Structure of the ArgRS–GlnRS–AIMP1 complex and its implications for mammalian translation

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In higher eukaryotes, one of the two arginyl-tRNA synthetases (ArgRSs) has evolved to have an extended N-terminal domain that plays a crucial role in protein synthesis and cell growth and in integration into the multisynthetase complex (MSC). Here, we report a crystal structure of the MSC subcomplex comprising ArgRS, glutaminyl-tRNA synthetase (GlnRS), and the auxiliary factor aminoacyl tRNA synthetase complex-interacting multifunctional protein 1 (AIMP1)/p43. In this complex, the N-terminal domain of ArgRS forms a long coiled-coil structure with the N-terminal helix of AIMP1 and anchors the C-terminal core of GlnRS, thereby playing a central role in assembly of the three components. Mutation of AIMP1 destabilized the N-terminal helix of ArgRS and abrogated its catalytic activity. Mutation of the N-terminal helix of ArgRS liberated GlnRS, which is known to control cell death. This ternary complex was further anchored to AIMP2/p38 through interaction with AIMP1. These findings demonstrate the importance of interactions between the N-terminal domains of ArgRS and AIMP1 for the catalytic and noncatalytic activities of ArgRS and for the assembly of the higher-order MSC protein complex.

Aminoacyl-tRNA synthetases (ARSs) catalyze the attachment of amino acids to their cognate tRNAs, which is an essential step of protein synthesis in all life forms (1). Higher eukaryotic ARSs contain additional motifs or domains that are believed to endow noncanonical functions supplementary to their primary roles in protein synthesis, such as angiogenesis and inflammatory and apoptotic responses (2, 3). Importantly, these extended domains also play key roles in organizing various ARSs and their accessory proteins into the evolutionarily conserved and essential multisynthetase complex (MSC) (3, 4).

The MSC is believed to facilitate protein synthesis through a channeling mechanism and to regulate the balance between translation and nontranslational regulatory functions (5). Deletion of the MSC scaffold proteins leads to lethality or multiple pathological symptoms, demonstrating the importance of this complex to cellular functions (6, 7). The MSC is composed of nine different ARSs and three accessory proteins (aminoacyl tRNA synthetase complex-interacting multifunctional proteins, AIMP5) and is thought to consist of two subcomplexes (Fig. 1 S1). One complex is composed of arginyl-tRNA synthetase (ArgRS, hereafter “RRS”), glutaminyl-tRNA synthetase (GlnRS, hereafter “QRS”), and AIMP1/p43; another complex includes the rest of the components. These two subcomplexes are connected via AIMP2/p38.

The ternary MSC subcomplex comprising RRS, QRS, and AIMP1 (referred to here as “ROA1”) is of particular interest because, in addition to their canonical contribution to translation, all three components have cell-regulatory activities. AIMP1 is secreted to regulate angiogenesis, glucose homeostasis, immune responses, and tissue regeneration (8, 9). QRS controls cell death via an antiapoptotic interaction with apoptosis-signal regulating kinase 1 (ASK-1) (10). RRS can occur in two forms depending on the presence or absence of a 12-kDa hydrophobic domain at its N terminus (Fig. 1A) (11, 12); the high molecular weight (HMW) protein containing the additional domain is assembled into the MSC, whereas the protein lacking this domain (ΔN-RRS) exists in a free form. It has been proposed that HMW-RRS in the MSC produces arginyl-tRNA for protein synthesis, whereas ΔN-RRS generates arginyl-tRNA for protein modification (5, 13). Truncation of the N-terminal extension of human RRS reduces translational activity in eukaryotic cells and impairs cell growth (5). Furthermore, knockdown of HMW-RRS in Caenorhabditis elegans reduces protein synthesis and unfolded protein toxicity, resulting in resistance to hypoxic stress (14); however, it is unclear why the N-terminal domain of HMW-RRS is critical for protein synthesis and cell growth. Similarly, deletion of AIMP1 results in multispecies pathologies. Biochemical analyses of MSC from rabbit liver suggested that AIMP1, RRS, and QRS form a complex with a stoichiometry of 2:2:1 (15, 16). However, it is not yet understood specifically how the three proteins interact and how this interaction would influence the catalytic activity of RRS and QRS. Structural information about the interaction of RRS, QRS, and

Significance

In higher eukaryotes, aminoacyl-tRNA synthetases (ARSs) are assembled to form a multisynthetase complex (MSC), which plays critical roles in translation and nontranslation functions essential for cell growth and survival of organisms. The MSC complex is comprised of nine different ARSs and three accessory proteins. The crystal structure of the arginyl-tRNA synthetase (ArgRS)–glutaminyl-tRNA synthase–aminoacyl tRNA synthetase complex-interacting multifunctional protein 1 (AIMP1) subcomplex reveals that the N-terminal domains of ArgRS and AIMP1 form an extended coiled-coil structure, which provides a central depot for the assembly of a ternary complex. The stability of the N-terminal helix of ArgRS is critical for its ARS activity and noncanonical function of the subcomplex, explaining the significance of the MSC structure in translation and cellular functions.

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AIMP1 would provide significant insight into the control of the mammalian translation and also their roles in the assembly of MSC. With this motivation, we solved the crystal structure of the one critical subcomplex of MSC composed of RRS, QRS, and AIMP1, and investigated the role of the interaction pairs among RRS, QRS, and AIMP1 in catalysis and how this subcomplex can be linked to the other subcomplex via AIMP2.

Results

The N-Terminal Domains of RRS and AIMP1 Form a Coiled-Coil Structure.

We obtained a crystal for the RQA1 complex that diffracted to a 4.0-Å resolution (Table S1). The crystal contained one molecule each of RRS, QRS, and AIMP1 in the asymmetric unit (Fig. 1B).

Analysis of the crystal structure of RQA1 revealed that these three proteins form an elongated complex measuring 100 Å (length) by 85 Å (width) by 92 Å (height). The long coiled-coil helices of RRS and AIMP1 are positioned at the center of the complex, where the C-terminal cores of QRS and AIMP1 are packed (Fig. 1B and Figs. S2A and B and S3A). The helices are extended away from the RRS core. The first half of the C-terminal domain of QRS is composed of two α/β structures containing an active site and the signature motif (HVGH/FKT) and a helical bundle at the C terminus (Fig. 1B and Figs. S2A and B and S3A). The second half of the AIMP1 helix (yellow). Also see Fig. S7A. The figure is shown in the same orientation as in B. (D) Close-up view of the coiled-coil interaction between the core of RRS (blue) and the second half of the AIMP1 helix (yellow). The opposite face of the leucine-rich surface of AIMP1 binds to the core of RRS through ion pairs and hydrogen bonds. (E) Close-up view of the interface between QRS (magenta) and the Hb helix of RRS (blue). A hydrophobic cluster at the center is supported by ion pairs at each end of the interface.

The trimeric RQA1 complex is formed primarily between AIMP1 and RRS, as well as between RRS and QRS. In the RRS–AIMP1 interface at the center of the structure, the second half of the N-terminal helix of AIMP1 forms a coiled-coil interface with the N-terminal helix of RRS through a leucine zipper interaction (Fig. 1B). This interface contains the most extensive interactions in the RQA1 complex in a buried surface area of 2,756 Å². At one face of the second half of the AIMP1 helix, Leu48, Leu55, Ile59, Leu62, and Leu66 are interdigitated with Leu44, Leu51, Leu55, Leu58, and Leu62 from helix Hb of RRS (Fig. 1C). At the opposite face, polar groups, including Lys54, Glu61, Glu65, and Gln68, form hydrogen bonds and ion pairs with Asp94, Lys89, His86, and Arg79 of the C-terminal core of RRS, reinforcing the interaction between AIMP1 and RRS (Fig. 1D).

The N-terminal domain of human QRS is known to interact with RRS in the MSC (18). However, QRS is anchored to the complex by the interaction of its C-terminal core with the Hb helix of RRS. This interaction, which is formed by hydrophobic groups and two ion-pairs, contains ~1,443 Å² of buried surface area (Fig. 1E).

The N-terminal Helix of RRS Forms a Platform to Anchor QRS. The crystal structure revealed that protein–protein interfaces within the trimeric RQA1 complex are formed primarily between AIMP1 and RRS, as well as between RRS and QRS. In the RRS–AIMP1 interface at the center of the structure, the second half of the N-terminal helix of AIMP1 forms a coiled-coil interface with the N-terminal helix of RRS through a leucine zipper interaction (Fig. 1B). This interface contains the most extensive interactions in the RQA1 complex in a buried surface area of 2,756 Å². At one face of the second half of the AIMP1 helix, Leu48, Leu55, Ile59, Leu62, and Leu66 are interdigitated with Leu44, Leu51, Leu55, Leu58, and Leu62 from helix Hb of RRS (Fig. 1C). At the opposite face, polar groups, including Lys54, Glu61, Glu65, and Gln68, form hydrogen bonds and ion pairs with Asp94, Lys89, His86, and Arg79 of the C-terminal core of RRS, reinforcing the interaction between AIMP1 and RRS (Fig. 1D).

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Although large portions of the extended helices of RRS and AIMP1 are involved in the formation of the complex, the first half of the N-terminal helix of AIMP1 and most parts of the RRS
and QRS core, including the active site, are fully exposed in the trimeric RQA1 subcomplex.

**The RQA1 Subcomplex Also Can Form a Hexameric Structure.** In a gel-filtration analysis, the RQA1 complex was eluted at a peak corresponding to ~280 kDa (Fig. S2E), suggesting that the following structural organizations can occur: (i) the three subunits of the RQA1 complex are present in 1:1:1 (190 kDa) stoichiometry but are elongated; (ii) the three subunits are present in a 1:1:1 stoichiometry, but the complex contains additional subunit(s); or (iii) the complex exists in equilibrium between a 1:1:1 and a 2:2:2 (380 kDa) stoichiometry.

Although RRS, QRS, and AIMP1 form a 1:1:1 ternary complex in the asymmetric unit, these proteins also could form a 2:2:2 hexameric complex by a twofold crystallographic-symmetry operation. The hexameric complex form is more similar to the previous biochemical analysis, although it contains one additional QRS (15). In the hexameric structure, a twofold symmetry axis intersects the center of the AIMP1 helix such that an X-shaped arrangement of the two AIMP1 N-terminal helices is formed at the center of the complex (Fig. 2 A and B). The two QRS proteins are located at each side of the butterfly-shaped hexameric structure, and the two RRS core regions are located at the top of the complex. The N-terminal helix of AIMP1, which is fully exposed in the trimeric complex, becomes stabilized by the Ha helix of RRS in the symmetry-related complex. These two helices form a parallel coiled-coil structure by leucine-zipper interactions. Specifically, Ile19 and Ile26 of the Ha helix of RRS form a hydrophobic cluster with Leu32, Ile37, and Leu25 of the first half of AIMP1 (Fig. 2C). Notably, Ile21 and Ile22 of the AIMP1 helix and Leu12 and Leu13 of the Ha helix of RRS are exposed in the hexameric structure, suggesting that additional subunits of the MSC could interact with these helices. In the hexameric architecture, the two RRS cores are located close to each other, although there is enough space for Arg or ATP to access the active site. However, the space between the RRS core and the symmetry-related RRS core is not sufficient to accommodate tRNA. We speculate that the highly flexible loop (residues 30–39), B factor, 221 Å)

![Image](image.png)

**Fig. 2.** Hexameric structure of the RQA1 subcomplex. (A) Stereodagram of the hexameric RQA1 subcomplex. The figure is shown in the same orientation as in Fig. 1B. (B) A cartoon representation of the overall shape of hexameric RQA1, shown in same orientation as in A. The invisible structures of the N-terminal domain of QRS (orange) and the C-terminal EMAPII domain of AIMP1 (yellow) were modeled. The linker between the N- and C-terminal domains of AIMP1 is indicated by a yellow dashed line. The active sites are marked with stars. (C) Close-up view of the coiled-coil interface between the Ha helix of RRS (blue) and the first half of the AIMP1 helix (yellow) in the hexameric structure. Also see Fig. S7B. (D) The SAXS profile of the solution structure (empty circles) of the RQA1 subcomplex. The solid red and blue lines are the theoretical SAXS curves calculated from the crystal structures of the trimeric and hexameric forms of human ARS, respectively. The solution structures of RQA1 in other concentrations also displayed similar SAXS profiles and are omitted here for clarity. The Guinier plot is shown in the Inset. (E) Pair distance distribution functions (p(r)) for the RQA1 complex based on an analysis of the experimental SAXS data (empty circles). The electron distribution functions for trimeric and hexameric crystal structures are shown by red and blue lines, respectively. (F) Structural superposition of the hexameric RQA1 subcomplex onto the molecular envelope calculated from the SAXS data showing two different views of the model. To compare the overall shape and dimensions, the crystal models were superimposed onto the solution models using the SUPCOMB program (30).
C-terminal domain of AIMP1 and/or the N-terminal domain of QRS.

The N-Terminal Helices of RRS Are Crucial for Formation of the RQA1 Subcomplex. To determine the importance of the leucine-zipper interactions between the second half of the AIMP1 helix and the Hb helix of RRS in the formation of the RQA1 subcomplex, we generated two AIMP1 mutants (M1 and M2) and determined their abilities to form a complex with RRS and QRS (Fig. S5A). The M1 mutant, in which the second half of the AIMP1 helix (residues 44–72) was deleted, failed to form a complex with RRS and QRS and was eluted at a later position in a gel-filtration analysis (Fig. 3A). To determine if the coiled-coil interaction between the AIMP1 helix and the Hb helix of RRS occurs in a sequence-dependent manner, the first (residues 7–43) and second halves of the AIMP1 helix were exchanged in the M2 mutant. Although the effect of this swapping mutation was not as severe as that of the M1 deletion, the AIMP1 M2 mutant failed to form a stable complex with RRS–QRS, because AIMP1 was eluted first, followed by the RRS–QRS complex. This result demonstrates the importance of sequence specificity in the coiled-coil formation between AIMP1 and RRS (Fig. 3A). Although the RRS-binding region was present in the M2 swapping mutant, it is likely that an altered arrangement of the AIMP1 helix prevented the formation of the correct ternary complex.

To examine the significance of the interface between QRS and the Hb helix of RRS, five residues (Lys50, Tyr53, Arg54, Ile57, and Glu65) in the Hb helix of RRS were mutated to create the R1 mutant (Fig. S5A). Among these residues, Glu65 is located close to Lys394 of QRS, which undergoes posttranslational modification (Fig. 1E) (19). A binding analysis revealed that QRS dissociated from the ternary complex containing the R1 mutant (Fig. 3A), indicating that this complex could compensate for AIMP1 M3 and M4 mutations. We also analyzed the solution structure of the RQA1 complex with the AIMP1 M3 or M4 mutant. The solution scattering data for the RQA1 complex with the M3 or M4 mutant showed that the complex adopts a structure that is close to that of a hexamer, although the structure is clearly different from the overall structure of wild-type RQA1 (Fig. S5D and E). These results show that local structural changes, including the movement of the Hb helix of RRS induced by the M3 or M4 mutation, have a negative effect on QRS catalytic activity, suggesting that the proper assembly and spatial arrangement of the RRS and its scaffold subunit are important for its catalytic activity.

Interestingly, free full-length RRS possessed RRS catalytic activity comparable to that of the RQA1 or RQKA1A2 complex. Circular dichroism analysis showed that the N-terminal region of RRS (residues 1–74) is ordered and forms a helix structure (Fig. S6A). RRS Ha (residues 1–30) fused to GST did not interact with FLAG-tagged RRS Hb (residues 38–68), suggesting that the N-terminal helices of the free, full-length RRS are highly mobile and move without constraint (Fig. S6B).

The First Half of the N-Terminal Helix of AIMP1 Binds to AIMP2. Previous studies reported that the RQA1 subcomplex is anchored to the MSC via interaction with AIMP2 (6, 22). Because both AIMP1 and AIMP2 contain a leucine-rich region, they may form a coiled-coil interface through leucine-zipper interactions. Therefore, we hypothesized that AIMP2 recruits the RQA1 subcomplex via interaction with the N-terminal helix of AIMP1. This interaction was examined directly by using a helical fragment of QRS.
AIMP2 (residues 40–80), the N-terminal helix (residues 1–73) of AIMP1, and the N-terminal helix (residues 1–74) of RRS. The three helical fragments formed a stable ternary complex, demonstrating that the helix (residues 40–80) of AIMP2 binds to the N-terminal helices of RRS and AIMP1 (Fig. 4A).

To determine which part of the N-terminal helix of AIMP1 interacts with AIMP2, a GST-fused AIMP2 (residues 40–100 containing the putative helix) was generated and tested in pull-down assays using wild-type or M3 or M4 mutant AIMP1. The results of the pull-down assays showed that the AIMP2 fragment binds directly to the N-terminal helix of AIMP1 and recruits the RQA1 complex (Fig. 4B). In contrast, the M3 and M4 mutant proteins did not interact with GST-AIMP2, confirming that the N-terminal helix of AIMP1 interacts with an AIMP2 fragment in a sequence-specific manner and participates in formation of the MSC. These results also suggest that the AIMP2 helical fragments do not associate with each other. GST-AIMP2 did not form a detectable complex with the exposed Ha helix of RRS, suggesting that the two proteins have weak or no interaction. Therefore we propose that—rather than forming triple coiled-coil structures comprising the RRS (Ha) helix, AIMP1, and AIMP2—AIMP2 and AIMP1 interact directly each other and that the Ha helix of RRS interacts with the coiled-coil structure via AIMP1.

Based on the biochemical information about the AIMP1 and AIMP2 complexes in conjunction with the structures of the RQA1 complex and the tRNA synthetase (KRS)–AIMP2 (KA2) complex, we built a model for the subcomplex formed by the five components, RRS, QRS, AIMP1, KRS, and AIMP2. We first determined the solution structure of the subcomplex using SAXS (Fig. 4C and Table S2). We then superimposed the structure of a hexameric RQA1 onto the molecular envelope calculated from the SAXS data (Fig. 4D). On top of each QRS molecule, the envelope has a space to accommodate a dimeric KRS molecule bound to an AIMP2 peptide, where we placed the KA2 complex.

Discussion

During the course of evolution, ARSs have recruited unique functional domains to their catalytic core domains (2, 15). These domains play roles in the control of their catalytic activities as well as in the control of cellular signaling pathways (2–4). In this work we report the structure of the RQA1 complex that forms a part of MSC and demonstrate the functional significance of the interactions between the N-terminal extensions of RRS and the AIMP1.

Stable Assembly of the N-Terminal Domains of RRS and AIMP1 Is Crucial for the Catalytic Activity of RRS. To our surprise, the structure revealed the role of the RRS N-terminal extension as a binding site for AIMP1 and QRS. In this complex, we have shown that the perturbation of the interface between N-terminal helices of RRS and AIMP1 abrogated the catalytic activity of RRS, demonstrating the importance of the stable assembly among the component proteins for the catalysis. The significance of the N-terminal extension of RRS is consistent with the previous reports showing that the N-terminal extension containing HMW-RRS, but not ΔN-RRS, is critical for the translation and cell growth in eukaryotic cells and organisms (5, 13).

Complete truncation of the N-terminal extension of RRS does not affect its catalytic activity (23). However, truncation or replacement of the first half of the AIMP1 helix almost completely abrogated the catalytic activity of HMW-RRS in the subcomplex. RRS activity was recovered fully upon removal of the Ha helix of RRS, which is stabilized by the first half of the AIMP1 helix in the hexamer (Fig. 3 B and C and Table S3). These results highlight the importance of the correct positioning of the Ha helix of HMW-RRS within the subcomplex to its catalytic activity. In the complex containing the AIMP1 M3 or M4 mutant, the Ha helix of RRS probably rotates freely, whereas the Hb helix of RRS is restrained by AIMP1. In contrast, active full-length RRS possesses highly mobile Ha and Hb helices. Thus, we speculate that the restricted movement of the N-terminal region of RRS in the complex may place the Ha helix in positions that impede the access of substrates to the active site of RRS.

The Molecular Architecture of the RQA1 Subcomplex. Although a trimeric form of the RQA1 subcomplex was observed in the asymmetric unit, the biochemical and SAXS analyses suggest that the complex is more likely to form the hexameric form. Furthermore, deletion of the first half of the AIMP1 helix, which is not involved in the interaction with RRS in a trimeric form, abolished the RRS activity of the subcomplex, suggesting that it interacts with RRS in trans. Because the interaction between the Ha helix of RRS and the first half of the AIMP1 helix is possible only in the hexameric form, these results, taken together with those of previous biochemical analyses (15), support the hexameric RQA1 model. Nevertheless, considering the dynamic nature of the MSC, as shown previously in the case of the KA2 complex (24, 25), the trimeric and hexameric subcomplexes of RQA1 may coexist within the MSC under physiological conditions. Previous studies showed that QRS forms a stable complex with AIMP2 only in the presence of both RRS and AIMP1, which is consistent with the structure presented here (22).

With the structure of the KA2 complex, we propose a plausible model showing how the RQA1 subcomplex can be anchored to MSC (Fig. 4 C and D). In this model, two RQA1 complexes are at the center of the structure, and two KRS molecules bound to an AIMP2 are located above each QRS molecule. The first half of AIMP1 is expected to interact with the AIMP2 helical segment (residues 40–80), forming the central building block of the MSC; hence, each RQA1 complex is recruited to the MSC via the interaction of AIMP1 with AIMP2. Because the KRS-binding region and the AIMP1-binding helix of AIMP2 are connected by a flexible

Fig. 4. A proposed model for the MSC. (A) Gel-filtration chromatography analysis of formation of the complex comprising the N-terminal helices of RRS, AIMP1, and AIMP2. The two fractions corresponding to the highest peak are shown in the Inset. The size difference between RRS (N-74) and AIMP1 (N-73) results from the 20-residues-long His-tag on RRS. (B) GST pull-down analysis of the ternary complex containing RRS, QRS, and wild-type or mutant (M3 or M4) AIMP1 using a GST-fused AIMP2 fragment. (C) The SAXS profile of the solution structure (empty circles) of the RQA1–KA2 complex. The solid blue line is the theoretical SAXS curve calculated from the structural model of the human RQA1–KA2 complex. The Guinier plot is shown in the Inset. (D) Structural superposition of the hexameric RQA1 complex and the KA2 complex onto the molecular envelope calculated from the SAXS data showing two different views of the model. The model was generated from the structures of the subcomplex comprising RRS (blue), QRS (magenta), and AIMP1 (yellow) and the subcomplex comprising KRS (gray) and AIMP2 (green).
in cis, the KA2 complex may undergo dynamic movement in the MSC.

The N-Terminal Extension of RRS Serves as a Binding Site for QRS. Because of the scaffolding role of AIMP1, it has been long predicted that QRS would be recruited to the MSC by AIMP1. In contrast, the structural analysis described here revealed that QRS is anchored to the N-terminal extension of RRS rather than to AIMP1, suggesting that the RRS N-terminal domain has a role as a station in the MSC. However, the structural evidence and binding affinities suggest that QRS is associated less tightly with the other components and thus may be more dynamic in the complex. In this regard, it is worth noting that one QRS was thought to be present in the rabbit MSC (15). The dynamic nature of the QRS association with the MSC may also have significance in its noncanonical function in the control of cell death. QRS released from the MSC was shown to bind to ASK-1 in a glutamine-dependent manner (10). QRS binds to the Hb helix of RRS via only a few interactions, which could allow its dissociation from the MSC by simple modifications. Indeed, a recent analysis revealed that Lys394 of QRS, which is located close to the interface of QRS, RRS, and AIMP1, can be ubiquitylated (Fig. 1) (19). In addition, Lys309 of QRS, which is located ~6.0 Å away from Glu42 and Glu46 in the Hb helix of RRS, is predicted to serve as an acetylation site (Glu46 in the Hb helix of RRS, is predicted to serve as an acetylation site (www.phosphosite.org)). This process resembles the phosphorylation-mediated liberation of KRS from the MSC (25). Further analysis is required to understand the mechanism by which QRS is dissociated from RRS.

In summary, the structure of the RQA1 subcomplex described here provides insights on how the noncatalytic domain of RRS and the accessory protein AIMP1 contribute to the function of MSC. In cis, these domains function as a molecular glue to attach into the MSC, but in trans they function as separate entities in the community of ARSs for efficient catalysis and higher-order communications.

Materials and Methods

Details of all biochemical, crystallization, data collection, and SAXS analysis are described in SI Materials and Methods.

The structure of the RQA1 complex was determined by the molecular replacement method. We initially determined the structures of human RRS and QRS core in the subcomplex with the PHENIX program using yeast RRS [Protein Data Bank (PDB) ID code 1BS2] and QRS (PDB ID code 4H3S) as a search model (26, 27). After density modification, an electron density map generated at a resolution of 4.0 Å clearly revealed the presence of N-terminal helices for AIMP1 and RRS. Successive rounds of model building using COOT (28) and refinement using PHENIX (29) were performed to build the complete model. The statistics are summarized in Table S1.

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