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A structural perspective on copper uptake in eukaryotes

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Abstract Over a decade ago, genetic studies identified a family of small integral membrane proteins, commonly referred to as copper transporters (CTRs) that are both required and sufficient for cellular copper uptake in a yeast genetic complementation assay. We recently used electron crystallography to determine a projection density map of the human high affinity transporter hCTR1 embedded into a lipid bilayer. At 6 Å resolution, this first glimpse of the structure revealed that hCTR1 is trimeric and possesses the type of radial symmetry that traditionally has been associated with the structure of certain ion channels such as potassium or gap junction channels. Representative for this particular type of architecture, a region of low protein density at the center of the trimer is consistent with the existence of a copper permeable pore along the center three-fold axis of the trimer. In this contribution, we will briefly discuss how recent structure–function studies correlate with the projection density map, and provide a perspective with respect to the cellular uptake of other transition metals.

Keywords Copper · Channel · Transporter · Structure · cryoEM

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Introduction

Copper ions are essential because their coordination and redox properties allow them to participate in important metabolic reactions such as the destruction of reactive oxygen species, the synthesis of ATP, the neurotransmitter dopamine, and connective tissue. However, the same chemical reactivity that is harnessed by enzymes, such as superoxide dismutase, also represents a potential biohazard and is the reason why cellular copper status is tightly regulated by cellular transporters and an array of intracellular copper binding chaperones (Fig. 1) (for reviews Huffman and O'Halloran 2001; Puig and Thiele 2002; Rosenzweig 2002; Rees and Thiele 2004). Traditionally, copper research has taken place in the wake of efforts to understand the biochemistry and pathology of superoxide dismutase. However, the discovery that mutations in copper transporting ATPases cause Wilson's and Menkes disease, emerging links between aberrations in copper metabolism and neurodegenerative diseases, and the recent finding that platinum-based

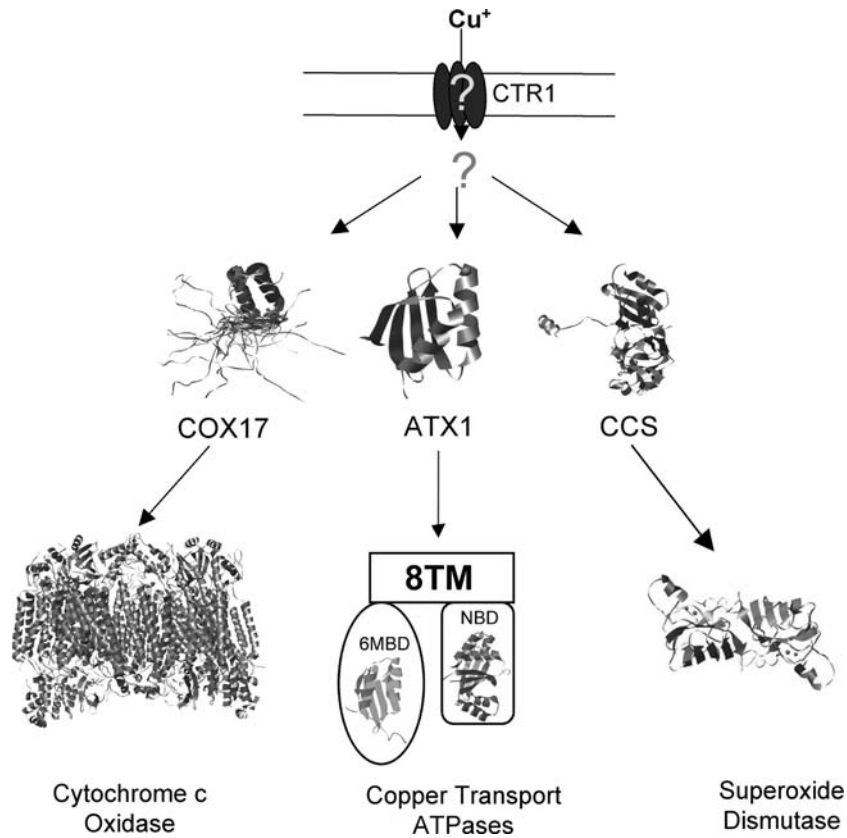

Fig. 1 Structural perspective on copper homeostasis. The figure gives a simplified view of what is known about the structures of proteins involved in copper homeostasis and emphasizes how gaining insights about the detailed structures of copper transporting membrane proteins is essential to complete the emerging picture. After cellular uptake, involving CTR-proteins and possibly reductases, Cu(I)-ions are passed on to cytosolic chaperones by mechanisms that have not yet been firmly established. After loading, each of the specific chaperones delivers Cu(I)-ions to the active site of its target enzyme through a ''handshake'' mechanism. Note, the Cox17/Cytc Oxidase-branch is still subject to debate and thus only a snapshot of this branch is shown. PDB accession codes for (domain) structures used: 1U96 (Cox17), 1CC8 (Atx1), 1JK9 (CCS), 10CC (CytC oxidase), 1KVJ (first metal binding domain of Menkes ATPase), 2ARF (Wilson disease protein N-domain), and 1SDY (superoxide dismutase). TM: transmembrane α-helices

chemotherapeutics hijack cellular copper uptake transporters to cross the plasma membrane have turned the study of copper homeostasis into an independent and exciting field of investigation (for reviews Waggoner et al. 1999; Mercer 2001; Cox and Moore 2002; Llanos and Mercer 2002; Brewer 2003; Safaei and Howell 2005).

In contrast to the biology of copper binding chaperones and their target enzymes, the mechanisms of copper translocation across cellular membranes remain largely unknown (Fig. 1) (Djinovic et al. 1991; Tsukihara et al. 1996; Rosenzweig et al. 1999; Lamb et al. 2001; Abajian et al. 2004; Dmitriev et al. 2006). This lack of knowledge is due to the difficulties that are associated with determining the structures of integral membrane proteins, and the fact that no in vitro systems have been devised to date to systematically study copper transport across membranes. Consequently, the majority of what is known about copper transport—cellular copper acquisition in particular—is based on genetic complementation strategies and measurements of copper transport in vivo (Dancis et al. 1994b; Lee et al. 2002; Eisses and Kaplan 2005) (for reviews Lutsenko et al. 2002; Voskoboinik and Camakaris 2002; Rees and Thiele 2004). Over the course of one and a half decades, these

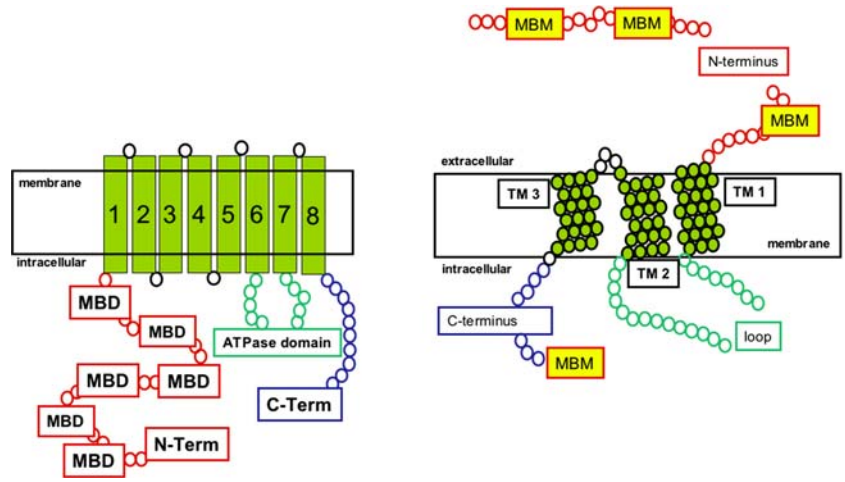

Fig. 2 Domain organization and topology of copper transporters. Genetic studies have established two fundamentally different families of copper transporting membrane proteins: copper transporting ATPases, ATP7A and ATP7B, whose overall design is shown to the left, are involved in copper sequestration and secretion from the cell, while the ATP-independent CTR-proteins, for which a bubble-diagram is shown to the right, are involved in copper uptake. Proteins in each family can be subdivided into four functionally distinct subdomains (color coded: N-terminus = red; membrane embedded domain = lime; intracellular loop/ATPase = green; C-terminus = blue). The N-termini in both families contain either metal binding domains (MBD) or metal binding motifs (MBM). Similarly, the membrane embedded domains are involved in copper translocation. However, the mechanism of copper movement is quite different, presumably occurring within the monomer and depending on ATP-hydrolysis in the case of the ATPases, while being nucleotide independent and presumably occurring at the central interface between subunits in CTR-trimers. The function, if any, of the intracellular loop of CTR-proteins is unknown. However, the short loop connecting TM2 and 3 is essential for function and enforces a very tight spatial relationship between these two membrane-spanning segments at the extracellular side of the membrane. Black circles in the diagram for the ATPases represent the connectivity between helices, not the actual number of residues. While the N- and C-termini as well as the ATPase domain of the transport ATPases are always facing the cytoplasm, the ''outer'' side of the protein can face different environments depending on subcellular localization

approaches have identified two fundamentally different families of transporters that are involved in copper shuttling across the plasma membrane and copper sequestration into intracellular compartments (Fig. 2). Of these two families, the copper transporting ATPases that catalyze copper sequestration/secretion have been studied more extensively than the ATP-independent transporters involved in cellular copper uptake, partially because of their direct link to Menkes and Wilson's disease, but also because easily discernible nucleotide and metal binding domains provide reasonable templates for both biochemical and limited structural analysis (for reviews Lutsenko et al. 2002; Voskoboinik and Camakaris 2002). However, the recent finding that both the yeast and human high-affinity copper transporters, yCTR1 and hCTR1, are able to mediate cellular uptake of platinum-based chemotherapeutics cast a spotlight on this family of copper transporters, emphasizing their importance for both basic cellular physiology and therapeutic intervention (Ishida et al. 2002; Lin et al. 2002; Holzer et al. 2004; for review Safaei and Howell 2005). The purpose of this brief review is to use the recently published projection density map of hCTR1 as guide for putting into perspective what is known about structure–function relationships of the membrane embedded domain of CTR-proteins and to create a framework for future biochemical studies of the detailed mechanism of copper and cisplatin transport by CTR-proteins.

General molecular design of CTR-proteins

While the link to cancer greatly increased interest in CTR-proteins, their primary amino acid

sequences provide little to no clues how these transporters accomplish copper and/or cisplatin translocation across the membrane. For instance, CTR-proteins vary in length from ~150 residues to almost 500 amino acid residues suggesting that some aspects of copper transport and/or transporter regulation may differ between different family members. In support of this view, both hCTR1 and yCTR1 are proteolyzed and/or cleared from the cell surface in response to a sudden increase of extracellular copper in certain cell types, while no such behavior has been observed for other CTRs, such as yCTR3 (Ooi et al. 1996; Peña et al. 2000; Klomp et al. 2002; Petris et al. 2003; Guo et al. 2004). Yet despite these functional differences, the large differences in molecular size, and a lack of extensive sequence conservation, CTR-proteins seem to share a global overall design that subdivides each transporter into four domains: (1) an extracellular N-terminal domain, (2) an intracellular loop of variable length, connecting the first and second putative membrane-spanning helices, (3) a membrane embedded domain predicted to have 3 transmembrane helices, and (4) an intracellular C-terminal domain of variable length.

Of these four domains, contributions of the extracellular N-terminus and the intracellular C-terminus to copper uptake, and CTR turnover have been studied the most, possibly because clustering of metal binding amino acid residues strongly suggested that these domains/residues play an important role in the function of CTR. Indeed, mutagenesis studies of what have become known as ''Mets-motifs'' in the N-terminus have established that one of these motifs plays a pivotal role in the mechanism of copper uptake (Puig et al. 2002; Guo et al. 2004; Eisses and Kaplan 2005; Beaudoin et al. 2006). Similarly, a contribution to copper transport of a cluster of metal binding residues at the very C-terminus has also been recently demonstrated (Eisses and Kaplan 2005), as was a direct transfer of Cu(I) from the C-terminal domain of yCTR1 to the yeast copper binding chaperone ATX1 (Xiao and Wedd 2002; Xiao et al. 2004). In contrast to this body of knowledge, the role—if any—of the intracellular loop in copper uptake is unknown, and only few studies have addressed the contribution of the membrane embedded domain to copper transport. This scarcity of information about the membrane embedded domain may in part be due to the lack of clear sequence conservation (Fig. 3), which makes it difficult to guess what residues may be important determinants for copper uptake. The sole exception is what could be considered to represent a CTR-signature motif MxxxM-X_{12}-GxxxG, which correlates a strictly conserved MxxxM-motif (MM4-motif) at the extracellular end of the second transmembrane α-helix (TM2) with an almost entirely conserved GxxxG-motif (GG4-motif) in the center of the third membrane-spanning α-helix (TM3). The stunning degree to which the MM4- and GG4-motifs are conserved above what otherwise appears an almost random assembly of residues (see Fig. 3) suggests that these two motifs and hence both TM2 and TM3 contribute to the copper permeable pore and/or the mechanism of copper translocation. Indeed, mutational analysis of the MM4-motif showed the metal binding properties of residues in these

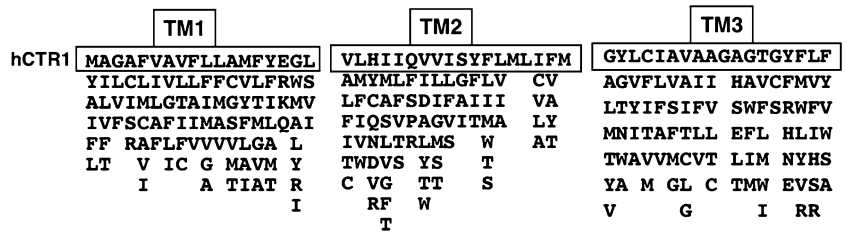

Fig. 3 CTR TM-segments are poorly conserved. Using the sequence of hCTR1 as reference, this figure illustrates which amino acid residues are found in the corresponding position of the putative TM-segments in other CTR-proteins. This listing does not include sequence data from recently identified CTR-proteins, but still emphasizes how divergent the sequences of their membrane embedded domains are. The MxxxM- and GxxxG-motifs in TM2 and TM3 are the most highly conserved motifs in the family and establish its MxxxM-X_{12}-GxxxG signature

positions to be important but not essential for copper transport by all CTRs (Puig et al. 2002; Eisses and Kaplan 2005). Similarly, studies of other residues in TM1, TM2, TM3 provided further evidence for the critical roles these helices play in CTR structure and function (Peña et al. 2000; Aller et al. 2004; Eisses and Kaplan 2005; Beaudoin et al. 2006; De Feo et al., manuscript in preparation). Interestingly, however, calculations by Spencer and Rees illustrated that a bundle of three ideal transmembrane helices cannot generate a pore large enough to allow passage of ions (Spencer and Rees 2002). As an immediate implication and without drawing on any biochemical data, these model calculations predict that CTRs need to oligomerize in order to function in copper uptake. In support of this notion, several groups demonstrated that oligomerization of CTR-proteins does occur in vivo and in vitro (Dancis et al. 1994a; Peña et al. 2000; Zhou and Thiele 2001; Eisses and Kaplan 2002; Lee et al. 2002; Puig et al. 2002; Klomp et al. 2003; Aller et al. 2004). Based on these studies, a trimeric organization appeared to be the most likely, but other models have also been proposed (Klomp et al. 2003). Our recent projection density map of hCTR1 provided a definitive answer to this question by unambiguously showing that hCTR1 exists as a trimer in the membrane (Fig. 4) (Aller and Unger 2006). The observed trimeric arrangement

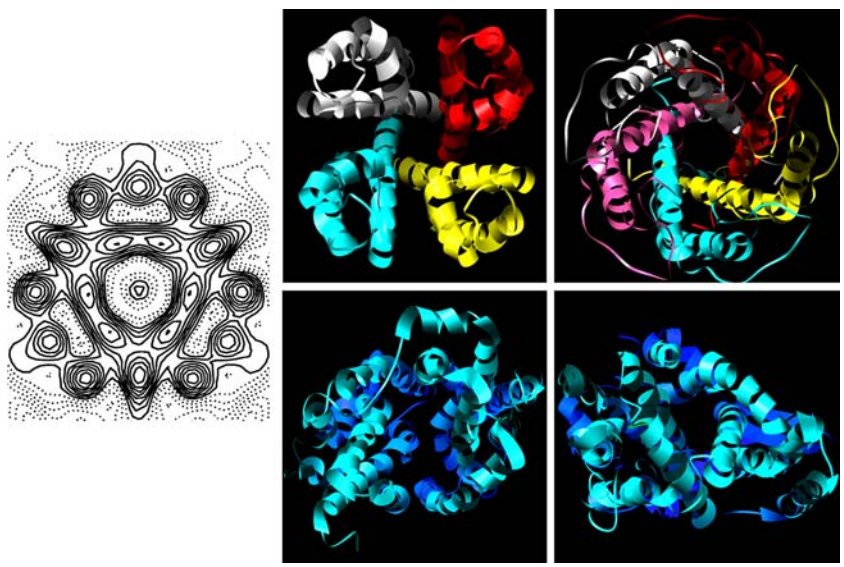

Fig. 4 hCTR1—Is it a ''Trannel''? The recently solved projection density map of a double-layered 2D-crystal of membrane embedded hCTR1 is shown to the left. Black solid lines represent protein related densities. Lipid, regions of low protein density, and/or aqueous pores are shown by dotted lines. Note, the double-layered nature of the crystals makes the projection a complicated superposition of two hCTR1 trimers that are stacked on top of each other in the two adjacent layers of the crystal. Therefore, no unambiguous assignment can be made which one of the strong circular features along the outer perimeter of the molecule belongs to which one of the two trimers. However, the projection clearly shows that regions of low densities are aligned with each other in the two stacked trimers—presumably indicating the location, but not the precise shape, of a copper permeable pore. For comparison, ribbon diagrams for the KcsA potassium and mechanosensitive channels are shown in the top row (PDB accession codes: 1BL8 and 1MSL, respectively). Individual subunits of the channels are colored differently, emphasizing that in these channels, ion conductive pores are formed at the interface between subunits. Shown in the bottom row are ribbon diagrams of lac-permease and the Na^+/H^+-antiporter NhaA (PDB accession codes: 2CFQ and 1ZCD, respectively) that represent ''traditional'' transporters. Based on this comparison, the overall architecture of hCTR1 seems more closely related to that of those ''traditional'' ion channels in which a pore is formed at the center of a radially symmetric oligomer. Nevertheless, the potential interplay of hCTR1 with metal reductases and/or intracellular copper chaperons mechanistically may place CTR-proteins closer to ''traditional'' transporters. Thus, based on the projection alone it cannot be decided whether CTRs function as transporters, channels or a hybrid combining aspects of both classes of membrane proteins

of subunits, containing three putative transmembrane helices each, certainly provides a sufficiently large number of potentially pore forming helices. However, ever since the structure of the bacterial multidrug transporter EmrE was solved, it has become clear that oligomericity is not necessarily correlated with symmetry because in EmrE a path for hydrophobic substrates is formed along an asymmetric interface between the two subunits of the dimeric complex (Ubarretxena-Belandia et al. 2003). Interestingly, the first glimpse of the CTR1 structure reveals a symmetric trimer whose radial symmetry places CTR-proteins closer to the known structures of ion channels like the KcsA potassium channel than to the structures of traditional transporters, such as lac-permease for instance (Fig. 4) (Doyle et al. 1998; Chang et al. 1998; Hunte et al. 2005; Mirza et al. 2006). Putting this design into context and remembering that three ideal α-helices cannot form a functional pore, the projection map of hCTR1 indicates that more than one of the membrane-spanning α-helices must contribute to the copper-conducting pathway. While the signature motif bearing TM2 and TM3 make for promising candidates, it cannot be ruled out categorically that the first membrane-spanning α-helix, TM1, also plays a role in organizing the copper conduction pathway through the membrane, and simple steric considerations would argue that contributions of any of the helices are not evenly distributed over the entire length of the pore because a barrel shaped pore that is equally lined by nine TM segments would be too large to be specific for copper. The remainder of this review will briefly discuss what is known about structure–function relationships of the membrane embedded domain of CTR-proteins and how its design relates to the structure and function of other channel and transporter proteins.

Unity despite diversity?

With exception of the MxxxM-X_{12}-GxxxG signature motif, primary sequences of CTR-proteins are poorly conserved. Specifically, <10% of the residues that constitute the membrane embedded domain are strictly conserved throughout the entire family. Per se, low degrees of sequence identity are not unheard of in the realm of membrane proteins because the large lipid-exposed surface areas of membrane proteins are not subject to selection pressure other than to retain hydrophobic residues. However, while a low degree of conservation can easily be tolerated in large helical bundles (e.g., >5 helices) where only a small number of helical packing contacts and functional residues need to be conserved, CTR-proteins need to fold into stable monomers, need to assemble into trimers and at the same time, need to support a potentially gated/regulated copper conduit through the membrane. In sight of these multiple layers of architectural and functional constraints, the observed low degree of sequence identity is suggestive of two hypotheses: (1) while maintaining the same overall fold/organization, different CTRs have evolved locally different solutions to allow folding, oligomerization and copper transport and (2) there has to be a (partial) overlap in functionality for certain residues, i.e., one would expect some residues to be equally important for establishing CTR-architecture, while at the same time contributing directly to copper transport and its regulation in the context of a ''transport cycle.'' While plausible, experimental proof for the first hypothesis has not yet been published. However, recent work in our lab suggests that this hypothesis is likely to hold (De Feo et al., manuscript in preparation), suggesting that mechanistic details of CTR-function may vary within the family. In contrast, mutational studies of TM2 and TM3 provide some evidence in support of the second hypothesis that certain residues are subject to ''multitasking.''

Does the highly conserved GG4-motif play multiple roles in CTR structure and function?

Previous studies from our lab established that two opposing faces of TM3 are sensitive to the insertion of the large side chain of tryptophan (Aller et al. 2004). While the observed phenotypes differed in severity, disruption of the GG4-motif by Trp-insertions was not tolerated at all,

consistent with the role for GG4 motifs in close helix packing (MacKenzie et al. 1997; Senes et al. 2001). Even less drastic replacements, such as introduction of leucine, resulted in noticeable defects. However, in these cases phenotypes varied depending on which glycine was replaced and whether the replacement was for hCTR1 or yCTR3. For instance, a G171L substitution resulted in only a partial loss of function in hCTR1 while the corresponding G206L mutation in yCTR3 was non-functional in a yeast genetic complementation assay. This latter observation reinforces the idea that despite their overall similarity, each CTR has converged on a slightly different solution to accommodate requirements for folding, assembly and copper transport. Yet, the overall picture that emerged from this study is that the position of the first Gly, which is universally conserved within the CTR-family, was the most constrained. Further examination of TM3 identifies that this glycine occupies the middle position of an extended glycine motif that Bowie and coworkers termed the ''glycine zipper motif'' (Kim et al. 2005). However, in contrast to a perfect glycine zipper, GxxxGxxxG, which was found to be one of the most abundant triplet sequence motifs in predicted transmembrane domains, hCTR1 features a serine residue preceding the first glycine, creating a modified version that nevertheless conforms to the general consensus of small side chains every four residues, as defined by Bowie and colleagues (Senes et al. 2000; Kim et al. 2005). Interestingly, in channel proteins glycine zippers often are responsible for driving helix packing around the pore and can partake in conformational changes (Kim et al. 2005; Edwards et al. 2005). However, in some channel proteins the motif is important for packing helices that are not involved in pore formation (Fig. 5) (Chang et al. 1998; Murata et al. 2000).

Keeping the concept of glycine zippers in mind, it is interesting to note that in hCTR1 even the middle glycine in the zipper motif can be replaced by alanine or serine, but not cysteine without completely disrupting hCTR1 function (unpublished). While not completely at odds with the role of glycine zippers in the close packing of

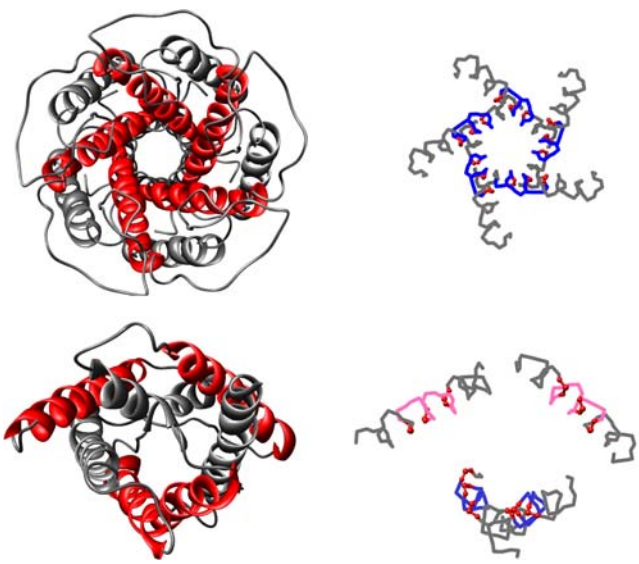

Fig. 5 Role of glycine zippers in the structure of membrane proteins. Using the mechanosensitive channel and aquaporins as examples, this figure illustrates how glycine zippers can play different roles in the structures of membrane proteins. In the structure of MscL (top, PDB access code: 1MSL), the glycine zippers pack against the backside of the same helix from an adjacent monomer to help create the pore. In the structure of aquaporin 1 (bottom, PDB access code: 1FQY), glycine zippers are used to stabilize packing of helices within the monomer. The pink and blue indicate the motifs from the pseudo-symmetric related helices in aquaporin, or from equivalent TM from different monomers for MscL. Actual glycine zipper residues are highlighted in red

transmembrane helices, the easiest explanation for why even the least malleable position in the glycine zippers of CTRs does tolerate introduction of some steric bulk would be to postulate that the glycines are turned inward, pointing towards the center three-fold axis of the trimer. Such a design would still aid close helix packing, but more importantly, it would be the combination of close packing and the absence of large side chains that could create the narrow constriction needed to form a copper selective pore. This idea is illustrated in Fig. 6. Alternatively, and similar to the role of glycines in the structure of the pore helix in the potassium channels KcsA, the glycines could serve as hinges for conformational changes that lead to the opening and closing of an intermittent pore allowing the coordinated passage of copper in the context of a more complex transport cycle (Jiang et al. 2002). Notably, either of these two models predict a dual role for the residues in the CTR glycine zipper: (1) support of trimer assembly, and (2) active participation in copper translocation.

While attractive, the model shown in Fig. 6 seems to contradict what was mentioned earlier on, i.e., that three ideal α-helices cannot form a pore large enough to pass ions. The only escape from this apparent conflict is to assume that TM3 does not adopt ideal helical geometry. Granted, this argument is somewhat hand waiving, but there are two additional considerations that could support the idea of why CTRs may get away with an ''unorthodox'' design of their selectivity filter and translocation pathway. First, as shown in Fig. 6, the model depicts only the central part of TM3 and therefore neglects the almost certain contribution of TM2 and its MM4-motif to the entrance of the pore at the extracellular side of the membrane (thus increasing the number of helices lining the pore in this part of CTRs). Second, copper ions are much smaller than, for example, potassium or sodium ions. And consequently, ''less protein components'' are required to create an exquisitely small pore. Moreover, TM3 of all CTR-proteins contain a cluster of large and often aromatic side chains in the last

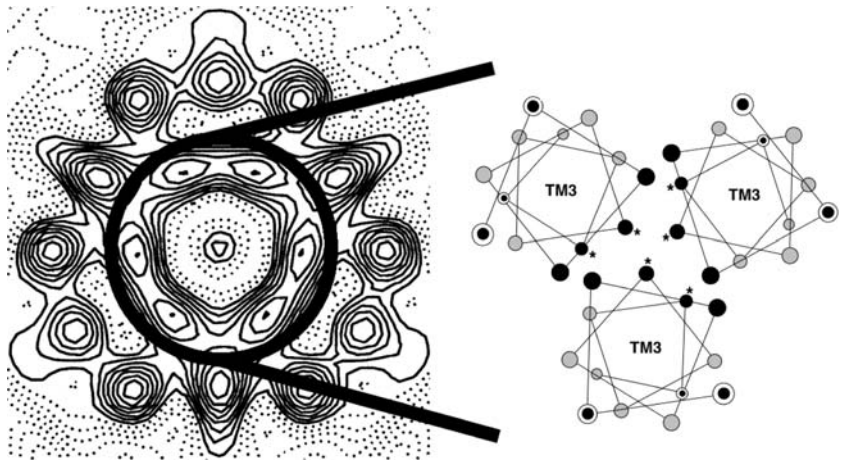

Fig. 6 Hypothetical model for the contribution of TM3 to the pore region of hCTR1. The figure illustrates how the glycine zipper found in TM3 of all CTR-proteins could contribute to the formation of a copper permeable pore. Shown are the projection density map of hCTR1 (left) and helical wheel presentations for the central part of the three TM3-segments from one of the two, stacked trimers (right). Black circles indicate positions that did not tolerate insertion of a bulky tryptophan. These positions included the highly conserved glycine residues of the GG4-motif (marked by *). Black dots with white rim indicate positions that were somewhat sensitive to the introduction of bulky Trp-residues, while insertion of Trp into the positions marked by grey circles did not cause any noticeable phenotypes. Note, this model is meant only to illustrate the basic idea of how the GG4-motif at the center of TM3 could facilitate both close helical packing and pore formation, it does not claim, exclusivity (other models are equally possible based on biochemical data), or accuracy and is grossly oversimplified. For simplicity, the model is drawn for only one of the stacked trimers, while the area circled in the projection density map contains contributions from both trimers (=presumably, there is no 1:1 correlation between the three TM3 segments and the 6 peaks that are visible within the circled region)

predicted 1.5 turns of this membrane-spanning segment. This is of potential interest because the structure of lac-permease illustrates how aromatic residues that point at neighboring helices can cause very significant bends in the helical backbone of the adjacent membrane helix (Abramson et al. 2003). If a similar design were at work in CTR-proteins, then the presence of several aromatic side chains could add sufficient bulk to keep the three TM3 segments spaced apart just far enough to create a copper permeable exit path from the membrane. Other than for steric reasons, the partial negative charges of the aromatic side chains also have the potential to create a suitable environment to ''solvate'' copper ions within the pore. While these postulates could explain the formation of a minimal copper-conducting pore, they do not explain how yCTR1 and hCTR1 can facilitate uptake of cisplatin, which structurally is much larger than copper ions. The forthcoming intermediate resolution 3D-structure of hCTR1 as well as ongoing experiments in our lab, testing this idea, will shed more light on this important aspect of CTR structure and function.

What is the mechanism of copper transport by CTR-proteins?

This is one of the remaining key questions in CTR biology, and despite the exciting progress towards determining the structure of hCTR1, the answer cannot come from just looking at the structure alone. Had the projection structure been solved in the late 1990-ties, spotting radial symmetry most certainly would have biased ones thoughts towards assuming that CTR-proteins function as copper channels. However, steady progress in the determination of membrane protein structures have begun to erode this seemingly clear-cut picture to the point that it has become impossible to make mechanistic assignments based on structure. To understand this one only has to look at ''non-traditional channels'' like aquaporins, which are passive channels that facilitate movement of water across biological membranes. At the level of quaternary structure, aquaporins are tetrameric, just like potassium channels. However, water-conducting pores lie within the four independent monomers, each of which has six transmembrane helices and a seventh, split helix that is formed by two reentrant loops carrying the family's NPA-box signature motifs (Murata et al. 2000). Similarly, ammonia channels AmtB are trimeric, just as CTRs (Khademi et al. 2004). Yet unlike CTRs, the high-resolution structure of the AmtB trimer clearly shows that this protein conducts ammonia through a pore that resides within the monomeric units. And consequently: while radial symmetry is associated with a channel mechanism in some cases, an increasing number of examples exist where channel pores reside within larger helical bundles of the monomer. At the other end of the spectrum is the case of the chloride channel. While some of the eukaryotic channels still appear to be channels, both the high-resolution structure and biochemical studies revealed that mechanistically, the *Escherichia coli* CLC functions as a chloride/proton antiporter with a glutamate residue coupling substrate transport (Dutzler et al. 2002, 2003; Accardi and Miller 2004; for review Miller 2006). With this in mind and refocusing on CTR: the radial symmetry seen in the projection density map certainly supports that copper could be channeled along the interface between the three monomers. However, CTR-proteins may oscillate between defined structural states that are associated with copper delivery to the pore—for instance by copper reductases that are present in the plasma membrane—and copper handoff from CTR to the intracellular chaperons which deliver copper ions to their final cellular targets. Hence, it is quite possible that mechanistically, CTRs act as transporters. Only additional structural and mechanistic studies will be able to answer this open issue.

CTRs—how related will their structure be to that of other ATP-independent transition metal transporters?

Despite the sometimes subtle distinctions that may set apart channels from transporters, an architectural theme that seems to be shared between a growing number of solute transporting membrane proteins is that substrate translocation

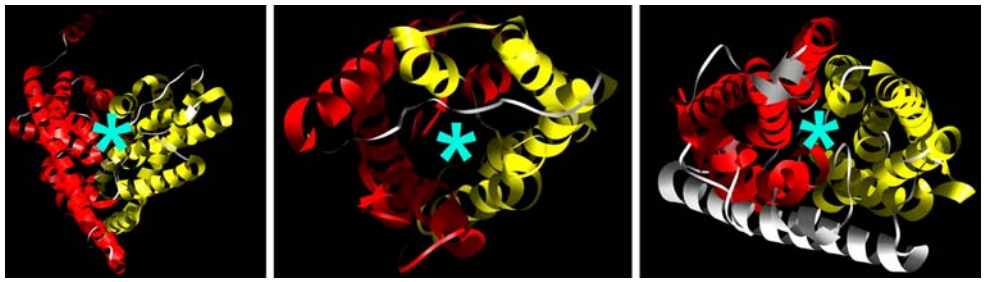

Fig. 7 Internal pseudo-two-fold symmetry, an important design principle in integral membrane proteins. Shown are ribbon diagrams for three membrane proteins whose architecture features an ''intramolecular interface'' between two halves that are related by pseudo-two-fold axis of symmetry within the membrane plane. In each of these proteins—*Salmonella typhimurium* H^+/Cl^- antiporter (PDB accession code 1KPL), human aquaporin 1 (PDB accession code 1FQY), and the AmtB ammonia channel (PDB accession code 1U77)—the substrate(s) are transported along the interface between the two related halves (indicated by *). Note that each of these proteins forms higher order oligomers in the membrane, but in contrast to CTRs and some traditional ion channels, no solute permeation pathways reside at the interface between monomers, rather they are along the interfaces of the pseudo symmetric halves of the proteins

occurs at an intramolecular interface of seemingly smaller building blocks, either at the interface of an oligomeric assembly, as mentioned earlier, or along a path corresponding to a pseudo two-fold axis of symmetry that correlates the two halves of the protein. Such internal structural symmetry is prevalent in many other channels and transporter structures solved to date including the ammonia channel, MFS transporters and the leucine transporter, which has led to the hypothesis that gene fusion events between simple packing motifs has given rise to the large variety of helical membrane proteins that exist today (see Fig. 7 for examples) (Murata et al. 2000; Dutzler et al. 2002; Abramson et al. 2003; Huang et al. 2003; Khademi et al. 2004; Yamashita et al. 2005; Yin et al. 2006). Interestingly, some of these fusion events involved the duplication and fusion of groups of three TM-segments. The arrangement of the aquaporin's six regular TM-segments was the first example where this design had been observed (Murata et al. 2000). Amazingly, even more complicated ''cutting-and-pasting'' is evident in the major facilitator family of transporters where the initial duplication of a three-helix bundle has gone one step further, adding two of the newly created 6TM-bundles into an extremely symmetric 12 TM-bundle with a $(2 \times 3) \times 2$ design. Yet even in this case, substrate is transported along the interface between the two 6TM-bundles, suggesting that exploitation of interfaces plays as important a role in membrane bound processes than it does in enzymes where catalysis often occurs in clefts between domains. With this in mind, it will be interesting to see how the structure of CTR compares to that of other transition metal transporters that have larger helix bundles of six and 12 (Dix et al. 1994; Gunshin et al. 1997; Kambe et al. 2004). It would not be at all surprising if the metal conducting pathways of these larger membrane proteins were to structurally resemble the copper-conducting pore in CTRs, which given the small number of TM segments could only be realized by exploiting the chemical and structural propensities of the CTR signature motif, MxxxM-X_{12}-GxxxG, to build what is a highly complex and sophisticated molecular machine, catalyzing the transport of copper ions across the plasma membrane.

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