SUPPORTING INFORMATION

SUPPLENTAL DISCUSSION

Mechanism for controlling fidelity of oligomerization—We have demonstrated that incorrect homodimers are never formed in the dimerization step (1, 2). If two identical molecules associate into a dimer, electrostatic repulsion will occur between the residues, forming inter-protomer ion pairs in the cap – rim interface (Fig. 2B). These residues would contribute to the fidelity control of multimerization by obstructing homodimer formation as well as stabilization of the inter-protomer interaction.

In the structure model of pre-pore, the steric restraint at interface 2 covered a wider range than that at interface 1 (Supplementary Fig. S3B), suggesting that energy required for the conformation change of the amino-latch and pre-stem at interface 2 is larger than that of interface 1. The difference may also be a determinant for the priority of dimerization.

Functions of individual molecules in pore formation—Biochemical data and the present octameric pore structure clearly showed that LukF is important for binding to the cell surface and is responsible for the structural transition from pre-pore to pore (1, 3). In contrast, the role of Hlg2 is obscure, although it was demonstrated that Hlg2 recognizes proteinaceous factor(s) located at the erythrocyte surface (4). In the present study, octameric pores were formed by MPD without membranes, suggesting that neither pore formation nor oligomerization require contact with the membrane. Crystallization was performed at extremely high protein concentration in comparison with biochemical assays, suggesting that γ-HL can form a pore structure spontaneously if the concentration is sufficiently high and the lipid head group or MPD is captured by Trp177 of LukF. In the previous biochemical assay, γ-HL could form a pore on an artificial membrane not containing the receptor molecule of Hlg2, although it required an approximately 100-fold higher concentration than for that on erythrocytes (5). Under these conditions, γ-HL probably forms pores due to the property of spontaneous assembly at high concentration. On the other hand, only about 10,000 molecules each of the γ-HL component (which forms 2500 octameric pores) are sufficient for complete hemolysis of a human erythrocyte (4). Assuming that this amount of each molecule is distributed evenly on the erythrocyte surface, the density of the toxin components is expected to be very low for

pre-pore formation. Hlg2 may contribute to effective condensation of heterodimers on the cell surface through binding with its receptor molecule(s). In fact, leukocidin forms pores on lipid rafts of leukocyte membranes (6). Taken together, it is reasonable to propose that components of γ-HL share roles in pore formation as follows; LukF is responsible for initial cell binding and induction of the structural change from pre-stem to stem, whereas Hlg2 acts to increase the local concentration of heterodimers by capturing receptor molecules.

SUPPLENTAL METHODS

Preparation of LukF and Hlg2—Two different DNA fragments encoding LukF and Hlg2 without the signal sequence were amplified using KOD-Plus DNA polymerase (Toyobo, Osaka, Japan), and inserted into the *Nco*I and *Xho*I sites of the pET26 vector (Merck, Whitehouse Station, NJ). A His6-tag was fused at the N-terminus to facilitate purification. Transformed *Escherichia coli* strain B834(DE3) harboring the expression vector of the desired protein and pRAREII (Merck) was grown at 37°C in LB medium supplemented with 25 μg mL^{-1} kanamycin and 34 μg mL^{-1} chloramphenicol until the logarithmic growth phase. To induce expression of the desired protein, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM, and culture was continued for 18 h at 25°C. Cells were harvested by centrifugation at 4500 × *g* for 10 min at 4°C, and then disrupted using a sonicator (Branson, Danbury, CT) in 20 mM Tris-HCl (pH 8.0), 300 mM NaCl. The cell debris was removed by centrifugation at 40000 × *g* for 1 h at 10°C, and the supernatant was loaded onto a Ni sepharose 6 Fast Flow column (GE Healthcare Biosciences AB, Uppsala, Sweden). After washing with the same buffer, the adsorbed protein was eluted with a gradient of imidazole. Fractions containing the desired protein were further purified on a HiLoad 26/60 Superdex 200-pg column (GE Healthcare Biosciences AB). Purified monomeric proteins were stored at 4°C until use.

Crystallization and X-ray diffraction data collection—The 1:1 mixtures of LukF and Hlg2 monomers were concentrated to 4 mg mL^{-1}. Crystals suitable for further experiments were grown by the sitting-drop vapor diffusion method from a solution

containing 0.1 M sodium acetate (pH 4.6), 0.5 M ammonium acetate, and 50% (v/v) MPD. X-ray diffraction experiments were performed on the beamline BL41XU at SPring-8 (Harima, Japan). The γ-hemolysin crystal belonged to space group $C222_1$ with unit cell parameters a = 206.45 Å, b = 206.14 Å, c = 190.30 Å. Diffraction data were indexed, integrated, scaled, and merged with the program XDS (7). This crystal was pseudomerohedrally twinned with twin operators (-k, -h, -l) and the twin fraction was estimated as 42% by Britton analysis performed with the program phenix.xtriage (8). It should be noted that the apparent lattice symmetry was tetragonal; however, in such a space group, the resultant model from molecular replacement clashed with its symmetry mates. R *vs.* R statistics (9) indicated that the crystal was pseudomerohedrally twinned with rotational pseudosymmetry. Data collection statistics are shown in Supplementary Table 2.

The structure of γ-hemolysin was determined by the molecular replacement method with the program Phaser (10) using the structures of monomeric LukF (PDB ID: 1lkf (11), Hlg2 (PDB ID: 2qk7 (12), and the stem region of α-hemolysin protomer (PDB ID: 3anz (13)) as search probes. The pre-stem regions of LukF and Hlg2 were removed before molecular replacement. Molecular replacement resulted in the octameric complex in the asymmetric unit. To monitor the refinement, a random 3.0% subset from all unique reflections was set aside for R-free evaluation, taking lattice symmetry into account with the program phenix.reflection_file_editor (8). Jelly body refinement with local NCS restraints was performed with the program REFMAC5 (14) after rigid body refinement. After several cycles of manual model fitting and building with Coot (15) and refinement with REFMAC5, individual atomic coordinate refinement and individual ADP refinement were performed with phenix.refine (8). The twin operator (k, h, -l) was applied during every round of refinement and the twin fraction was refined. Finally, R-work and R-free values converged to 20.68% and 23.64%, respectively. The stereochemical qualities of the final refined model were analyzed with phenix.validate including MolProbity analysis (16). The buried surface area was calculated with the program PISA (17). The number of atoms participating in inter-protomer van der Waals interaction and inter-protomer polar interaction were calculated with the program CONTACT and PISA in the ccp4 program package (18), respectively. The refinement statistics are summarized in Table S2.

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SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Oligomer formation by MPD. Purified monomeric LukF and Hlg2 were incubated with or without MPD. Oligomer formation was confirmed by SDS-PAGE. SDS-resistant hetero-oligomer was formed with 20% MPD.

Figure S2. Comparison of the pore structures among γHL (center), VCC (left), and αHL (right). Diameter and height are indicated. The bottom figures show the outline of the central vestibule and channel of pores calculated using the program HOLE(1). Constriction and the residues responsible are also indicated. The central axis is shown as a yellow line.

Figure S3. Residues mentioned in the Discussion section. (A) Lys222 of Hlg2 (green) and Ser46 of LukF (red) are shown as spheres. In the study reported by Nguyen *et al.* (2), TMR and IC5 were introduced into these residues for the FRET experiment. The distance between these residues in the dimer through interface 1 is 21 Å, whereas that though interface 2 is 43 Å. Upon dimer, tetramer, hexameric assembly, the FRET intensity increased proportionally (2), showing that the initial heterodimer is formed through interface 1. **(B)** Thr28 (orange) and Thr21 (green) of Hlg2, and Asn157 (red) and Thr158 (yellow) of LukF are shown as spheres. In the study reported by Joubert *et al.* (2), Thr28 (Hlg2) – Asn157 (LukF), and Thr21 (Hlg2) – Thr158 (LukF) were covalently linked. These mutants could effectively form pores. **(C)** Tyr72, Trp257, Phe260, and Tyr261 of LukF, necessary for the initial cell binding (3-5), are shown as orange spheres. The phospholipid head group binding residues, Trp177 and Arg198, are also shown as green spheres (6). The vertical interval between Phe260 and Trp177 was 14 Å, suggesting the inclining orientation of LukF upon attachment to the cell surface.

Fig. S4 Pre-pore model. (A) Top and side view of the pre-pore structure constructed by superposing monomeric LukF and LukS onto protomers of LukF and Hlg2, respectively.

As the amino-latch was not constructed in the crystal structure of monomeric Hlg2, LukS monomer was used as the S-component. Red, cap and rim domains of LukF; blue, those of LukS; green, pre-stem of LukF; cyan, that of LukS; yellow, amino-latch of LukF; orange, that of LukS. **(B)** Steric collisions between protomers. Clashing atoms at interfaces 1 and 2 are shown as magenta and purple surfaces, respectively. Close-up view around the top of the interfaces is also shown. Colors in the cartoon correspond to those in Fig. S4A.

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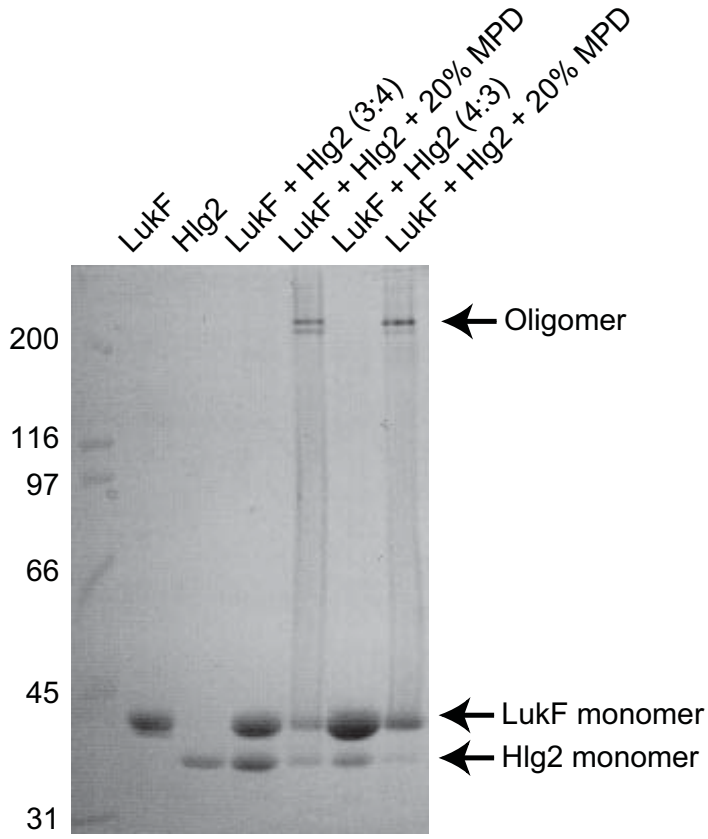

LukF
Hlg2
LukF + Hlg2 (3:4)
LukF + Hlg2 + 20% MPD
LukF + Hlg2 (4:3)
LukF + Hlg2 + 20% MPD
200
116
97
66
45
31
Oligomer
LukF monomer
Hlg2 monomer

Fig. S1

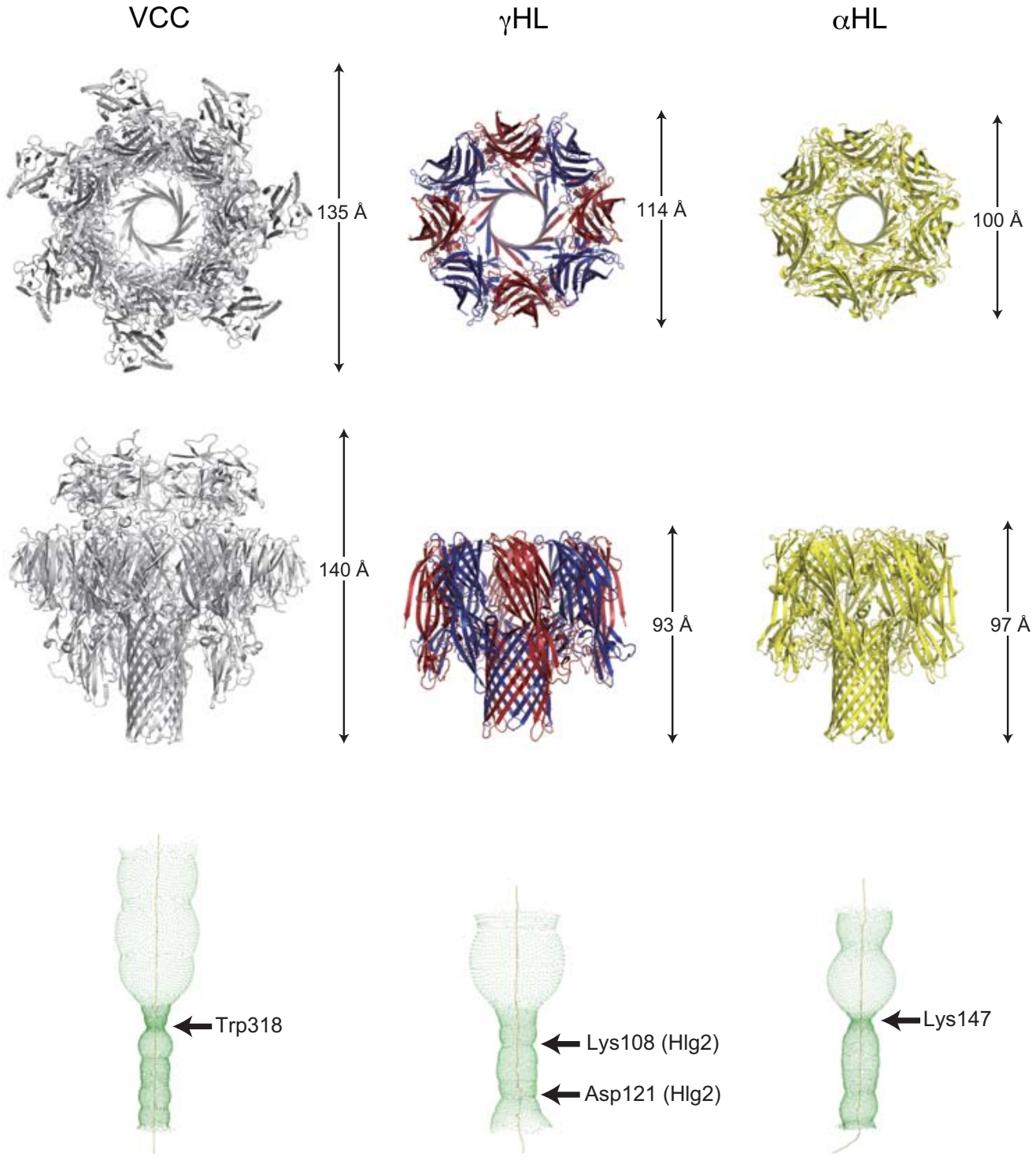

VCC
γHL
αHL
135 Å
114 Å
100 Å
140 Å
93 Å
97 Å
Trp318
Lys108 (Hlg2)
Asp121 (Hlg2)
Lys147

Fig. S2

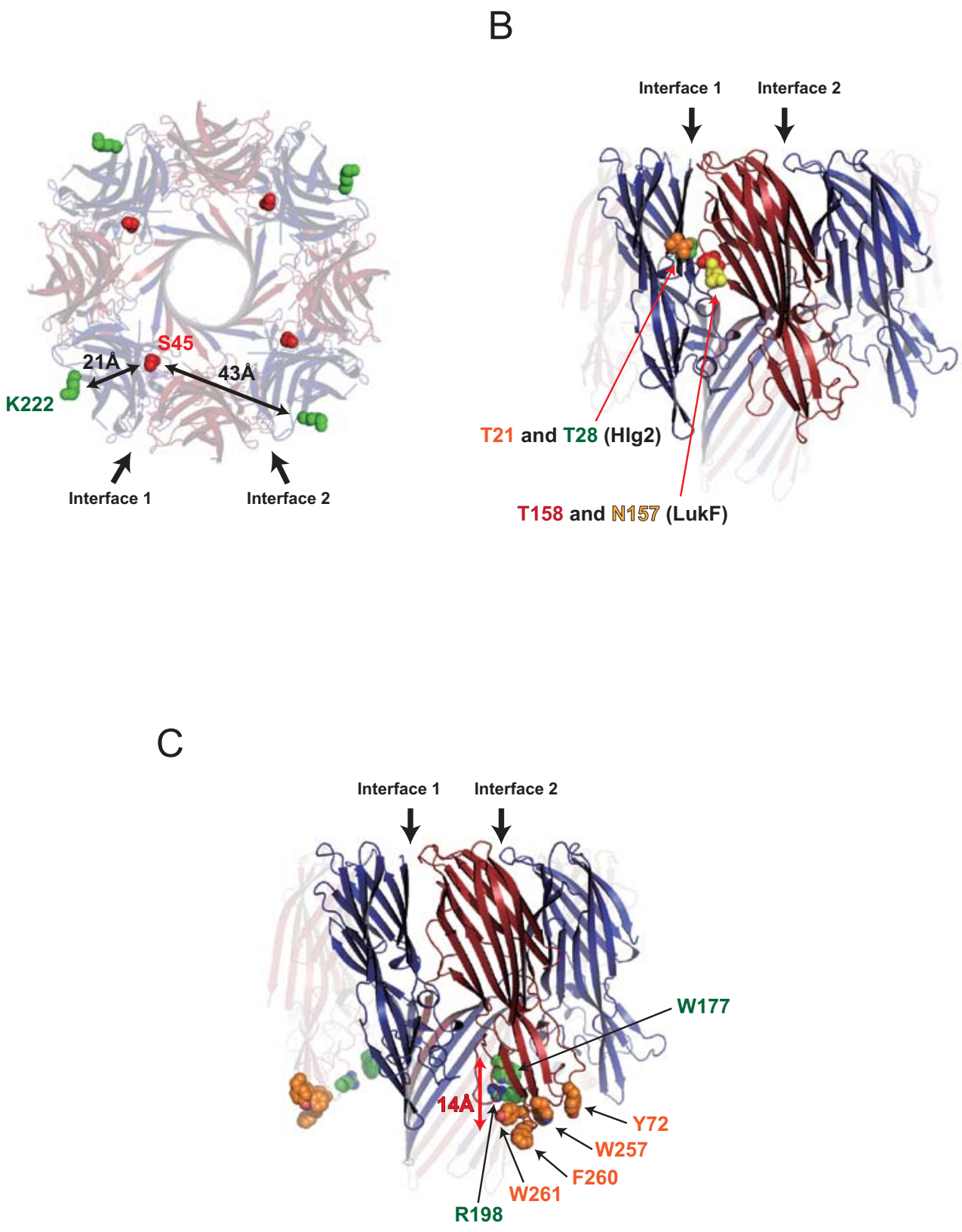
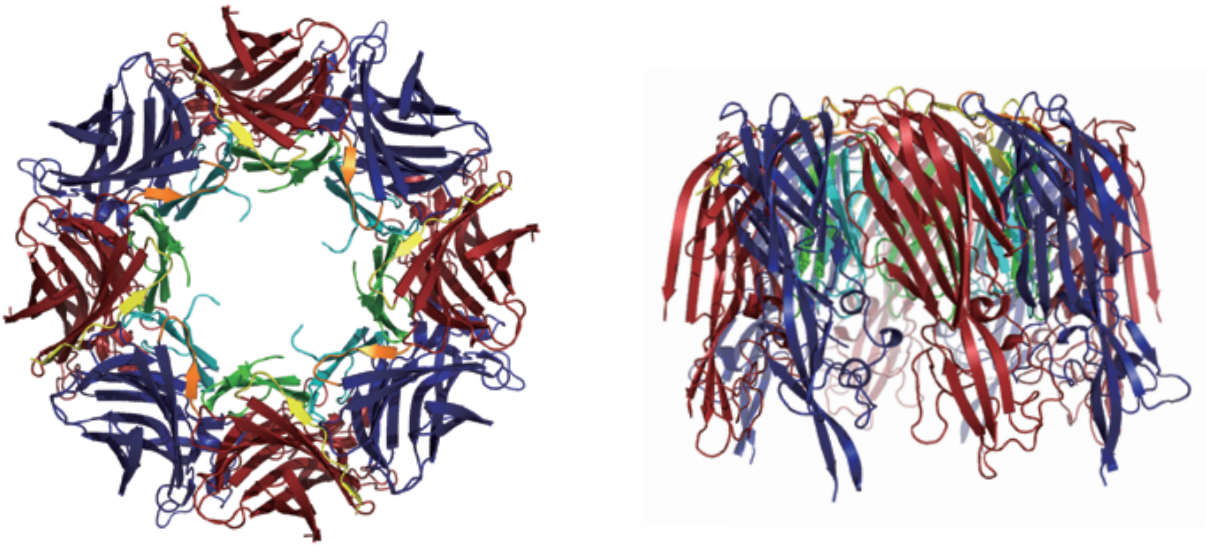

A
S45
21Å
43Å
K222
Interface 1
Interface 2
B
Interface 1
Interface 2
T21 and T28 (Hlg2)
T158 and N157 (LukF)
C
Interface 1
Interface 2
W177
14Å
Y72
W257
F260
W261
R198

Fig. S3

A



B

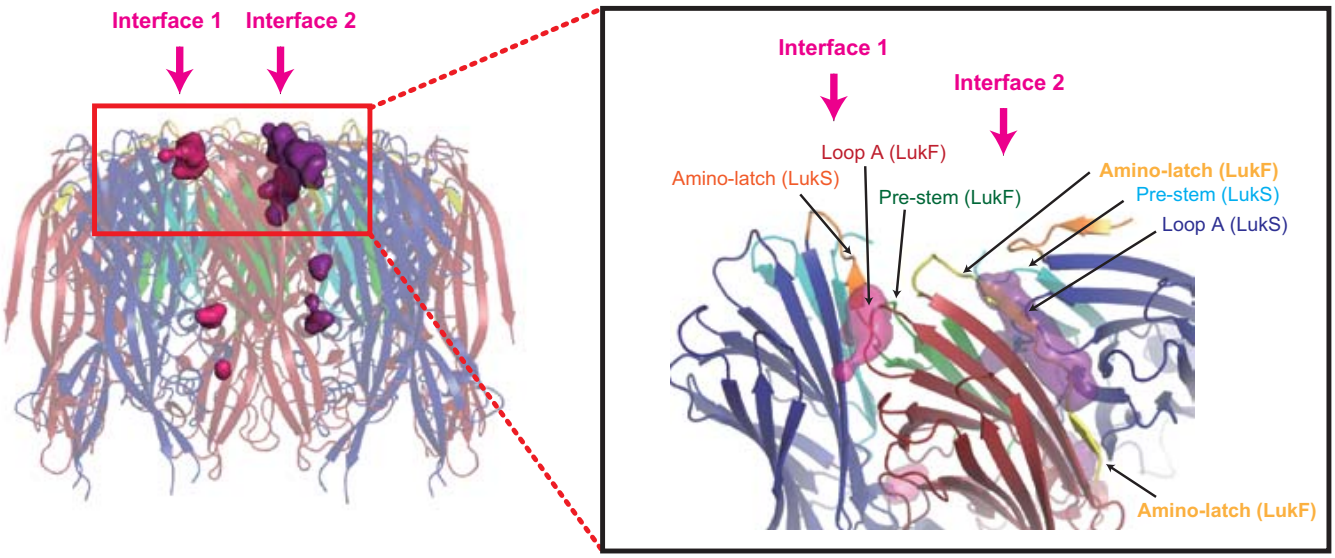

Interface 1
Interface 2
Interface 1
Interface 2
Loop A (LukF)
Amino-latch (LukS)
Pre-stem (LukF)
Amino-latch (LukF)
Pre-stem (LukS)
Loop A (LukS)
Amino-latch (LukF)

Fig. S4

Table S1. Observed inter-protomer interactions

Interface			Number of atoms participating in the inter-protomer interaction			
			van der Waals interaction		polar interaction	
Interface 1						
	LukF	Hlg2	LukF	Hlg2	LukF	Hlg2
	cap	cap	177	169	38	46
	cap	rim	66	94	24	23
	cap	stem	0	0	0	0
	cap	triangle loop	0	0	0	0
	rim	cap	0	0	0	0
	rim	rim	59	38	7	7
	rim	stem	0	0	0	0
	rim	triangle loop	0	0	0	0
	stem	cap	0	0	0	0
	stem	rim	56	40	8	8
	stem	stem	290	298	87	95
	stem	triangle loop	0	0	0	0
	triangle loop	cap	48	49	16	16
	triangle loop	rim	22	25	4	4
	triangle loop	stem	4	10	0	0
	triangle loop	triangle loop	28	24	6	6
	total[a]		750 (460)	747 (449)	190 (103)	205 (110)
Interface 2						
	LukF	Hlg2	LukF	Hlg2	LukF	Hlg2
	cap	cap	219	205	59	49
	cap	rim	0	0	0	0
	cap	stem	0	0	0	0
	cap	triangle loop	41	40	12	8
	rim	cap	16	16	8	8

rim	rim	24	36	3	3
rim	stem	11	6	1	1
rim	triangle loop	8	22	0	0
stem	cap	0	0	0	0
stem	rim	0	0	0	0
stem	stem	291	277	78	78
stem	triangle loop	8	4	0	0
triangle loop	cap	0	0	0	0
triangle loop	rim	0	0	0	0
triangle loop	stem	0	0	0	0
triangle loop	triangle loop	16	21	4	8
total[a]		634 (343)	627 (350)	165 (87)	155 (77)

a The values in parentheses refer to total number except stem-stem interaction.

Supplementary Table S2. Data collection and refinement statistics

	γHL (3B07)
Data collection	
Beamline	SPring-8 BL41XU
Detector	Rayonix MX225HE
Wavelength (Å)	1.00000
Space group	$C222_1$
Cell dimensions (Å)	*a*=206.45, *b*=206.14, *c*=190.30
Resolution range (Å) [a]	43.30-2.49 (2.65-2.49)
R_{sym} (%) [a]	11.1 (65.1)
$<I/\sigma(I)>$ [a]	14.3 (3.5)
Completeness (%) [a]	98.9 (96.3)
Multiplicity [a]	7.6 (6.8)
No. of observed reflections [a]	1,054,332 (147,704)
No. of unique reflections [a]	138,838 (21,660)
Refinement	
Resolution (Å)	43.30-2.49
R-work (%)	20.68
R-free (%)	23.64
Twin fraction (%)	44.3 (-k, -*h*, -*l*)
$R_{twin, obs}$, $R_{twin, calc}$ (%) [b]	6.5, 43.4
No. of protein atoms	17,896
No. of ligand atoms	32
No. of water molecules	449
Average B-factors (Å^2)	
protein atoms	43.78
ligand atoms	70.40
water molecules	31.30
r.m.s.d. from ideal	
bond lengths (Å)	0.009
bond angles (°)	1.125
Ramachandran plot [c]	
residues in favoured region	2,136 (97.1%)

residues in allowed region	64 (2.9%)
residues in outlier region	0 (0.0%)

a The values in parentheses refer to data in the highest resolution shell.
b $R_{twin} = \Sigma | I(\boldsymbol{h}) - I(S_{twin}\,\boldsymbol{h}) | / \Sigma (I(\boldsymbol{h}) + I(S_{twin}\,\boldsymbol{h}))$, where S_{twin} is the twin operator (1).
c Ramachandran plot analyses were performed using *RAMPAGE* (2).

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