

C α -trace model of the transmembrane domain of human copper transporter 1, motion and functional implications

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The trimeric human copper transporter 1 (hCTR1) is essential for copper uptake and is implicated in sensitivity to chemotherapy drugs. Using the cryoelectron microscopy (cryoEM) map of hCTR1 and evolutionary data, we constructed a C α -trace model of the membrane region. The model structure, supported by mutagenesis data, was used to investigate global dynamics through elastic network models. Identified as dominant hinge regions, hCTR1's MxxxM and GxxxG motifs were shown to have significant roles in functional movements characterized by the two slowest modes of motion. Both modes predicted significant changes at the wide cytoplasmic pore region; the slowest mode introduced a rotational motion around the pore central axis, whereas in the following mode the cytoplasmic parts of the helices approached and moved away from the pore center. In the most cooperative mode, the MxxxM motif in the extracellular narrow region remained static. The second mode of motion, however, predicted a cooperative rotational motion of this copper-binding motif, possibly reflecting activation at the pore's extracellular entrance. We suggest a molecular mechanism of copper transport in which this motif serves both as a gate and as a selectivity filter. We also suggest residues that are responsible for pH activation.

dynamics | hCTR1 | mechanism | model structure | structural bioinformatics

Copper ions are vitally important in key biological processes such as clearance of free radicals and respiration in eukaryotes (1). Increased cellular concentration of copper, however, is toxic and causatively linked to Menkes and Wilson diseases (2, 3). Furthermore, copper was found to induce the formation of amyloidic protein structures, and excess copper has also been proposed to play roles in prion disorders (3, 4). Uptake of Cu⁺ is mediated by membrane proteins of the copper transporter (CTR) family, ubiquitously present in eukaryotic organisms (5). Human CTR1 (hCTR1) is considered the main copper uptake machinery (6) and is of particular interest owing to its role in accumulation of platinum-based chemotherapy agents (7). In the cytoplasm, Atox1 and other specialized chaperone proteins deliver the closely regulated copper ions to target proteins, including ATP7A/B (6). Interestingly, when hCTR1 was transfected in human cells, its activity was shown to be elevated in acidic pH relative to alkaline pH; more details regarding this detected pH dependency remain to be elucidated (8).

A three-dimensional (3D) intermediate-resolution structure of hCTR1 was recently determined using cryoEM techniques (9), providing a first glance into the unique fold of this homotrimeric protein. It was proposed that the central pore of the structure is mainly shaped by TM2 [the second transmembrane (TM) helix] from each of the subunits. Members of the CTR family are characterized by two previously identified sequence motifs: an MxxxM motif in TM2 and a GxxxG motif in TM3 (Fig. S1A and ref. 6), both entirely conserved. De Feo and coworkers approximately mapped the residues constituting these motifs onto the cryoEM map (9). Met150 and Met154 of the MxxxM motif, implicated in copper binding, were suggested to reside at the narrow extracel-

lular entrance to the pore, whereas Gly167 and Gly171 of the GxxxG motif were proposed to mediate a tight interface between TM3 and TM1.

Many of the molecular and functional properties of the TM domain have remained obscure, because the positions of the individual residues could not be detected in the intermediate-resolution map. More elaborate structural data can provide additional clues needed to decipher the copper transport mechanism of hCTR1 and to gain insight into regulation and functional motion. Toward this goal, we explore the structure and dynamics of hCTR1 using computational approaches. We generated a C α -trace model based on the method of Fleishman et al. (10) and subsequently, to provide insight into functional motion, carried out a normal mode analysis using elastic network models (11, 12).

An intermediate-resolution map for producing a TM model structure was first employed by Baldwin and coworkers, who constructed a C α -trace model of vertebrate rhodopsin (13). The model was generated from a structure at 7 Å resolution in the membrane plane using sequence-based and biochemical constraints. When the crystal structure of higher resolution was later determined, the orientations of TM helices in the model were found to be quite accurate (3.2 Å rmsd) (14). Expanding on this pioneering approach, Fleishman and coworkers developed an automatic method for TM-model prediction from cryoEM maps based essentially on evolutionary conservation (10). This approach was used to model the bacterial multidrug resistance transporter EmrE (15). The model superimposed well on the X-ray structure, which came out later, with core rmsd of 1.4 Å (16).

Currently of renewed interest, normal mode analysis using elastic network models can provide valuable insight into equilibrium dynamics of TM proteins (12). Specifically, the Gaussian network model (GNM) (17, 18) and the anisotropic network model (ANM) (19) provide coarse-grained mode analysis to estimate the magnitudes and directions of thermal fluctuations. These techniques have proven useful in demonstrating long-time or large-scale functional motion of proteins. It is now recognized that structural rearrangements revealed by the most cooperative (or slow) modes of the normal mode analysis often correlate with functional motions in TM proteins; such motions include pore opening, ion gating, and signal transduction (12).

We present herein a study of the structure-function relationship of hCTR1. Our model structure proposes the precise locations and interactions of highly conserved sequence motifs, along with molecular-level interpretation of biochemical data.

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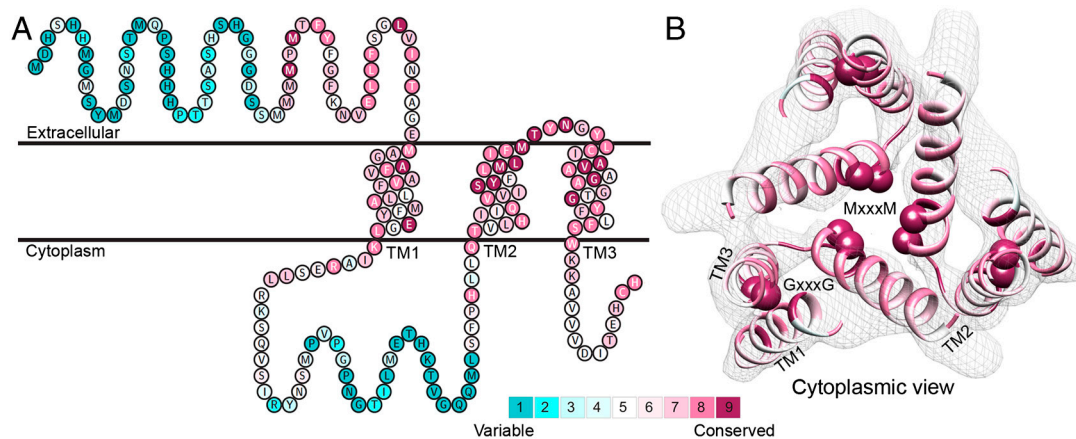


Fig. 1. Evolutionary conservation profile of the hCTR1 full sequence and TM model. In both panels, the coloring is according to the evolutionary conservation scores calculated via the ConSurf server (34, <http://consurf.tau.ac.il>), with turquoise-to-maroon signifying variable-to-conserved positions (shown by the color bar). (A) The predicted topology of hCTR1. The three TM segments are marked. (B) Intracellular view of the α -trace model of the TM domain hCTR1, fitted onto the cryoEM map (9) (mesh). Residues of the MxxxM and GxxxG motifs are shown as spheres, and the three TM helices are marked on one of the subunits.

Our analysis of cooperative dynamics reveals potential functional motions that may offer insight into both the copper transport mechanism and pH regulation.

Results

Computing the α Model. After manually extracting the principal axes of the TM segments from a cryoEM map, we constructed the TM helices, incorporating minor kinks in TM1 and TM3 to better match the contours of these helices in the density map (Fig. 1). We then investigated possible combinations of helix assignments and topological orientation of the helices to the principal axes. Reassuringly, the highest ranked helix assignment and topology were in agreement with those previously presented by De Feo et al. (9) (Table S1). Refining our initial model, we added the short, highly conserved loop between TM2 and TM3 (Fig. 1).

In the final model, highly conserved residues populated packed interfaces between helices or lined the central pore (Fig. 1B). Specifically, residues of the TM2 MxxxM and TM3 GxxxG motifs were predicted to reside in strategic locations. Met154 and Met150 formed two sequential threesomes of pore-facing methionine residues, designated herein as the “Met triads.” The first triad was composed of Met154 from each of the three subunits; the three Met154 residues were situated on a single plane at the pore’s extracellular end. Similarly, the second triad, comprising Met150, was located one helix turn deeper into the membrane core. Gly167 and Gly171 resided at the interface of TM3 with neighboring TM1, mediating the most densely packed contact region of the structure. Less conserved residues were situated in the slacker interfaces, whereas variable residues were exposed to the lipids, as anticipated. The physicochemical characteristics of the hCTR1 model are discussed in *SI Text* (Fig. S1).

Model Is Consistent with Experimental Data. To investigate the model’s validity, we turned to published site-directed mutagenesis (Table S2), which was not used for model building. In addition to the elaborate mutagenesis data available for residues of the

MxxxM and GxxxG motifs (20–23), we used data from Eisses and Kaplan, who performed mutations in several polar and charged residues in the TM domain (20), and from Aller et al., who initiated a Trp scan of the third TM helix of a homologous yeast protein, yCTR3 (23). The various studies, however, employed different experimental systems to assess the effects of mutations on protein structure and function. To simplify the comparison of the diverse biochemical data, we roughly divided the mutations into three groups: deleterious, neutral (nondeleterious), and partial (Table S2). In several positions, different substitutions had conflicting effects. We designated mutations in these positions as deleterious if at least one of the performed substitutions resulted in functional impairment.

Mapped on the model, sensitive-to-mutation positions lined the pore, resided in the tight TM1-3 interface or accommodated the conserved TM2-3 loop at the narrow extracellular entrance (Fig. 2 and Table S3). The majority of the nonsensitive residues, on the other hand, faced the lipids. Whereas some partially sensitive positions resided at wider pore-facing segments, the remaining ones, along with a few nonsensitive positions, were found at relatively slack helix–helix interfaces. As this pattern is anticipated for a TM protein, we conclude that our hCTR1 model is in good agreement with prior experimental results (Fig. 2 and Table S3). The agreement with experimental data implies that the model structure correctly depicts the essence of the elements of the native structure and the interactions between them.

Global Movements and Cooperative Dynamics. Hinge regions governing global movements and the directions of functional motion are often identified through the lowest-frequency GNM and ANM modes, respectively (11, 12, 24). In addition to revealing the relative mobility of each residue, reflected in the GNM-derived mean-square fluctuations, the GNM analysis discloses interresidue positive and negative dynamical cooperativity. In this study, we examined fluctuations and cooperative dynamics of hCTR1 revealed by the five slowest GNM modes, along with

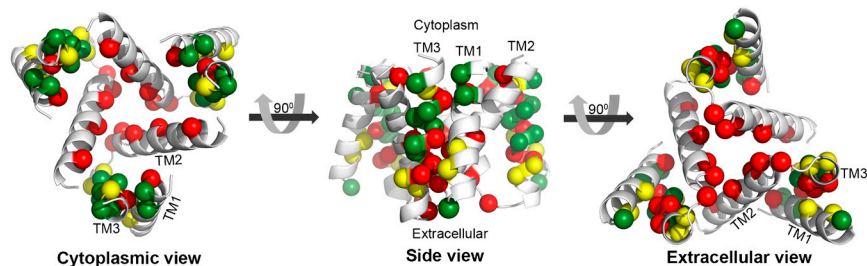


Fig. 2. Validation of the model structure via mutagenesis data. Mutagenesis data mapped on the hCTR1 model. Three different views are presented. α -atoms of residues for which mutational data are available are shown as spheres and colored according to the functional effect of the mutation, with red and green depicting positions sensitive and nonsensitive to substitutions, respectively. Positions in which mutations induced partial function are colored yellow.

Pore-Related Movements. Two orthogonal motions affected the pore conformation, especially at its cytoplasmic end (Figs. 4A and 5C and *Movie S1*, *Movie S2*, and *Movie S3*). In one mode of motion, helices from neighboring subunits fluctuated cooperatively in a torsion-like movement around the pore center, and in the second the cytoplasmic portions of all helices approached and moved away from the pore center. The alternative “deformed” conformations sampled by both ANM modes conformed to the expected conservation pattern and hydrophobic nature of a TM protein, just as the original model structure did (Fig. 1B and Fig. S1B). This indicates that such structural dynamics could be accommodated by the unique architecture of hCTR1. In view of the cone-like structure of hCTR1, narrowing of the wide-cytoplasmic pore region depicted by these movements would facilitate a more compact translocation pathway for copper ions, embedded mainly in the hydrophilic residues of TM2 as well as in polar and charged residues at the edges of TM1 and TM3. Such conformation could be more suitable for transport and may disclose the relationship between possible inactive and active states.

Two Met Triads, One Ion? The strategic positioning of the known copper-binding residues Met150 and Met154 at the extracellular entrance of the pore (Fig. 1B), forming two sequential triads, intuitively suggests that the residues function as the Cu^+ selectivity filter; clearly, this proposition must still be supported by atomic-resolution structural data followed by detailed simulations. Indeed, hCTR1 is selective for copper over other ions (8), and three Met residues are capable of specifically stabilizing Cu^+ (28). The findings of our dynamics investigation were in line with this notion, designating Met150 and Met154 as the structural hinges in the most cooperative mode (Fig. 3A), whereas the following mode revealed positive correlation in the motion of these positions (Fig. 5B and C). Interestingly, in another study, the selectivity filter residues of five potassium channels, and in particular the GYG signature motif, emerged as the prominent hinge regions in the most cooperative mode as well (29).

Recently, De Feo et al. showed that the two Met triads evidently bind a single ion at a time, and the researchers indeed suggested that transport could involve conformational change in this region (9). This still raises an obvious question: If one triad is sufficient for a single ion, what is the functional significance of the two sequential triads of the CTR family? We suggest a potential explanation. Cooperative rotational movement of the extracellular end of TM2 alters the structural disposition of the Met triads (Fig. 5C and *Movie S2*), and a copper ion, initially bound to the (external) Met154 triad, may proceed along the pore to the Met150 triad, whereas the Met154 triad blocks the entrance of additional ions as an external gate (Fig. 6). The ions are transported one at a time, thus avoiding the disproportionation reaction between two colliding Cu^+ ions that leads to Cu_2S and Cu^+ . Moreover, passing a single ion in each cycle seems like an effective way to tightly regulate the transport process, preventing free copper ions from readily entering living cells and generating reactive, toxic radicals (30). The suggested translocation pathway is complementary to previous findings regarding the functional role of the extramembrane domains at the N- and C-terminal regions (9, 20, 22).

Glu84 and His139, Active State and pH Dependence. As Glu84 and His139 are highly conserved, hydrophilic residues embedded in the TM domain, one might intuitively infer that both possess functional significance. On the basis of mutagenesis findings, Eisses and Kaplan indeed designated these residues as functional (20), although the residues’ specific functional roles are yet to be determined. Building the side-chains of these amino acids in the model structure, we realized that Glu84 of TM1 and His139 of TM2 from adjacent subunits could possibly interact. Moreover, these adjacent positions showed positively correlated dynamics

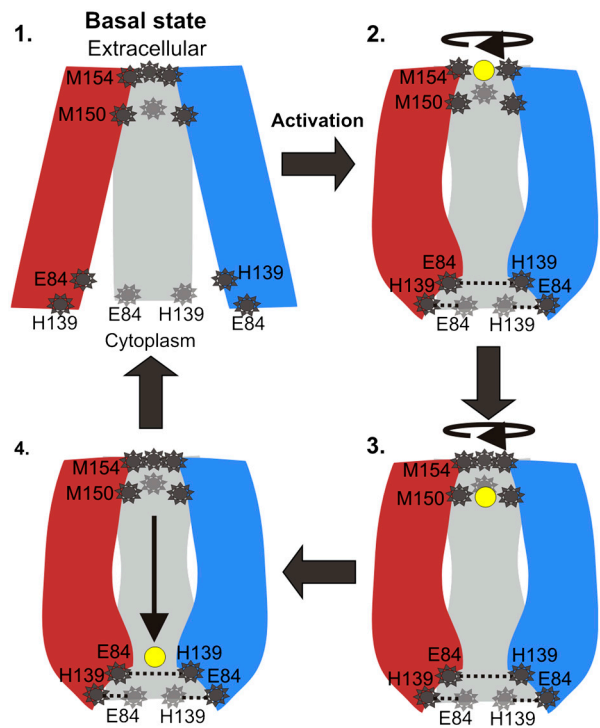


Fig. 6. Transport mechanism. In the proposed mechanism the copper ions are transported one at a time, and the TM region of hCTR1 alternates between four conformations. The three subunits are shown in red, blue, and gray, with residues of interest depicted in dark gray and the copper ion in yellow. Step 1: In the basal state, ions do not bind to the TM domain, the Met154 triad serves as an external gate and (unprotonated) His139 does not interact with Glu84. Our model structure is suggested to depict the basal conformation, because it was computed from a cryoEM map determined at pH 7.4 (9), a condition in which hCTR1 exhibits low activity according to Lee et al. (8). Step 2: Activation resulting, for example, from pH shift or ion binding, involves conformational change at the cytoplasmic end, along with a rotational movement at the extracellular end. A copper ion binds to the Met154 triad, and (protonated) His139 interacts with Glu84, stabilizing the conformation. Step 3: Following another conformational change at the extracellular end, the copper ion is passed on to the Met150 triad and the Met154 closes the pore entrance, thereby preventing the entry of a second ion. Step 4: The copper ion passes freely through the polar pore. It is yet to be revealed how copper ions are passed through to the C-terminus and from there to the copper chaperones.

in GNM mode 1,2 (Fig. 3C), suggesting that they move together in the most global mode. Interestingly, hCTR1’s activity is pH-regulated; the transporter is more active in pH 5.5 than in pH 7.5 (8). Considering the above and assuming that His139 maintains its physiological pKa in the protein environment, we suggest that an interaction between Glu84 and His139 might stabilize an active conformation of hCTR1 in low pH, where His139 would probably be protonated. In higher pH, however, His139 would be deprotonated, resulting in a weaker interaction with Glu84 and destabilization of the putative active state. Indeed, this may clarify not only the molecular basis of hCTR1’s pH-regulated activity but also the obscure hyperactivity observed for the H139R mutant (20), which can interact with Glu84 in a higher pH range as well.

Mechanism of Cu^+ Transport. Combining structural data, functional motion, cooperative dynamics, and available mutagenesis, we attempted to provide additional insight into the transport mechanism of hCTR1’s TM region (Fig. 6). The Met150 and Met154 triads, by controlling gating and selectivity, regulate the passing of a single ion in every cycle, possibly via the directed motion presented in Fig. 5C and in *Movie S2*. Fluctuations at the cytoplasmic ends could modify the pore conformation (Figs. 4A and 5C, *Movie S1*, and *Movie S3*), adjusting the hydrophilic

environment encountered by the copper ion for optimal transport. The intersubunit contact Glu84-His139 may stabilize such an active conformation, favorable at low-pH conditions. Notably, the positive correlation between the TM2-3 loop, TM1 and TM3 in the slowest mode (Fig. 3C) might hint at vital allosteric interactivity between the cytoplasmic and extracellular parts of the TM domain. This interactivity would be crucial for transducing signals stimulated, for example, by binding of an ion in the extracellular side, or, alternatively, by pH change. These suggestions can be useful to guide future experimental efforts and should be further assessed in light of emerging empirical investigations. Solid empiric validation for these structure-based mechanistic interpretations, however, would be best obtained through at least one, but preferably more, experimentally determined high-resolution conformational states of hCTR1.

Methods

Building the Model Structure. We approximated the helix boundaries in the hCTR1 sequence and calculated the conservation scores of each amino acid position. We also manually extracted the principal axes of the helices from the hCTR1 cryoEM map, introducing kinks to follow the helix density rods as precisely as possible (9). Because TM1 and TM3 are partly bent in the map, we introduced into the original algorithm (10) a new feature that allowed incorporation of the kinked axes. Using the same scoring function, which favored conformations in which conserved or charged amino acids were buried in the protein core while variable or uncharged amino acids were exposed, we constructed C α -trace helices and separately rotated each helix around its principal axis using a 5° increment (Fig. S7). The final model was based on the best-scored rotational angle for each of the nine (3 × 3) helices. We then generated the short TM2-3 loop (positions 155–160) using the protein local optimization program (31). Further elaboration regarding the model-building procedure is provided in *SI Text*.

Normal Mode Analysis with Elastic Network Models. In this study, we employed the slow modes of motion as detected by the GNM and ANM models (17–19, 32). As the underlying potential function of the GNM is more physically real-

istic than that of the ANM, the former model is more robust in the prediction of fluctuations (11, 24). We therefore employed the slowest modes from the GNM analysis to detect the magnitudes of fluctuations and to identify cooperative dynamics (11, 17, 18). In accordance with previous definitions (12, 33), we designated regions of lowest mobility (as determined by the GNM); i.e., minima, as “hinges” in cases in which the regions were located between segments of opposite dynamical correlation. The normalized correlations between the residue fluctuations describe the dynamical cooperativity; two positively-correlated residues move in the same direction, whereas two negatively-correlated positions move in opposite directions from each other. Through the analysis of positively and negatively correlated fluctuations in the slowest modes, we identified the dynamic domains and the cooperation between them, implicated in the overall motion of the protein. As the slowest modes often reflect functional motion (11, 12, 24), the dynamically-correlated domains were expected to represent functional units.

GNM is isotropic and cannot account for the direction of the fluctuations. Therefore, we incorporated ANM-derived slow modes (19) for predicting the directions of motion and conformations, in compliance with the fluctuations described by the matching GNM modes (Table S4 and Fig. S3). Additionally, the predicted global modes for symmetric structures may include modes that maintain the structural symmetry with the same type of deformation in all monomers or in a subgroup of monomers (e.g., refs. 29 and 33). Thus, we superimposed the fluctuations of detected degenerative modes, obtaining symmetrically shaped deformations that more accurately described the global motion. More details are available in *SI Text*.

Note Added in Proof. While the manuscript was in review, a full Trp scan of TM1 and TM2 of hCTR1 was published (35). Our model structure is in excellent agreement with this data (Fig. S8).

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