Supplemental information and Supplemental Figure 1:

Cryo-EM methods:

2 μl of 1 mg/ml RNA in 20 mM trisHCl, pH 8, 1 mM $MgCl_2$ was deposited onto a Quantifoil 200 mesh carbon grid and frozen in liquid ethane. Before data collection, each grid was examined for appropriate vitreous ice conditions (thickness, adequate sample deposition within the holes). Data was collected on a JEM2010F 200kV electron microscope using a Gatan 4k x 4k CCD camera and the JAMES data collection software.[1] The specimen stage was cooled to liquid nitrogen temperature. The magnification of the microscope was set to 60,000x with a resolution of 1.81 Å per pixel. The targeted defocus range was 1.5-2.5 μm under focus and the dosage was set between 16 and 22 e/Å^2. For each data set, ~100 CCD frames were collected. In some cases, focal pairs were collected to aid in particle selection.[2] Particles in each frame were selected semi-automatically.

The selection of single particles from each image is of utmost importance. The small size of the S-domain particles made particle selection very challenging. Particles in each CCD frame were first selected with an automatic particle selection program based on projections created from a reference image. The exact conformation of this reference image was not particularly important because 50-60 kD particles appear on the raw CCD images as only bright spots above background. Manual inspection of each selected particle was performed to remove any obviously erroneous selections such as those including multiple particles or ice abnormalities (bubbles, thickness, etc). In the case of 50-60 kD particle selection, user bias based on particle shape is unlikely because low particle contrast in the image means there are few such details to discern by eye prior to averaging. More than 11,000 particles were selected and processed for each

construct. Collection of data sets using focal pairs did significantly improve the manual inspection of selected particles.

Due to the small size and low contrast of the particles, we were unable to accurately determine the defocus value for the particles with sufficient accuracy to perform even phase-flipping CTF correction. This initial study uses uncorrected data, and the resolution is effectively limited to ~26 Å, the approximate resolution at which the first zero crossings appear. FSC curves (0.5) criterion indicate that we have achieved even better resolutions than this (Fig. S1E), but anything beyond 26 Å will be subject to phase reversal errors, thus all maps have been low-pass filtered such that at the presented level of detail, the models are accurate.

References:

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(3) Baird, N. J.; Westhof, E.; Qin, H.; Pan, T.; Sosnick, T. R. *J Mol Biol* **2005**, *352*, 712-22.

(4) Baird, N. J.; Gong, H.; Zaheer, S. S.; Freed, K. F.; Pan, T.; Sosnick, T. R. *J Mol Biol* **2010**, *397*, 1298-306.

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Fig. S1: Modeling the S-domain folding intermediate based on cryo-EM reconstruction. (A) Overlay of the cryo-EM reconstruction with the representative model previously created using SAXS data as global constraints.[3,4] (B) The representative model (blue) largely fits within the cryo-EM map although an adjustment of the P12 arm is warranted. The adjusted structure (red) fits the cryo-EM map better than any of the original family of models (representative model in blue). (C) The robustness of cryo-EM SPR is tested by generating a map starting from a Gaussian ellipsoid as the initial model. The model from Fig. 1D (red) is overlaid with this reconstruction. (D) The reconstruction starting from the Gaussian ellipsoid (gray) is in overall agreement with the reconstruction initialized from the 2D classes (red). The model generated from the ellipsoid has less structural features as it does not utilize information taken from the (model-free) 2D class averages. (E) Fourier Shell Correlation curve of two independent reconstructions.[5]