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Abstract

Ion channels are frequently organized in a modular fashion and consist of a membrane-embedded pore domain and a soluble regulatory domain. A similar organization is found for the CIC family of Cl- channels and transporters. Here, we describe the crystal structure of the cytoplasmic domain of CIC-0, the voltage-dependent Cl- channel from T. marmorata. The structure contains a folded core of two tightly interacting cystathionine beta-synthetase (CBS) subdomains. The two subdomains are connected by a 96 residue mobile linker that is disordered in the crystals. As revealed by analytical ultracentrifugation, the domains form dimers, thereby most likely extending the 2-fold symmetry of the transmembrane pore. The structure provides insight into the organization of the cytoplasmic domains within the CIC family and establishes a framework for guiding future investigations on regulatory mechanisms.
Crystal Structure of the Cytoplasmic Domain of the Chloride Channel CIC-0

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Summary

Ion channels are frequently organized in a modular fashion and consist of a membrane-embedded pore domain and a soluble regulatory domain. A similar organization is found for the CIC family of Cl− channels and transporters. Here, we describe the crystal structure of the cytoplasmic domain of CIC-0, the voltage-dependent Cl− channel from T. marmorata. The structure contains a folded core of two tightly interacting cystathionine β-synthetase (CBS) subdomains. The two subdomains are connected by a 96 residue mobile linker that is disordered in the crystals. As revealed by analytical ultracentrifugation, the domains form dimers, thereby most likely extending the 2-fold symmetry of the transmembrane pore. The structure provides insight into the organization of the cytoplasmic domains within the CIC family and establishes a framework for guiding future investigations on regulatory mechanisms.

Introduction

Ion channel proteins catalyze the flow of ions across cellular membranes, a mechanism that underlies many physiologically important processes. To achieve this, channels usually combine two important properties: selective ion conduction at high rates, and the ability to regulate the conduction in a process called gating (Hille, 2001). These proteins are therefore frequently organized in a modular fashion and consist of a transmembrane catalytic domain, which allows the diffusion of ions through an aqueous pore, and an additional regulatory domain that controls the opening and closing of the pore in response to external stimuli (Jiang et al., 2002). With the exception of voltage-sensing domains, most regulatory domains resemble soluble proteins facing either the cytoplasm or the extracellular solution (MacKinnon, 2004). These soluble domains are commonly found to specifically bind ligands, and thereby to activate or inhibit ion permeation by opening and closing of the pore (Hille, 2001).

An important class of ion channel proteins that exhibits this modular organization is the CIC family. The CIC channels constitute a large protein family of chloride (Cl−) channels and transporters that is ubiquitously expressed from bacteria to man (Jentsch et al., 2002). Nine isoforms of the family are expressed in human and serve as key players in various physiological processes. Mutations in those isoforms have been identified to cause familial diseases such as myotonias, nephropathies, and osteopetrosis (Jentsch et al., 2005). The CIC proteins are unique, as they are not related to any other class of ion transport proteins. Within this family the members are closely related in sequence and architecture. All CIC proteins contain a conserved membrane-inserted catalytic pore domain, which is, in many cases, followed by a large cytoplasmic domain (Figure 1A). The X-ray structure of the prokaryotic CIC homolog from E. coli (EcCIC) provides a structural framework for the membrane-inserted domain (Dutzler et al., 2002, 2003). The CIC proteins are homodimers of two identical subunits, and each subunit contains an ion translocation pore. The topology of the pore domain is complex (Figure 1A). Two roughly repeated halves span the membrane in opposite directions and thereby contribute residues from different parts of the protein to form a selectivity filter for chloride ions in the center of the membrane. This selectivity filter harbors closely spaced ion binding sites that bridge the intra- and extracellular solution. Although EcCIC shares the conserved membrane-embedded pore domain with other CIC family members, it lacks the cytosolic domain frequently found at their C terminus. This domain is present in all eukaryotic and some prokaryotic CIC proteins, regardless of whether they function as chloride-dependent ion channels or as secondary chloride transporters (Estevez and Jentsch, 2002). Up until now, there was no structural information of this regulatory domain available.

In vertebrates, the length of the cytoplasmic domain is variable and ranges from 160 to 315 amino acids. A common feature in all family members is the presence of two CBS subdomains, CBS1 and CBS2, each about 50 residues long (Estevez and Jentsch, 2002). These small, compact protein domains consist of a three-stranded β sheet and two α helices and were originally discovered in the enzyme cystathionine-β-synthetase (Bateman, 1997). The large size difference in the CIC C terminus between different family members results from a variable linker region (linker) between the two CBS domains and the sequence that follows CBS2 (C-peptide). The topological organization of the CIC subunit is schematically depicted in Figure 1A. The domains share the closest homology within the different branches of the CIC family. One important branch includes the chloride channels CIC-0, CIC-1, and CIC-2 and the kidney channels CIC-Ka and CIC-Kb (Jentsch et al., 2002). All family members of this branch reside in the plasma membrane of various tissues and function as gated chloride channels. Figure 1B shows a sequence alignment of the two closely related channels: CIC-0 from the torpedo electric ray, and CIC-1, a human channel that is expressed in skeletal muscle cells. The strong conservation within the CBS domains is evident from the alignment, although there are large differences in the linker and the C-peptide, both being significantly longer in CIC-1.

The role of the cytoplasmic domains for CIC function is still obscure, but several experiments suggest that
they are vital for proper functioning of the proteins (Ben- 
etts et al., 2005; Denton et al., 2003; Estevez and 
Jentsch, 2002). The ClC family members show a com- 
plex functional behavior, and this is underlined by the 

fact that a very similar protein architecture encodes 
both coupled Cl⁻ transporters and gated Cl⁻ channels. 
The Cl⁻ channels CIC-0 and CIC-1 are the best-charac-
terized members of the CIC family. Two modes of gating 
have been described for these proteins: gating of the in-
dividual pore of each subunit, and a common gating 
mechanism that opens and closes both pores at once 
(Accardi and Pusch, 2000; Chen, 2005; Miller, 1982).

While the gating of the individual pore occurs at the 
selectivity filter, the molecular mechanism underlying 
common gating is so far not known. Its large tempera-
ture dependence suggests substantial conformational 
changes, and mutational studies indicate an involve-
ment of the cytoplasmic domain (Estevez et al., 2004; 
Fong et al., 1998; Pusch et al., 1997). In that respect, 
the location of the domain in relation to the selectivity 
filter is intriguing. It directly follows the last helix of the 
pore domain, which itself contributes a coordinating 
side chain to a specific ion binding site in the selectivity 
filter (Figure 1A). This peculiar architecture immediately 
hints at a possible interaction with the selectivity filter, 
thus providing a possible mechanism by which the do-

mains can modulate the channel’s opening and closing 
(Dutzler et al., 2002).

In the absence of a structure, it is difficult to achieve 
detailed insight into the molecular mechanisms underly-
ing gating and into the role of the cytoplasmic domains 
for channel function. Here, we report the structure of 
the cytoplasmic domain of the Cl⁻ channel CIC-0 from 
Torpedo marmorata (Jentsch et al., 1990). The two CBS 
subdomains are found to interact tightly in a well-folded 
core. A 96 residue long mobile linker connecting the two 
subdomains, in contrast, is disordered in the crystals.

As revealed by analytical ultracentrifugation, the do-

mains form dimers in solution, thereby most likely ex-
tending the 2-fold symmetry of the transmembrane 
pore. To our knowledge, the structure describes for 
the first time the architecture of the soluble part of any 
member of the CIC family and thus makes an important 
contribution to the understanding of CIC function.

Results

Domain Architecture

To gain insight into the structural organization of the cy-
toplasmic domains of CIC channels, we have deter-
mined the structure of the domain of CIC-0 at 3.1 Å res-
olution by X-ray crystallography (Table 1; Figures S1A 
and S2A; see the Supplemental Data available with this 
article online). The construct containing residues 525– 
805 was expressed in E. coli as a soluble hexahistidine 
fusion protein. Limited proteolysis after purification not 
only removed the hexahistidine tag, but it also removed 
the residues of the C-peptide following CBS2. As con-
firmed by mass spectrometry, the crystallized construct 
consisted of the residues from 525 to 774 and included 
the N terminus that follows the R helix, both CBS subdo-

mains, and the connecting linker region (Figure 1). The 

crystal structure was determined by multiple isomor-
phous replacement, including the anomalous scattering 
contribution of a Pt and a Hg heavy metal derivative for 
phasing. The crystals were of space group P2₁3, with 
two copies of the CIC-0 domain in the asymmetric unit. 
Unexpectedly, there is no 2-fold relationship between 

Figure 1. Subunit Organization and Sequence Alignment

(A) Schematic overview of the CIC-0 subunit. The construction prin-
ciple is general for eukaryotic CIC family members. The α helices of 
the transmembrane pore domain are shown as yellow cylinders, and 
the approximate position of the membrane is shown as a gray box. 
The last, partly membrane-embedded α helix of the pore domain is 
labeled (R helix); the arrow points at the position of the residue in-
volved in ion binding. The cytoplasmic domain is shown attached 
to the pore, and the two CBS subdomains are depicted as blue 
and red spheres, respectively.

(B) Sequence alignment of the cytoplasmic domains of CIC-0 and 
CIC-1. Identical residues are highlighted in yellow, and similar resi-
dues are highlighted in green. Secondary structure and numbering 
(CIC-0) are indicated above and below the sequences, respectively. 
The R –helix, with the Cl⁻–coordinating tyrosin residue (arrow) pre-
ceding the domains, is included in the alignment. The first residue 
of the crystallized construct is highlighted (*). Amino acid sequences 
are CIC-0 T. marmorata (GenBank: X56758) and CIC-1 H. sapiens 
(GenBank: M97820).

they are vital for proper functioning of the proteins (Ben- 
netts et al., 2005; Denton et al., 2003; Estevez and 
Jentsch, 2002). The CIC family members show a com-
plex functional behavior, and this is underlined by the
interacting proteins found in the crystals, which would be anticipated from the symmetry of the transmembrane domain. The well-ordered core structures of the two proteins in the asymmetric unit are very similar, and there are no large differences apparent at this resolution.

Figure 2 shows the topology and the structure of the CIC-0 domain. The two CBS subdomains (CBS1, CBS2) are well defined in the electron density. As anticipated, they share the typical topology with other CBS domain-containing proteins (Miller et al., 2004; Zhang et al., 1999). As with those proteins, the two triangular-shaped subdomains are related by pseudo 2-fold symmetry and interact via the β-sheets formed by β-strands 2 and 3 (Figures 2B and 2C). The N terminus (after residue 536) preceding CBS1 forms a well-ordered loop that specifically interacts with residues on the surface of CBS2 (Figure 2C). As it is commonly observed in other proteins with the same fold, this interaction positions the N and C termini of the protein in close proximity. On the opposite side of the protein, 26 residues of the linker region (Figures 2B and 2C) are not well defined in the electron density. The residues form an α-helix followed by an extended loop, which resembles the N terminus in its interaction with CBS1 (Figure 2C). Additional weak electron density is found for residues at the N terminus that appear to interact in extended conformation with symmetry-related proteins in the crystal, and for several additional residues of the linker region (Figure S1B). The remainder of the linker between CBS1 and CBS2 lacks electron density and is therefore most likely unstructured. The fact that a large fraction of the protein is disordered is reflected in the high average B factor and the somewhat elevated R factors of the otherwise well-refined structure (Table 1).

The absence of well-ordered electron density in the linker motivated us to investigate its amino acid composition. Within the CIC family, this region shows large variation in length and only poor sequence conservation. When analyzing the CIC-0 domain with algorithms predicting the propensity of a sequence to form a well-ordered structure, we found a strong correlation between order in the crystal and the probability of being structured (Figure 2D) (Ward et al., 2004). The region of the CBS subdomains and the end of the linker have strong electron density and are predicted to be ordered. The unstructured part of the linker lacks electron density, and the C-peptide is found to be susceptible to proteolysis. Both regions show a high propensity for disorder (Figure 2D). A similar propensity distribution is found for CIC-1. This indicates that the absence of well-ordered electron density in the linker region is not an artifact of crystallization, but rather reflects the intrinsic flexibility of the domains.

### Oligomeric State in Solution

In the high-resolution structures of ion channel proteins and of their soluble domains, we generally observe two properties: the soluble domains are involved in mutual tight interactions and mirror both the symmetry and oligomeric states of the transmembrane channel domain (Brejc et al., 2001; Jiang et al., 2002; Nishida and Mackinnon, 2002; Zagotta et al., 2003). Neither of these two features were found in the CIC-0 domain crystals, in which the proteins appear to be monomeric, in contrast to the homodimeric architecture of the transmembrane CIC pore domain. We were therefore particularly interested to investigate whether the oligomeric state of the domains in the crystal corresponds to their state in solution and to their organization when attached to the channel.

The first hints of the domain size in solution came from gel filtration, which showed that the protein eluted with an apparent molecular weight of a dimer (~60 kDa). To quantitatively determine the oligomeric state of our construct in solution, we studied its sedimentation properties with analytical ultracentrifugation. The distribution of sedimentation coefficients obtained from sedimentation velocity experiments clearly shows a predominant peak with a maximum of 2.17 ± 0.02 S (SD averaged over six experiments) (Figure 3A). The corrected sedimentation coefficient (S_{20,w}) of 3.56 S corresponds well with the dimeric form of the domain. A second minor peak with about half the sedimentation coefficient most certainly represents a single subunit, possibly due to a slow monomer-dimer equilibrium (Figure 3A). The molecular weight of the domain was subsequently determined in a sedimentation equilibrium experiment (Figure 3B). Fitting the data to a single-species model (S_{20,w} value of 3.50 S; rms deviation of fit, 0.00606 ± 0.00026) resulted in a molecular weight of 55.3 kDa, which is in good agreement with the 55.5 kDa calculated for the dimer. The sedimentation data clearly show that the domains are dimers in solution, rather than monomers, as seen in the crystal. It is likely that the protein dissociated into monomers at the high salt concentration present during crystallization.

### Table 1. Data Collection and Model Refinement Statistics

<table>
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<td>50–3.4</td>
<td>50–3.7</td>
<td>50–4.1</td>
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<td>99.7</td>
<td>99.8</td>
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<tr>
<td>Rmsd bond angle (Å)</td>
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Values for the highest-resolution shell are given in parentheses. Native2 was used as the native data set for calculating initial phases. R_{sym} = \sum_i (-1)^i \langle |I_i| \rangle / \langle |I_i| \rangle, where |I_i| is the scaled intensity of the ith measurement and \langle |I_i| \rangle is the mean intensity for that reflection. R_{work} = \sum_i \langle |F_i| - |F_o|/\langle |F_i| \rangle, where |F_i| and |F_o| are the observed and calculated structure factor amplitudes, respectively. R_{free} was calculated by using a randomly selected 5% sample of the reflection data omitted from refinement.
Quaternary Structure

The oligomeric state of the cytoplasmic domains in solution suggests that the domains also tightly interact when attached to the transmembrane pore. Although the crystal structure does not reveal the interactions in the dimer, a possible mode of dimerization is found in the crystal structure of TM0935, a CBS domain-containing protein from *T. maritima* (Figure 4A) (Miller et al., 2004). Two intimately interacting subunits of this homologous protein are related by 2-fold symmetry. The extended dimer interface involves helices $a_1$ and $a_2$ of the four CBS subdomains in the crystal and buries more than 2000 Å$^2$ of the monomer surface. A dimer of two CIC-0 domains, which was generated by superposition with the protein from *T. maritima*, is shown in Figure 4A. The respective subunits of the two proteins superimpose with an rms deviation of 4.2 Å. The model shows good packing in the subunit-subunit interface without major clashes between protein residues. Despite the similarity of the two structures, it has to be pointed out that the two equivalent surfaces involved in dimerization differ in their distribution of hydrophobic and charged residues (Figure 4B).
While the dimer interface in the *T. maritima* protein is found to be predominantly hydrophobic, the equivalent surface in ClC-0 contains several charged side chains. If, despite the difference in the contact surface, the ClC-0 domains share the same mode of interaction, the entire channel could be assembled as shown in Figure 4C. The model emphasizes the size relationship between the transmembrane pore and the cytoplasmic domain. The surface of the domain in contact with the channel, however, remains ambiguous. A remarkable feature of the domain structure is the spatial proximity of the N and C termini. The position of the N terminus with respect to the pore is constrained by its attachment to the R helix. Consequently, CBS2 and the C terminus have to be closer to the pore region, while CBS1 is more remote (Figure 4C). This arrangement is interesting with regard to the influence of CBS2 and the C terminus on channel function (Estevez et al., 2004; Hebeisen and Fahlke, 2005).

**Discussion**

We have solved the structure of the cytoplasmic domain of the voltage-dependent Cl⁻ channel ClC-0 from the electric ray *T. maritima*. About two-thirds of the protein forms a well-folded core structure, and its two CBS subdomains interact tightly. In contrast to the folded core, the linker between the two CBS domains and the residues at the very C terminus, which constitute about one-third of the protein, are highly mobile and do not fold into a defined structure. In aqueous solution, the ClC-0 domains form dimers, which most likely extend the 2-fold symmetry of the transmembrane pore domain. The structure, which is conserved within the family, provides insight into the architecture of the water-soluble part of ClC channels and transporters. However, even with this first structure in hand, our understanding of how the cytoplasmic domains contribute to proper ClC function is far from complete. In the following paragraphs, we will discuss the currently available functional data in light of the structure.

After the first sequences of the ClC family became available, the region at the C terminus immediately came into focus for functional characterization (Jentsch et al., 1990). Several mutations in the domains have been identified in familial diseases, and the role of the cytoplasmic domains in ClC function has been studied by a combination of mutagenesis and electrophysiology (Jentsch et al., 2002). Maduke et al. (1998) used a split channel approach to investigate the role of the CBS domains for ClC-0 channel function. While channels lacking the domains were not functional when expressed in *Xenopus* oocytes, constructs in general showed wild-type-like behavior if the channel was split at a residue in the linker region between CBS1 and CBS2. A similar phenotype was also observed for the homologous muscle channel ClC-1 (Estevez et al., 2004; Schmidt-Rose and Jentsch, 1997). Interestingly, channel function was not affected by the removal of residues that were part of the disordered linker region. In contrast, no functional channels were obtained if the truncation was made in the structurally well-defined part of the linker region preceding CBS2 or within the CBS subdomains (Estevez et al., 2004). These observations are well explained by the structure, which shows a strong interaction between the two CBS subdomains. They also imply that the structured C-terminal part of the linker is necessary for assembly, while the largest part of the disordered region is not interacting with the folded core.

Mutations in the cytoplasmic domains have been shown to interfere with gating in ClC-0 and ClC-1, particularly the common gating. This regulatory process affects both subunits in concert and is still not well understood. The structure of the domains and their arrangement with respect to the transmembrane pore give several leads for interpretation of the available biochemical data. In general, most mutations that change gating are located within CBS2 and the C-peptide that follows CBS2 (Estevez et al., 2004). This is in agreement with the higher sequence conservation of CBS2 within the ClC family when compared to CBS1. The proximity of CBS2 and the C-peptide to the pore region (Figure 4C) points toward a possible interaction during the gating process. Several residues affecting gating in ClC-1 are...
located at the surface in a cleft between $\alpha$ helices 1 and 2 of CBS2 (Estevez et al., 2004). Interestingly, a cluster of residues in the same region of the more distantly related ClC-7 is found to be mutated in the bone remodeling disease osteopetrosis (Estevez and Jentsch, 2002). It is possibly not a coincidence that the mutations are in proximity to both the N and C termini of the domain. Interaction with the termini or the pore domain may thus prove to be important for regulatory processes.

Next to CBS2, residues of the C-peptide appear to play an important role in the gating process. A naturally occurring mutation of ClC-1 in the myotonic goat (A885P) concerns a residue that is located only 10 residues after CBS2 (Beck et al., 1996). This mutation and the corresponding mutation in ClC-0 both have a large effect on gating by shifting the voltage dependence of opening toward more positive voltages (Beck et al., 1996; Maduke et al., 1998). Moreover, several truncations of the C terminus of ClC-1 after CBS2 show large shifts in the open probability of the channels (Hebeisen and Fahike, 2005; Hryciw et al., 1998). The influence of the C-peptide on gating remains puzzling. In ClC-0, the C-peptide following CBS2 includes a region of 33 amino acids (Figure 2B). The C-peptides in both channels have an unusual amino acid composition and are predicted to be disordered (Figure 2D). The protein in our crystals did not include this region since it was found to be susceptible to proteolysis, and since other constructs that included the C-peptide failed to crystallize. The position of the C-peptide in the domain structure is found close to the proposed dimer interface; thus, a change in the sequence close to CBS2 could interfere with dimerization (Figures 4A and 4C). At present, we do not understand the mechanism by which the disordered C terminus affects gating. It is conceivable that the residues interact with the pore region, and that during gating the access of the C-peptide to the pore is modulated.

Soluble domains are frequently found in different ion channel families to regulate ion permeation in response to ligand binding. Recent studies suggest that the CBS domains of certain CIC family members bind ATP and thereby regulate ion permeation and transport (Bennetts et al., 2005; Scott et al., 2004; Vanoye and George, 2002). The measured binding affinity for the nucleotide is relatively low ($\sim 1$ mM). In an attempt to investigate the interaction of ligands with the CIC-0 domain, we studied ATP binding with radiolabeled nucleotides in equilibrium dialysis experiments, and we crystallized the domain in the presence of 10 mM ATP (data not shown). Both attempts failed to show binding, which could either

Figure 4. Model of the CIC-0 Domain in Its Context in the Channel

(A) Proposed quaternary structure of the domain. The dimeric crystal structure of the related protein TM0935 (T. maritima) viewed along the 2-fold axis (left) and a model of the CIC-0 dimer obtained from a least square fit of the two CBS subdomains onto the respective subunit of TM0935 (right) are shown as a Ca trace. The subunits are colored in red and blue for TM0935 and in green and blue for CIC-0, and the two halves of each subunit are shown in different shades of the same color.

(B) View on the dimer interface of a single subunit of TM0935 (left) and of the CIC-0 domain (right). The surface was calculated with MSMS (Banner et al., 1996). Residues at the surface are colored according to their physicochemical properties (acidic residues are in red, basic residues are in blue, hydrophobic residues are in yellow, and all other residues are in white).

(C) Model of the CIC-0 domains relative to the transmembrane pore. The views are from within the membrane—the extracellular side is on top, and the cytoplasm is on the bottom (left)—and from the cytoplasm (right). The structure of the EcCIC dimer (PDB code: 1OT5) serves as model for the pore domain. Both EcCIC and the dimeric CIC-0 domain are shown as ribbon models. The two subunits are colored in blue and green. The C terminus of the EcCIC subunit and the N and C termini of the CIC-0 domain are colored in red. The residues connecting the pore (R) and the cytoplasmic domain and the disordered C terminus are depicted by dashed, red lines. The disordered linker between two CBS subdomains is indicated by a dashed line in the respective color of the subunit. Ions bound to the selectivity filter within each subunit are shown as red spheres.
mean that the construct we used does not bind the ligand in the absence of the transmembrane domain, or, alternatively, that CIC-0 is not regulated by ATP. More experiments will be needed to clarify the interaction of ligands with the cytoplasmic domains of CIC channels and transporters.

In the CIC-0 domain, 96 residues of the linker region between the two CBS domains are found to be disordered in the crystal. The role of this linker for CIC function is currently unknown. Interestingly, disordered regions are frequently found in the sequences of eukaryotic proteins, while they are much less frequent in their homologs from prokaryotic organisms (Ward et al., 2004). It was proposed that these highly mobile loops are involved in the regulation of protein function by providing binding sites for kinases or other regulatory proteins (Dyson and Wright, 2005). Indeed, several studies suggested a regulation of CIC family members by kinases (Denton et al., 2005; Rosenbohm et al., 1999). It will be interesting to gain insight into the role of these disordered linker regions with respect to channel function from future studies.

It remains to be shown whether and how the cytoplasmic domains of CIC channels receive signals from the intracellular environment and how such signals are transmitted from the domains to the transmembrane pore to influence gating. A similar mechanism might also modulate function in the secondary active H⁺/Cl⁻ transporters within the family in a yet unknown way. The model shown in Figure 4C offers some hypotheses. The soluble domains are close to the transmembrane pore. A conformational change in the domains could thus trigger a rearrangement in the ion conduction path. Two possible ways to transduce a conformational change could be imagined: either via the N terminus of the domain, which is bound to a helix that contributes a residue to the selectivity filter, or through the C terminus in a way similar to inactivation processes seen in voltage-dependent K⁺ channels (Dutzler et al., 2002; Zhou et al., 2001). In order to gain further insight into the mechanism, we will need to see a structure of a channel with C-terminal domains attached.

The structure of the C-terminal domain of CIC-0 has closed a gap in our understanding of the structural organization of the CIC channels and transporters by outlining the molecular architecture of the soluble part of these physiologically important ion transport proteins. In combination with the multitude of biochemical data, the structure offers initial insight into the regulatory processes these domains confer. Although it will ultimately be necessary to reveal the relation of the domains with respect to the transmembrane pore, the structure of the isolated domain from CIC-0 already presents an important step in the elucidation of the molecular mechanisms of CIC function, and it provides a framework for guiding further experiments.

Experimental Procedures

Cloning and Expression

Residues 525–805 of CIC-0 from Torpedo marmorata (Jentsch et al., 1990) were cloned into the pET28b+ expression vector (Novagen) with a C-terminal thrombin cleavage site and a hexa-histidine tag. Transformed E. coli BL21 (DE3) cells were grown at 37°C in LB medium containing 50 mg/l kanamycin to an OD600 of 1.5. Expression was induced by the addition of 0.5 mM isopropyl-D-thiogalactopyranoside (IPTG) and proceeded overnight at 20°C. Harvested cells were lysed by sonication in 50 mM Tris-HCl (pH 7.5), 400 mM NaCl, 5 mM MgCl₂, 5 mM 3-mercapto-ethanol, 200 mg/l lysozyme, 20 mg/l DNase, leupeptin, pepstatin, and 1 mM phenylmethyl sulfonyl fluoride. The lysate was cleared by centrifugation and loaded onto a Ni-affinity column (Chelating Sepharose, Pharmacia Biotech). The column was washed with buffer containing 15 mM imidazole, and the pure protein was eluted with 300 mM imidazole. The protein was dialyzed into gel-filtration buffer and digested with thrombin until protease cleavage was complete. After concentrating, the protein was run on a Superdex 200 column in 10 mM Tris-HCl (pH 7.5), 400 mM NaCl, 5 mM MgCl₂, 5 mM 3-mercapto-ethanol. Pooled peak fractions were concentrated to a protein concentration of 20–30 mg/ml.

Crystal Preparation

CIC-0 domain crystals were grown in sitting drops at 4°C by equilibrating a 1:1 mixture of protein and reservoir solution against the reservoir. The reservoir solution contained 3.25–3.5 M sodium formate. The crystal for the best native data set was grown in the same conditions with additional 10 mM MgCl₂. Crystals were in space group P2₁2₁2₁ (a = b = c = 126 Å, α = β = γ = 90°), with two copies of the domain in the asymmetric unit. Heavy metal derivatives were obtained by soaking native crystals for 20 hr in mother-liquor containing either 1 mM methyl mercury chloride or 1 mM Pt(II)-terpyridine chloride (Aldrich). Crystals were prepared for cryocrystallography by successive transfer into higher concentrations of sodium formate up to a final concentration of 4.2 M and were subsequently flash frozen in liquid propane and stored in liquid nitrogen. For the analysis of the protein after crystallization, a crystal was washed twice in mother liquid and submitted to MALDI-TOF mass spectrometry.

Structure Determination and Modeling

Data were collected on frozen crystals on a Mar CCD detector at the microdiffrafractometer at the X06SA beamline at the Swiss Light Source (SLS) of the Paul Scherrer Institute (PSI). For collecting anomalous differences, the wavelength was set to 1.008 Å and to 1.072 Å for the Hg and the Pt derivatives, respectively. The data were indexed and integrated with DENZO and SCALEPACK (Otwinowski and Minor, 1997) and were further processed with CCP4 programs (CCP4, 1994). Heavy metal positions for the first derivative were determined by a Patterson search with RSPS (CCP4, 1994), and, for the second derivative, positions were determined by difference Fourier methods (CCP4, 1994). Heavy atom positions were refined, and phases were calculated by using SHARP (dela Fortelle and Bricogne, 1997). Initial phases were improved by solvent flattening with SOLOMON (Abrahams and Leslie, 1998), and by 2-fold non-crystallographic symmetry (NCS) averaging with DM (Cowtan, 1994). A model containing residues 536–602 and 696–770 was built into the electron density with the program O (Jones et al., 1991). Refinement was carried out by several cycles of simulated annealing in CNS (Brünger et al., 1998) alternated with inspection and manual rebuilding with O. Two-fold NCS constraints were used in the initial stages of the refinement. In later stages, the strict constraints between the two copies in the symmetry unit were loosened, and restraint individual B factors were refined. R_meas (calculated from 5% of the reflections omitted during refinement) was monitored throughout. Residual weak electron density in a 2Fₐ – Fᵦ map allowed for tracing of the backbone of several additional residues of the N terminus and the linker region of the molecule (Figure S1B). Including those residues, which are found to interact with symmetry-related molecules, in the model had little influence on the statistics. The final model contains 2472 atoms with good geometry and no outliers in the Ramachandran plot. The slightly elevated R factors for this resolution (R = 27.3/Rfree = 30.9) and the high B factors are probably due to the large solvent content. The dimer of the CIC-0 domain was generated by fitting the subunit to each chain of the dimeric CBS domain-containing protein from T. maritima (Tm0935, PDB code: 1o50) by using the program O. A total of 81 Cα atoms were found to superimpose with a rms deviation of 4.22 Å. Analysis of the sequence with respect to its...
propensity to form structure was performed with the program DRIPRED (http://www.sbc.su.se/~macallr/disorder/).

Analytical Ultracentrifugation
Experiments were performed with a Beckman XL-I analytical ultracentrifuge with an An 50-Ti rotor at 4°C. For the sedimentation velocity experiments, 12 mm epon double-sector cells were filled with 400 μl 150 mM NaCl, 10 mM Tris (pH 7.5), 5 mM MgCl₂, 5 mM β-mercapto-ethanol, and protein sample. Sedimentation velocity experiments were performed at protein concentrations of 18, 35, and 75 μM. Data were acquired at 280 nm in continuous scan mode in 0.003 cm intervals at a rotor speed of 42,000 rpm. The data analysis was performed with the C(s) module of Sedfit (Schuck, 2000; Schuck et al., 2002). The buffer parameters, partial-specific volume of the protein, and the corrected S₀20,w were calculated by using Sednterp, partial-specific volume of the protein, and the corrected S₀20,w were calculated by using Sednterp (Lauje et al., 1992). Sedimentation equilibrium experiments were conducted at protein concentrations of 18 and 75 μM at rotor speeds of 19,000, 23,000, and 27,000 rpm, 4°C. Data were modeled with the program Sedphat (Vistica et al., 2004).

Supplemental Data
Supplemental Data including stereoviews of the electron density and of the asymmetric unit are available at http://www.structure.org/cgi/content/full/14/2/299/DC1/.

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Coordinates and structure factors have been deposited with the Protein Data Bank with accession code 2D4Z.