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STRUCTURE NOTE

Structure of human CLIC3 at 2 Å resolution

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INTRODUCTION

The chloride intracellular channels (CLICs) are an unusual family of primarily soluble proteins that are thought to dynamically transition into a membrane spanning state to mediate ion conductance. The CLIC family consists of six human members, and is a subgroup of the glutathione-*S*-transferase (GSTs) superfamily. Physiologically, the CLICs have been associated with biological processes as diverse as angiogenesis,[1] macrophage activation,[2] lactation and gastric acid secretion,[3] the response to DNA damage,[4] maintenance of membrane structure,[5] and bone resorption.[6] The CLICs were first discovered when a chloride ion channel identified in bovine kidney, was purified to homogeneity, cloned and named p64 (now CLIC5).[7–9] Subsequent *in vitro* experiments revealed a direct role for the CLICs in ion channel formation[10–15]; purified protein from several members of the CLIC family were shown to form functional chloride channels when the soluble form of the protein was added to artificial lipid bilayers. However, though the soluble GST-like state of some CLIC family members is now well characterized, the nature of their structural transition into a membrane spanning ion channel remains unknown.

This article presents the structure of the soluble form of CLIC3, a family member that was first identified in a yeast two-hybrid screen as a potential binding partner of a mitogen-activated protein kinase.[16] Our knowledge about CLIC3 is limited; a great deal of the biochemical analysis and cellular localization studies performed on it must now be considered inconclusive. This is because these studies were unwittingly carried out with a truncated fragment of the CLIC3 protein with coding commencing at Met-30,[16] a fragment that at the very least would be unable to fold its N-terminal domain in the soluble state. Although our molecular understanding of CLIC3 is unclear, detailed histological experiments have shown it to be very prominently expressed in placental tissue, possibly suggesting a role in fetal development.[17] In this article, we present the crystal structure of a soluble form of CLIC3 in reduced and oxidized states, it possesses a GST-like form with a more open and polar active site than that of other CLIC proteins. This open conformation suggests that the CLIC3 interaction partners or substrates may be family-member specific. Like CLIC2, the CLIC3 active cysteine can form an internal disulfide bond with its partner within a thioredoxin-like CxxC motif. Additionally, the CLIC3 foot-loop, a region that is highly flexible in the other family members, appears uniquely ordered in this structure. Consideration is given as to how each of these differences may affect the behavior of CLIC3.

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METHODS

Cloning

Human *clic3* cDNA (BC007012) was cloned into a modified pET-28 expression vector producing a construct consisting of residues 1–230 and an N-terminally fused hexahistidine tag (MAHHHHHHSAALEVLFQ-GPG). Protein was expressed in *E. coli* BL21 (DE3) grown in LB at 37°C. Once an OD_{600} of 0.8 was reached the temperature was lowered to 14°C and expression allowed to occur overnight after induction with 1 m*M* IPTG.

Protein purification

Cells were harvested, resuspended in buffer A (200 m*M* NaCl, 5 m*M* imidazole, 0.5 m*M* TCEP, 20 m*M* HEPES pH 7.5) then lysed by sonication. Cell debris was pelleted by centrifugation and the resulting soluble fraction applied to Ni^{2+}-NTA affinity resin (Qiagen). After washing (10 m*M* imidazole), the protein was eluted from the resin with 400 m*M* imidazole (in buffer A). The eluent was then applied to a HiLoad S200 16/60 gel filtration column (GE Healthcare) equilibrated in buffer A. CLIC3 eluted as a single monomeric peak and was concentrated to 12–15 mg/mL.

Crystallization and data collection

Crystals were grown at 18–20°C using the hanging drop method. Three microliters of (15 mg/mL protein was added to 3 μL of reservoir on a glass slide that was suspended over 1 mL of reservoir (1.05*M* $(NH_4)_2SO_4$, 0.225*M* $LiSO_4$, and 0.1*M* Tris-HCl pH 8.5). Large multilayered crystals appeared after a week. The oxidized CLIC3 structure came from a crystal harvested and transferred into a drop containing reservoir solution supplemented to 3*M* $(NH_4)_2SO_4$ as a cryoprotectant before vitrifying in liquid nitrogen. Diffraction data were collected at 100 K at the European Synchrotron facility on beamline ID23-1 on a corner of a multilayered crystal using the microfocus beamline. The reduced CLIC3 structure came from a crystal grown in drop consisting of 0.95*M* $(NH_4)_2SO_4$, 0.225*M* $LiSO_4$, and 0.1*M* Tris-HCl pH 8.5, the initial drop was seeded with crushed crystals which accelerated the growth. This crystal was frozen using 20%v/v ethylene glycol as a cryoprotectant with data collected on a Bruker Microstar generator. Both crystals diffracted to 1.95 Å in the space group P1 with cell dimensions $a = 48$ Å, $b = 48$ Å, $c = 60$ Å, and $\alpha = 69°$, $\beta = 81°$, and $\gamma = 75°$ (see Table I for data collection and refinement statistics).

Model building and refinement

A model of CLIC3 was built after obtaining phases by molecular replacement using AMoRe and a single protein subunit of CLIC4 (PDB 2AHE) as a probe.[20] The electron density was clear for the majority of the molecule, allowing rounds of manual building and automatic refinement using the programs Refmac 5,[21] Coot,[22] and Phenix.[23] The CLIC3 model consists of two molecules within the asymmetric unit, with residues 5–45 and 59–230 visible in molecule A; and 4–45, 59–230 visible in molecule B. Additional ordered hexahistidine tag residues are also present adjacent to s2. In the oxidized structure, both subunits show density indicative of a disulfide bond between Cys-22 and Cys-25. Additionally, there are cis-peptide bonds for all instances of Pro-63 and Pro-89. Structure factors and the models themselves have been deposited with PDB codes 3FY7 (oxidized state) and 3KJY (reduced state).

Table I
Data Collection and Refinement Statistics

	Oxidized (2FY7)	Reduced (3KJY)
Data collection statistics		
Number of crystals	1	1
Space group	P1	P1
Unit cell		
a, *b*, *c* (Å)	48.29, 48.67, 60.15	48.08, 48.38, 59.76
α, β, γ (°)	69.1, 80.97, 74.8	69.6, 80.89, 74.60
Resolution limits (high)	28.3–1.95 (2.06–1.95)	28.3–1.95 (2.06–1.95)
Completeness (%)	94 (86.6)	92.5 (89.4)
Number of unique reflections	33742 (4518)	32724 (4604)
Multiplicity	1.9 (1.9)	2.2 (2.2)
R_{merge}	0.086 (0.464)	0.036 (0.406)
$<I/\sigma>$	8.6 (1.9)	15.7 (2.1)
Wilson *B*-factor (Å^2)	24.9	31.8
Model refinement statistics		
R_{factor}[a]	0.231	0.198
R_{free}[a]	0.287	0.245
Number of protein atoms	3552	3539
Number of waters (SO_4^{2-})	140 (4)	146 (7)
Ramachandran plot[b]		
Favored	97.2%	98.4
Outliers	0.5% (Cys-22A and B)	0.5% (Cys-22A and B)
RMS bond (Å)[a]	0.008	0.007
RMS angle (°)[a]	1.040	1.021

[a]Calculated within Refmac 5.[18]
[b]Calculated with Molprobity.[19]

RESULTS AND DISCUSSION

We have solved the crystal structure of a soluble form of CLIC3 at 2 Å resolution in oxidized and reduced states [Fig. 1(A)]. The construct that crystallized had residues 231–236 truncated and 19 additional residues deriving from an N-terminal hexahistidine tag. The P1 crystal form has two molecules within the asymmetric unit but gel-filtration experiments indicate that CLIC3 is monomeric in solution. The two molecules are identical (RMS deviation of 0.6 Å over 219 residues) and CLIC3 adopts a fold similar to that of other CLIC family mem-

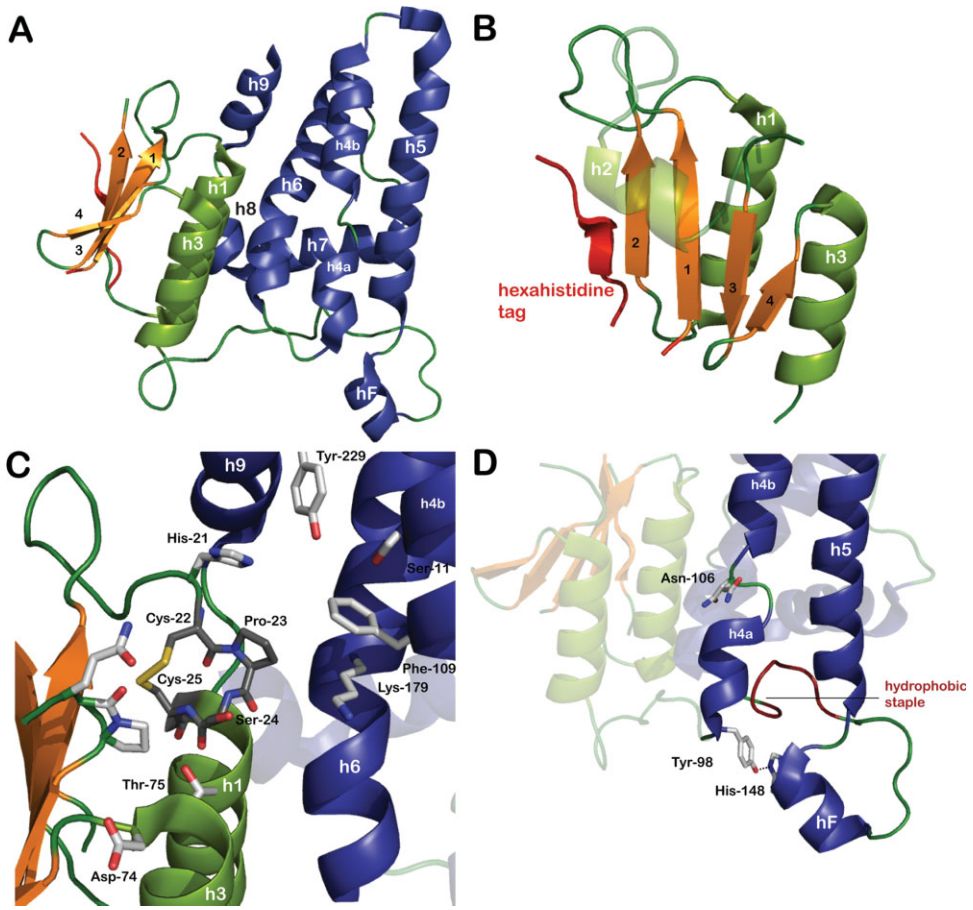

Figure 1

A: Cartoon representation of CLIC3 A-molecule; loop regions are colored dark green, β-strands orange, and α-helices light green within the N-terminal domain or blue within the C-terminal domain. All secondary structural elements are labeled. **B**: A similar representation of the N-terminal thioredoxin domain alone (residues 5–88), red highlights the hexahistidine tag attached to the β-sheet. Also shown is a transparent depiction of helix 2 within the position in which it is observed in CLIC4. This entire segment is disordered in this structure. **C**: The residues within the CLIC3 active site whose function is currently unclear, the region displayed is analogous to glutathione binding and substrate binding regions of the GSTs. **D**: The ordered CLIC3 foot loop and the split helix 4.

bers.[10,11,20,24] CLIC3 has an additional helix F between h5 and h6 but is otherwise identical, allowing us to refer to secondary structural elements as originally defined in the crystal structure of the soluble monomeric form of CLIC1.[24] The CLIC-fold consists of two domains: an N-terminal thioredoxin like domain, and a C-terminal all helical domain, which together form a glutathione transferase-like fold. This fold is contained within the C-terminal 230 amino acids of each CLIC protein and is highly conserved throughout the family [the structures of CLIC3 and CLIC4 have a RMSD of 0.8 Å over 223 C_α and share 52% sequence identity, see Fig. 2(B)]. Despite structural homology with the omega GSTs, this domain in the CLICs is sufficient to elicit ion conductance in membranes *in vitro*.[24] Given the CLICs proposed function as ion channels, the reason for their evolutionary connection to the GSTs is unclear. Canonical GSTs are well-studied enzymes that catalyze the conjugation of glutathione to a broad range of compounds such as xenobiotic molecules or metabolic by-products, thereby facilitating their inactivation and elimination. However, the GST-fold is also found in a number of proteins that have redox-related functions including: disulfide reduction in *E. coli* glutaredoxin 2[25]; thiol oxidoreductase and arsenic biotransformation activity in the GSTOs[26]; catalyzation of isomerization reactions in bacterial GSTs[27,28]; as crystallins in squid eyes[18] and in the prionic yeast regulatory protein Ure2.[29] These noncanonical roles are often redox-related but, being mostly enzymatic, are still far removed from the ion-channel activity attributed to the CLIC-branch of the GST-superfamily. Given the lack of classical transmembrane regions within the CLIC family and also the lack of evidence for an evolutionary connection to classical ion-channel fami-

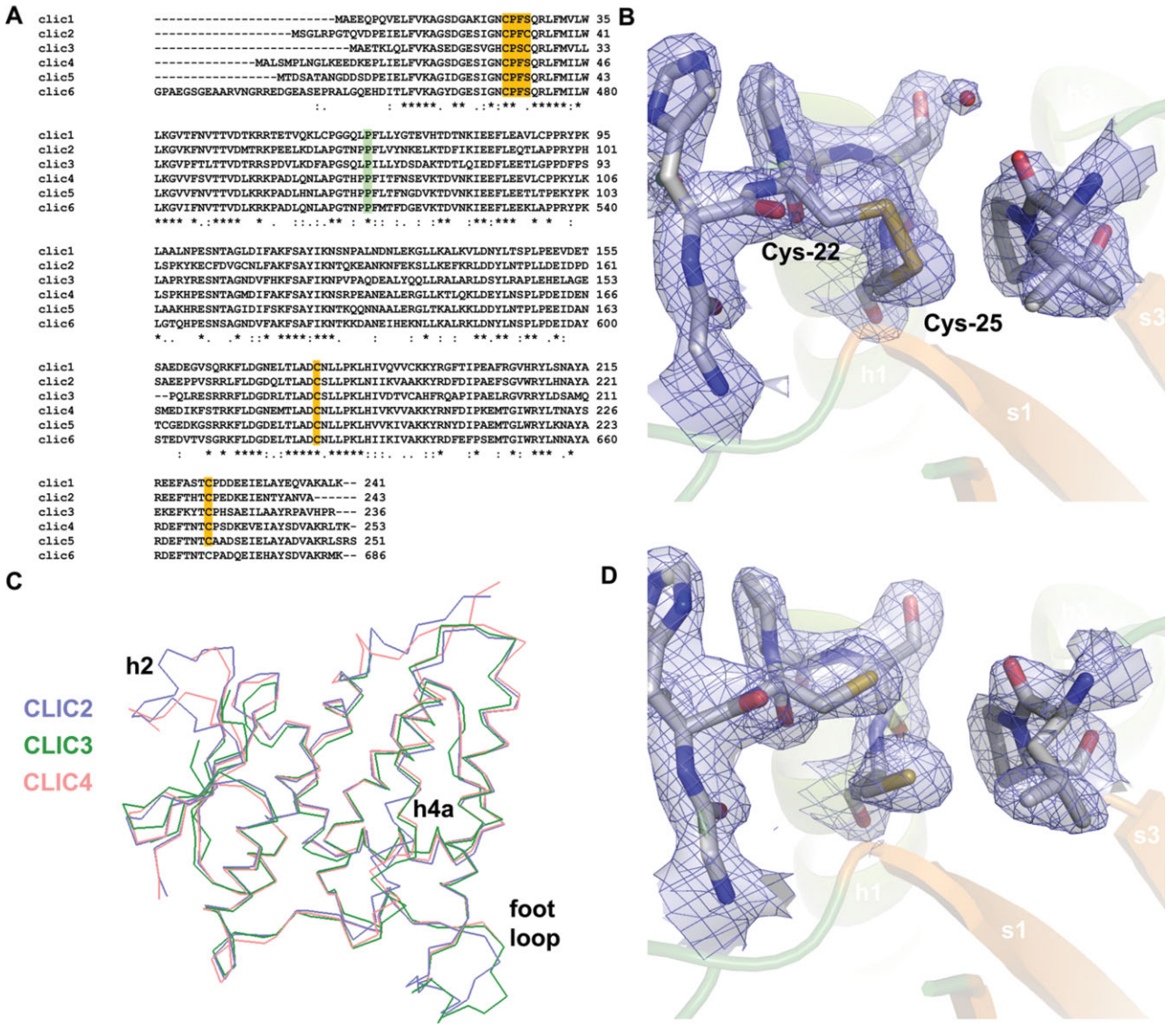

Figure 2

A: ClustalW sequence alignment of human CLIC family members highlighting the conserved cysteines, CxxC motif, and cis-proline residue. **B**: The electron density of the active site cystine is shown contoured at 1.4σ for the oxidized form of CLIC3. **C**: Alignment of human CLIC2 (blue), CLIC3 (green), and CLIC4 (pink) structures is shown. **D**: As in panel (B), the electron density of the active site cysteine is shown for the reduced state of CLIC3.

lies, the designation of the CLIC proteins as direct mediators of ion conductance has remained controversial. However, even the canonical GSTs have recently been found to have membrane-active properties. Morris et al. demonstrated that canonical GST proteins could rapidly transit from the extracellular milieu into the cell through an, as yet undefined, endocytotic mechanism.[30] Ten different proteins with the GST-fold were shown to be capable of rapid internalization into cells, with a CLIC member (CLIC2) having the highest rate of import. Thus, the GST-fold itself may function in some way as a membrane transduction domain.[30] These findings imply some inherent ability to associate with membranes in the GSTs, thus reducing the apparent evolutionary functional discontinuity between the CLIC-branch and the remainder of the superfamily.

In the enzymatic GSTs, the thioredoxin domain is primarily responsible for binding glutathione and the C-terminal domain the hydrophobic substrate. Unlike the GSTs, the CLICs show low affinities for glutathione under reducing conditions,[24] suggesting that the CLIC N-terminal domain has another function. The thioredoxin fold is also found in a number of other redox-related proteins such as thioredoxins, glutaredoxins,

disulfide bond oxidoreductases, and alkylhydroperoxidases.[31] Thus, it is intriguing that experiments suggest that the CLIC ion channel is regulated by redox status.[13] Like the thioredoxins and glutaredoxins, the CLIC N-terminal domain has an active cysteine that is capable of forming mixed disulfides. Moreover, the C-terminal substrate binding region of the CLICs appears large enough to accommodate peptide substrates raising the possibility that a redox-regulated protein interaction may form part of the channel forming process. Indeed, in CLIC4, it was shown that membrane translocation is abolished by mutating the putative substrate binding residues equivalent to Tyr-229 and Phe-109 in CLIC3.[32]

The topology of the thioredoxin fold consists of a four-stranded mixed β-sheet and three α-helices in the order βαβαββα. Together these elements result in a structure in which the four-stranded β-sheet has helices 1 and 3 running parallel to the strands on one face and helix 2 running perpendicular on the other. In our structure, due to crystal packing, we also observe the hexahistidine tag forming an outside β-strand next to β2 [Fig. 1(B)].

A central feature of the thioredoxin fold of the CLICs, the glutaredoxins and the noncanonical omega, tau, and beta classes of GST is a catalytic cysteine residue that can form a mixed disulfide with substrates. This cysteine is frequently contained within a CxxS or CxxC motif located at the N-terminus of helix 1 in the fold[31] [see Fig. 2(A)]. The remainder of the domain thereby serves to fine-tune the reactivity and enzymatic action of the cysteine toward the substrate. Most of the CLICs utilize a CPFS motif, but CLIC2 and CLIC3 are exceptional in that they contain the thioredoxin-like double cysteine motifs CPFC and CPSC, respectively. Two structures of CLIC3 were obtained from different crystals: one structure has electron density that indicates a predominantly oxidized CPSC motif [Fig. 2(B)] and the other a reduced motif [Fig. 2(D)]. Both CLIC3 crystals were grown from protein purified in the presence of reducing agent; however, the oxidized form of the protein nucleates more readily. In standard wells multilatticed crystals begin to grow after 5–14 days as the reducing agent dissipates, this is how the oxidized structure was obtained. If additional reducing agents (5 m*M* TCEP) are added the growth process is slowed while oxidizing agents (5 m*M* NiCl) accelerate it. To obtain a reduced CLIC3 structure, crystals were grown in a fresh drop seeded with crushed 20-day-old crystals. These reduced crystals grow only rarely but are well ordered, in contrast the standard oxidized crystals grow readily but are multilatticed and need to be collected on a microfocus beamline to target well-ordered subdomains. The electron density of the oxidized CLIC3 structure clearly shows a disulfide linkage between these two CxxC cysteines [2.0 Å between sulfhydryl groups, see Fig. 2(B)], a feature also observed in the CPFC motif of CLIC2.[10,33] In contrast the reduced structure shows distinctly separate electron density for the sulfhydryl groups and is analogous to the structure of the reduced CxxC motif of *Dm*CLIC[11] [3.3 Å between sulfhydryl groups, see Fig. 2(D)]. The differences between the two states are subtle and limited to a rotation of the His-21-Cys-22 peptide bond that allows the latter residue to move toward a relatively static Cys-25. Interestingly in both states Cys-22 is found within a relatively unusual region of Ramachandran space. It remains unclear if and under what circumstances this disulfide bond would be formed *in vivo* and how this oxidized form would affect the proteins function.

The environment immediately surrounding the active cysteine presumably also contributes to the recognition of any CLIC substrates/binding partners. Most CLIC family members have highly conserved active sites suggesting they would bind similar substrates. However, CLIC3 appears unique in that many of these putative active site residues differ. One site of note is within the active cysteine motif. The phenylalanine residue in the CPFS motif of the other family members results in a hydrophobic cleft near the active cysteine similar to that observed in the omega class GSTs, where it is involved in glutathione binding.[34] In the CLIC3 structure, a serine residue (Ser-24) replaces this conserved phenylalanine thereby yielding a more open and polar mouth to the active site with easier access to the active cysteine. This could indicate that CLIC3 has subtly different interaction partners to the other CLIC proteins.

A structured foot-loop

Additional structural differences exist between the human CLICs and other GST-superfamily members. One prominent feature is an insertion in the CLIC fold of ~15 residues within the loop that connects h5–h6. In the classic GSTs, this loop is 8–10 residues long and progresses straight from h5 into a conserved hydrophobic staple motif, which is important for folding.[19] The additional 15 CLIC residues are inserted between this motif and h5 where they form a "foot-loop" that projects away from the body of the molecule. This loop feature shows low-sequence conservation amongst the CLIC family, and has consequently been observed to differ in structure; despite this the CLIC foot-loops do display some common features such as the inclusion of a number of charged residues and an inherent degree of flexibility. Indeed, the foot-loop of CLIC4 is often disordered in crystal structures,[20,35] whereas those of CLIC1[24] or CLIC2[10] have been found to crystallize in multiple conformations.

In contrast to the other CLICs, the h5–h6 foot-loop insertion of CLIC3 forms a uniquely ordered and identical structure in both crystallographic subunits. After a proline-induced kink following h5, CLIC3 continues into a unique two-turn helix that extends outward. The backbone then loops back on itself to return to its origin near

the C-terminus of h5 [Fig. 1(D)]. This structure is stabilized by the formation of a small hydrophobic core that is absent in the more dynamic foot-loops of the other CLIC members. This hydrophobic core is comprised from the pyrrolidine ring of Pro-145 and the side chains of Leu-150 from within hF and Leu-156 from the returning loop. The orientation of hF is secured by an interaction between the helix and the remainder of the GST-fold through a hydrogen bond formed between His-148 from the N-terminus of the helix and Tyr-98 from h4b [see Fig. 1(D)].

This interaction between the foot-loop and helix 4 is interesting as the CLIC proteins generally have an S-bend that splits helix 4 into two halves. The dynamic formation and collapse of this disrupting bend may be utilized as a regulatory mechanism that alters the GST-like active site. The bistable nature of the helix is best illustrated for CLIC2 in which a structure solved at pH 8.0 shows h4 to be contiguous while it is split at pH 7.5[10] [see Fig. 2(C)]. This transition leaves h4b relatively static while h4a undergoes a 1.5 Å rigid-body shift toward its C-terminus. Within the S-bend of CLIC2, the transition to a single helix influences the position of residues within the middle of the bend rotating them into the mouth of the active site (Asn-106 in CLIC3). In CLIC3, if h4 were to collapse into a single helix then Tyr-98 would be expected to move along with the remainder of h4a. Its connection with the foot-loop may therefore suggest a connection point between these two dynamic parts of the molecule that could affect the GST-like substrate binding site. In turn, this could influence protein–protein interactions that may be part of the channel forming process.

Crystal packing and the GST-dimer

The glutathione transferases are physiologically dimeric enzymes in which residues from each subunit contribute to the two active sites. These GST dimers are arranged about a twofold axis such that helices h3 and h4 from each subunit align parallel to their counterparts forming a core part of the interaction interface. In contrast, the reduced soluble form of the CLIC proteins is monomeric; however, physiological stimuli may still promote oligomerization[12] and because of this possibility the packing interactions within CLIC crystals are often carefully analyzed.[11,35] In CLIC3, the two molecules within the asymmetric unit contact each other via the same face of the protein that would be expected to mediate a GST-like dimerization, albeit the arrangement of molecules is both rotated and offset with respect to that of the GSTs. A PISA analysis of the crystal interface indicates that it is polar and could conceivably be attained by chance.[36] In the absence of further evidence suggesting a CLIC3 dimerization mechanism, we consider this interface most likely to be purely induced by crystallization.

CONCLUSIONS

The soluble monomeric form of human CLIC3 is globally similar to those of other known CLIC family members. Uniquely, the CLIC3 GST-like fold includes a well-ordered foot-loop between h5 and h6, this forms a defined helical structure instead of the more flexible loops seen in other CLICs. Additionally, the CLIC3 active site is more open and polar than that of other family members due in part to the replacement of a conserved phenylalanine residue with Ser-24. The reactive cysteine within the active site is contained within a thioredoxin or glutaredoxin-like CxxC motif and, like CLIC2, the two cysteines within this motif are capable of forming an intramolecular disulfide bond. Cataloging these structural differences will help us understand how the CLIC proteins function and what makes each of the family members unique.

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