The essential double-ring eukaryotic chaperonin TRiC/CCT (TCP1-ring complex or chaperonin containing TCP1) assists the folding of ~5–10% of the cellular proteome. Many TRiC substrates cannot be folded by other chaperonins from prokaryotes or archaea. These unique folding properties are likely linked to TRiC’s unique heterooligomeric subunit organization, whereby each ring consists of eight different paralogous subunits in an arrangement that remains uncertain. Using single particle cryo-EM without imposing symmetry, we determined the mammalian TRiC structure at 4.7-Å resolution. This revealed the existence of a 2-fold axis between its two rings resulting in two homotypic subunit interactions across the rings. A subsequent 2-fold symmetrized map yielded a 4-Å resolution structure that evinces the densities of a large fraction of side chains, loops, and insertions. These features permitted unambiguous identification of all eight individual subunits, despite their sequence similarity. Independent biochemical near-neighbor analysis supports our cryo-EM derived TRiC subunit arrangement. We obtained a Cα backbone model for each subunit from an initial homology model refined against the cryo-EM density. A subsequently optimized atomic model for a subunit showed ~95% of the main chain dihedral angles in the allowable regions of the Ramachandran plot. The determination of the TRiC subunit arrangement opens the way to understand its unique function and mechanism. In particular, an unevenly distributed positively charged wall lining the closed folding chamber of TRiC differs strikingly from that of prokaryotic and archael chaperonins. These interior surface chemical properties likely play an important role in TRiC’s cellular substrate specificity.

asymmetric reconstruction | atomic model | subunit structure

Defective protein folding is emerging as the molecular basis underlying a growing number of human diseases, ranging from cancer and heart disease (1) to aggregation-linked neurodegenerative diseases such as Alzheimer’s, Huntington’s, and mad cow disease (2, 3). The eukaryotic group II chaperonin TRiC (also known as CCT) is a central mediator of cytosolic protein folding and assembly (4, 5). TRiC also appears important for the prevention of protein aggregation and toxicity (6–8). TRiC is essential for cell viability, as it assists the folding of many essential proteins, including actin, tubulin, and many cell cycle regulators and signaling molecules (9, 10). Notably, a number of TRiC substrates cannot be folded by other chaperonins, suggesting that TRiC possesses unique structural and mechanistic properties that distinguish it functionally from other chaperonins (11, 12).

All chaperonins share a double-ring architecture, where each ring contains a central cavity that binds and folds substrate proteins. TRiC, a 1 MDa group II chaperonin, facilitates folding through the ATPase driven closure of a built-in lid that encloses the substrate in the central chamber (13–16). Each ring of TRiC consists of eight distinct but related subunits sharing 27–39% sequence identity (Fig. S1) (13, 17). In contrast, bacterial (18) and archael chaperonins (19) only have 1–3 different types of subunits, and for those archaea with three types of subunits, it is unclear whether in the natural organism they form homo- or heterooligomeric chaperonins (20). The divergence of TRiC subunits occurred early in the evolution of eukaryotes, because all eukaryotes sequenced to date carry genes for all eight subunits; orthologs of the various subunits across species are more similar than paralogs within a single species (17). Having eight distinct subunits may have allowed the diversification of substrate binding sites and activities within the ring of TRiC. Actin, tubulin (21, 22), Huntingtonin (8), and the von Hippel-Lindau tumor suppressor (23) are all recognized by distinct subsets of TRiC subunits through specific motifs; this directed binding may provide specificity for TRiC in the folding process. Accordingly, elucidating the subunit arrangement within the complex is essential to determine how TRiC affects the conformation and folding pathway of its substrates.

Understanding the molecular basis of the specific folding capacity of TRiC has been hindered by the paucity of structural information on this complex. An intraring subunit arrangement proposed from analysis of spontaneously dissociated TRiC complexes remains untested (24). Previous cryo-EM studies of TRiC achieved only up to ~15-Å resolution with imposed 8-fold symmetry (13, 25, 26), inadequate to resolve the asymmetry between the eight structurally similar subunits. Here we present a high-resolution cryo-EM structure of mammalian TRiC, derived without imposing any symmetry among the eight subunits. Our analysis reveals (i) a 2-fold axis of symmetry between the two rings of TRiC, and (ii) its intraring subunit arrangement. The structure-derived subunit arrangement of TRiC is supported by cross-linking analysis. Our study provides a structural baseline to elucidate the complicated mechanisms of substrate recognition, folding and cooperativity in TRiC.
Results

4.7-Å Resolution Map with No Symmetry Enforced. We used cryo-EM to determine the structure of bovine TRiC in the presence of ATP-AlF4. This biochemical preparation generated a homogeneous both-ring-closed conformation in all the particles (Fig. 1A). Using ~101,000 particle images, we calculated a density map without any imposed symmetry employing EMAN, a single particle reconstruction package (27, 28). At 4.7-Å resolution of this asymmetric map (Fig. 1B and Fig. S2A), it was possible to unambiguously delineate the 16 individual subunits in the complex. Moreover, the α-helix pitch, β-strand separation, and some of the bulky side-chain densities and loop regions were resolved in all subunits (Fig. S2B). Of note, this level of detail is sufficient to observe relatively small structural differences, i.e., insertions and differences in amino acid composition, among the subunits. To avoid any initial arrangement bias in our analysis, we have arbitrarily named the rings a, encompassing subunits ai–aiii, and b, with subunits bi–biv (Fig. 1B), labeled clockwise looking down on either end of the map.

One outstanding question about the organization of TRiC is whether both rings have the same subunit arrangement and, if so, what is the relative orientation of the rings. Because the structure was determined with no symmetry imposed, we were able to compare the two rings as well as use unique structural features in each subunit to answer these questions unambiguously.

An exhaustive correlation analysis, carried out by pairwise comparison between each computationally extracted subunit in one ring and each of the eight subunits in the other ring, reveals that the ordering of the subunits in the two rings is identical. A subsequent rotational correlation analysis between the two rings shows one pronounced peak at 0° (Fig. 1C), pinpointing the location of the 2-fold axis between the rings (Fig. 1B). These analyses identify the relative orientation of the rings and localize the 2-fold symmetry axis between them. In this arrangement, two opposing subunits (ai and aj) are directly aligned with the same subunits in the opposing ring (b, and b,). As a result, two subunits (ai and aj) are related 180° azimuthally from their equivalent subunits in b ring and the remaining four subunits (ai, aj, ai, and aii) are related 90° azimuthally to their equivalents in the b ring (Fig. 1D). This two-ring arrangement pattern is different from the previous report hypothesized from low-resolution cryo-EM maps (26).

Identification of Intraring Subunit Arrangement. Exploiting the existence and position of the 2-fold symmetry axis, a new TRiC structure was computed enforcing the 2-fold symmetry. This produced an improved map with ~4.0-Å resolution (Fig. S2), in which a large proportion of the side-chain densities was visible in all the subunits (Fig. 2). These identifiable structural features were used to unambiguously localize subunit-specific sequences and thus assign which subunits corresponded to the densities within the ring.

To start the identification process, we first constructed an initial homology model for each subunit of bovine TRiC based on the homologous group II chaperonins (13): the thermosome [1A6E, (19)] and KS-1 [1Q3O, (29)]. Herein we denote the TRiC subunits by both their CCT number and a Greek letter, i.e., CCT1 (α), CCT2 (β), CCT3 (γ), CCT4 (δ), CCT5 (ε), CCT6 (ζ), and CCT8 (η). Because all eight TRiC subunits share a high level of sequence identity (Fig. S1A), we would anticipate that the subunit densities are quite similar. However, sequence alignment of the eight bovine TRiC subunits (Fig. S1A) reveals substantial variations in the apical domain, particularly helix H8 and the adjacent connecting long loop (Fig. 2A and B). In addition, unique insertions are found in several subunits, including CCT1 (α) (equatorial domain, 471NPERKNL478), and CCT4 (δ) (apical domain, 283ILRDA287) (Fig. S1A). These insertions are predicted to form loops based on our homology models and provide characteristic features that, together with the diagnostic apical domain regions, allowed us to identify the individual subunits in the maps.

Next, each subunit density was evaluated against each of the eight possible homology models, with a special focus on the most divergent sequence regions in the apical domain (Fig. 2A and B). For instance, the side-chain densities of the subunit ai map gives the best match to the model CCT8 (η) as shown in Fig. 2C. The unique sequence stretches of CCT8 (η) located in the helix H8 and connecting long loop including 251NFSK254, 262AO263, and 265KAIAD269 (red, highlighted in Fig. 2B) matched the larger side-chain densities unambiguously visualized in the ai map (red characters in Fig. 2C). The side-chain densities in these apical regions were similarly matched to unique sequences in the other seven subunits (Fig. 2D–J).

Analyses of other unique sequence insertions (Fig. S3) and characteristic sequence stretches (Fig. S4) provided independent...
evidence for the identification of the eight subunits. For instance, CCT6(ζ) has a unique insertion loop (473 VCGDSDKGF 481) right after helix H8 (Fig. S1A). Fig. S3C demonstrates that the subunit αiV map most closely matches the CCT6(ζ) model; indeed the αiV map clearly depicts the extra density in the corresponding insertion loop region, along with the unique stretch 270 KK 271 of CCT6(ζ) and some of the bulky side-chain densities (i.e., D278, K279, and F281). In contrast, fitting any of the other seven models did not produce a match, leaving this insertion loop density unoccupied (Fig. S3D shows that CCT2(β) did not fit to the αiV subunit map). Furthermore, the insertion loops of several other subunits, including CCT3(γ) (Fig. S3E), CCT1(α) (Fig. S3E), and CCT4(δ) (Fig. S3G), were also identified as the best match to the corresponding densities. The above two sets of independent evidence (Fig. 2 and Figs. S3 and S4) provide a consistent intraring subunit arrangement of TRiC.

We next optimized the Ca model for each subunit from the initial homology model with the constraint of the corresponding cryo-EM subunit map, concurrently improving the peptide geometry. The optimized Ca model of the TRiC complex is shown in Fig. 3A. Viewed from the top of the map (Fig. 3A and B), the subunits follow the following clockwise arrangement: CCT8(θ), CCT3(γ), CCT2(β), CCT6(ζ), CCT1(α), CCT7(η), CCT5(ε), and CCT4(δ). The arrangement is identical in the trans ring, thus creating two pairs of homotypic interring interactions: CCT1(α)–CCT1(α) and CCT8(θ)–CCT8(θ) (Fig. 3B). Our structure-based subunit ordering differs substantially from the previous proposal based on the analysis of spontaneously dissociated dimers in extracts (24).

Validation of Subunit Arrangement by Biochemical Near-Neighbor Analysis. An independent assessment of the TRiC subunits

![Fig. 2. The match of the side-chain densities in an apical domain region with the corresponding optimized model for each of the eight subunits. (A) Location of this stretch (Sky Blue, including the protruding helix H8 and the connected loop) in a complete TRiC subunit (Gray). The three domains are labeled. (B) Sequence alignment of bovine TRiC eight subunits in that apical domain region as shown in A. Unique sequence stretches of each subunit are highlighted by red characters. Either one or a combination of several such characteristic stretches can serve as a fingerprint for each TRiC subunit. (C) Subunit aiV map (Blue meshes) with the optimized model of the best matching CCT8(θ) (Red). The residues with the clearly observable side-chain densities are labeled in black or red. Here the red labels correspond to the residues in the unique stretches of CCT8(θ) as in A. (D–J) Similar rendering style as in C for each of the subunit maps and the corresponding optimized models in the equivalent region.](image-url)
Optimization and Validation of the TRiC Model. Our cryo-EM structure clearly resolves many side chains within the subunits. In X-ray crystallography, density maps determined at ~4.0-Å resolution range are often considered marginal for determining the atomistic structures (32). However, recent studies have shown that it is indeed possible to reliably build a de novo Cα model directly from cryo-EM density map in this resolution range (28, 33). It should also be noted that our density map (Fig. 1B) was directly computed from the raw images (Fig. 1A) and not biased by any atomic model.

In order to assess the ultimate quality of our map and model, we optimized the full atomic model from the initial Cα trace by matching it to the map and the visible side-chain densities for three randomly chosen subunits: CCT2(β), CCT1(α), and CCT7(η). Fig. 4A shows the atomic model of the CCT2(β) subunit. The Ramachandran plot of this optimized model shows that over 95% of the main chain dihedral angles fall within allowable regions (Fig. 4B), demonstrating the quality of the final model to follow the protein stereochemistry. Optimized atomic models of the other two subunits show similar quality.

To further validate our model, we compare the only available TRiC domain crystal structure [i.e., the apical domain of mouse CCT3(γ) (34)] with our corresponding model. Because the crystal structure was not used as a template to build our model, 1GML can serve as a quality check. The good match between our model and 1GML (Fig. S6) validates the reliability of our model and thus, the quality of our map.

Discussion

Unlike most chaperonins, which have one to three distinct subunits, TCP1-ring complex or chaperonin containing TCP1 (TRiC/CCT) has eight distinct, but similar, subunits. The subunits are sufficiently similar that determining individual particle orientation with sufficient precision would require very high resolution. Considering also the roughly spherical shape of the both-ring-closed conformation (Fig. 1A and B), the determination of particle orientation of TRiC presented even more challenges than other asymmetric structures studied so far by cryo-EM. Approximately 35% of the ~160,000 particles lacked sufficient contrast even at this resolution to unambiguously assign their orientation. Identifying and eliminating these particles using custom software
converting resolution for an asymmetric structure by cryo-EM and our recently developed 2D fast rotation matching method (FRM2D) (36, 37) for the image alignment was critical in achieving the final resolution. This approach provided unprecedented resolution for an asymmetric structure by cryo-EM and allowed the unambiguous identification of the TRiC subunit arrangement.

Our TRiC structure has a number of important biological and mechanistic implications. First, it shows that each type of subunit has a fixed position within the ring, as suggested previously based on biochemical and genetic data (38). It also identifies the location of a 2-fold axis between the rings and reveals a unique interring arrangement that produces three pairs of heterotypic interring contacts, [CCT3(γ)–CCT4(β), CCT2(β)–CCT5(ε), and CCT6(ζ)–CCT7(θ)], and two sets of homotypic interring contacts, namely, CCT8(θ)–CCT8(θ) and CCT1(α)–CCT1(α). This unexpected subunit arrangement has important implications for the assembly mechanism and allosteric regulation of TRiC. Like other chaperonins, TRiC exhibits positive intraring cooperativity and strong negative interring cooperativity (15, 39). Interestingly, close analysis of the interring contacts for the various subunit pairs reveals different types of interfaces, with some pairs exhibiting a more extensive set of contacts (Fig. 3A). These data may open the way for understanding the molecular basis of the negative cooperativity, likely achieved through interring contacts (15). Furthermore, it has been proposed that the positive intraring cooperativity follows a sequential KNF model, whereby the conformational change is initiated at a specific subunit (39). Because this would be determined by the chemical and enzymatic properties of the individual subunits within the ring, as well as the substrate, identifying the subunit arrangement is critical to test this mechanism.

Our model also provides insights into the unique ability of TRiC to fold certain substrates. The eight different subunits appear to bind different motifs within nonnative substrates and thus may position their folding intermediates in a set of defined orientations. Lid closure confines the substrate to the inner chamber, where folding of stringent substrates takes place (14). In the group I bacterial chaperonin GroEL-ES, the inner chamber of the closed cis-ring is polar, and the pattern of charges in the chamber is important for the folding ability of GroEL (40).

We thus computed the electrostatic surface property of TRiC from our model and compared it with those of the thermosome, an archaeal group II chaperonin, and GroEL-ES (Fig. 5). TRiC, like the thermosome (Fig. 5E and Fig. S7B) and GroEL-ES (Fig. 5F and Fig. S7C), has a large proportion of hydrophilic residues lining the inner surface of the closed chamber (Fig. 5D and Fig. S7A). Notably, both TRiC (Fig. 5D) and thermosome (Fig. 5E) have a lower percentage of hydrophobic residues lining the chamber than GroEL-ES (Fig. 5F). Although the charge distribution in both thermosome (Fig. 5B) and GroEL-ES (Fig. 5C) is symmetrical, we observe varying surface properties among the different subunits of TRiC (Fig. 5A); e.g., CCT6(ζ) is more positively charged, whereas CCT2(β) is more negatively charged. Notably, the wall of the TRiC chamber is dominated by positively charged patches (Fig. 5A), particularly on the side containing subunits CCT1(α), CCT6(ζ), CCT2(β), and CCT3(γ). The thermosome (1A6E) closed cavity is also dominated by positive charges, but unlike TRiC, the charge distribution is symmetric (Fig. 5B). In contrast, the inner chamber of GroEL-ES is dominated by negatively charged patches (41).

The differences between TRiC and other simpler chaperonins, particularly the diversified surface properties of the inner chamber of TRiC, might be related to TRiC’s differential ability to fold some substrates that cannot be folded by other chaperones. Our structure will open the way to understand the mechanism and physical properties that underlie this selectivity.

Fig. 5. Surface property of the central cavity of TRiC, thermosome (1A6E) and the cis-ring of GroEL-GroES (1AON). (A–C) The inner cavity electrostatic potential of the TRiC/thermosome/GroEL-GroES are shown in cutaway views of the apical and intermediate domains, including GroES for C. Blue represents positively charged patches, red negatively charged patches, and white neutral patches. The smaller panel illustrates the viewing angle. Of note, the surfaces are approximate and variances due to model/map resolution may affect the fine details of surface potential. (D–F) Inner wall surface property of TRiC/thermosome/ GroEL-GroES with side-chain properties is shown: hydrophilic (Sky Blue), hydrophobic (Yellow), and main chain (White).
Materials and Methods

TRiC sample purification (42) and cryoEM sample preparation follow our established procedures (14). Data were collected on a JEM3200FSC electron microscope with an in-column energy filter (energy slit of 15 eV) under the following conditions: 300 kV, ~500,000 magnification, ~20 electrons/Å² dose, and 101 K specimen temperature. Images were recorded on Kodak SO163 film and digitized on a Nikon 9000 ED scanner with a 1.2 A/pixel sampling. The majority of the defocus ranges from 1.2–2.7 μm.

Approximately 160,000 particles were selected from 1,500 micrographs with the EMAN2 tool e2boxer. Contrast transfer function parameters were determined using cftf of EMAN (27, 28, 43). A recently developed FRMZD algorithm for image alignment (36, 37, 44) available in EMAN 1.8– suffers from a small-to-moderate performance on cryoEM images, was adopted in the refinement steps. We used a previously determined 15-Å resolution 8-fold symmetrized map of closed TRiC (13) as the initial model of the reconstruction. Other than that, in the asymmetric reconstruction and refinement process, no symmetry was imposed. The final map was computed from ~101,000 particle images, after eliminating particles that were not consistently classified in the same orientation between iterations. The map resolution was based on the 0.5 Fourier shell correlation (FSC) criterion (45). The final map was filtered and scaled to optimized map resolvability (46, 47).

Detailed procedures about map similarity analysis, homology model building and model optimization, and cross-linking and nearest-neighbor analysis are provided in SI Materials and Methods.

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Supporting Information

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SI Text

SI Materials and Methods.

Cryo-EM sample preparation. TRIC was purified according to established procedures (1, 2). Purified TRIC was incubated with 1-mM ATP-ALF for 1 h at 30 °C (3) prior to vitrification on a glow-discharged 200-mesh Quantifoil holey grid with 1.2 × 1.3-μm hole size (Quantifoil Micro Tools) using a Vitrobot (FEI Company).

Map segmentation and similarity analysis. The individual subunits from both the asymmetric and the 2-fold enforced cryo-EM maps were segmented using the EMAN program segment3d (4). Rigid body fitting of the individual model into subunit densities was carried out using Fit in Map module in Chimera [http://www.cgl.ucsf.edu/chimera; (5)].

We calculated the pairwise correlation score between each individual subunit map in the a ring with each of the eight subunit maps in the b ring of the asymmetric map. This allowed us to identify the corresponding subunit pairs between the rings and thus reveal whether the subunits in the two rings were arranged with the same ordering. The location of the 2-fold axis between the rings of TRIC in the asymmetric map was then confirmed by calculating the rotational correlation coefficient between the two rings of this map using EMAN2 (6). To enhance the significance of the similarity score difference, we subtracted the 8-fold symmetrized density from the asymmetric TRIC map. We then calculated the rotational correlation score from this difference map between the a ring and the b ring with a ring being fixed and b ring rotated every 1° clockwise up to 360° (Fig. 1C). The origin of this curve is arbitrary; we positioned the highest peak at the origin. But no matter where we place the origin of the curve, the location of the 2-fold axis in the map deduced from this curve is the same.

Homology model of bovine TCP1-ring complex or chaperonin containing TCP1 (TRIC/CCT) closed state. We built the initial homology models of the eight bovine TRIC subunits in the both-ring-closed conformation using MODELLER (7). We utilized the available sequences of the eight bovine TRIC subunits, and the crystal structures of homologous group II chaperonin, including thermosome (1A6E) (8) and KS-1 (1Q3Q) (9), served as template structures. Details were described in our previous study (3). Briefly, the eight bovine TRIC subunits have 35 ~41% sequence identity with the template structures; with this level of sequence identity, the models are expected to be quite reliable.

Model optimization. After the subunits were identified, each homology model was adjusted to the corresponding subunit density using the Regularize Zone module in COOT (10) to optimize the agreement of the model with cryo-EM density map, while maintaining correct stereochemistry. During this model optimization step, priority was given primarily to placement of Cα atoms to the density. For three subunits [CCT2(β), CCT1(α), and CCT7 (β)], further model improvement was carried out for the entire subunit by optimizing the side-chain atom placement to putative side-chain density, as well as the best possible main chain dihedral angles (4, 11). In the remaining subunits, this additional optimization was carried out only on helix H8 and the adjacent loop, and the apical domain of CCT3(γ).

The electrostatic potential was calculated with CHIMERA’s Coulombic Surface Coloring module (5).

TRIC cross-linking rationale and approach. Defining the overall subunit arrangement of TRIC using only cross-linking or only MS is extremely challenging, because all of the eight subunits are close in size and isoelectric point, and many of them are modified posttranslationally. Our approach was to identify the most probable neighbors for the subunits using a short, ideally zero-length, cross-linker. We chose formaldehyde as a cross-linker because it has one of the shortest cross-linking spans (~2–3 Å) of any cross-linking reagent (12, 13), thus making it an ideal tool for our approach. The cross-links obtained with formaldehyde are nucleophilic attack reactions, including side-chains containing sulfhydryls, amines, phenols, and imidazoles (12). The cross-linking reaction yields an ensemble of variants that adds to the complexity of TRIC itself. Therefore, we separated the individual cross-linking reaction products using a 2D gel electrophoresis analysis to maximally exploit the slight differences in size and charge. We excised from the 2D gel the few well-separated spots corresponding in size to dimers. As this region is complex, approximately 20 selected spots were excised (Table S1). We next used MS to identify the subunit content of the excised spots. All TRIC subunits could be identified by this analysis. We considered as positive neighbors only to those arising from well-separated spots that had multiple peptides corresponding to only two subunits. Such decision is based on the assumption that the separation of the two subunits is <3 Å apart as expected from the formaldehyde cross-linker length (12, 13). Importantly, these results provide an unbiased and independent confirmation in the arrangement of the eight subunits obtained separately by cryo-EM modeling (Fig. 3).

TRIC cross-linking and nearest-neighbor analysis. TRIC cross-linking was performed in 50 mM KHepes (pH 7.4), 50 mM KCl, 10 mM MgCl2, and 10% glycerol. To initiate cross-linking, formaldehyde (0.1% vol/vol final) was added to 2 μM TRIC and incubated for 20 min at room temperature. The low concentration of TRIC ensured that TRIC was fully monomeric and that no intercomplex cross-links could be obtained. The formaldehyde concentration was empirically determined to maximize the formation of dimers and not higher-order cross-links. The low yield of cross-links ensured that few nonspecific cross-links were produced, and these would not result in discernible spots in the 2D gel. The reaction was quenched by adding 70 mM TrisCl (pH 8.0) (Fig. S5A). Crosslinked TRIC (300 μg) was separated by 2D electrophoresis according to the carrier ampholine method of isoelectric focusing (14) by Kendrick Labs (Fig. S5B). Isoelectric focusing was performed using pH 5–8 ampholines (GE Healthcare), and proteins were then separated by SDS-PAGE using 7% polyacrylamide and stained with Coomassie blue. In-gel digestion was performed essentially as in ref. 15. Few well-separated spots corresponding in size to dimers were excised from the 2D gel and analyzed by MS on an ESI-QTOF Mass Analyzer (QSTAR® Elite Hybrid Quadrupole TOF, Applied Biosystems). TRIC subunits in each spot were identified from the MS data using ProteinPilot 2.0.1 (Applied Biosystems). Only proteins identified with 2× high confidence peptides were considered. For nearest-neighbor analysis, spots consistent with a dimer size and containing predominantly two or fewer subunits were considered (Fig. S5C).

A summary of data and complete methods is provided in Table S1. An overlapping or mixed set of neighboring cross-links, which would overlap in the gel, would yield peptides from multiple subunits (e.g., spot G in Table S1). These data demonstrate that we can detect all subunits of TRIC excised from a 2D gel by...
MS. Western blot analysis of the 2D gels using subunit-specific antibodies, similar to a previous approach (16), also confirmed specific cross-links [e.g., CCT2(β)–CCT5(ε)]; however, the MS approach is more sensitive and specific and does not suffer from the lack of specificity and affinity of some of the commercially available TRIC subunit-specific antibodies.

Methods for in-gel digest, LC-MS data acquisition, and data analysis. In-gel digestion for excised gel slices followed a standard protocol (15) with modifications as follows: DTT was replaced with 5 mM tris(2-carboxyethyl)phosphine in 100 mM ammonium bicarbonate; 200 mM iodoacetic acid in 100 mM sodium hydroxide; a sufficient volume of trypsin buffer was added to completely cover each gel slice and incubated overnight. Resulting samples were vacuum concentrated for 1 h, and a sufficient volume of trypsin buffer was added to completely cover each gel slice and incubated overnight. Resulting samples were vacuum concentrated for 1–2 h but generally not taken to dryness. For dried samples, 50 μL of 10% acetonitrile (ACN) in 100 mM ammonium bicarbonate solution was added and the sample was resuspended by vortexing for 2 min.

For chromatographic separation, 10 μL of the extracted peptide solution from each gel spot was autoinjected using an Eksigent Nano-LC (Applied Biosystems) onto a PepMap100 trapping column (0.3 × 5 mm) and washed for 20 min at 15 μL/min. Reverse-phase separation was completed on a PepMap100 column (75 μm × 15 cm) at a flow rate of 300 nL/min using buffers 2% ACN, 0.1% FA (A), and 98% ACN, 0.1% FA (B). The gradient was run as follows: 2–30% B in 15 min, 30–80% B in 2 min, 80% B for 3 min, 80–2% B in 2 min, and 2% B for 30 min. The column flow was directly connected to an ESI-QTOF Mass Analyzer (QSTAR® Elite Hybrid Quadrupole TOF, Applied Biosystems) using electrospray ionization at 2350 V. Precursor ions were scanned from 350 to 1,800 m/z. Three product ion scans were set to be collected for each cycle and the mass window for transmission was set to low. Only ions exceeding 35 counts were selected as parent ions for fragmentation, and fragments were scanned from 70 to 1,800 m/z. Parent ions and their isotopes were excluded from further selection for 90 s. A mass tolerance was set to 100 ppm. The fragment intensity multiplier was set to 4.0, with a maximum accumulation time of 2.0 s. Collected mass spectra were analyzed using ProteinPilot 2.0.1 (Applied Biosystems). A FASTA file containing all the ORF protein sequences of the TRIC complex was added to the UniProtKB/Swiss-Prot database [downloaded Feb. 2008 (17)]. ProteinPilot was run in rapid identification mode, with trypsin as the cleavage enzyme.

Fig. S1. (A) Sequence alignment of eight bovine TRiC subunits. For each subunit, a unique insertion or sequence stretch is highlighted by yellow and red frames, which is further illustrated in Figs. S3 and S4. (B) Ribbon diagram showing the general architecture of a TRiC subunit, with α-helices longer than two turns and β-sheets being labeled.
Fig. S2.  (A) Resolution assessment of the asymmetric map (Blue Curve) and the 2-fold imposed map (Red Curve) based on Fourier shell correlation of two independent reconstructions. Using the 0.5 criteria (dashed line), a resolution of 4.7 Å was obtained for the asymmetric map, and 4.0 Å for the 2-fold imposed map. (B) Representative features of the 4.7-Å resolution density map with no symmetry imposed.

Features of the 4.7 Å asymmetric map of TRiC

β-strands separation  α-helix pitch feature
Fig. S3. (A, C, E, and G) Examples of a unique fit between the map and the identified model in a characteristic insertion region for four TRiC subunits with such insertions: CCT3(γ) in A, CCT6(ζ) in C, CCT1(α) in E, and CCT4(δ) in G. The insertion sequence is highlighted in dark purple in both the model and the sequence stretch. Unique sequence stretches are highlighted in red characters. For each of the insertion shown in A, C, E, and G, we also show a comparison case (B, D, F, and H) by fitting CCT2(β) model into the same subunit map as in A, C, E, and G. CCT2(β) does not have any significant insertion in the entire sequence, which allows it to serve as a control case. For example, CCT3(γ) fits into the a_map very well (A), whereas fitting CCT2(β) into the same map will have an obvious unoccupied density as shown in B, highlighted by orange dotted circle and red arrowhead.
Fig. S4. Examples of a unique fit between the subunit map and the identified model in a characteristic sequence stretch for the four TRiC subunits without unique insertion: CCT8(θ) in A, CCT2(β) in B, CCT7(η) in C, and CCT5(ϵ) in D. The unique sequence stretches are highlighted in red characters in both the sequence and the model, which are also shown in Fig. S1A.

Fig. S5. Cross-linking and nearest-neighbor analysis. (A) Formaldehyde cross-linking of TRiC results in the production of dimeric and oligomeric species. Cross-linked (+) and control (−) TRiC samples were incubated in parallel in the presence and absence of cross-linker and separated by 8% acrylamide SDS-PAGE and stained with Sypro ruby. (B) Two-dimensional gel electrophoresis was used to separate the cross-linked species. Spots from the region consistent with dimers (−110 kD) were examined to identify linked pairs. (C) Magnification of the dimeric region of the gel. Gel spots were excised and examined by MS to identify TRiC subunits. Given the similarity of molecular weight and isoelectric point among TRiC/CCT subunits, most dimeric cross-links were overlapping in the gel. Five spots from the −110 kD region of the gel corresponded to unique cross-linked subunit dimers, as indicated in the gel. We attribute CCT8(θ)–CCT8(θ) to a homodimeric cross-link as it runs at the expected dimer molecular weight and is the only spot analyzed containing a single TRiC subunit. Of note, CCT8(θ) also has the lowest isoelectric point of all TRiC subunits, consistent with its position at the acidic end of the gel.
**Fig. S6.** Model quality validation. (A) Conformation comparison of mouse CCT3(γ) apical domain crystal structure (1GML chain A, Purple) with our corresponding optimized model of bovine CCT3(γ) (Green). (B–D) Detailed comparison between the two models in the H8 (B) and H10 (C) α-helix regions, and S11 and S12 (D) β-strand regions with side chains being illustrated. We zoomed in these helices and beta strands for comparison because these are well resolved in our map. Our model agrees very well with 1GML. Of note, 1GML is the crystal structure of apical domain only without the constrains of intermediate domain, therefore, the bottom beta strands underneath S11 and S12 in A appear to have a conformation variation from our model, which is from a complete CCT3(γ) subunit.

**Fig. S7.** Central cavity residue properties of TRIC (A), thermosome (1A6E, in B), and the cis-ring of GroEL-GroES (1AON, in C). Cutaway views of the apical and intermediate domains of the three complexes showing various side chains: positively charged (Blue), negatively charged (Red), polar (Green), hydrophobic (Yellow), and main chain (White). The viewing angle is illustrated in the lower left small panel.

**Other Supporting Information Files**
Table S1 (DOCX)