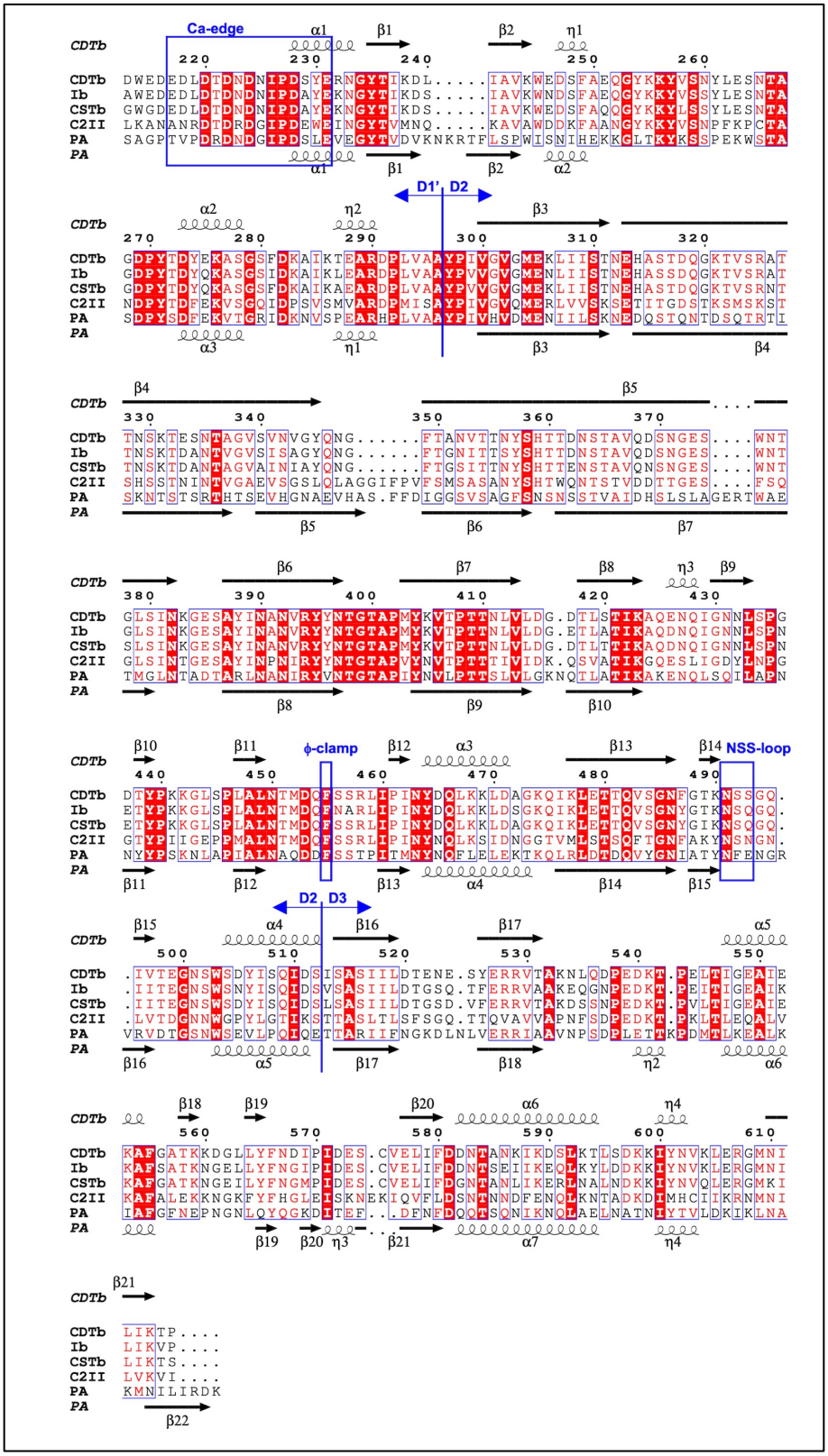
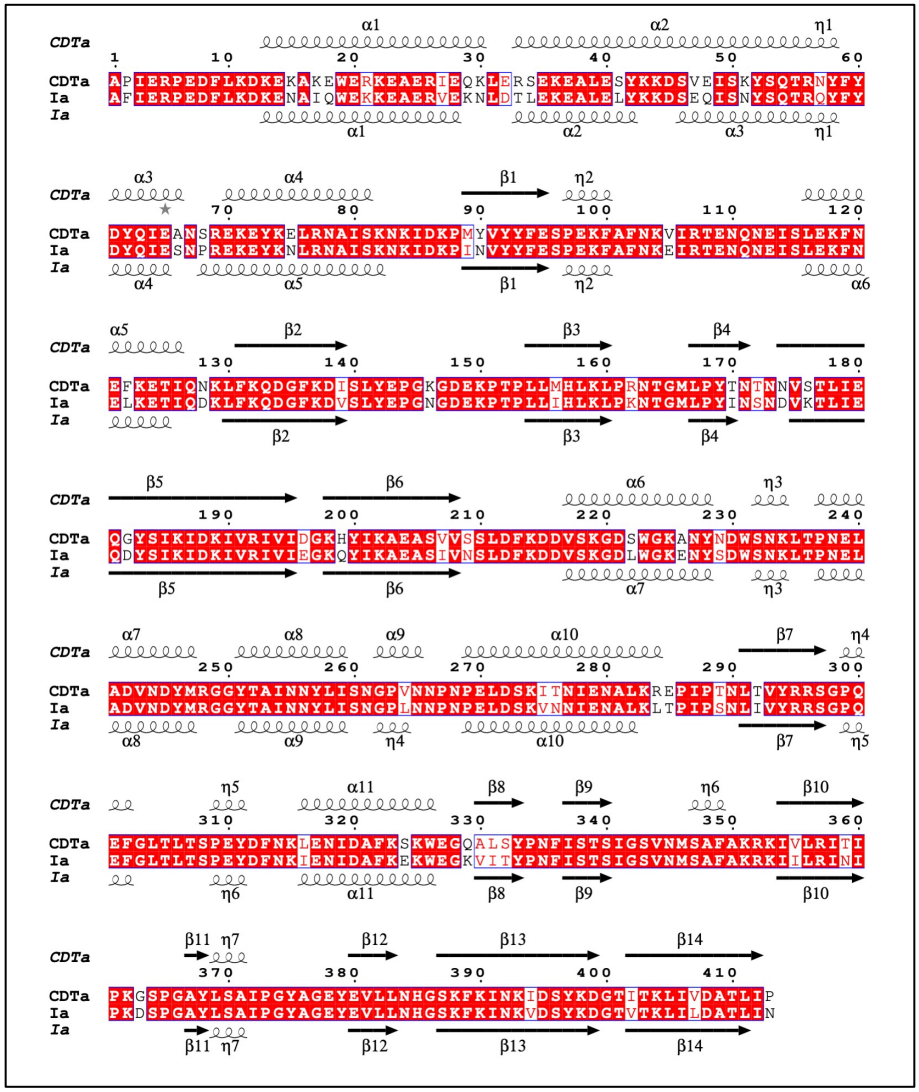
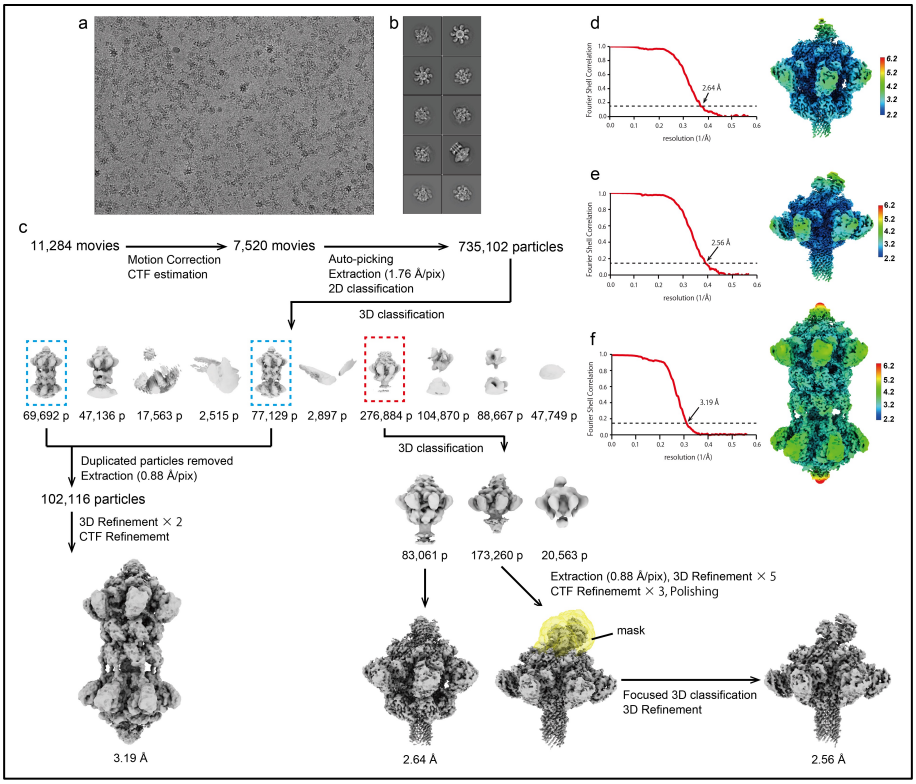


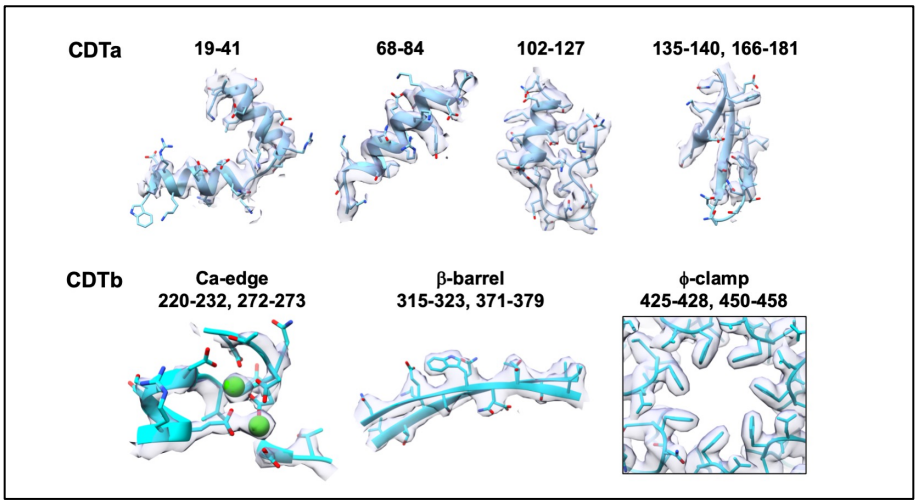
Supplementary Fig. S1a: Sequence alignment of binary toxins
The sequence alignment results with secondary strucrues were made using ESPript server.[61] **a** Sequence alignment of binary toxin B components with the secondary structure. The borders of domains and the residues that form constriction sites are indicated. Uniprot IDs: CDTb, o32739; Ib, Q46221; CSTb, o06498; C2II, o86171; PA, P13423



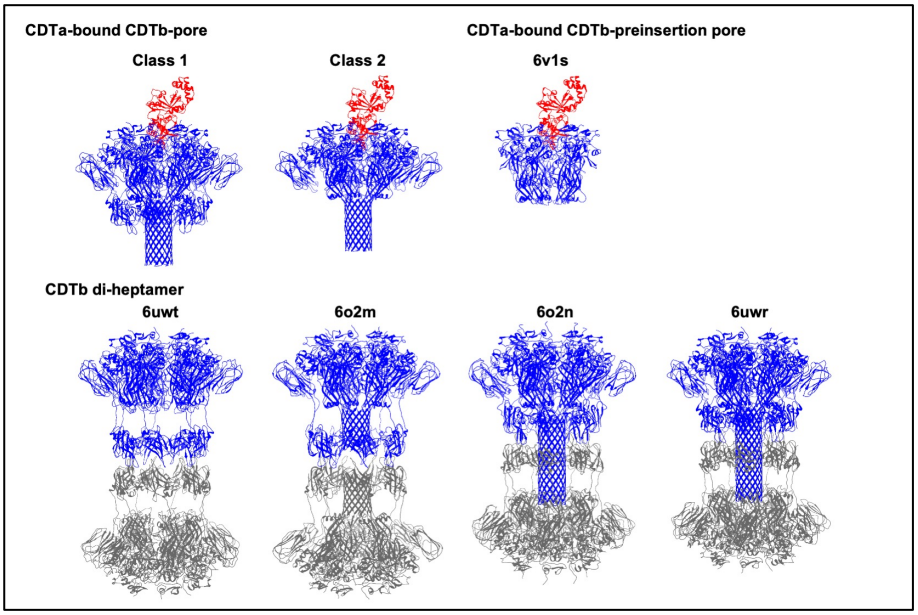
Supplementary Fig. S1b: Sequence alignment of binary toxins
The sequence alignment results with secondary strucrues were made using ESPript server.[61] **b** Sequence alignment of binary toxin A components with the secondary structure. Uniprot IDs: CDTa, Q9KH42 ; Ia, Q46220.



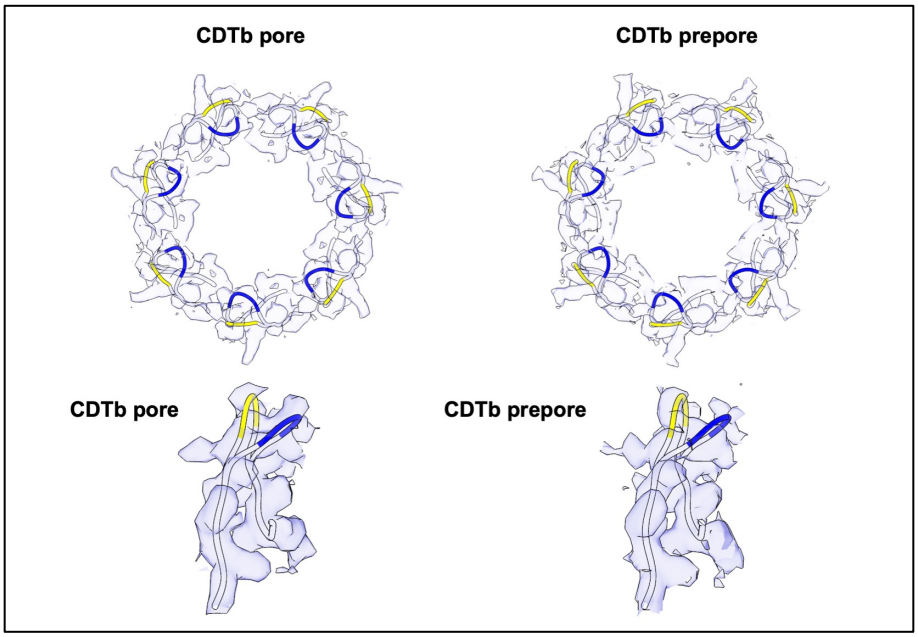
Supplementary Fig. S2: Single particle analysis of CDTa-bound CDTb-pore
a Representative microscope image. **b** 2D classification. **c** Flow chart of cryo-EM image processing. **d,e** Gold-standard FSC curve of the final map and final 3D reconstruction map of class1 and 2 CDTa-bound CDTb-pores with color-coded according to local resolution. f.



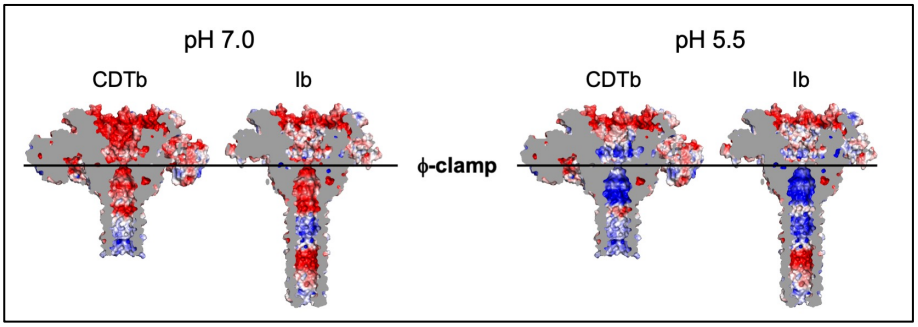
Supplementary Fig. S3: Cryo-EM density maps and models of CDTa-bound CDTb-pore (class 2)
Representative cryo-EM maps and model of CDTa-bound CDTb-pore at 2.56-Å resolution (class 2).



Supplementary Fig S4: Reported structures of CDTb including CDTa-bound CDTb-pores
CDTa is coloured as red, and CDTb is shown as blue or grey. CDTa-bound CDTb-pore (Class 1) (PDB ID: 7VNN (2.64-Å)), CDTa-bound CDTb-pore (Class 2) (PDB ID: 7VNJ (2.56-Å)), CDTa-bound CDTb-pre-insertion state (PDB ID: 6VLS (3.8-Å)), di- heptamer (PDB ID: 6UWT (3.1-Å), 6O2M (6.3-Å), 6O2N (3.7-Å), and 6UWR (2.8Å)).



Supplementary Fig. S5: Map and model of the di-heptamer CDTb consisting pore and pre-pore (PDB ID: 6UWR)
Each NSS-loop are coloured as blue (in) or yellow (out), respectively. Cryo-EM map is shown as white. *top*, Top view of the NSS-loops. *bottom*, Close up side view of a representative NSS-loop.



Supplementary Fig. S6: Electrostatic potential of CDTb and Ib
Electrostatic potential of CDTb (class 2) and Ib at pH 7.0 and pH 5.5 (positive, red; negative, blue).

Residues of Ca-edge
Residues of NSS-loop (in)
Residues of NSS-loop (out)

Subunit A (in)

Interfacing Residue	Bond	Binding CDTa residue
A:SER 492 (in)		

Subunit A (out)

Interfacing Residue	Bond	Binding CDTa residue
There is no interfacing residues.		

Subunit B (in)

Interfacing Residue	Bond	Binding CDTa residue
B:SER 492 (in)	H	H:GLY 147
B:SER 493 (in)		
B:GLY 494		
B:GLN 495		

Subunit B (out)

Interfacing Residue	Bond	Binding CDTa residue
B:SER 493 (out)		
B:GLN 495		

Subunit C (out)

Interfacing Residue	Bond	Binding CDTa residue
C:ASP 218		
C:LEU 219		
C:ASP 220		
C:ASN 223		
C:ASN 225	H	H:ASN 112
C:PRO 227		
C:ASP 239		
C:LEU 240		
C:ILE 241		
C:ALA 242		
C:THR 272		
C:TYR 274		
C:GLU 275	HS	H:LYS 146
C:LYS 490		
C:ASN 491 (out)		
C:SER 492 (out)		
C:SER 493 (out)		

Subunit D (in)

Interfacing Residue	Bond	Binding CDTa residue
D:TRP 214		
D:GLU 215		
D:ASP 216		
D:ASP 218	HS	H:ARG 192 H:LYS 123
D:LEU 219		
D:ASP 220	H	H:ARG 192
D:THR 221	H	H:VAL 194
D:ASP 222		
D:ASN 223	H	H:GLU 143
D:ASN 225	H	H:LYS 202
D:ASN 491 (in)		
D:SER 492 (in)		
D:SER 493 (in)		
D:GLN 495		
D:VAL 497		
D:GLU 499		

Subunit D (out)

Interfacing Residue	Bond	Binding CDTa residue
D:TRP 214		
D:GLU 215		
D:ASP 216		
D:ASP 218	HS	H:ARG 192 H:LYS 123
D:LEU 219		
D:ASP 220	H	H:ARG 192
D:THR 221	H	H:VAL 194
D:ASP 222		
D:ASN 223	H	H:GLU 143
D:ASN 225	H	H:LYS 202
D:ASN 491 (out)		
D:SER 492 (out)		
D:SER 493 (out)		
D:VAL 497		
D:GLU 499		

Subunit E (in)

Interfacing Residue	Bond	Binding CDTa residue
E:ASP 220		
E:ASN 223	H	H:LYS 159
E:ASP 224		
E:ASN 225	H	H:ASP 86
E:LEU 240		
E:ILE 241		
E:SER 264		
E:THR 272		
E:TYR 274	H	H:ARG 162
E:GLU 275	HS	H:LYS 87
E:ASN 491 (in)	H	H:ARG 26
E:SER 492 (in)	H	H:ARG 26
E:SER 493 (in)	H	H:ARG 26
E:GLN 495		
E:GLU 499		
E:GLY 500		

Subunit E (out)

Interfacing Residue	Bond	Binding CDTa residue
E:ASP 220		
E:ASN 223	H	H:LYS 159
E:ASP 224		
E:ASN 225	H	H:ASP 86
E:LEU 240		
E:ILE 241		
E:SER 264		
E:THR 272		
E:TYR 274	H	H:ARG 162
E:GLU 275	HS	H:LYS 87
E:LYS 490		
E:ASN 491 (out)		
E:SER 492 (out)		
E:GLN 495		
E:GLU 499		
E:GLY 500		

Subunit F (iout)

Interfacing Residue	Bond	Binding CDTa residue
F:ASP 218		
F:ASN 223		
F:THR 489	H	H:LYS 30
F:LYS 490		
F:ASN 491 (out)	H	H:GLN 29
		H:LEU 31
F:SER 492 (out)	H	H:GLU 32
F:SER 493 (out)	H	H:ARG 33
F:GLN 495	H	H:LYS 36
F:VAL 497		

Subunit G (in)

Interfacing Residue	Bond	Binding CDTa residue
G:ASN 223	H	H:ARG 33
G:ASP 224		
G:ASN 225	H	H:ARG 33
G:SER 264		
G:THR 272		
G:SER 492 (in)	H	H:GLU 40

Subunit G (out)

Interfacing Residue	Bond	Binding CDTa residue
G:ASN 223	H	H:ARG 33
G:ASP 224		
G:ASN 225	H	H:ARG 33
G:SER 264		
G:THR 272		

Supplementary Table 1

CDTb-pore residues interfacing with CDTa

The CDTb residues interfacing with CDTa are shown. The residues are coloured as cyan (Ca-edge), blue (NSS-loop (in)) and yellow (NSS-loop (out)), respectively. The CDTb residues forming hydrogen bond or salt bridge with CDTa are shown as H and S, respectively.