Supplementary Information

Molecular basis of cyclic oligoadenylate processing by small stand-alone CRISPR-Cas Ring Nucleases

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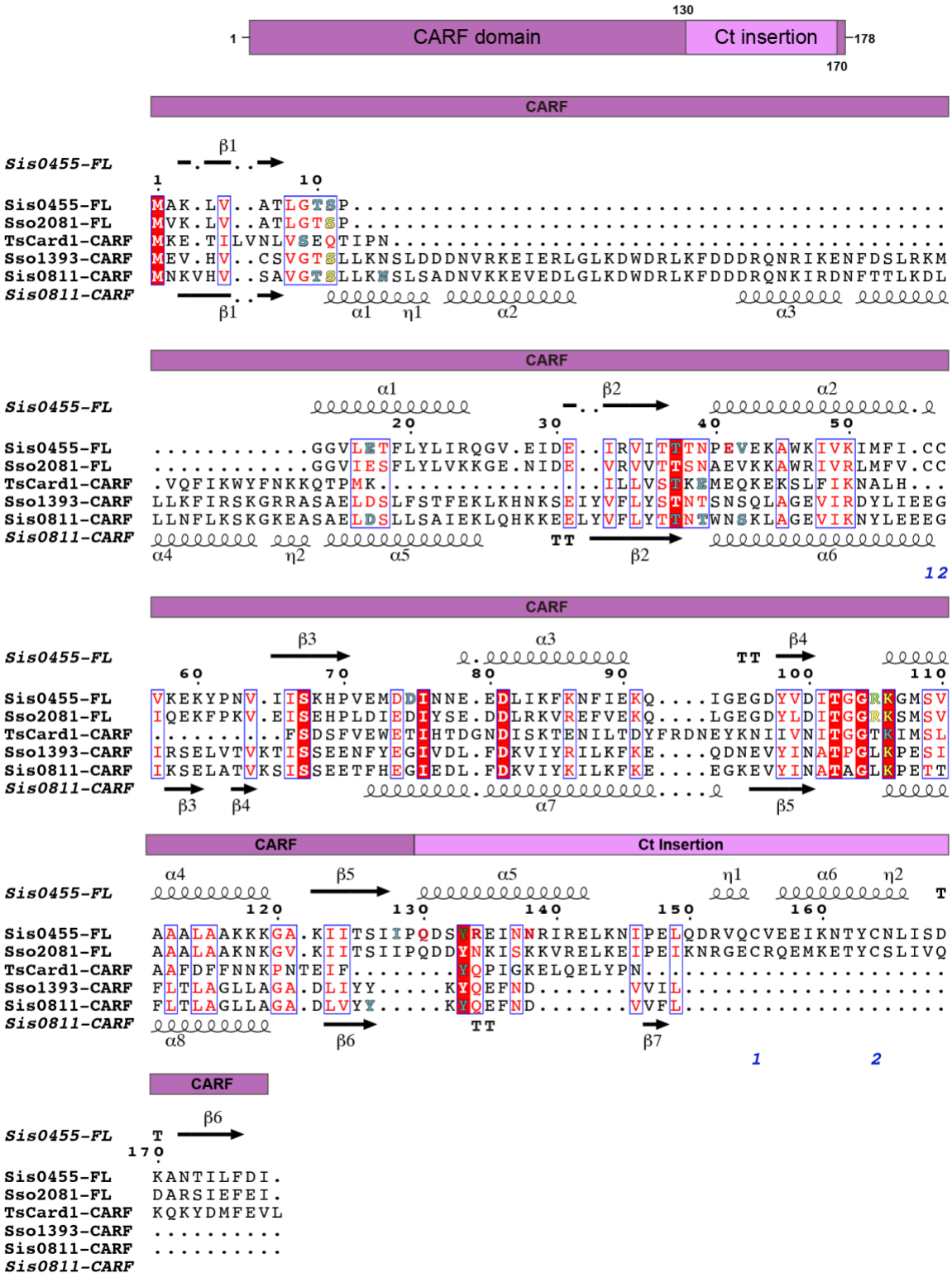
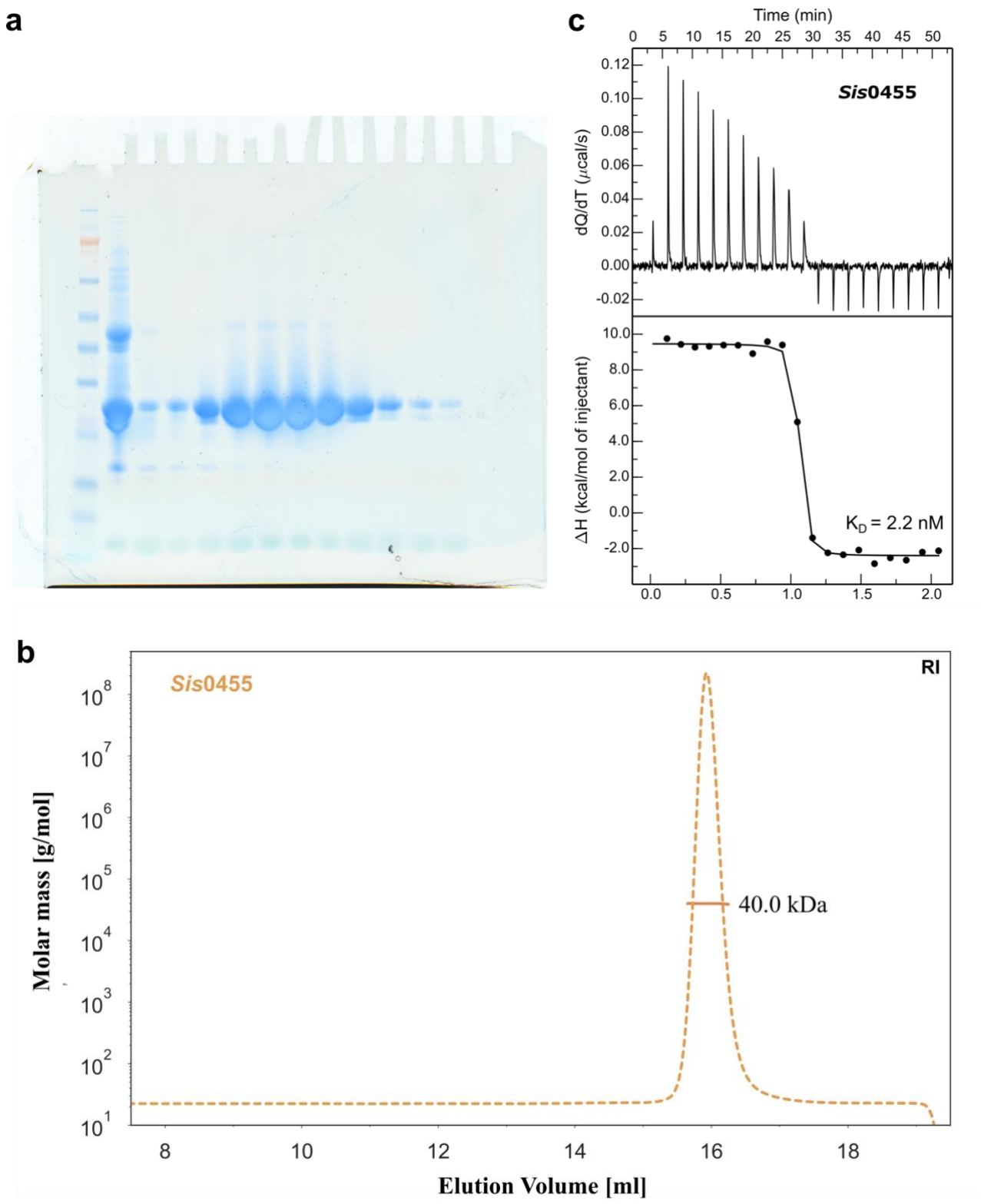
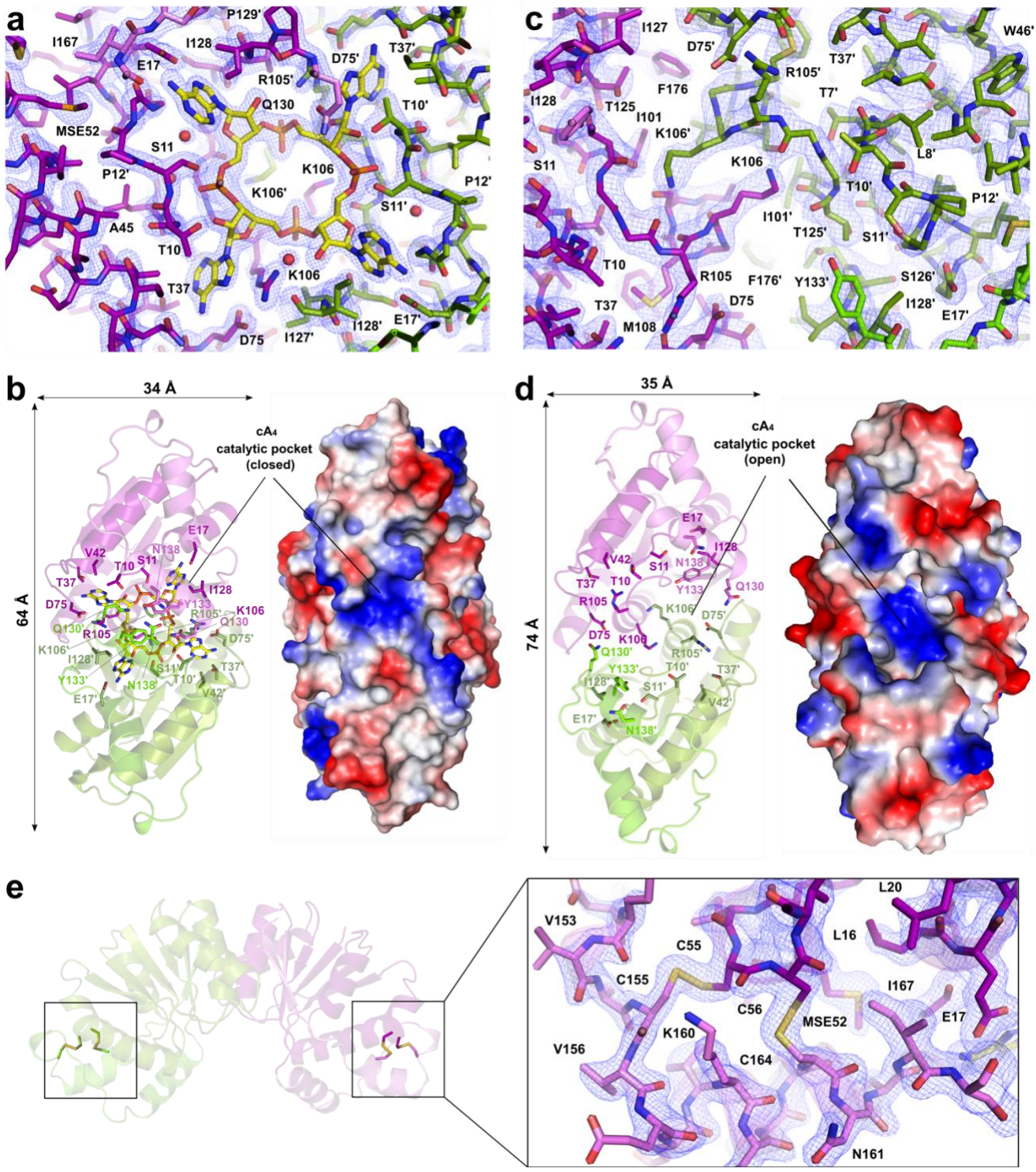


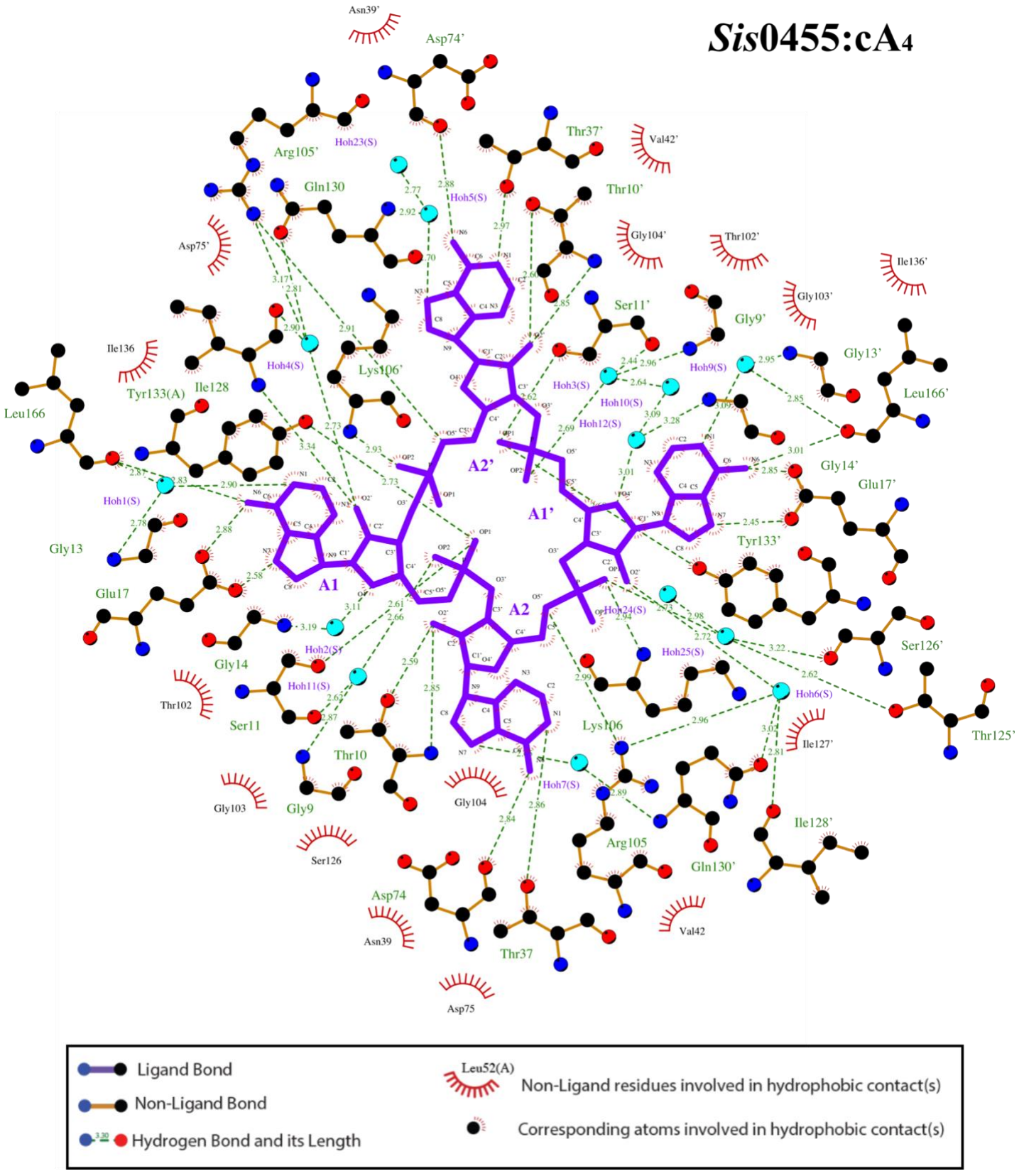
Supplementary Figure 1. Structure-based sequence alignment of stand-alone Ring Nucleases involved in cA_4 binding/cleavage. The amino acid sequences of *Sis*0455 from *S. islandicus* (*Sis*0455-FL, UniProt Entry: F0NGX6, PDB code: 7Z55), *Sso*2081 from *S. sulfataricus* (*Sso*2081-FL, UniProt Entry: Q7LYJ6), *Ts*Card1 from *T. succinifaciens* (*Ts*Card1-CARF, UniProt Entry: , PDB code: 6WXX), CARF-*Sso*1393 from *S. sulfataricus* (*Sso*1393-CARF, UniProt Entry: Q97YD2, PDB code: 3QYF) and CARF-*Sis*0811 from *S. islandicus* (*Sis*0811-CARF, UniProt Entry: F0NH89, PDB code: 7PQ3) were aligned by using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/tcoffee). The figure was prepared using ESPript (http://espript.ibcp.fr/ESPript/ESPript/index.php). Residue numbers are labelled according to the *Sis*0455 sequence. Key residues for cA_4 binding are bolded as blue; key residue for catalysis is bolded in yellow; key residues in stabilizing the closed state are bolded in red; key residue for both stabilizing the closed state and cA_4 binding is bolded in dark green. key residue for both cA_4 binding and catalysis is bolded in light green. 1,2 numbers depicted in dark blue represent the sulphur bridge bonds present within the *Sis*0455 structure.



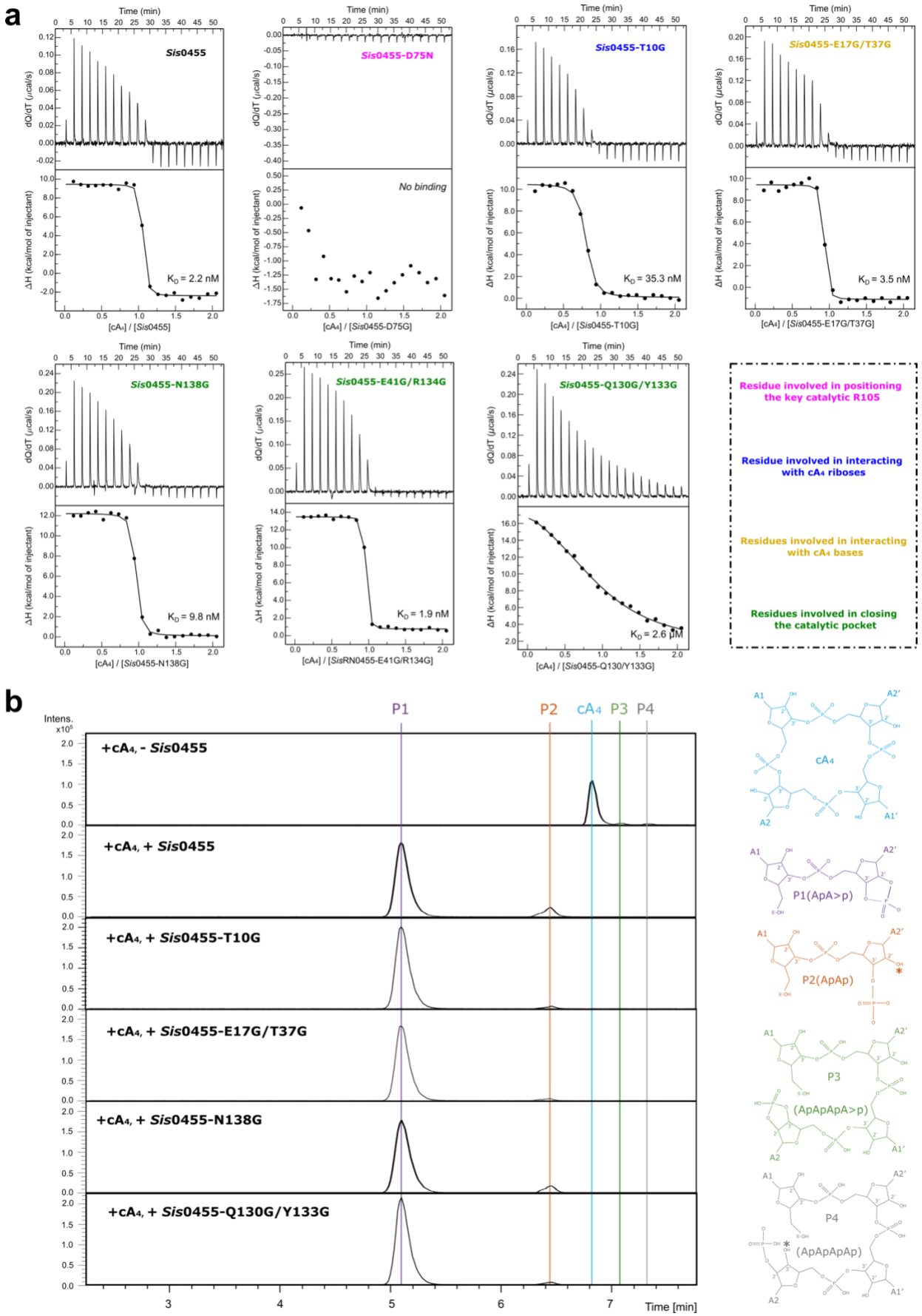
Supplementary Figure 2. *Sis*0455 sample characterization. (a) PAGE-SDS gel sampling last purification step displaying GF fraction purity. **(b)** SEC-MALS analysis. Ring nuclease *Sis*0455 variant was injected into a Superdex 200 Increase 10/300 GL column. The change in refractive index as a function of protein concentration was used to compute the molar masses for the different samples. The discontinued orange line plotted on the right axis scale correspond to the RI traces from the SEC column scaled across the greatest magnitude of all chromatogram data. The molar mass across the eluting peak is plotted as lines on the left axis scale (molar mass). The average molecular weight is displayed on the figure. **(c)** *Sis*0455 substrate binding assay analysed by ITC.



Supplementary Figure 3. *Sis*0455 and *Sis*0455: cA_4 catalytic pocket. **(A)** The zoom shows 2fo-fc electron density map at the catalytic pocket of the *Sis*0455, generated by their CARF (purple and green) domains, in complex with its substrate (cA_4, yellow). **(B)** Top view of the catalytic dimer CARF domains from *Sis*0455:cA_4 complex crystal structure displaying the residues involved in cA_4 binding/catalysis (left panel) and those responsible of stabilizing the catalytic pocket. Overall electrostatic potential representation pointing the cA_4 binding/catalytic pocket is shown at the right panel. **(C)** The zoom shows 2fo-fc electron density map at the catalytic pocket of the *Sis*0455 in its apo form. **(D)** Top view of the catalytic dimer CARF domains from *Sis*0455 apo crystal structure depicting the proposed residues involved in cA_4 binding/catalysis (left panel) and its electrostatic potential representation showing the cA_4 binding/catalytic pocket (right panel). The ´ symbol differentiates residues from the two monomers. 2fo-fc electron density maps are contoured at 1.5 σ. **(E)** Location of the S-S bridges within the *Sis*0455 overall dimer structure (left panel) and zoom view showing its 2fo-fc electron density map.



Supplementary Figure 4. Schematic representation of cA_4 interactions in *Sis*0455 catalytic pockets. The interactions were depicted by LigPlot.



Supplementary Figure 5. Binding and cleavage activity characterization of *Sis*0455 mutants. **(a)** Substrate binding assays analysed by ITC. **(b)** Cleavage assays analysed by Mass Spectrometry. The mass spectrometry profile of D75N mutant is not included since binding was not detected in (a).