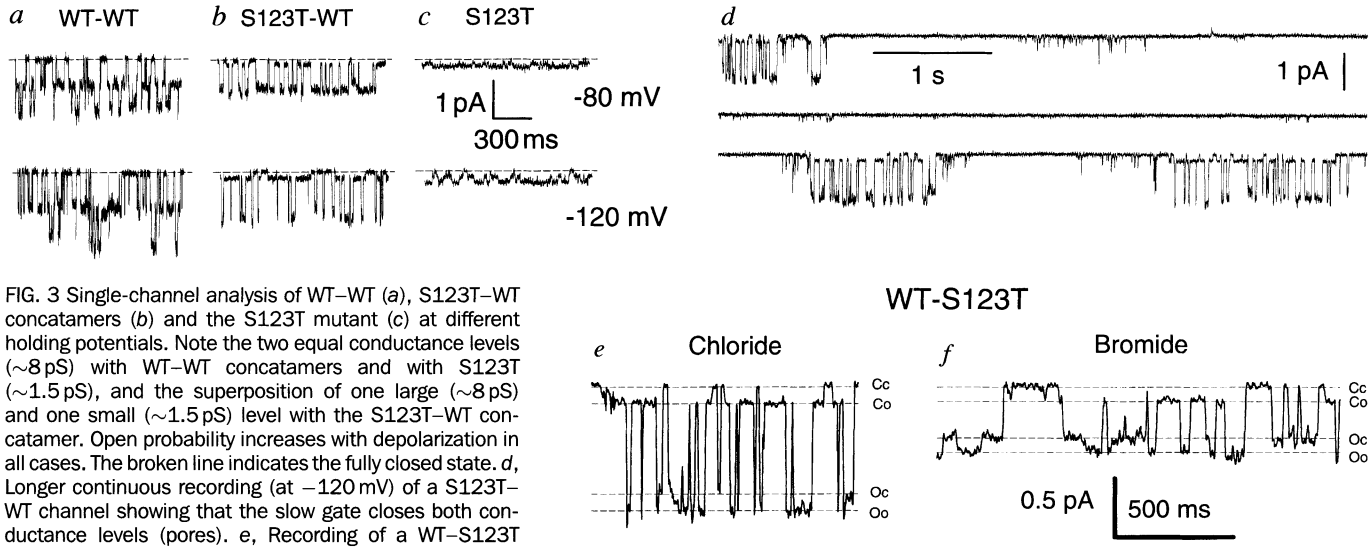

FIG. 3 Single-channel analysis of WT–WT (*a*), S123T–WT concatamers (*b*) and the S123T mutant (*c*) at different holding potentials. Note the two equal conductance levels (~8 pS) with WT–WT concatamers and with S123T (~1.5 pS), and the superposition of one large (~8 pS) and one small (~1.5 pS) level with the S123T–WT concatamer. Open probability increases with depolarization in all cases. The broken line indicates the fully closed state. *d*, Longer continuous recording (at −120 mV) of a S123T–WT channel showing that the slow gate closes both conductance levels (pores). *e*, Recording of a WT–S123T channel with high cytoplasmic chloride concentration (160 mM). *f*, The same patch after replacement of chloride by bromide; the conductance of the large (wild-type) pore is reduced by 50%, and the mutant pore by only 20%. Similar effects were seen in 5 patches. Nomenclature (Cc,Co,Oc,Oo) is explained in the Fig. 4 legend.

To exclude the possibility that these observations are specific for the S123T mutant, we studied a mutant[14] (K519E) at a very different position (Fig. 1). It yielded a bursting channel with two superimposed conductance levels of ~1 pS (Fig. 5*a*). With a WT–K519E concatamer, we again observed three conductance levels (~1, ~8 and ~9 pS) (Fig. 5*b*), suggesting the presence of independent ~8-pS and ~1-pS pores.

Our experiments indicate a simple dimeric structure of ClC-0 (Fig. 6*A*). With alternative tetramer models, where each pore is formed by two subunits (Fig. 6*C–F*), one would expect to find (WT/WT), (mutant/mutant), and (mutant/WT) pores with different conductances. However, when co-injecting monomeric mutant S123T and wild-type subunits, we only observed 8/8 pS, 1.5/1.5 pS, and 8/1.5 pS channels (8 patches), never double-barrelled channels with other conductances. With S123T–WT and WT–S123T concatamers (injected singly), we again only observed 8/1.5 pS channels (14 patches). In principle, S123T subunits may associate preferentially with themselves, but this seems unlikely as WT–K519E concatamers again had one large and one small pore ($n = 3$). Another explanation could be that this linkage forces the subunits to assemble into mutant–mutant and WT–WT pores (Fig. 6*D*, *F*). We therefore co-injected oocytes with S123T–WT and WT–S123T concatamers at a 1:1 ratio. One now would expect to find 8/1.5 pS channels in half of the cases (as a result of WT–S123/WT–S123 (Fig. 6*D*) and S123T–WT/S123–WT complexes (Fig. 6*F*), and S123T–WT/WT–S123T channels in the remaining 50% (Fig. 6*E*). These latter channels should have two identical conductance levels of unknown (but probably intermediate) magnitude. We only observed 8/1.5 pS channels in all 12 patches displaying 'double-barrelled' channels. Assuming equal association efficiencies, this reduces the probability of the tetramer model to 0.03%. Thus, consistent with a sedimentation analysis of ClC-0 protein[15], our experiments strongly favour a homodimeric structure of ClC-0. However, analysis of dominant-negative mutants suggested that ClC-1 has more than two subunits[5]. It seems impossible that the number of subunits lining a pore differs between these channels, but the interactions between individual pores may be different. Indeed, several dominant-negative mutations were found[5,6] in ClC-1, but we have not yet been able to construct dominant-negative mutants in ClC-0.

In a homodimer, either each single subunit forms one pore by

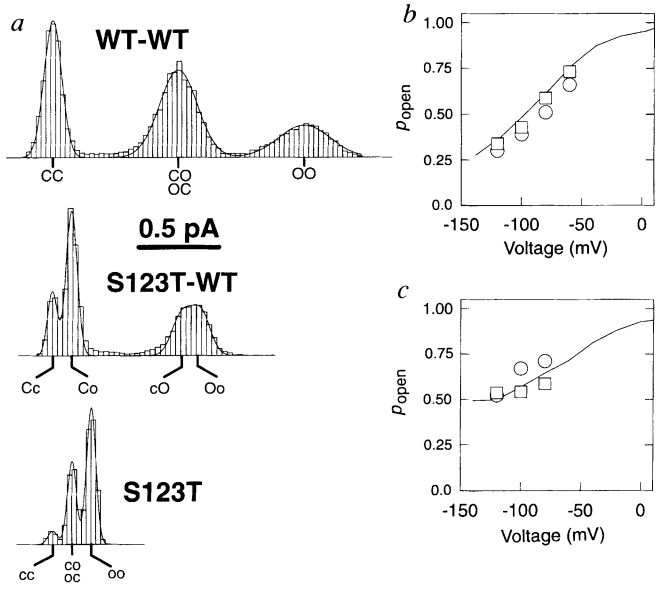

FIG. 4 Analysis of single-channel data. *a*, Amplitude histograms of single-channel recordings (within bursts, that is, with open 'slow gate') of WT–WT and S123T–WT concatamers, and the S123T mutant at −100 mV. With WT–WT concatamers (and with wild-type ClC-0 (ref. 10)), and with S123T mutants, three distinct, equidistant conductance levels are seen, which are due to two closed pores (CC), one closed and one open pore (CO or OC, which are indistinguishable because both pores have equal conductance), and two open pores (OO). The two pores of the S123T–WT concatamer have different conductances, yielding different currents for Co and Oc (lower-case letters refer to the smaller S123T pore), giving four current levels. The transitions between Oc and Oo levels cannot be clearly resolved owing to the low conductance of the S123T pore and the noise from the open wild-type pore. Lines indicate fits of independent gaussian distributions, with four distributions fitted for the heteromers. Marks indicate maxima of these fits. Conductances are nearly unaffected by the second pore: for the wild-type (which can be resolved quite clearly) conductance was 7.8 ± 0.4 pS ($n = 3$) in WT–WT dimers, and 8.6 ± 0.4 pS ($n = 6$) in S123T–WT dimers. *b*, *c*, Comparison of open probability (p_{open}) of the fast gate from macroscopic measurements (line) with those derived from single-channel analysis (data points) for WT (*b*) and S123T (*c* pores. Results are for pores within homomeric channels (WT–WT concatamers or S123T channels) (circles) and pores within the heteromeric S123T–WT channel (squares).

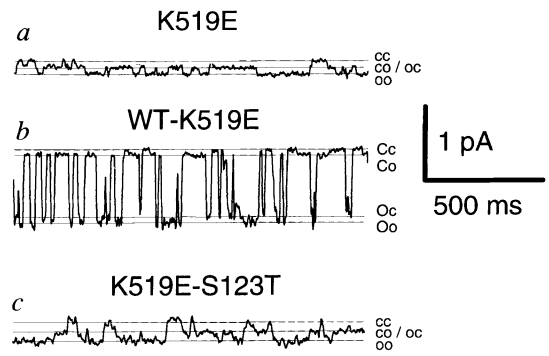

FIG. 5 Analysis of the K519E mutant, and of the WT–K519E and K519E–S123T concatamers. *a*, A single K519E channel held at −120 mV has two ~1 pS pores; WT–K519E concatamers (*b*) yield channels with one ~8 pS (wild-type) pore and one ~1 pS mutant pore. The ~1 pS conductance is consistent with previous noise analysis[14]. *c*, The K519E–S123T tandem channel yields two small (~1–1.5 pS) pores. Macroscopic currents were strongly outwardly rectifying (data not shown), as expected for S123T and K519E pores. (A linear wild-type pore with its larger conductance would dominate the current (see Fig. 2*b* for the S123T–WT concatamer).) This supports the model in Fig. 6*Aa*, *Ba*. Lettering as in Fig. 4.

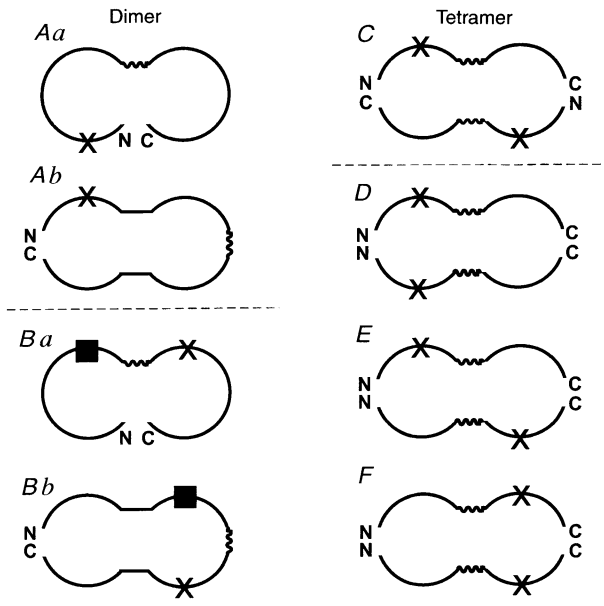

FIG. 6 Possible models for the multimeric structure of ClC-0 based on experiments using various concatamers. The most simple models assumes a dimer, with either one pore per monomer (*Aa*) or a pore formed together by different portions of both subunits (*Ab*). A concatamer of two ClC-0 monomers connected by a linker (wavy line) is depicted. Amino (N) and carboxy (C) termini are shown. In both models, a single mutation (indicated by X) will affect only one pore. The models in *A* can be differentiated when mutations are inserted in the second part of the first subunit and the first part of the second one (panels *B*) (for example: X, S123T; square, K519E). *Ba* predicts two mutant pores; *Bb* predicts one wild-type and one mutant pore. Our experiments (Fig. 5*c*) support model *Aa*, *Ba*, *C–F*, Hypothetical tetramers, in which each pore is formed by two ClC monomers. The symmetrical (and more feasible) model in *C* predicts channels with two identical pores; this seems to be excluded, as we always observed two different 'barrels' with S123T–WT, WT–S123T and WT–K519E concatamers. The alternative model (*D–F*) is *a priori* much less likely, as it violates symmetry (as shown, different 'sides' of the protein would face the pore). However, the models in *D* and *F* both predict channels with two different barrels, as observed experimentally. Assuming equal association efficiencies, half of the channels produced by coexpressing both concatamers should assemble as in *E*. These will have two equal (heteromeric) barrels, which we never observed. Thus our experiments strongly favour the simple dimeric model shown in *Aa*, *Ba*.

itself (Fig. 6*Aa*), or both ClC-0 monomers contribute (with different portions) to each of the two pores (Fig. 6*Ab*). A K519E–S123T dimer should discriminate between these possibilities (Fig. 6*B*). Single-channel analysis revealed two small pores (Fig. 5*c*), strongly suggesting (but not proving) that a single subunit forms a single pore.

Our experiments have also identified a new region determining pore properties and fast gating which, at the primary sequence level, is remote from the end of D12 that also affects these properties[14]. As one pore is formed by a single subunit (or, less likely, by two different parts of the two subunits), one would indeed expect that several regions of the protein participate in pore formation. This is in contrast to the situation found in cation channels[16].

Note added in proof: Similar results were obtained by Middleton *et al.* (*Nature*, this issue pp. 337–340). □

Methods

Functional expression in *Xenopus* oocytes. Mutant and concatameric ClC-0 cDNAs were constructed by recombinant PCR. Construction of concatamers introduced four residues (GTTS) between the monomers. Capped cRNA was transcribed *in vitro* from constructs in the vector pTLN[6], 5–10 ng of which was injected into *Xenopus* oocytes[1]. Currents were measured after 2–4 days by two-electrode voltage-clamping or patch-clamping at room temperature. Standard saline was ND96 (ref. 5) and contained 104 mM Cl^-. For Fig. 2*b*, 80 mM Cl^- was replaced by equivalent amounts of other anions. Single-channel recordings were performed in the inside-out configuration of the patch-clamp technique. The external solution contained (in mM): 95 *N*-methyl-D-glucamine-Cl (NMDG-Cl), 5 $MgCl_2$ and 5 HEPES, pH 7.3. The internal solution contained 95 NMDG-Cl (or 160 NMDG-Cl or NMDG-Br for Fig. 3*e*, *f* and Fig. 5), 2 $MgCl_2$, 5 EGTA and 5 HEPES, pH 7.3. Currents were filtered at 300 Hz (S123T and K519E) and 500 Hz (other constructs).

Data analysis. Amplitude histograms were constructed from single-channel traces of 7 s duration using the Biopatch program (Biologic, Grenoble). For kinetic analysis, idealized current traces (>300 events) were constructed with a 50% threshold criterion, followed by visual inspection of each transition. From this, mean dwell times were determined (τ_{cc}, τ_{oc}, and τ_{oo}, for homomeric channels; τ_{cC}, τ_{oC}, τ_{cO} and τ_{oO} for heteromeric channels). For homomeric channels, opening rate α, and closing rate β were calculated as $\alpha = 1/(2\tau_{cc})$, and $\beta = 1/(2\tau_{oo})$. See Fig. 4 legend for nomenclature. Because open wild-type pores have an increased noise level, openings of the small mutant pore on top of open wild-type pores could not be analysed quantitatively (that is, τ_{cO} and τ_{oO} were unreliable). Gating rates could, however, be obtained using the following procedure. First, we determined the well-defined dwell times of the lumped states τ_{cC+oC} and τ_{cO+oO} (ignoring transitions of the small mutant pore) from which we calculated $\alpha_{WT} = 1/\tau_{cC+oC}$, $\beta_{WT} = 1/\tau_{cO+oO}$. From these values we obtained α_{mutant} and β_{mutant} from τ_{cC} and τ_{oC} assuming that $\tau_{cC} = 1/(\alpha_{WT} + \alpha_{mutant})$, and $\tau_{oC} = 1/(\alpha_{WT} + \beta_{mutant})$. The probability of being open (p_{open}) was calculated as $p_{open} = \alpha/(\alpha + \beta)$. This measure of p_{open} was indistinguishable from values obtained from the gaussian fits to the amplitude histograms.

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