The Intracellular Chloride Ion Channel Protein CLIC1 Undergoes a Redox-controlled Structural Transition

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Most proteins adopt a well defined three-dimensional structure; however, it is increasingly recognized that some proteins can exist with at least two stable conformations. Recently, a class of intracellular chloride ion channel proteins (CLICs) has been shown to exist in both soluble and integral membrane forms. The structure of the soluble form of CLIC1 is typical of a soluble glutathione S-transferase superfamily protein but contains a glutaredoxin-like active site. In this study we show that on oxidation CLIC1 undergoes a reversible transition from a monomeric to a non-covalent dimeric state due to the formation of an intramolecular disulfide bond (Cys-24-Cys-59). We have determined the crystal structure of this oxidized state and show that a major structural transition has occurred, exposing a large hydrophobic surface, which forms the dimer interface. The oxidized CLIC1 dimer maintains its ability to form chloride ion channels in artificial bilayers and vesicles, whereas a reducing environment prevents the formation of ion channels by CLIC1. Mutational studies show that both Cys-24 and Cys-59 are required for channel activity.

Chloride ion channels control a variety of cellular processes that are central to normal function and disease states (1). The CLIC1 family is a recently identified class of Cl− channel proteins that consists of seven members (p64, parchorin, CLIC1–5) (2, 3). A conserved C-terminal CLIC module of ~240 amino acids is present in each member of the family with several members containing additional, unrelated N-terminal domains. Most CLICs are localized to intracellular membranes and have been linked to functions including apoptosis, pH, and cell cycle regulation (4–6). The CLIC ion channels are unusual in that they possess both soluble and integral membrane forms (2). In this regard they are similar to some bacterial toxins and several classes of intracellular proteins including Bcl-xL and the annexins (7). Our understanding of how such dual natured proteins enter the membrane is limited by the dearth of high resolution structures for key states in this process.

We have recently determined the crystal structure of a soluble monomeric form of CLIC1 (8) and found that it is a structural homologue of the GST superfamily of proteins (9). This soluble form of CLIC1 consists of two domains, the N-domain possessing a thioredoxin fold closely resembling glutaredoxin and an all α-helical C-domain, which is typical of the GST superfamily. CLIC1 contains an intact glutathione-binding site that was shown to covalently bind glutathione via a conserved CLIC cysteine residue, Cys-24. This led to the suggestion that CLIC1 function may be under redox control, possibly via reactive oxygen or nitrogen species.

The structure and stoichiometry of the integral membrane form of the CLIC proteins is still unclear. Electrophysiology of purified, soluble (Escherichia coli-expressed) recombinant CLIC1 in reconstituted artificial bilayers shows that CLIC1 alone is sufficient for chloride ion channel formation (8, 10–12). Electrophysiological studies of FLAG epitope-tagged CLIC1 in the plasma membranes of Chinese hamster ovary K1 cells suggest an extracellular N terminus and a cytoplasmic C terminus (13). These experiments imply that in the channel form of the protein, CLIC1, crosses the membrane an odd number of times.

One or possibly two putative transmembrane helices have been postulated to form the membrane-spanning region(s) of the CLIC module (2). The most conserved of these putative TM regions (residues 24–46; CLIC1 numbering) is located within the N-terminal domain and contains Cys-24, which is at the center of the glutathione-binding site. In the structure of the monomeric soluble form of CLIC1, this putative N-terminal transmembrane segment forms an α helix (h1) and β-strand (s2) within the glutaredoxin-like N-domain (8). Thus, CLIC1 is...
We present the crystal structure of this soluble, oxidized lent dimerization. This transition is reversible on reduction. Formation of an intramolecular disulfide bond and non-covalent activity. Either Cys-24 or Cys-59 to serine results in the loss of channel lipid bilayers under reducing conditions and that mutation of we show that purified CLIC1 cannot form ion channels in lipid bilayers that are similar to the native channel. Finally, the glutaredoxin-like N-domain has undergone a radical rearrangement to expose an extended hydrophobic surface, which forms the dimer interface. This transition is stabilized through the formation of an intramolecular disulfide bond between two originally distant cysteine residues, Cys-24 and Cys-59. The dimer forms chloride ion channels in artificial lipid bilayers that are similar to the native channel. Finally, we show that purified CLIC1 cannot form ion channels in lipid bilayers under reducing conditions and that mutation of either Cys-24 or Cys-59 to serine results in the loss of channel activity.

**EXPERIMENTAL PROCEDURES**

**Expression, Purification, and Oxidation of CLIC1—**Recombinant GST-CLIC1 fusion proteins (wild type and mutants) were expressed in *E. coli* BL21(DE3)pLysS using the pGEX-4T-1 vector (2). The fusion protein was purified from the cell lysate supernatant by binding to glutathione S-Sepharose (Amersham Biosciences) and cleaved using bovine plasma thrombin (Sigma). Protein was further purified at 4 °C by gel filtration on a Superdex G75 column (Amersham Biosciences) and preequilibrated with 10 mM Hepes, 100 mM KCl, 0.5 mM CaCl2, 1 mM DTT, and 1 mM NaN3 at pH 7.0. The resulting peak at 30 kDa corresponding to monomeric CLIC1 was dialyzed at 4 °C into 1× phosphate-buffered saline and concentrated to 15 mg/ml. The final product consisted of the wild-type CLIC1 sequence with an extra two residues at the N terminus (Gly-Ser) as a result of the thrombin cleavage site in the fusion construct.

**Oxidation Experiments—**To examine the effect of hydrogen peroxide treatment on CLIC1, a 20 mM stock solution of hydrogen peroxide was prepared immediately before use then added at a final concentration of 2 mM to typically 20–50 μl of the purified protein (~2 mg/ml) in 20 mM potassium phosphate buffer, pH 7.0, containing 150 mM NaCl and incubated for various time periods at room temperature. After incubation the proteins were either analyzed directly by gel filtration chromatography as described below or reduced by the addition of DTT to a final concentration of 50 mM and incubated for a further 1 h at room temperature before gel filtration.

For gel filtration chromatography analysis untreated and oxidized proteins were diluted to 200 μl final volume before injection onto a Superose 12 (HR 10/30, Amersham Biosciences) column preequilibrated in phosphate-buffered saline, pH 7.4, at 0.5 ml/min. Proteins were detected by absorbance at 280 nm. The column was calibrated with the standard proteins carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), yeast alcohol dehydrogenase (150 kDa), β-amylose (200 kDa), ferritin (440 kDa), and the column void volume was determined from the elution time of blue dextran (2×106 kDa).

Electrophoresis was performed either under non-denaturing conditions using 10% native polyacrylamide gels or under denaturing...
conditions by SDS-PAGE on 15% gels. Samples were electrophoresed either unreduced or reduced by the addition of 50 mM DTT to the sample buffer. Bands were visualized by Coomassie Brilliant Blue staining.

**Crystallization of the Oxidized CLIC1 Dimer**—CLIC1 was oxidized by the addition of H2O2 to a final concentration of 2 mM in phosphate-buffered saline solution. The protein was incubated under oxidizing conditions for 5 min at 18 °C before dialyzing against 10 mM Hepes, 100
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Table I
Data reduction and refinement statistics

|                               | Reflections (unique) | Completeness (1.86-1.8Å shell) | I/σ (1.86-1.8Å shell) | Rmerge (1.86-1.8Å shell) | Protein (water) atoms | R factor (Rmerge) | r.m.s.d. bond lengths | r.m.s.d. bond angles | Ramachandran plot | Most favored region | Additionally allowed | Disallowed |
|--------------------------------|----------------------|---------------------------------|-----------------------|--------------------------|-----------------------|-------------------|----------------------|---------------------|-------------------|-------------------|---------------------|------------|
|                               | 163,232 (40,808)     | 95.3 % (85.1%)                  | 10.7 (2.8)            | 0.05 (0.26)              | 3346 (268)            | 0.196 (0.228)     | 0.011 Å              | 1.44°               | 94.1%             | 5.6%             | 0.3 % (Ala-125, A subunit) |

* From Procheck (31).

\[
D = \sqrt{(\phi_\alpha - \phi_\beta)^2 + (\theta_\alpha - \theta_\beta)^2}
\]

(Eq. 1)

where subscripts D and M refer to the dimer and monomer structures.

Chloride Efflux Experiments—400-nm unilamellar liposomes (soybean phosphatidylcholine:cholesterol 9:1 w:w; Sigma P-5638 and C-8662, respectively) containing 200 mM KCI, 2 mM HEPES, pH 6.5, were prepared by extrusion (Avestin Lipofast extruder) and extraveicular chloride removed by desalting on Bio-Gel P-6DG spin columns (Bio-Rad) equilibrated in “assay buffer” (330 mM sucrose, 2 mM HEPES, pH 6.5). A chloride selective electrode (Radiometer Pacific) was used to monitor chloride efflux from the vesicles upon the addition of freshly prepared CLIC1 equilibrated in assay buffer. In each experiment protein (30 μg/ml final concentration) was added to the liposomes followed by 3 μM valinomycin to initiate potential driven

\[\text{FIG. 3. Structural transition of CLIC1 between the monomeric and the dimeric forms.} \]

Representations of reduced monomeric form of CLIC1 (A) and a subunit of the oxidized dimeric form (B). C, backbone superposition of CLIC1 for the reduced monomeric (green) and the oxidized dimeric (magenta) states. Ramachandran distances for residues 23–234 are mapped onto the backbone of the monomeric (D) and dimeric (E) forms. The color gradient, from gray to pink, represents Ramachandran distances from 0° to 180°. Residues not observed in the dimer are colored gold. F, Ramachandran plot of residues within the N-domain with Ramachandran distances greater than 35° between the two structures. Monomer ϕ-ψ co-ordinates are plotted as orange squares, and dimer co-ordinates are in black with a connecting line. The figures were made with SETOR (25), MOLSCRIPT (26), RASTER3D (27), and GRASP (29).
Chloride efflux is measured as a percentage of chloride released from the 400-nm unilamellar liposomes that occurs within 120 s after the addition of valinomycin (± S.D). Numbers in parentheses refer to the number of trials. Each set of experiments was done with protein from at least two independent preparations.

The first numeral indicates the number of tip dip bilayer experiments where chloride ion channels were observed within 20 min of protein addition, and the second numeral is the total number of trials. Where no channels were observed within the experimental time, a second sample of native CLIC1 monomer was added as a positive control to ensure the integrity of the system. For the mutant proteins the number in parentheses indicates the probability (as a percentage) that the result observed would be obtained if the protein had the same properties as the wild type. The calculated probabilities assume a binomial distribution.

### RESULTS

**Reversible Redox-induced Dimerization of CLIC1**—A feature of CLIC1 is that it possesses a glutaredoxin-like active site, suggesting that its function may be under redox control (8). To investigate this we have studied the effect of reactive oxygen species on CLIC1. Purified recombinant CLIC1 was incubated at room temperature in the presence of 2 mM hydrogen peroxide (H$_2$O$_2$) for various times and then analyzed by gel filtration chromatography. Untreated protein eluted as a monomer (Fig. 1A), whereas the addition of H$_2$O$_2$ resulted in the appearance of a CLIC1 dimer (Fig. 1B) comprising approximately two-thirds of the total protein (the proportion was unchanged between 5 min and 6 h of incubation with H$_2$O$_2$). Dimerization was completely reversed by reduction with 50 mM DTT (1 h, room temperature; Fig. 1C). Electrophoresis showed that the observed dimer is not primarily due to an interchain disulfide bond(s) (Fig. 1D), although a minor amount of such material is always present in CLIC1 samples (Fig. 1D, non-reducing SDS-PAGE).

**Structure of the Oxidized Dimer of CLIC1**—The structure of the oxidized dimeric form of CLIC1 was determined at 1.8 Å of resolution (Fig. 2, Table I). The crystal contains one dimer per asymmetric unit, with the two subunits nearly identical (root mean square deviation of 0.34 Å excluding residues 147–164). The dimer is 75 Å long and 25 Å wide and tapers in height from 45 Å at each end to 20 Å in the middle (Fig. 2D). The structure is all helical, and the dimer interface occurs between the two newly configured N-domains. The arrangement of the subunits bears no relationship to that seen in GST dimers (20).

**Comparison of the CLIC1 Monomer and Dimer Structures**—The formation of the oxidized dimer has produced a dramatic change in the conformation of CLIC1 (Fig. 3, A–C). The two C-domains (residues 92–241) show only minor alterations. In contrast, the N-domain has undergone a radical structural rearrangement, including the formation of an intramolecular disulfide bond between Cys-24 and Cys-59 (Fig. 2, B and C). In the monomer, the sulfhydryl groups of these two residues are separated by 13.1 Å (Ref. 8). Cys-24 is conserved in all CLICs, where it is at the center of the glutathione-binding site in the monomeric form, whereas Cys-59 is unique to CLIC1, corresponding to a conserved Ala in all other CLICs (Fig. 2B).

The most apparent structural change between the monomer and dimer is the disappearance of the β-sheet (Fig. 3, A–C). In...
are plotted for the dimer (amplitude histogram current values at different membrane potential showing the distribution of closed (left panel) and monomer on the right). The right panel shows the open probability plot (right panel) obtained from 3 s of data each show similar values to those obtained previously (10). C, the current amplitude histograms showing the distribution of closed (0) and open (1) states obtained from 3 s of data for the dimer (left panel) and monomer (right panel). D shows the iV curve (left panel) in which the peak amplitude histogram current values at different membrane potential are plotted for the dimer (squares) and for the monomer (triangles). The combined data can be fit to a single channel conductance of 28 ± 0.9 picosemians. The right panel shows the open probability plot (squares for dimer and triangles for monomer), which is comparable with previous analysis (10). E, percentage of electrophysiological experiments (tip dip patches) where CLIC1 channel activity was observed after the addition of CLIC1 monomer or dimer at 5 mM DTT. Three different protein preparations of monomer and of dimer were used to test the effect of DTT. A null result indicates that no chloride ion channels were observed within 20 min of protein addition to a patch with a gigaseam. Error bars represent 1 S.D., computed by assuming Poisson statistics (σ = √n). Numbers in parentheses indicate total number of trials for each condition.

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5m M DTT. Three different protein preparations of monomer and of dimer were used to test the effect of DTT. A null result indicates that no chloride ion channels were observed within 20 min of protein addition to a patch with a gigaseam. Error bars represent 1 S.D., computed by assuming Poisson statistics (σ = √n). Numbers in parentheses indicate total number of trials for each condition.

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channels seen in inside-out patches from Chinese hamster ovary cells transfected with CLIC1 (13). The channel characteristics for the monomer, dimer, and inside-out patches from transfected Chinese hamster ovary cells (10) are as follows: conductance, $28 \pm 9$, $28 \pm 9$, and $30 \pm 2$ picoSiemens (Fig. 5D); mean open time, $28.8, 30.4$, and $26.9$ ms (Fig. 5B); open probability, $0.53 \pm 0.03$, $0.50 \pm 0.06$, and $0.53 \pm 0.03$ (Fig. 5D), respectively. All three types of channels are inhibited by IAA94.

Tip dip channel recording experiments were repeated in the presence of DTT. The probability of observing channel activity was reduced dramatically for both monomer and dimer in the presence of 5 mM DTT (Fig. 5E). Presumably the presence of DTT converts CLIC1 to the monomer form, which is then non-functional under reducing conditions. As a corollary of this, the channel activity observed for CLIC1 monomer is likely due to the effect of oxidation occurring in the absence of reducing agents.

**Cys-24 and Cys-59 Are Essential for Dimer Formation and Channel Activity**—To investigate whether disulfide bond formation is essential for channel activity the following mutant proteins were produced: C24S, C59S, and C89S. On the addition of H$_2$O$_2$ neither C24S nor C59S produced a CLIC1 dimer (Fig. 1E). Chloride flux measurements show that both C24S and C59S are inactive as channels, whereas C89S is indistinguishable from wild-type CLIC1 monomer (Table II). The same mutants were examined by tip dip electrophysiology. In keeping with the chloride efflux experiments channels were only observed for C89S (Table II). Statistical analysis using a binomial distribution shows that in the electrophysiological experiments C24S and C59S are inactive as channels, whereas C89S is non-functional under reducing conditions. As a corollary of this, the channel activity observed for CLIC1 monomer is likely due to the effect of oxidation occurring in the absence of reducing agents.

**DISCUSSION**

Our results imply that oxidation is essential for the transition of CLIC1 from the monomer to the integral membrane chloride channel form, requiring both Cys-24 and Cys-59. This suggests that the formation of the intramolecular disulfide bond between these residues is essential for the transition. The dimer structure shows that oxidation results in a large structural alteration in CLIC1 that exposes a new hydrophobic surface that is masked in vitro by non-covalent dimerization. In vivo this hydrophobic surface may represent the membrane-docking interface.

Based on these results we propose the following model for the transition between monomeric, soluble CLIC1 and the integral membrane channel form. The monomeric form maintains a GST-like structure, but the N-terminal domain occasionally transitions to the conformation observed in the dimer structure. In the presence of reactive oxygen species, this altered conformation becomes trapped via the formation of the intramolecular disulfide bond between Cys-24 and Cys-59. This new, monomeric state will be unstable in solution due to the large exposed hydrophobic surface. In the presence of a lipid bilayer the conformationally altered CLIC1 monomer will dock to the membrane, whereas in the absence of lipids it will dimerize. Competition between these two processes will depend on the concentration of CLIC1 and the effective concentration of lipid bilayers. Once CLIC1 docks to the membrane it can undergo further structural changes to produce the integral membrane chloride channel form of the protein. A precedent for ion channels that are controlled by local concentrations of intracellular reactive oxygen species is the Ca$^{2+}$ ion channel in plant root hair cells of Arabidopsis thaliana (21). Our working model for CLIC1 is speculative and will require further experimental verification. In vivo the membrane integration of CLIC1 is likely to be more complex and will probably involve other molecules.

A major unanswered question is, what is the structure of the integral membrane CLIC ion channel? Several alternative models have been proposed for the structure of the membrane inserted CLIC proteins (8, 11). Sequence analyses indicated that Cys-24 to Val-46 might form a transmembrane helix in the channel state (13). Previously, this seemed unlikely due to the GST-like monomer structure (8), which would require an unfolding of the N-domain on membrane insertion. Our present structure indicates that such an unfolding is indeed possible. However, membrane integration may also require unfolding of the C-domain (11).

The structural change observed in the monomer-to-dimer transition in CLIC1 is quite radical. Similar large scale conformational editing through redox switching has been observed in the E. coli H$_2$O$_2$ transcription factor, OxyR (22). Furthermore, reconciliation of biochemical evidence and the location of catalytic cysteine residues in the thioredoxin-like structure of peroxiredoxin 5 necessitate a conformational change during peroxide reduction (23). The rearrangement of the $\beta$-sheet for CLIC1 may, therefore, not be a unique feature of the CLIC protein family but, rather, a structural duality capable of being tolerated by the thioredoxin fold of some proteins.

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