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Trimeric structure of the wild soluble chloride intracellular ion channel CLIC4 observed in crystals ☆

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Abstract

The crystal structure of a wild type of the human soluble chloride intracellular ion channel CLIC4 (wCLIC4) has been determined at a resolution of 2.2 Å. The structure shows a homotrimer in an asymmetric unit, which is first observed in CLICs. The assembly of the trimer takes a unique triple interaction mode between three monomers with a hydrogen-bond network and hydrophobic contacts. Through such complicated interactions, the homotrimer of wCLIC4 is firmly stabilized. The structure shows an oligomeric mode with a unique assembly mechanism by which the oligomerization of CLIC4 can be performed without any intramolecular disulfide bond formation. It indicated a possibility that CLIC4 may take a unique structural organization distinct from CLIC1 for docking with lipid bilayers. In addition, the structure shows distinct conformational states of the h2 region for respective monomers of the trimer, which reveal an intrinsic conformational susceptibility for this significant region in the structural transition.

Keywords: Chloride intracellular ion channel; CLIC4; Crystal structure; Trimeric structure

Chloride ion channels are critical for various physiological processes, including control of secretion and absorption of salt, regulation of membrane potentials, organellar acidification, and cell homeostasis [1]. Some functional abnormity of these channels can induce severe diseases [2]. These channels are located in the plasma membrane and other internal cell membranes. They are classified into several classes based on their sequence relationship [3,4]. The chloride intracellular channels (CLICs) are recently characterized at a molecular level as a new class of chloride ion channels. To date, six human CLIC family members referred as to CLIC1–CLCI6 have been identified [3–7] (Fig. 1). It is an unusual feature that all of these proteins exist as soluble globular proteins that can constitute the integral ion channels in organellar and plasma membranes [3–6,8–11]. Each CLIC protein contains a conserved C-terminal 240-residue module with a rather variant N-terminal (Fig. 1), in which CLIC6 possess an additional N-terminal domain with 440 residues. The 3D-structural studies on CLIC family were focused on CLIC1. At first, the crystal structure of CLIC1 was determined at 1.4 Å resolution, which showed that CLIC1 has a GST fold with a covalent binding site for glutathione [12]. Recently, Littler et al. [13] reported that on oxidation CLIC1 would undergo a noticeable conformational change, in which the N-domain of CLIC1 is completely rearranged, resulting in the exposure of a hydrophobic surface and a concomitant formation of an intramolecular disulfide bond between Cys24 and Cys59. In vitro, this new conformation is stabilized by non-covalent dimerization.

Most recently, the crystal structure of a mutant form of human CLIC4 with a C-terminal extension, referred to as CLIC4(ext), has been determined where the last two amino

☆ *Abbreviations:* CLIC, chloride intracellular ion channel; wCLIC4, wild CLIC4; CLIC4(ext), mutant CLIC4 with C-terminal extension of 16 residues.

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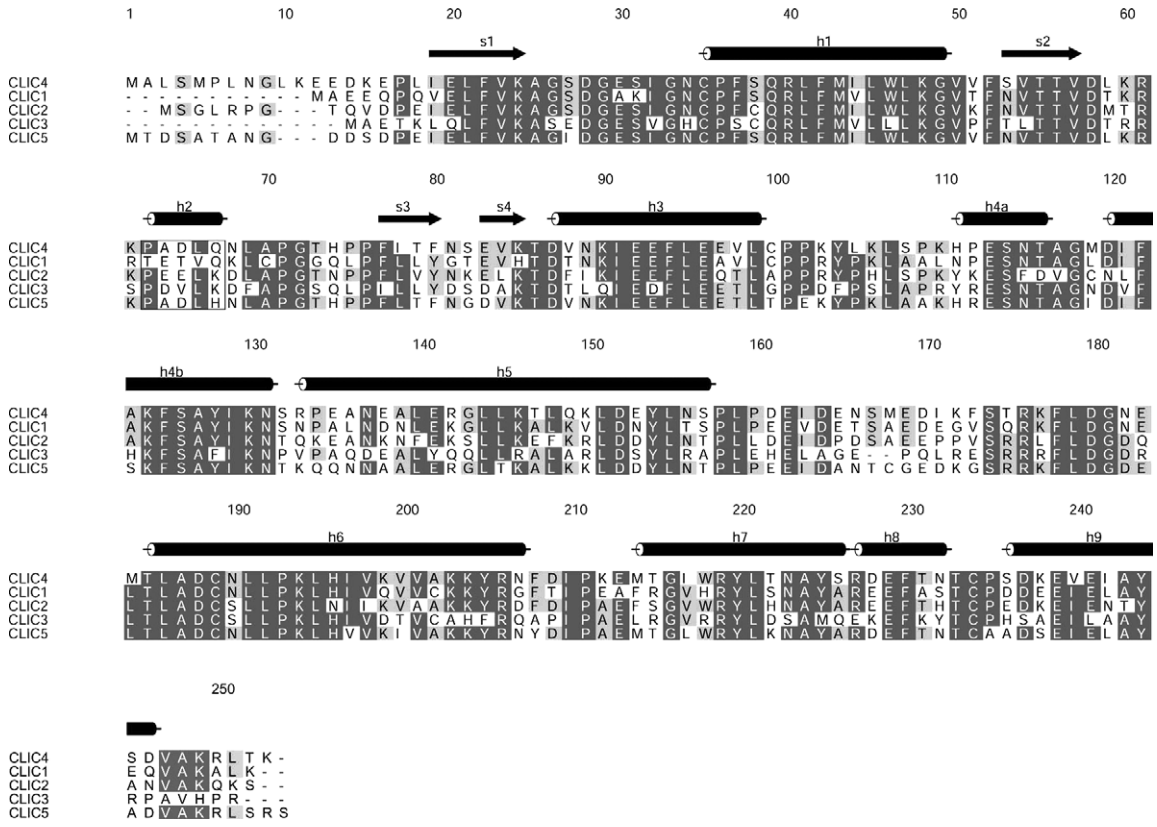

Fig. 1. Sequence alignment of CLIC1-5. Identical and conserved residues are shaded in black and gray, respectively. H2 region takes distinct conformational states in the monomeric CLIC1 and CLIC4(ext) (α-helix with three turns) and the trimeric wCLIC4 (disorder–Mol A, loop–Mol B, one turn–helix–Mol C), which is highlighted with box.

acids of the wild CLIC4 have been serendipitously replaced by a 16-residue peptide [14]. The CLIC4(ext) structure is monomeric and closely similar to CLIC1. The major differences between these two structures localized in some sensitive regions of the molecule, including the helix 2 and related loop (residues 58–74), the hairpin connecting β-strands 3 and 4, as well as the flexible foot loop [13]. The structural differences correlate with the sequence variations. Especially, in CLIC4 the counterpart residue corresponding to Cys59 in CLIC1 was replaced by residue Ala, which destroyed the possibility to form the intramolecular disulfide bond between this position and Cys24 like in CLIC1. It has been demonstrated that the formation of this intramolecular disulfide bond (Cys24–Cys59) would induce the structural transition of CLIC1 from a monomeric to a non-covalent dimeric, which is critical for constituting the membrane docking form of CLIC1 [13]. Therefore, the question is open: whether CLIC4 could be also oligomerized in the absence of the intramolecular disulfide bond. Evidently, more structural information of CLIC4 in certain unique states is required. In this paper, we report the crystal structure of a complete wild type of CLIC4, referring to it as wCLIC4, at 2.2 Å resolution. The structure shows a unique trimeric organization of wCLIC4 and different conformational state around the area of hlix2 in the trimeric wCLIC4, which is speculated to be a hinge region for the structural transition from soluble to membrane docking form. Besides, the N- and C-terminal segments of wCLIC4 more naturally appeared in the molecules. These new structural information, especially the self-assembly behavior of wCLIC4, provide some clue to understand the possible way by which the oligomeric form of wCLIC4 in the structural transition can be stabilized without the intramolecular disulfide.

Materials and methods

Expression, purification, and crystallization of wCLIC4. The coding sequence for wCLIC4 protein was amplified from RT-PCR products of human hemopoietic stem cell using the polymerase chain reaction (PCR) method. The PCR products was restricted with *Nde*I and *Xho*I, and ligated into the pET22b(+) vector (Novagen) with a His-tag. The recombinant plasmid was transformed into *Escherichia coli* strain BL21(DE3) for expression. The overexpressed protein was purified by Ni–NTA affinity chromatography and size-exclusion chromatography. The purified protein was concentrated to about 30 mg/ml for crystallization. wCLIC4 has been crystallized by the hanging-drop vapor-diffusion technique using trisodium citrate as the precipitant. The best crystals were obtained by microseeding method in 1 μl drop with a 400 μl reservoir solution both containing 1.1 M trisodium citrate, 0.1 M Tris, pH 7.5, with 0.1 M magnesium chloride, and 3% ethanol as additive at 293 K.

Data collection and processing. Diffraction data were collected at RIGAKU RAXIS IV with seal-tube X-ray source using Cu Kα radiation ($\lambda = 1.5418$ Å) from a rotating anode operating at 40 kV and 20 mA with 0.1 mm cofocus incident beam diameter. The data were collected at 85 K with a crystal-to-detector distance of 100 mm, $\Delta\varphi = 1°$ and 300 s exposure time. A total of 180 frames were collected. Data were processed with the

programs *MOSFLM* [15] and *SCALA* [16]. The crystal parameters and data collection statistics are listed in Table 1.

Structure determination and refinement. The structure of wCLIC4 was determined by the molecule replacement method. When we resolve the structure of wCLIC4, the CLIC4(ext) structure has not been reported, so the CLIC1 monomer structure (1K0M) was used as a molecule replacement probe using the CCP4 program *AMoRe* [17]. An initial phasing model consisting of three molecules is built, with CLIC1 residues 6–165, 175–241. After phase refinement by CNS [18], the resulting electron density map was clear, against which then the CLIC4 sequence was built up in the model with the program *O* [19]. It was refined using maximum likelihood methods. The final model consists of residues A16–A61, A66–A160, A172–A253, B6–B162, B171–B253, C13–C160, C173–C255 plus 764 water molecules. Residues Pro76 and Pro102 have *cis* peptide bonds. The structure was refined to a resolution of 2.2 Å with *R* factor of 0.216 and *R* free of 0.262 using CNS version 1.1 [18]. The quality of the structure was checked using PROCHECK. The statistics are summarized in Table 2. The CLIC4 coordinates and structure factors have been deposited in the Protein Data Bank (Accession Code 2D2Z).

Table 1
X-ray data collection and refinement statistics

Crystal	Space group (molecules/asu)	$P12_11$ (3)
	a (Å)	73.18
	b (Å)	86.05
	c (Å)	73.38
	β (°)	112.99
Data collection	Wavelength (Å)	1.5418
	Resolution range (Å)	23.49–2.20
	Observed reflections	130,311
	Unique reflections	42,522
	Redundancy	3.1
	Completeness	99.7% (99.9%)
	I/sigma(*I*)	7.80 (17.7)
	R_{merge}	0.066 (0.365)
	WILSON PLOT B (Å^2)	36.0
Refinement	Sigma cutoff	0.00
	R factor	0.216 (0.313)
	R free	0.262 (0.374)
Model	Protein non-H atoms	5474
	Water molecules	764
	Average *B* factors (Å^2)	38.3
Ramachandran plots	Most favored region	90.0%
	Additionally allowed	8.6%
	Generously allowed	1.0%
	Disallowed	0.4%

Values in parentheses are for the highest resolution shell (2.34–2.20 Å).

Table 2
Hydrogen bonds and hydrophobic interaction involved in the trimer contact

Hydrogen bonds		
B8Asn O	C199Lys NZ	3.00 Å
B9Gly O	C212Lys NZ	2.85 Å
B11Lys NZ	A92Glu OE2	2.74 Å
B11Lys O	A194Lys NZ	2.90 Å
B12Glu O	C212LYS NZ	2.48 Å
B13Glu OE2	A194Lys NZ	2.95 Å
Hydrophobic interactions		
B7Leu	C221Leu, C225Tyr, C218Trp	
B10Leu	C218Trp, A119Met	

Results and discussion

Unique trimeric organization

wCLIC4 consists of 253 residues which mostly appeared in the fine electron density maps. The general fold of wCLIC4 monomeric form resembles that of CLIC4(ext) consisting of four-stranded mixed β sheet (β1–β4) around three α-helices (h1–h3) in N-domain and all six α-helices (h4–h9) in C-domain (Figs. 1, 2A, 3A and B). Distinctly, the refined wCLIC4 model contains three molecules in the asymmetric unit that form a homotrimer through a unique organization (Fig. 2A). The structure of wCLIC4 is the first intracellular chloride ion channel observed as a trimer. The three molecules of the trimer are related to each other by rotation of about 135° (Molecule A–Molecule B), 75° (Molecule B–Molecule C), and 60° (Molecule A–Molecule C) around axis vertical with both two molecules' N-terminal to C-terminal vectors, respectively. The trimer interface in wCLIC4 displays a high degree of shape complementary, exhibiting a shape coefficient (S_c) of 0.620 and 0.687 ($S_c = 1.0$ for interface with geometrically perfect fits) [20], and a large buried surface area of 402 and 1769 Å^2, respectively, for assemblies of Mol A–Mol C and Mol A–Mol B–Mol C, relative to the overall surface area of a 253-residue protein.

The assembly of wCLIC4 trimer takes a unique way. Among three monomeric forms of wCLIC4, Mol A and Mol C seem essential as a framework of the oligomer and Mol B may play a role as a mediating molecule. Mol A and Mol C are close to each other to form a slot-like tertiary arrangement through h3, h4a, h4b, h6 of Mol A, and h7 of Mol C (Fig. 2A). There are a series of hydrophobic and polar residues distributed on the surfaces of the slot, including Glu92, Lys194, Met119 from Mol A and Lys199, Lys212, Trp218, Leu221, Tyr225 from Mol B (Fig. 2B). In oligomerization, the N-terminal segment of Mol B is inserted into this slot to interact with Mol A and Mol C through a hydrogen-bond network consisting of six hydrogen bonds and a number of hydrophobic contacts between six hydrophobic residues (Figs. 2A and B and Table 2). Besides, there are two main-chain hydrogen bonds to be formed between the C-terminal residue Leu251 from Mol C and residues Ser82 and Val84 from Mol B. In addition, residues Ala64, Asp65, and Asn68 on the flexible position h2 as well as residue Thr86 from Mol B are further hydrogen-bonded with the C-terminal residues Ser245, Asp246, Arg250, and Lys253 from Mol C (Fig. 2C). Evidently, the homologous trimer of wCLIC4 is firmly stabilized by such complicated interactions.

In the most recent report [13], Littler et al. 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 (Cys24–Cys59) and the crystal structure of this oxidized state was determined to show a major structural transition. It was identified that the oxidized CLIC1 dimer maintains its ability to

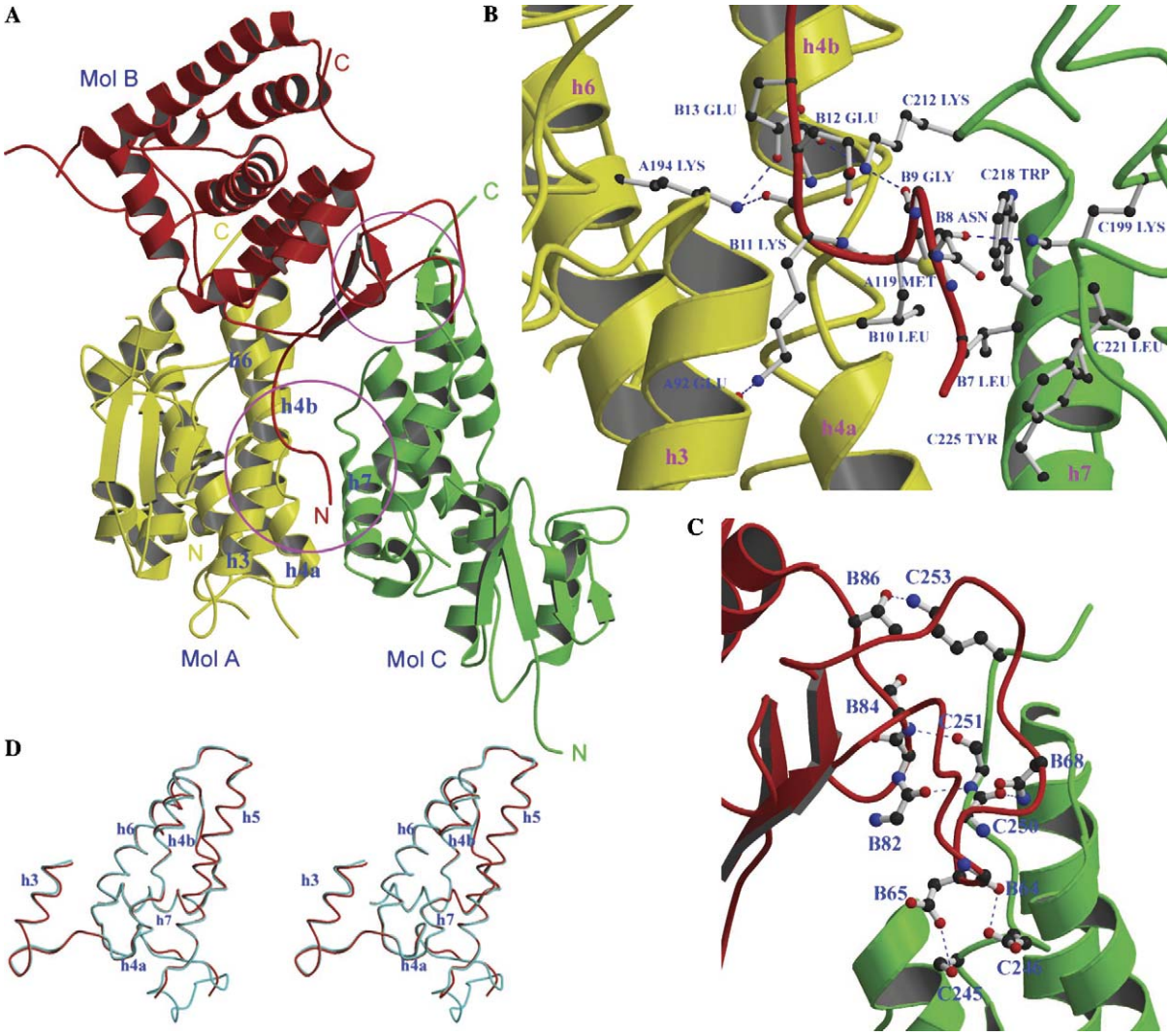

Fig. 2. Overall structures in wCLIC4 trimer is asymmetric unit and unique organization of the oligomer. (A) Ribbon diagram of the trimeric wCLIC4. The regions involved in intermolecular contacts are highlighted by circle, which are shown in detail in (B,C), respectively. (B) Triple interactions in wCLIC4 trimer. The N-terminal segment of Mol B is inserted into a slot to interact with both Mol A and C through six hydrogen bonds and hydrophobic contacts between six residues. (C) Interactions between C-terminal residues of Mol C and residues 64–68 and 82–86 of Mol B. Mol A, B, and C are shown in yellow, red, and green, respectively. (D) Structural comparison between the slot-forming helices (h3, h4a, h4b, h6, and h7) of wCLIC4 (red) and oxidized CLIC1 (green). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

form chloride ion channels in artificial bilayers and vesicles. Therefore, it was proposed that this dimeric form of CLIC1 should be the membrane docking form. Compared with oxidized CLIC1, the structural elements critical for formation of the tertiary slot between Mol A and Mol C in wCLIC4 trimer, including h3, h4a, h4b, h6, and h7, adopt the same conformation as that in oxidized CLCI1 (Fig. 2D). Furthermore, the residues contributed to the intermolecular contacts in this region, including Met119, Trp218, Leu221, Tyr225, and Glu92, Lys194, Lys199, all are conserved in sequence alignment (Fig. 1). It implies that the tertiary organization like in wCLIC4 trimer might have also appeared in certain specific conditions. Instead of the stabilizing mechanism with the intramolecular disulfide bonding, the assembly of wCLIC4 is stabilized by the intermolecular non-covalent interactions, where the N-terminal segment of one molecule Mol B is inserted into a dimeric interface (between Mol A and Mol C) to make all contacts that are needed for stabilization. Here it is not needed to form any intramolecular disulfide bond. Though the observations are from the crystallographic asymmetric unit and also we do not know any relationship of the wCLIC4 trimer with the possible membrane docking form of CLIC4 at the moment, the unique trimeric structure of wCLIC4 reported in this paper provides an oligomeric mode and an assembly mechanism without assistance of the intramolecular disulfide bond formation for CLIC4. It shows a possibility that CLIC4 may take a structural organization distinct from CLIC1 in specific conditions.

Distinct conformational state of helix 2 region

The general fold of three monomers of wCLIC4 trimer is similar (the r.m.s.ds of main chain atoms are 0.669,

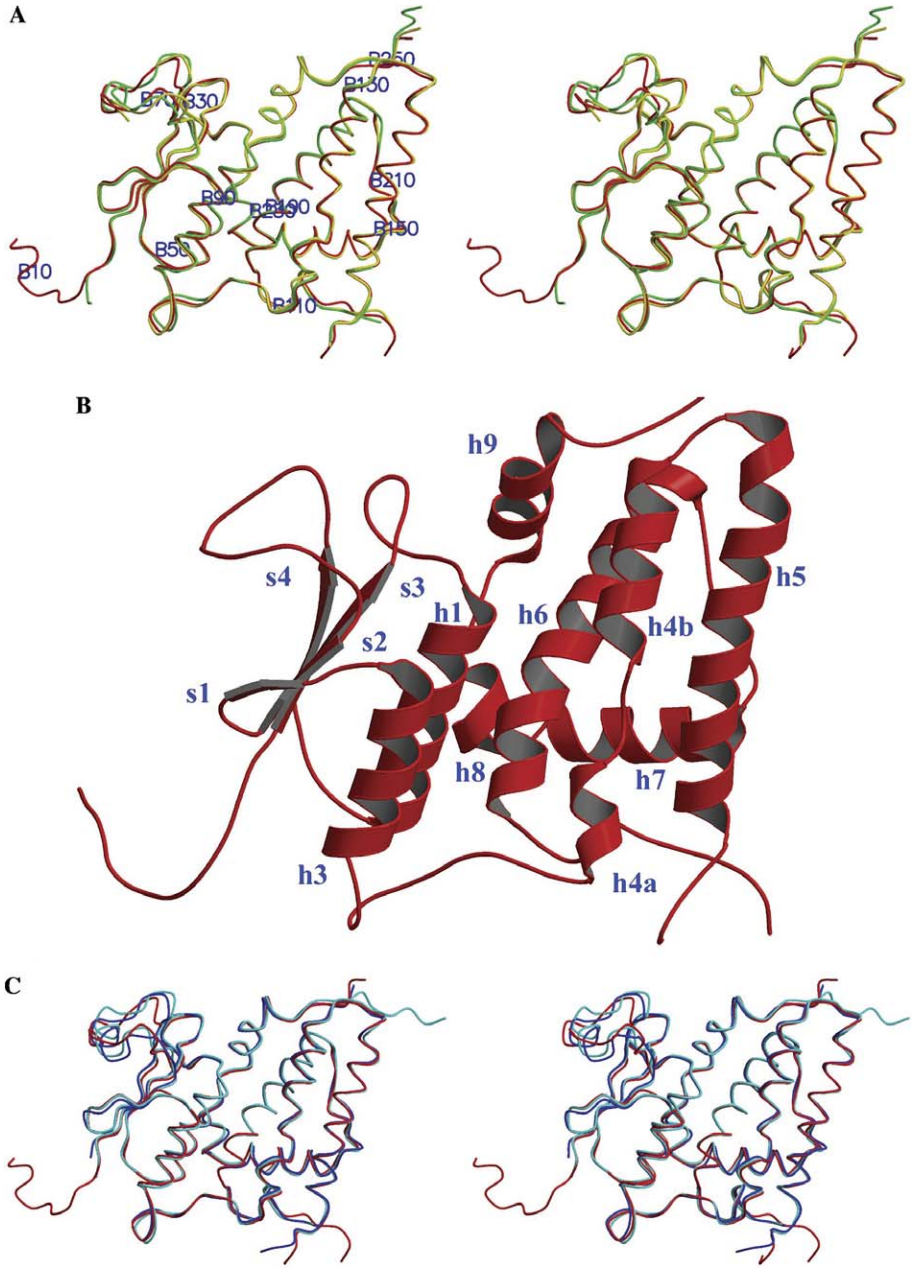

Fig. 3. General fold of wCLIC4 monomer and comparison with CLIC4(ext) and CLIC1. (A) Stereo drawing of superposition of Mol A (yellow), B (red), and C (green) of the wCLIC4 trimer. (B) General fold of Mol B of wCLIC4 as a representative monomer. (C) Stereo diagram of superposition between Cα traces of wCLIC4 (Mol B) (red), CLIC4(ext) (cyan), and CLIC1 (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

0.663, and 0.660 Å for Mol A–B, Mol B–C, and Mol C–A, respectively) and resembles that of CLIC4(ext) [14] (Fig. 3). It has the general fold of the GST superfamily including an N-terminal domain (residues 1–90) with a thioredoxin fold and an all α-helical C-terminal domain (residues 100–253). The detailed comparison between monomeric forms of wCLIC4 and CLIC4(ext) showed that the major differences centered in three regions: the helix 2 region, the flexible foot loop, and the N- and C-terminal segments. Among others, the conformational state of h2 region is more distinct.

In CLIC4(ext) and CLIC1 as monomeric form in crystals residues 63–70 (number referring to CLIC4) adopt a regular α helical conformation (h2), where there is a 15° relative rotation in these two structures [14]. However, in the structure of wCLIC4 trimer, the region corresponding to h2 of CLIC4(ext) takes distinct conformational states in the respective monomeric form. The electron densities definitely showed that this region was appeared as disorder, a loop or a short helix in Molecule A, B, and C, respectively (Fig. 4). Accompanying, the orientation of flanking segments connected h2 to β2 and β3 is also changed.

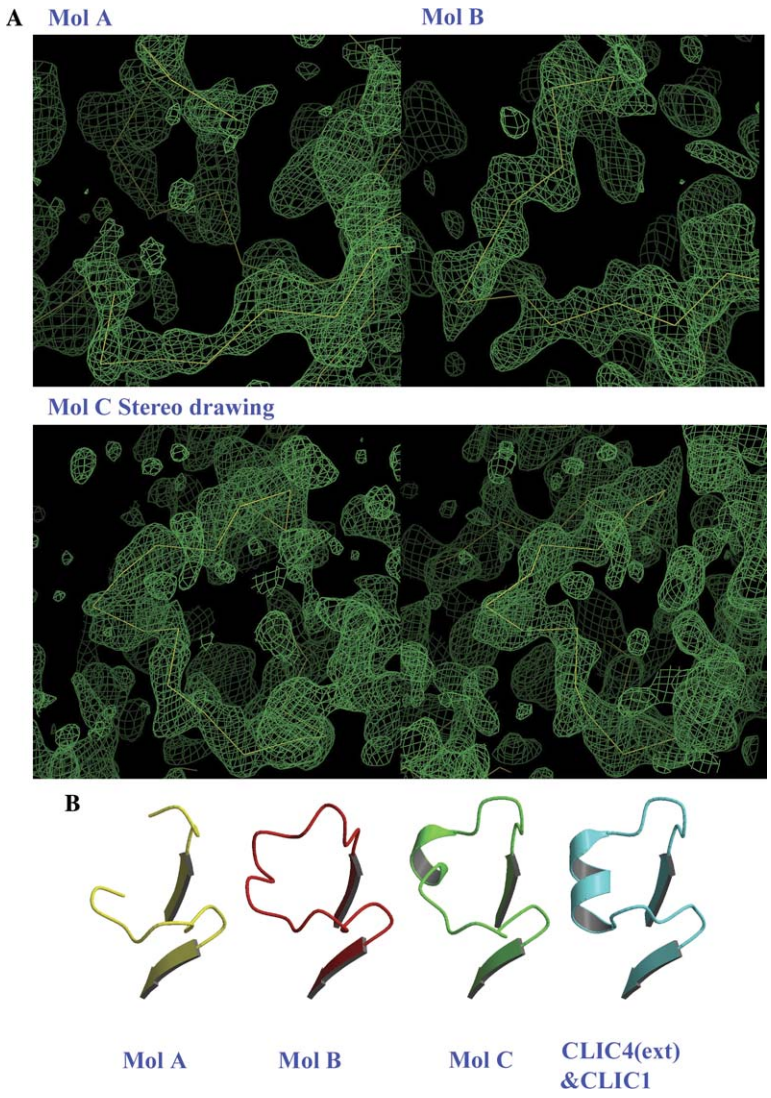

Fig. 4. Distinct conformational states around residue h2 region (residues 63–67). (A) $2F_o - F_c$ electron density maps around residues 63–67, which definitely show disorder, a loop, and a short helix for Mol A, B, and C of wCLIC4 trimer, respectively. (B) Ribbon diagrams of h2 region showing its different conformational states in Mol A, B, C of wCLIC4 trimer and CLIC4(ext) and CLIC1.

It indicated that the h2 region possesses an intrinsic conformational susceptibility.

The studies on the redox-controlled structural transition of CLIC1 have shown that on oxidation CLIC1 undergo a reversible transition from a monomeric to a non-covalent dimeric state and the latter one is proposed as a membrane docking form [13]. The crystal structure of oxidized dimer showed that the most apparent structural change between the soluble monomer and oxidized dimer is relative to the h2 region. In the dimer, the first 22 residues (β-strand 1) are disordered and helix 2 is extended by an extra 2 turns. In addition, the residues between helix 2 and 3 (strands 3 and 4 in monomer) from an extended loop are loosely packed against the C-domain in the dimer [13]. It seems that h2 region is a central structural clement for the redox-controlled structural transition. The structural variability of h2 region observed in wCLIC4 trimer may reflect that the intrinsic conformational susceptibility of h2 region could play a role in the initial step of the structural transition of CLIC4 in certain condition, like oxidation.

Specific N- and C-terminal segment and flexible foot loop

Like in CLIC4(ext) monomer, the N-terminal 15 residues are disordered in Mol A and C of wCLIC4 trimer. However, the N-terminal segment in Molecule C of wCLIC4 trimer displays a specific conformation, which is involved in the intermolecular interactions for trimeric organization (Fig. 2 and Table 2). The structure of wCLIC4 trimer further identified the intrinsic flexibility of the N-terminal segment, and also showed that this flexibility may play a critical role in mediating the oligomerization of CLIC4 or the interaction of CLIC4 with the certain structural element on the bilayer membranes.

In mutant CLIC4(ext), the C-terminus Thr252 and Lys253 were artificially replaced by a 16-residue peptide

(PSKVDKGEFQHTGGRY) [14] and the connecting residue Pro252 should alter the orientation of the C-terminal segment. Therefore, the C-terminal structure of wCLIC4 may show the more native conformational state for the CLIC4 C-terminal segment. For Mol A and Mol B the C-terminal eight residues His-tag did not appear in electron densities, but two more residues could be traced down for Molecule C because it was involved in the intermolecular contacts (Fig. 2C). The structural superposition showed that the general conformations of C-terminal segment of Mo A, B, and C of wCLIC4 are similar (Fig. 3A).

The flexible foot loop (residues 159–175) is distinct structure element in CLICs, where about 10 residues are disordered. The structure of the flexible foot loop in wCLIC4 closely resembles those of CLIC(ext) and CLIC1. There are 11, 8, and 12 residues being disordered in Mol A (161–171), Mol B (163–170), and Mol C (161–172), respectively. A fewer residues of Mol B are in disorder due to there being some intermolecular contacts between Ile171, Lys172 and a symmetric Glu153. The conformations of the hinge residues, Pro158 and Arg176 of wCLIC4, are similar to that in CLIC4(ext). It is noticed that the side chain of Ser174 can form hydrogen bond with the backbone of Arg176 and Asn156, which should also contribute to the stability of the foot loop. In fact, Ser174 is conserved in CLICs.

Acknowledgments

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