X-ray Crystal Structure of a TRPM Assembly Domain Reveals an Antiparallel Four-stranded Coiled-coil

Yuichiro Fujiwara and Daniel L. Minor Jr

Cardiovascular Research Institute, Departments of Biochemistry and Biophysics and Cellular and Molecular Pharmacology, California Institute for Quantitative Biosciences, University of California San Francisco, San Francisco, CA 94158-2330, USA

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Introduction

Transient receptor potential (TRP) channels comprise a large family of tetrameric cation-selective ion channels that respond to diverse forms of sensory input. Earlier studies showed that members of the TRPM subclass possess a self-assembling tetrameric C-terminal cytoplasmic coiled-coil domain that underlies channel assembly and trafficking. Here, we present the high-resolution crystal structure of the coiled-coil domain of the channel enzyme TRPM7. The crystal structure, together with biochemical experiments, reveals an unexpected four-stranded antiparallel coiled-coil architecture that bears unique features relative to other antiparallel coiled-coils. Structural analysis indicates that a limited set of interactions encode assembly specificity determinants and uncovers a previously unnoticed segregation of TRPM assembly domains into two families that correspond with the phylogenetic divisions seen for the complete subunits. Together, the data provide a framework for understanding the mechanism of TRPM channel assembly and highlight the diversity of forms found in the coiled-coil fold.

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Abstract

Transient receptor potential (TRP) channels form a diverse family of nonselective cation channels that contribute to a range of sensory processes including thermosensation, phototransduction, chemosensation, and nociception. These channels are members of the superfamily that includes voltage-gated channels for calcium, potassium, and sodium, as well as cyclic nucleotide-gated channels. As with other members of the superfamily, TRP channels are thought to be composed of four pore-forming subunits, an idea supported by the recent identification of TRPM subfamily tetrameric cytoplasmic assembly domains and low-resolution cryo-EM studies of TRPV1. Members of the TRPM subfamily include channels that respond to cold, TRPM8, which are involved in taste, TRPM5, and channel kinases that are important for divalent cation uptake, TRPM6 and TRPM7, and channels that act as redox sensors, TRPM2. Concomitant with their diverse functions, TRP channels have diverse C-terminal domains but share a C-terminal cytoplasmic tetrameric coiled-coil that self-assembles and directs channel assembly. Coiled-coils are a widespread protein-protein interaction structural motif and are found in disparate protein classes that include fibrous proteins, motor proteins, transcription factors, and membrane fusion proteins. Beyond the varied biological roles, the regularity of the coiled-coil structure has made coiled-coils a favorite model system for understanding protein sequence-structure relationships, deciphering interaction specificity determinants, and protein engineering efforts. The involvement of cytoplasmic coiled-coil domains as key elements of ion channel assembly is an emerging theme with examples found in diverse members of the voltage-gated ion channel superfamily, such as TRPM channels, Kv7 channels, Eag channels, polycystins, and in the voltage-sensor only proton channel Hv1.

Many ion channels are composed of multiple pore-forming subunits and recurrently, incorporation of...
different subunit types has profound effects on the functional properties of the resulting channel. Understanding how elements in the individual subunits guide channel assembly and specify subunit assembly preferences is an incompletely understood problem. To date, two cases have been characterized structurally, the T1 domain of Kv1-Kv4 channels, and coiled-coil domains from Kv7 (KCNQ) channels. The presence of cytoplasmic coiled-coil domains in diverse voltage-gated ion channel superfamilies suggests that this type of domain has a predominant role in directing pore-forming subunit assembly.

Here, we report the structure of the coiled-coil assembly domain from the channel kinase TRPM7, a divergent cation-permeable channel that is expressed in a wide variety of tissues including the brain, heart, kidney, and the hematopoietic organs. TRPM7 channels conduct calcium and magnesium ion influx into cells, are tonically inhibited by intracellular Mg\(^{2+}\) and Mg-complexed nucleotides, and are modulated by a variety of factors that include acidic pH, phosphatidylinositol bisphosphate (PIP\(_2\)), and phospholipase C pathways. TRPM7 activity supports multiple physiological functions that include Mg\(^{2+}\) homeostasis, cell viability and growth, neuronal cell death, intestinal pacemaking, and skeletogenesis. In addition to its plasma membrane roles, TRPM7 has been found in cholinergic synaptic vesicles, where its activity may regulate the amount of mobile transmitter contained therein. TRPM7 dysfunction is linked to familial Alzheimer’s disease, and may have a role in hypertension. Additionally, TRPM7 forms functional heteromultimers with TRPM6, a subunit in which mutations lead to the Mg\(^{2+}\) absorption/absorption disorder, Hypomagnesemia with secondary hypocalcemia. Thus, understanding how TRPM7 assembles alone and as heteromultimers with TRPM6 is important for understanding both the basic mechanisms of TRPM channel assembly and TRPM channelopathies.

We find that the TRPM7 assembly domain, TRPM7cc, is an antiparallel coiled-coil having an unprecedented coiled-coil architecture that is largely symmetric in the middle but that diverts at the ends into a diamond-shaped packing arrangement coincident with an alternating “knobs-against-knobs” packing arrangement of the core a and d positions. The D2 symmetry in the TRPM7cc antiparallel arrangement does not match the expected rotational fourfold symmetry of the pore but is in accord with the twofold symmetry found in the kinase domain that lies just C-terminal to the coiled-coil. Structure and assembly determinants of antiparallel coiled-coils are less well understood than their parallel counterparts due, in part, to a limited number of examples. The TRPM7cc structure highlights the remarkable structural diversity found in coiled-coils and should serve as a framework to begin to understand the structural basis for TRPM channel assembly specificity.

Results

Crystal structure of the TRPM7 assembly domain

TRPM C-terminal cytoplasmic domains have varied lengths and domain compositions (Fig. 1a). In the midst of this architectural diversity resides a coiled-coil domain that is well conserved in terms of its position in the C-terminal cytoplasmic domain, ~100 residues C-terminal to the last transmembrane segment of the pore-forming domain, and clear heptad repeat pattern, denoted (abcedfg), (Fig. 1a and b). Even though coiled-coils can be readily identified from sequence analysis by the presence of the distinctive heptad repeat, (abcedfg), in which a and d positions are hydrophobic amino acids, accurate prediction of coiled-coil three-dimensional structure and assembly preferences from sequence remains elusive. In order to begin to understand the architecture that underlies the TRPM assembly domain, we expressed, purified, and crystallized a rat TRPM7 coiled-coil domain construct, TRPM7cc (residues 1230–1282).

Vapor diffusion crystallization gave two TRPM7cc crystal forms, monoclinic C212, and orthorhombic C222, that diffracted synchrotron X-rays to 2.00 Å and 2.40 Å, respectively. Structure determination by molecular replacement using several types of parallel four-stranded coiled-coils failed. Therefore, we grew crystals of TRPM7cc in which selenomethionine was biosynthetically incorporated. Selenomethionine-substituted TRMP7cc crystals grew in the C222 form and diffracted X-rays to 2.8 Å resolution (Table 1). Structure solution using experimentally determined phases from a three-wavelength MAD experiment gave readily interpretable electron density maps in which the α-helical backbone, side chains, and selenomethionine positions were visible (Fig. 1c). The maps further revealed that the single selenium atoms from each chain were clustered into pairs at both ends of an antiparallel tetrameric coiled-coil (Supplementary Data Fig. 1) and provided a clear explanation for why molecular replacement using parallel four-stranded coiled-coils failed. An initial structural model from the C222 crystal form experimental electron density was built using Arp/Warp and refined for several cycles. The resulting model was used to solve the structure of the C212 crystal form, which diffracted X-rays to higher resolution than the C222 form, by molecular replacement using Phaser (Fig. 1d). The C212 asymmetric unit contains two tetramers. We were able to build all of the atoms for the eight polypeptides except for a few terminal residues and a few side chains (Supplementary Data Fig. 2), and refine the resulting model to an acceptable level (R/Rfree=18.8/24.8%) (Table 1).

Core packing analysis and comparison with other antiparallel tetratrams

The overall structure of TRPM7cc is that of a tightly twisted, symmetrical, antiparallel left-handed four-
stranded coiled-coil that is 25 Å wide and 77 Å long (Figs. 1e and 2a). The tetramer interface shows a classical antiparallel coiled-coil pattern in which the a and d positions comprise layers along the superhelical core composed of pairs of diagonally opposed a position residues and d position residues (Figs. 2a and b).

Three different anti-parallel four-stranded coiled-coils core-packing architectures have been described, the a–d core, e–a–d core, and a–d–g core (Fig. 2c),
Table 1. Data collection and refinement statistics

<table>
<thead>
<tr>
<th>Data collection</th>
<th>TRPM7cc native</th>
<th>TRPM7cc SeMet</th>
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<tbody>
<tr>
<td>Resolution (Å)</td>
<td>50.0–2.00 (2.07–2.00)</td>
<td>50.0–2.40 (2.44–2.40)</td>
</tr>
<tr>
<td>C121</td>
<td>C222</td>
<td>C222</td>
</tr>
<tr>
<td>Space group</td>
<td>C222</td>
<td>C222</td>
</tr>
<tr>
<td>Cell dimensions</td>
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<td>93.21, 185.45, 72.65</td>
</tr>
<tr>
<td>α, β, γ (°)</td>
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<td>90.00, 90.00, 90.00</td>
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<td>Rsym</td>
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<td>7.8 (52.3)</td>
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<td>Wavelength</td>
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<tr>
<td>I/σ</td>
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<td>18.3 (2.3)</td>
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<tr>
<td>Completeness (%)</td>
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<td>99.9 (99.8)</td>
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<tr>
<td>Redundancy</td>
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<td>4.2 (4.1)</td>
</tr>
<tr>
<td>Refinement</td>
<td>Resolution (Å) 2.01</td>
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</tr>
<tr>
<td>No. reflections</td>
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<td>23,978</td>
</tr>
<tr>
<td>Rwork/Rfree</td>
<td>18.8/24.8</td>
<td>22.7/29.4</td>
</tr>
<tr>
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<tr>
<td>Total water atoms</td>
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<td>297</td>
</tr>
<tr>
<td>Average B-factors</td>
<td>26.9</td>
<td>34.1</td>
</tr>
<tr>
<td>Protein (Å)</td>
<td>1.78</td>
<td>1.78</td>
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<tr>
<td>Water (Å)</td>
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<td>1.783</td>
</tr>
<tr>
<td>RMSD from ideal</td>
<td>Bond lengths (Å) 2.01</td>
<td></td>
</tr>
<tr>
<td>Bond angles (°)</td>
<td>Bond lengths (Å) 2.40</td>
<td></td>
</tr>
</tbody>
</table>

Values in parentheses are for the highest-resolution shell.

Each of which has a distinct buried surface area pattern among the heptad positions. Position-specific analysis of buried surface area relative to an isolated α-helical strand shows that the TRPM7cc quaternary structure completely buries the a and d positions as well as a substantial amount of the surface area of the e and g positions (Table 2). In contrast, b, c, and f positions along the exterior surface of the helical bundle remain highly exposed. These features classify TRPM7cc as an α-d core coiled-coil. Relative to the three other known a-d core four-stranded anti-parallel structures, WSPLP, GCN4-E20C, and Rop, the amount of burial at the TRPM7cc positions that flank the core (e versus g and b versus c), is much more uniform (Table 2, Supplementary Data Fig. 3).

Each TRPM7cc a-d layer comprises two a and two d position residues. Notably, there are substantial differences in the way the components of the layers pack as one progresses from the center layers to the layers near the strand termini. In the middle region, layers 1 and 2, the a and d position side chains interact in the classical knobs-into-holes packing (Fig. 2b). In this portion of the structure, the individual strands of the coil form an approximately square symmetrical shape. However, near the coiled-coil ends, layers 5–7, the core packing geometry changes to the knob-against-knob packing in which the a and d positions directly face each other (Fig. 2b). Furthermore, the side chain positions that oppose each other through the knob-against-knob interactions alternate from layer to layer (e.g. layer 5 d positions interact, whereas layer 6 a positions interact). Other a–d core antiparallel tetramers show only one type of the knob-against-knob packing involving either the side chains at a positions GCN4-E20C and WSPLP, or d positions, Rop. Thus, the TRPM7cc layer-by-layer alternation in packing is unique among antiparallel coiled-coils (Table 2).

Fig. 2. Core packing in the TRPM7 channel coiled-coil domain (a) Coiled-coil core hydrophobic layers. van der Waals spheres depicting the side chains of the a (blue) and d (red) layers on a ribbon backbone (gray) are shown. Layer numbers and N- and C-terminal ends of the coiled-coil are indicated. (b) Geometry of individual coiled-coil layers. Ball-and-stick representations show each layer of the core. The a and d positions are colored as in a. Layer numbers are indicated in black. (c) Helical wheel representations for the different antiparallel homotrameric coiled-coil core-packing arrangements. Heptad repeat positions are labeled a–g. Core-forming positions are highlighted in green and continuous or broken lines link positions lying in the same layer. d, Sausage diagram of TRPM7cc and GCN4-E20C a–d core antiparallel coiled-coils. Cross-sections of the helical arrangements are shown for the center and ends of the coils.
Fig. 2 (legend on previous page)
Along with the packing changes in the terminal layers, the arrangement of the individual strands differs from that found in the center of the helical bundle. Rather than the symmetrical square arrangement seen in layers 1 and 2, the terminal parts of the individual strands of the coil are arranged in a diamond shape in which the C-terminal strands are more peripheral whereas the N-terminal strands are in the center (Figs. 2b and d). Each of the three final layers (5–7) includes two buried, polar residues. In each case, the buried, polar residue is involved in hydrogen bonding interactions. S1271 (layer 5) makes an interaction with a buried water molecule that is bridged to either the side chain carbonyl of Gln1268 from the same strand or the backbone carbonyl of Val1236 from the adjacent strand. Each interaction is seen in two of eight possible incidences. In contrast, layers 6 and 7 hydrogen bond interactions are present in all eight possible occurrences in the asymmetric unit. Thr1264 in layer 6 makes a hydrogen bond to the carbonyl oxygen of Leu1270 from its own helix. Thr1232 (layer 7) is part of a hydrogen bond network (see below). It seems likely that the difference in the strand arrangement between the center and ends of the coil is linked to the incorporation of the hydrophilic residues in the core.

Comparison of the TRPM7cc superhelical parameters with previously determined antiparallel homo-tetramer coiled-coil structures shows that TRPM7cc is more tightly packed and more twisted than other antiparallel coiled-coils (Table 2). These factors, together with the unique packing and strand divergence indicate that the TRPM7cc structure is unique among the a–d core antiparallel tetrameric coiled-coils and underscores the structural diversity inherent in the coiled-coil fold.27

**Table 2. Comparison of coiled-coil parameters**

<table>
<thead>
<tr>
<th>Buried surface area (%)</th>
<th>a–d core</th>
<th>e–a–d core</th>
<th>a–d–g core</th>
</tr>
</thead>
<tbody>
<tr>
<td>Position</td>
<td>TRPM7cc</td>
<td>GCN4 E20C</td>
<td>WSPLP</td>
</tr>
<tr>
<td>a</td>
<td>98</td>
<td>99</td>
<td>100</td>
</tr>
<tr>
<td>b</td>
<td>15</td>
<td>22</td>
<td>8</td>
</tr>
<tr>
<td>c</td>
<td>16</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>d</td>
<td>98</td>
<td>95</td>
<td>96</td>
</tr>
<tr>
<td>e</td>
<td>62</td>
<td>76</td>
<td>74</td>
</tr>
<tr>
<td>f</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>g</td>
<td>61</td>
<td>45</td>
<td>54</td>
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</table>

<table>
<thead>
<tr>
<th>Knob-against-knob pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>a–d core</td>
</tr>
<tr>
<td>Position</td>
</tr>
<tr>
<td>a–d–d</td>
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</table>

<table>
<thead>
<tr>
<th>Coiled-coil parameters</th>
</tr>
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<tbody>
<tr>
<td>Superhelical parameter</td>
</tr>
<tr>
<td>Radius (Å)</td>
</tr>
<tr>
<td>Residues/turn per superhelix turn</td>
</tr>
<tr>
<td>Pitch (Å)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>α-Helical parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radius (Å)</td>
</tr>
<tr>
<td>Residues/turn</td>
</tr>
<tr>
<td>Rise/residue (Å)</td>
</tr>
</tbody>
</table>

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**Electrostatic interactions**

The TRPM7cc surface is composed predominantly of polar residues. A number of these are used in three distinct sets of side chain hydrogen bond networks that form across helix interfaces within the coiled-coil bundle (Fig. 3). The central component of Network 1 is Arg1235, a g position. This residue interacts with the side chain hydroxyl of Thr1277, a g position from the neighboring helix, and a buried water molecule that makes bridging hydrogen bonds to the Thr1274 carbonyl oxygen in the adjacent helix and the d position Thr1232 side chain hydroxyl from the Arg1235-bearing helix. This network is observed in all eight possible copies in the asymmetric unit. Network 2 involves three side chains; Asn1251 and Lys1254, b and e positions from one strand, interact with Asp1261 an e position, from the adjacent strand and is arranged in two configurations. Of the eight possible Network 2 occurrences, the tripartite arrangement occurs five times. The remaining three lack one substituent but retain cross-strand interactions that involve either the Asn1251–Asp1261 b–e or Lys1254–Asp1261 e–e pairs. Network 3 involves three sidechains: Glu1245 and Arg1249, c and g positions from the same strand, and Gln1263, a g position from the adjacent
strand. Network 3 displays two configurations in which the participating side chains interact. In one, Network 3a (Fig. 3), two substituents from the same chain interact (Glu1245 and Arg1249), and Arg1249 makes a bidentate hydrogen bond to Gln1263 from the adjacent helix. The Network 3a arrangement occurs in six instances; however, two of these lack the intra-subunit Glu1245–Arg1249 hydrogen bond. In the other configuration, Network 3b (Fig. 3), which is observed twice, Gln1263 is pinned between Arg1249 and Glu1245 from the adjacent strand. In addition to these intersubunit interactions, we observed an intrasubunit salt bridge between b and f positions Arg1230 and Glu1234 in the first turn of
the helix in six of the eight copies in the asymmetric unit.

**Structure-based comparison of TRPM coiled-coils uncovers two subfamilies**

Structure-based comparison of the coiled-coil regions from the TRPM subtypes (Fig. 4a) reveals that even though all possess a coiled-coil domain, the TRPM coiled-coil domains segregate into two groups: those having TRPM7 features (TRPM1, TRPM3, and TRPM6, denoted the TRPM7 group) and those that are not like TRPM7 (TRPM2, TRPM4, TRPM5, and TRPM8, denoted the TRPM8 group). In light of the TRPM7cc structure, the most obvious difference between the groups is that the TRPM8 group has coiled-coil domains that are shorter than the TRPM7 group by 1–1.5 heptad repeats (Fig. 1a).

Within the TRPM7 group, TRPM6 is most similar to TRPM7. Five of the seven a–d layers (layers 1, 2, 5, 6, and 7) are identical and the remaining two layers (3 and 4) have conservative, hydrophobic changes. Amino acids that form the three interhelix electrostatic interaction networks are also well conserved (Fig. 4a). The core and interhelix hydrogen bond compatibilities are consistent with the observation that TRPM7 and TRPM6 subunits interact to form heteromeric channels.18–20 With the exception of layer 2, TRPM1 and TRPM3 coiled-coil domains have a and d position side chains compatible with the TRMP7cc core. TRPM1 and TRPM3 retain residues that could form the Network 2 interaction but lack appropriate side chain chemistry for making Networks 1 and 3. Together, such differences may help to specify assembly preferences among the closely related TRPM7 group coiled-coil domains.

Consideration of the TRPM8 core residues in the context of the TRMP7cc antiparallel framework shows a remarkably poor conservation (Figs. 1b and 4a). The incompatibilities extend to the electrostatic contacts. Together with the apparent differences in coiled-coil lengths and earlier biochemical results that establish the tetrameric self-assembly properties of TRPM8 subgroup members,6 it seems likely that the TRPM8 subgroup has a coiled-coil quaternary structure different from that of the TRPM7 group. Interestingly, the class differences we find between the TRPM7 and TRPM8 groups from the structure-based comparison of the assembly domains are reflected in phylogenetic analysis of full-length TRPMs (Fig. 4b).11 Additionally, the close conservation of the TRPM7-TRPM6 and TRPM1-TRPM3 coiled-coil pairs is reflected by the full channel comparison. As the coiled-coils (~50 residues) are only a fraction of the total channel subunit sequence (~1000 residues), correspondence between the overall TRPM family tree and the relationships of the coiled-coils suggests that the relative divergence of the coiled-coil sequences has paralleled other changes within the channel subunit. This observation reinforces the idea that interactions between the coiled-coil strands are likely to be important TRPM assembly specificity determinants.

**Fig. 4.** Structure-based comparison of TRPM coiled-coils. (a) Comparative interaction mapping of TRPM subtypes. Column labels identify residue positions involved in the a–d core layers and electrostatic networks. Filled boxes indicate conserved positions. Shaded boxes indicate nonconserved residues that remain capable of supporting interactions similar to those observed in TRPM7cc. White boxes indicate nonconserved positions. (b) TRPM channel phylogentic comparison.11
TRPM7cc is an antiparallel tetramer in solution

There are a number of cases in which crystal structures of engineered coiled-coils did not represent the only, or even the major, species present in solution. Therefore, we used a number of biochemical and biophysical measures to probe the structural and assembly properties of TRPM7cc in aqueous solution. Measurement of the TRPM7cc circular dichroism (CD) spectrum reveals minima at 208 nm and 222 nm that are characteristic of a protein with high helix content. Estimation of helix content indicates that the peptide is 92.2% helical. This value is in good agreement with the structure where each helix contains 50 residues (Arg1230–Lys1279) that comprise 87.7% of the 57 residue peptide. Further, the temperature-dependence of the CD signal shows a cooperative loss of structure, consistent with a well-folded complex having an extensive hydrophobic core, as seen in the crystal structure.

Size-exclusion chromatography of TRPM7cc showed a single peak with an apparent molecular mass that is consistent with a tetramer in agreement with the crystallographically observed stoichiometry. Size exclusion relies on the hydrodynamic radius. Because comparison of an elongated protein assembly, such that formed by TRPM7cc, to the globular protein size standards

Table 3. TRPM7cc equilibrium sedimentation data

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>Observed molecular mass (kDa)</th>
<th>Oligomeric state</th>
<th>n</th>
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<tbody>
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<td>250</td>
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<tr>
<td>150</td>
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<td>70</td>
<td>23.7±0.7</td>
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<tr>
<td>30</td>
<td>25.4±1.0</td>
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<tr>
<td>Average</td>
<td>25.2±1.5</td>
<td>4.0±0.2</td>
<td>13</td>
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</tbody>
</table>

Monomer molecular mass is 6.3 kDa. Errors indicate standard deviation.
used in the size exclusion experiments has some uncertainty, we used sedimentation equilibrium experiments, a method that is shape-insensitive, to attain a precise measurement of the assembly state of TRPM7cc in solution. Over a range of concentrations, the TRPM7cc equilibrium sedimentation data were well-fit by a single species model having the molecular mass of a tetramer (Fig. 5d, Table 3). These data establish the tetrameric nature of TRPM7cc in solution.

To probe whether TRPM7cc tetramers assembled in the antiparallel orientation in solution, we used a disulfide exchange assay that reports on the relative helix orientation within the bundle (Fig. 6a). We expressed and purified TRPM7cc variants in which a single cysteine residue was added to the N terminus (N-TRPM7cc) or C terminus (C-TRPM7cc) via a Gly-Gly-Ser linker. These peptides were used in subunit exchange experiments in which complexes containing either oxidized N-TRPM7cc or C-TRPM7cc homodimers were mixed, reduced, allowed to exchange partners, and reoxidized. Figure 6a depicts the equilibria and expected species for the antiparallel and parallel cases following the exchange of reduced subunits and disulfide bond reformation. The presence of antiparallel tetramers results in the creation of a unique heterodimeric disulfide linked species N-TRPM7cc/C-TRPM7cc that is absent from the parallel arrangement. HPLC and mass spectrometry analysis showed that a new peak that corresponds to disulfide linked N-TRPM7cc/C-TRPM7cc heterodimer, N-C, appears following the initial mixing of N-TRPM7cc and C-TRPM7cc homotetramers composed of only N-N and C-C dimers (Fig. 6b). The N-C antiparallel peak becomes the dominant disulfide-bonded species as the system reaches equilibrium and provides an unambiguous signature of the presence of antiparallel quaternary structure in solution. Taken together, these data establish that the TRPM7 assembly domain

![Fig. 6](image-url)
is a helical antiparallel tetramer in solution that mirrors the crystal structure and the expected stoichiometry of tetrameric TRPM channels.

Discussion

The presence of cytoplasmic domains that direct channel assembly appears to be a feature of many voltage-gated ion channel superfamily members. Because the assembly of different combinations of pore-forming subunits can have profound effects on channel functional properties, it is critical for members of this channel class to encode assembly determinants that direct association with the appropriate partners.\(^4,19,71–73\) Despite the fundamental nature of this problem, the mechanisms that drive channel assembly and assembly specificity remain imperfectly understood. To date, two different types of assembly domains have been characterized structurally: the T1 domain, a tetramerization and assembly specificity domain found in Kv1-Kv4 channels,\(^38–41\) and the Kv7 (KCNQ) coiled-coil assembly specificity domain.\(^29,30\) Coiled-coils have been identified in a number of other voltage-gated superfamily members that include TRPM channels,\(^8,23,24\) Eag channels,\(^31,32\) CNG channels,\(^33,34\) polycystins,\(^35,36\) and in the voltage sensor-only proton channel Hv1.\(^37\) and suggest that the coiled-coil motif is the predominant means for mediating pore-forming subunit assembly in this superfamily.

TRPM7cc is a four-stranded antiparallel coiled-coil that is held together by extensive core packing and interstrand polar interactions. Three types of core packing have been reported for homotetrameric antiparallel coiled-coils, a–d, e–a–d and a–d–g (Fig. 3b). Two classes, e–a–d and a–d–g cores, have been proposed to arise when either the e positions,\(^74\) or g positions\(^79\) contain largely nonpolar residues, respectively. TRPM7cc has the a–d packing and bears e and g amino acids that are mostly polar and incompatible with the e–a–d and a–d–g modes. Comparison of a–d four-strand antiparallel bundles further shows the architectural diversity that can arise even when the basic core of the assembly is similar. The TRPM7cc structure buries the surface area of the e and g positions in a more symmetrical way than other a–d core coiled-coils (Table 2; Supplementary Data Fig. 3).

One of the striking features of the TRPM7cc structure is the gradual change in the strand arrangement from square-shaped symmetry in the middle layers to a diamond-shape in the end layers (Fig. 2d). This change occurs in conjunction with changes in core packing from knobs-into-holes to knobs-against-knobs and an unusual layer-to-layer alternation regarding which residues participate in the knobs-against-knobs packing. One possible source for the change is that the volumes of the side chains in the middle layers are similar, whereas there are larger mismatches (e.g. layer 5 Met/Ser) at the ends. Additionally, layers 5–7 contain polar core residues that are involved in a variety of interactions with positions outside of the core. Such features may contribute to the change in helix packing arrangement.

Even though coiled-coils are the most common and best understood protein-protein interaction domain\(^25,26\) and there are a number of robust algorithms for identifying coiled-coil sequences, the ability to predict the association state and assembly orientation of a given coiled-coil sequence remains elusive.\(^7\) The unique structural features of TRPM7cc highlight the diversity of assembly architectures that can be encoded in a coiled-coil sequence.

Insights into subunit assembly preferences

TRP channels form the largest and most diverse subfamily within the voltage-gated ion channel superfamily.\(^1\) In accord with other family members, TRP channels are thought to function as homotramers or heterotramers of pore-forming subunits. Analysis of TRPM coiled-coil assembly domains through the lens of the TRPM7cc structure reveals that TRPM coiled-coil assembly domains cluster into two families, the TRPM7 group (TRPM1, TRPM3, TRPM6, and TRPM7) and the TRPM8 group (TRPM2, TRPM4, TRPM5, and TRPM8). Gross differences in the coiled-coil lengths and incompatibilities in hydrophobic core compositions suggest that the TRPM7 and the TRPM8 groups have different quaternary structures and that there is unlikely to be co-assembly between group members. Because of the extensive differences, it is unclear from the comparison whether the TRPM8 group forms parallel or anti-parallel tetramers.

In contrast, the presence of extensively conserved side chain chemistries among a–d core and interstrand polar interaction network residues indicates good structural compatibility between TRPM7 and TRPM6 coiled-coils. This assembly domain similarity matches the ability of these two channel subunits to form functional heteromultimers.\(^18–20\) The other two members of the TRPM7 group, TRPM1 and TRPM3, appear more similar to each other than to TRPM7. Although the TRPM1 and TRPM3 coiled-coil a–d core residues have a good deal of similarity to TRPM7cc, the inclusion of bulky and polar amino acids Met and Glu in layers 1 and 2 (Fig. 4a) might be incompatible with the very regular square arrangement of the strands in this part of the coil. Further TRPM1 and TRPM3 lack the ability to form Network 1 and Network 3 interactions (Fig. 4b). The similarities between TRPM1 and TRPM3 assembly domains raise the possibility that these subunits form heteromeric channels. The most striking observation from the structure-based comparison of the TRPM7 assembly domains is that the relationships found in the assembly domains reflect the phylogenetic relationships among the full-length subunits (Fig. 4). This observation lends support to the idea that the assembly domains carry information for directing assembly specificity.
Fig. 7. Comparisons between TRPM7 and Kv7 channel assembly domains. (a) Cartoon model of TRPM7 showing the relationship between the antiparallel assembly domain, transmembrane domains, and dimeric kinase domains, 1IA9. For display purposes, the kinase domain dimer is shown linked to the C-terminal ends of antiparallel assembly domain strands. The other arrangement, kinase dimers linked to parallel strands, is also possible. Presently available data do not favor one arrangement over the other. (b) Cartoon model of a Kv7 channel. The parallel coiled-coil assembly domain, 1OVC, is shown as ribbons. In both panels, only two of the four transmembrane domains are shown.
Relationship of the TRPM7cc antiparallel assembly to other TRPM domains and ion channel assembly domains

Structural studies have elucidated three different assembly domain architectures employed to specify assembly and assembly preferences of individual pore-forming subunits within the voltage-gated channel superfamily: tetramers of the Kv1-Kv4 T1 domain,28,38,75 Kv7 channel parallel tetrameric coiled-coils,29,30 and TRPM7 antiparallel coiled-coils. Because Kv7 coiled-coil assembly domains are parallel tetramers having approximate $C_4$ symmetry, which matches the expected symmetry of the voltage-gated channel superfamily pore-forming domains, it was unexpected to find that the TRPM coiled-coil assembly domain TRPM7cc is an antiparallel coiled-coil having $D_2$ symmetry (Fig. 7). Although at first glance the antiparallel coiled-coil assembly domain might seem incompatible with the pore architecture, TRPM7cc is located ~100 residues distal to the end of the last transmembrane segment, S6. This intervening linker is sufficiently long to ease any requirement for an exact symmetry match between the assembly domain and the pore-forming subunit. It is notable that the arrangement of the TRPM7cc C-terminal ends is compatible with the symmetry of the immediately downstream kinase domains that forms dimers (Fig. 7a).55 Thus, the antiparallel coiled-coil assembly would be a suitable linker between the tetrameric transmembrane region and the dimer cytoplasmic enzyme domain. Given the similarities between TRPM7, TRPM6, TRPM1, and TRPM3 coiled-coils, it seems likely that similar antiparallel arrangements exist for this subclass.

Both Kv1-Kv4 T1 domains76-78 and Kv7 coiled-coil assembly domains79-81 form platforms for the recruitment of regulatory molecules to the channel. The importance of such protein complexes is underscored by the observation that some disease mutations reside on the exterior of the Kv7 coiled-coil assembly and rather than act by blocking channel assembly, interfere with regulatory protein binding.29,30 It seems likely that TRPM coiled-coil assembly domains will perform a similar role in the recruitment of TRPM modulatory proteins and may harbor channelopathy mutations. The structure of TRPM7cc that we present here should serve as a useful starting point for focusing questions about TRPM channel assembly, assembly specificity, interactions to cytosolic regulatory factors, and in understanding the roles of TPRM channels in human diseases.

Materials and Methods

Protein cloning, expression, and purification

DNA corresponding to the rat TRPM7 coiled-coil domain (residues 1230–1282) was amplified by PCR and ligated into the NarI/HindIII sites of a pET27 (Novagen) derived vector (pSV272) denoted HMT55 that contains, in sequence, a hexahistidine tag, the maltose-binding protein, and a cleavage site for the tobacco etch mosaic virus (TEV) protease. As a consequence of the cloning sites, the constructs retain an extra N-terminal sequence GAGS following TEV cleavage of the purified protein. Point mutations were made using oligonucleotide extension (Pfu Turbo Polymerase, Strategene), digested with DpnI (New England Biolabs), and transformed into DH5α cells, and verified by DNA sequencing.

HMT fusions were expressed in Escherichia coli [BL21 (DE3)pLysS] grown in 2YT at 37 °C and induced at $A_{600\text{ nm}}$ 0.4–0.8 with 0.4 mM IPTG for 4 h. SeMet-TRPM7cc protein was expressed in PASM-5052 auto-induction medium (50 mM Na2HPO4, 50 mM KH2PO4, 25 mM (NH4)2SO4, 2 mM MgSO4, 0.5% (v/v) glycerol, 0.05% (w/w) glucose, 0.2% (w/w) lactose, 0.2× trace metal mix, 200 mg/ml of each of 17 amino acids except cysteine and tyrosine, 10 mg/ml methionine, 125 mg/ml SeMet, and 100 mM vitamin B12) for 24 h.53 Cells were harvested by centrifugation at 5000g for 15 min, at 4 °C, and cell pellets were frozen at −20 °C. Thawed cell pellets were lysed by sonication in lysis buffer (20 mM Tris pH 8.0, 250 mM KCl, 10% (w/v) sucrose, 1 mM EDTA, 1 mM PMSF). Insoluble material was precipitated by centrifugation at 12,000g for 20 min at 4 °C. The resulting soluble fraction, which contained the HMT fusion protein, was applied to a 45 ml Poros20MC (Applied Biosystems) nickel-charged column, washed in wash buffer (10 mM PO4−3, pH 7.3, 250 mM KCl), and eluted on a linear gradient to 500 mM imidazole in the same buffer on an ÄKTA-FPLC system (GE Healthcare). Imidazole was removed by dialysis (SPECTRUMLABS.COM). Fusion proteins were then applied to a 60 ml Amylose (New England Biolabs) column, washed in wash buffer, and eluted in maltose buffer (10 mM PO4−3, pH 7.3, 250 mM KCl, 10 mM maltose). The buffer was exchanged to (10 mM Heps, pH 7.4, 50 mM KCl, 1 mM EDTA) by dialysis. The HMT-fusion protein was cleaved with TEV protease (~300 μM TEV protease for 10 h at 18 °C in the TEV buffer).84 Cleaved material was applied to a Poros20MC nickel column and coiled-coil peptides were collected in the flow-through. Needle-shaped crystals formed in the fraction tubes in this purification step. The flow-through was further concentrated in an Amicon centrifugal filter (Millipore) during which time more microcrystals formed. The needle-shaped crystals were collected and dissolved in one of three buffers, Mg2+ buffer (10 mM Tris, pH 8.0, 100 mM MgCl2), guanidine buffer (50 mM Tris, pH 8.4, 300 mM guanidine, 3 mM EDTA), or acidic buffer (20 mM sodium acetate, pH 5.0). Protein concentration was determined by absorbance (BCA Protein Assay Kit, PIERCE). For purification of SeMet TRPM7cc, 5 mM 2-mercaptoethanol was included in the purification buffer for each step. SeMet protein also formed microcrystals during purification. For SeMet TRPM7cc crystallization, 5 mM DTT was included in both the protein solution and reservoir solution.

Crystallization and data collection

TRPM7cc crystals were obtained by the hanging-drop, vapor-diffusion method at 20 °C. Space group C2121 crystals grew from mixtures of 1 μl of peptide (5 mg ml−1 dissolved in the Mg2+ buffer) and 1 μl of reservoir solution containing 100 mM Tris pH 8.0, 100 mM MgCl2, 5–10% (v/v) isopropanol. Space group C2221 crystals grew from mixtures of 1 μl of peptide (5 mg ml−1 dissolved in the acidic buffer) and 1 μl of reservoir solution containing 100 mM sodium acetate, pH 4.9, 70–140 mM (NH4)2SO4.
and 40–50% (v/v) glycerol. Crystals appeared within about one day and reached full size in three to four days. TRPM7cc SeMet crystals were grown in the C222 crystallography conditions. For data collection, the C121 crystals were transferred to Paratone-N and flash-cooled in liquid nitrogen. C222 crystals were frozen directly from the drop in liquid nitrogen. Data were collected at ≤100 K at Advanced Lightsource Beamline 8.3.1 (Lawrence Berkeley National Laboratory, Berkeley, CA) equipped with a Quantum 210 CCD detector (Area Detector Systems) and at the SSRL Beamline 9-2 (Stanford Synchrotron Radiation Laboratory) equipped with a MarUSA MarMosaic–325 CCD detector and were processed with HKL2000 (HKL Research).

Structure determination

Three-wavelength MAD experiments were performed on the SeMet C222 crystals. Fourteen selenium positions were located using SOLVE. After density modification, the figure of merit was 0.68. The initial model was built with RESOLVE and ARP/warp. The structure from the C121 crystal was solved by molecular replacement using the structure template from the C222 crystal by Phaser. Model building, including automatic and manual addition of solvent molecules, was done with COOT. The C121 crystal structure was refined to 2.01 Å using REFMAC5. 4

Structure analysis

Models were evaluated using the PDB evaluation server. No residue was found in Ramachandran plot disallowed regions. Buried surface areas were calculated from the difference of the accessible side chain surface areas of the tetramer structure and of the individual helical monomers using ArealMol of the CCP4 suite. Residues 1231–1277 of TRPM7, 4–31 of GCN4-E20C (PDB code 2CCN), 7–27 of chain A and 34–52 of chain B of ROP (PDB code 1ROP), 31–50 of WSPLP (PDB code 1YOD), 340–355 of Lac repressor (PDB code 1LBJ), 5–29 of GCN4-pAεLV (PDB code 2R2V), 5–29 of SARS-C44 (PDB code 1ZV7) and 6–27 of GCN4-PV (PDB code 2B22) were used in the calculations. Three or four residues at the most N-terminal and at the most C-terminal of the coiled-coil structures were omitted to minimize end effects. Coiled-coil parameters were calculated with TWISTER.

CD spectroscopy

Purified TRPM7cc (50 μM in 250 mM NaCl, 100 mM MgCl₂, 10 mM Tris, pH 8.9) was analyzed with a Aviv model 215 spectropolarimeter (Aviv Biomedical) equipped with a Peltier device. Wavelength scans from 315 nm to 190 nm were taken at 4 °C in a 2 mm pathlength cuvette. Thermal stability was assessed by monitoring [θ]222 every 2 °C from 4–98 °C with 1 min equilibration time at each step. The molar ellipticity per residue of the buffer-subtracted CD spectrum was calculated as a function of concentration, path length, and number of residues per monomer:

\[
[\theta]_{222} = \theta / (cN_R)
\]

where [θ]MRD is the molar ellipticity per residue in deg·cm⁻²·(mol·res⁻¹), θ is the experimental ellipticity (in millidegrees), M is the molecular mass of the peptide, c is the protein concentration (in μM), l is the cuvette path length (in cm), and NR is the number of residues in the peptide. The percentage helicity was estimated as:

\[
\%\text{helicity} = \frac{[\theta]_{222}}{[\theta]_{222}^\text{max} \times (1 - i_c/N_R)} \times 100
\]

where [θ]222 is the experimental molar ellipticity per residue at 222 nm, [θ]222^⁰ is the molar ellipticity for a helix of infinite length at 222 nm (i.e., −39,500 deg·cm²·(dmol·res⁻¹)). i is the number of helices, θ is a wavelength-specific constant with a value of 2.57 at 222 nm, and N_R is the number of peptide residues.

Size-exclusion chromatography

A 100 μl sample of TRPM7cc peptide (500 μM peptide in 300 mM KCl, 100 mM MgCl₂, 50 mM Tris, pH 8.0) was passed through a Superdex75 HR 10/30 column (GE Healthcare) in 300 mM KCl, 100 mM MgCl₂, 50 mM Tris, pH 8.0 on an AKTA-FPLC system (GE Healthcare) at 4 °C. Eluates were monitored by measuring the absorbance at 280 nm at a flow rate of 0.4 ml min⁻¹. Elution volumes from at least three runs were averaged.

Equilibrium sedimentation

Equilibrium sedimentation experiments were performed at 4 °C in a Beckman Optima XL-A analytical ultracentrifuge (Beckman Coulter). TRPM7cc peptide was dissolved at a concentration of 150 μM in 400 mM KCl, 200 mM MgCl₂, 80 mM Tris, pH 8.0 and loaded into a six-chamber analytical ultracentrifuge cuvette, using the dialyze as a blank in the adjacent chamber. The molecular mass was calculated from a single-species exponential fit (Excel) to the distribution of concentration over the radius of the chamber as follows:

\[
M = \frac{2RT}{[(1 - \nu R)\omega^2]} \left( \frac{d\ln(c)}{dr^2} \right)
\]

where M is the molecular mass (in g mol⁻¹), R is the gas constant (8.314 J mol⁻¹ K⁻¹), T is temperature (in K), ν is the partial specific volume of the protein (in ml g⁻¹), ω is the angular velocity (in rad s⁻¹), and r is the distance (in cm) from the center of the rotor to a given position in the cell. Partial specific volume was calculated from the sum of the partial specific volumes of the individual residues in the protein. Solvent density was calculated from the components in the buffer. Residuals were calculated as the difference between the measured absorbance value and the predicted value extrapolated from the calculated molecular mass.

Disulfide exchange assay

Variants of the TRPM7cc sequence with N-terminal Gly-Ser-Gly-Ser-Cys-Gly-Gly-Ser (N-TRPM7cc) or C-terminal Gly-Ser-Gly-Ser-Cys (C-TRPM7cc) were cloned into the pSV227-HMT vector. The Gly-Ser-Gly residues N-terminal to the cysteine in N-TRPM7cc were included to facilitate HPLC separation and unambiguous mass spec identification the heterodimer. N-TRPM7cc and C-TRPM7cc were expressed and purified separately. Purified peptides were mixed (1 mg ml⁻¹ each) and incubated in a reducing buffer (300 mM guanidine, 50 mM Tris–HCl pH 8.4, 3 mM EDTA, 1 mM DTT) for 0 h, 2 h and 48 h. After incubation, the buffer was exchanged to 300 mM guanidine, 50 mM Tris–
HCl pH 8.4, 3 mM EDTA using an Amicon centrifugal filter (Millipore) with several times of dilution and increased to a final volume of 30 ml. Disulfide bonds were generated by oxidation with 20 μM H2O2. Samples were concentrated to 100 μl and analyzed by HPLC using a linear water/acetonitrile gradient in 0.1% (v/v) tri-fluoroacetic acid and a C-18 218TP54 analytical column (Vydac) monitored by measuring absorbance at 230 nm. Identities of disulfide-bonded species were confirmed by mass spectrometry in-house or in the Vincent Coates Foundation Mass Spectrometry Laboratory (Stanford).

**Protein Data Bank accession code**

Coordinates and structure factors have been deposited with the Protein Data Bank with structure code 3E7K.

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**Supplementary Data**

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.jmb.2008.08.059

**References**


Supplemental Figure 2

A: GAGSRVTFERVEQMSIQIKEVGDRVNYIKRSLQSLDSQIIGHLQDSLALTVDTLKTLT
B: GAGSRVTFERVEQMSIQIKEVGDRVNYIKRSLQSLDSQIIGHLQDSLALTVDTLKTLT
C: GAGSRVTFERVEQMSIQIKEVGDRVNYIKRSLQSLDSQIIGHLQDSLALTVDTLKTLT
D: GAGSRVTFERVEQMSIQIKEVGDRVNYIKRSLQSLDSQIIGHLQDSLALTVDTLKTLT
E: GAGSRVTFERVEQMSIQIKEVGDRVNYIKRSLQSLDSQIIGHLQDSLALTVDTLKTLT
F: GAGSRVTFERVEQMSIQIKEVGDRVNYIKRSLQSLDSQIIGHLQDSLALTVDTLKTLT
G: GAGSRVTFERVEQMSIQIKEVGDRVNYIKRSLQSLDSQIIGHLQDSLALTVDTLKTLT
H: GAGSRVTFERVEQMSIQIKEVGDRVNYIKRSLQSLDSQIIGHLQDSLALTVDTLKTLT

(Missing Atoms)

Chain-A: GLU1237 Cδ Oε1 Oε2
          GLU1245 Cδ Oε1 Oε2
Chain-B: LYS1244 Cε Nζ
          GLU1245 Cγ Cδ Oε1 Oε2
          LYS1254 Cδ Cε Nζ
          LYS1279 Cδ Cε Nζ
          THR1280 Cβ Oγ1 Cγ2
Chain-C: LYS1244 Cε Nζ
Chain-D: LYS1244 Cε Nζ

Chain-E: GLU1237 Cγ Cδ Oε1 Oε2
          LYS1244 Cε Nζ
          LYS1279 Cε Nζ
Chain-F: GLU1234 Cδ Oε1 Oε2
Chain-G: LYS1244 Cδ Cε Nζ
Supplemental Figure 1

Crystal packing and heavy atom positions in a tetramer coiled-coil.

MAD experimental density map for the TRMP7cc C222 crystal form contoured at 1.0 σ. Two heavy atoms are clustered at each end of one coiled-coil tetramer. Heavy atom positions are shown as orange spheres. Sequence alignment (bottom) indicates the selenomethione position.

Supplemental Figure 2

Summary of TRPM7cc structure

Residues absent from the TRPM7cc refined model are shown in grey. Missing atoms from sidechain residues are indicated.

Supplemental Figure 3

Comparison of the buried surface area among ‘a-d’ core antiparallel coiled coils positions