Crystal structures of a double-barrelled fluoride ion channel

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To contend with hazards posed by environmental fluoride, microorganisms export this anion through F̶-specific ion channels of the Fluc family4-7. Since the recent discovery of Fluc channels, numerous idiosyncratic features of these proteins have been unearthed, including strong selectivity for F̶ over Cl̶ and dual-topology dimeric assembly8-13. To understand the chemical basis for F̶ permeation and how the antiparallel subunits convene to form a F̶-selective pore, here we solve the crystal structures of two bacterial Fluc homologues in complex with three different monobody inhibitors, with and without F̶ present, to a maximum resolution of 2.1 Å. The structures reveal a surprising ‘double-barrelled’ channel architecture in which two F̶ ion pathways span the membrane, and the dual-topology arrangement includes a centrally coordinated cation, most likely Na+. F̶ selectivity is proposed to arise from the very narrow pores and an unusual anion coordination that exploits the quadrupolar edges of conserved phenylalanine rings.

The fluoride anion, ubiquitous in the aqueous biosphere throughout evolutionary time, is a xenobiotic inhibitor of essential phosphoryl-transfer enzymes2. unicellular organisms directly exposed to environmental F̶ counteract the toxicity of the anion through the action of F̶-exporting membrane transport proteins that keep cytoplasmic F̶ below inhibitory levels14-16. Two recently discovered phylogenetically unrelated families of F̶ exporters carry out this task: the CLC F̶/H̶ antiporters, a strictly bacterial clade of the CLC superfamily of anion transporters, and small-membrane proteins of the Fluc family (also known as CrcB or FEX). Fluc genes are also found in plants, fungi, primitive marine chordates, and sponges, but not in mammals. We recently established17 that Fluc proteins are ion channels with two unusual properties: an exceedingly high specificity (>106) for F̶ over Cl̶, and a dual-topology dimeric architecture, in which the two subunits forming the active channel associate in antiparallel transmembrane orientation. Dual-topology dimeric construction is known in small multidrug transporters18-20, but has not been previously observed in ion channels. These inferences regarding function and structure of Flucs provoke fundamental questions about their mechanisms, such as (1) how the channel achieves such extreme selectivity for F̶, arguably the highest selectivity of any ion channel to our knowledge; (2) whether the protein contains a single pore on the subunit interface or two pores, one in each subunit, as in CLC Cl̶ channels12; and (3) whether the channel homodimer is symmetrical, or the two subunits adopt different conformations, as in the multidrug resistance transporter EmrE21-23.

In previous work, the antiparallel transmembrane topology of Fluc channels was intimated by the distribution of positively charged residues in Fluc sequences13, was strongly suggested by crosslinking and functional reconstitution2, and was established definitively by two-sided block of single channels by ‘monobodies’, engineered proteins selected as high-affinity binding partners from combinatorial libraries11. We used these monobodies in crystallization trials to form complexes with a Fluc channel from Bordetella pertussis, denoted Bpe. Crystals could not be grown unless monobodies were present; however, diffraction to 3.6 Å Bragg spacing was obtained with the monobody Mb(Bpe–S7) (ref. 6), hereafter denoted S7. A structure was solved, with phases initially determined by single-wavelength anomalous diffraction (SAD) of Bpe labelled with Hg at a unique cysteine residue, and improved with Hg-labelled selenomethionine-substituted samples (Extended Data Table 1). A view of the crystal lattice highlights the importance of the monobodies as crystallization chaperones, which exclusively mediate crystal contacts in all structures presented here (Extended Data Fig. 1).

Although devoid of F̶ ions and at low resolution, the Bpe–S7 structure reveals the overall architecture of the channel (Fig. 1a). Bpe is an antiparallel homodimer in which each subunit consists of
four transmembrane helices (denoted TM), with an overall fold that is novel among membrane proteins. The 1,700 Å² dimer interface is almost completely membrane-embedded. The third, highly conserved helix (TGXXXGLTFTSFXXE, in which X denotes any amino acid) is broken into two halves, TM3a and TM3b, by a six-residue non-helical segment located roughly at the centre of the membrane. These two segments, one from each subunit, cross each other near the two-fold axis of the channel running parallel to the membrane plane. The channel is hourglass-shaped, with wide vestibules symmetrically opening to two aqueous solutions (Fig. 1b) separated by a solid plug of protein 10–15 Å thick. A conspicuous universally conserved TM1 arginine residue (R23) protrudes into each vestibule, suggestive of an electrostatic lure for F⁻. No aqueous pore connecting the vestibules is visible in this low-resolution structure.

The channel is capped on both ends by the S7 monobody (Fig. 1c). This monobody was selected from a library designed to target convex protein surfaces¹⁴, and indeed its interaction surface, consisting largely of the residues diversified in the library, wraps around a protrusion formed from the TM1–TM2 and TM3b–TM4 connecting loops of the channel. An eight-residue loop on the monobody plunges deeply into each vestibule, contacting the channel mainly via side chains. Channel–monobody interactions are mostly hydrophobic, aromatic, and hydrogen bonded, the paucity of salt-bridges rationalizing the rather weak ionic strength dependence of monobody binding⁴⁵. Most of the aqueous-exposed surface of the channel is covered by monobody, consistent with S7 block of Bpe seen in single-channel recordings (Fig. 1c).

It is tempting to imagine that a central pore connects the two vestibules in an unseen ‘open’ conformation. But the low resolution of the structure and the absence of F⁻ preclude identification of the ion-permeation pathway. We therefore attempted in meso crystallization in the hope of identifying bound F⁻ ions. Crystals diffraacting to 2.1 Å in the presence of 20 mM F⁻ were obtained with a different monobody, L2, which is also a blocker (Extended Data Fig. 2). Structures solved by molecular replacement (Extended Data Table 1) again show the channel with a monobody on each end (Extended Data Figs 1 and 2). The backbone conformation of the channel is identical to that in the lower-resolution structure (Cα root mean squared deviation (r.m.s.d.), 0.4 Å), and L2, although binding in a different orientation than S7, also extends a long loop of 8–10 Å into the vestibule, occluding much of the channel’s water-exposed surface.

This higher-resolution structure reveals five intriguing electron densities (Fig. 2a and Extended Data Fig. 3). First, a prominent density resides in the centre of the plug separating the vestibules, precisely on the homodimer’s two-fold non-crystallographic axis (Fig. 2b). We identify this as a Na⁺ ion on the basis of its coordination by four backbone carbonyl groups from residues in each subunit associated with the conserved TM3 break (G77 and T80). This coordination is inconsistent with a F⁻ ion, a water, a divalent metal, or a K⁺ ion¹⁶,¹⁷. Although coordination by only four oxygen ligands is uncommon for Na⁺, it is nevertheless seen in ~15% of Na⁺-binding sites in the protein database¹⁸. This deeply buried cation could not exchange with aqueous solution if the plug remained intact during functional activity, and indeed, Bpe channels with familiar behaviour are readily recorded in solutions with Na⁺ completely substituted by N-methyl glucamine (Extended Data Fig. 4). We propose that the ion is an important structural component incorporated irreversibly upon dimer assembly.

A second notable detail in the Bpe–L2 structure is a set of four electron densities located in crevices between TM2, TM3b, and TM4 near the periphery of the channel, distant from the vestibules and the central plug (Fig. 2a, c). We provisionally identify these as F⁻ ions, labelled F1 and F2 in non-crystallographic-symmetry-related pairs, according to their distinct chemical environments. The putative liganding atoms embracing these densities are consistent with a halide coordination shell. In particular, the surround is composed of electro-positive side chains, which would engage the strong H-bond-accepting tendency of the F⁻ ion. Prominent among these are a strongly conserved asparagine (N43) in TM2, and two conserved serines (S108 and S112) in TM4. In addition, two pairs of conserved phenylalanine rings (F82 and F85) near the TM3 break approach these densities in a side-on orientation that presents the electropositive carbons of the quadrupolar ring to the F⁻ ion (Fig. 2d). These four aromatic rings appear to be mutually stabilized in a notable ‘box’ assembly. Edge-on aromatic liganding of anions is rare but not unprecedented in proteins¹⁹, and F⁻ coordination by aromatic edges appears in many small-molecule structures²⁰. This type of coordination is reminiscent of the phenylalanine rings of a proposed Cl⁻ binding site in the bestrophin channel¹⁷. With a deficit of H-bond acceptors, the coordination shells observed here would be chemically iminal to ordered waters, which cannot be distinguished from F⁻ based on X-ray scattering alone.

The two F⁻ ions of each pair lie in a vertical line tilted slightly off normal to the membrane plane, possibly marking a narrow permeation pathway. If these densities do indeed represent F⁻, then their positions lead to a surprising conclusion: the Bpe channel contains two pores running in antiparallel orientation along opposite sides of the dimer, rather than a single central pore connecting the vestibules through the plug. Two-pore behaviour is not apparent in single-channel recordings as both are nearly always open⁶. The structures here would represent the monobody-blocked state similar to that observed electrophysiologically⁶,¹⁵.

Figure 2 | Bpe–L2 structure. a, Bpe homodimer with mFobs — DF, electron density map contoured at 4σ (green), viewed from solution (left) and the membrane (centre and right). Aqueous volumes of the vestibules are shown (grey). Dashed boxes in right- and left-most structures indicate zoomed regions in b and c, respectively. b, Na⁺ coordination sphere, indicated by blue-dashed lines, viewed from solution. TM3 is represented as a cartoon. c, The same view at 90°, showing Na⁺, F⁻ ions, N43, and the phenylalanine box (F82 and F85). Cartoons indicate TM2 and TM3. d, F⁻ coordination shells, indicated by blue dashed lines, with F82, S112 (at F1), N43, S108, and F85 (at F2) shown as sticks. Stereo images are shown in Extended Data Fig. 3.
Our reading of the structure as a double-barrelled channel depends crucially on identifying these four densities as F\(^{-}\) ions; however, we do not consider the above evidence sufficient to accept such an unusual picture as firmly established. Accordingly, two additional experimental approaches were pursued. First, a structure was determined for a different Fluc homologue, Ec2, complexed with a monobody S9 (Extended Data Fig. 5). This homologue of only 33% identity shows similar electrophysiological behaviour to Bpe, and is blocked by S9 at nanomolar concentrations\(^6\). Ec2–S9 crystals diffracting to 2.6 Å were grown from detergent in the presence of F\(^{-}\), and to avoid model bias the structure was solved using SAD phasing with selenomethionine-labelled protein (Extended Data Table 1). The Ec2 and Bpe folds are identical (C\(_{\alpha}\) r.m.s.d. 0.6 Å), with the inferred Na\(^{+}\) density appearing in equivalent locations. Two strong difference densities lie at precisely the same locations as the F1 ions in Bpe, coordinated identically (Fig. 3a). The appearance of these densities, in a separate homologue under very different crystallization conditions, strengthens our hypothesis that the four densities in Bpe represent F\(^{-}\) ions. Additional densities also appear in Ec2 in the general vicinity of the F2 ions in Bpe, but at this lower resolution and without supporting experimental evidence, these densities in Ec2 cannot be unambiguously assigned.

The chemical nature of the crevices housing the densities makes sense for narrow diffusion pathways that are welcoming to F\(^{-}\) ions in both homologues (Fig. 3b). In particular, the crevice-facing surface of TM4 is lined with H-donating side chains, as manifested by every fourth residue in the sequences of Bpe (Y104, S108, S112, and T116) or Ec2 (S102, H106, S110, and T114). These particular residues are only modestly conserved among Fluc channels, but H-bond donors consistently appear here throughout the family. These could plausibly contribute to a polar track, along which largely dehydrated F\(^{-}\) ions move across the membrane. Because these pathways are extremely narrow, protein dynamics may be necessary to allow F\(^{-}\) permeation, and the monobodies might force a conformation in which the two pores are less ‘open’ than in the fully conducting state.

We next examined the functional consequences of mutating each of the three conserved Bpe residues coordinating the F\(^{-}\) densities: F82, F85, and N43. We did not observe single-channel activity in electrical recordings, but more sensitive ‘anion-dump’ experiments\(^{22,23}\) reveal notable changes in F\(^{-}\) permeation (Fig. 3c). In these experiments, Bpe-reconstituted liposomes loaded with KF are suspended in low-F\(^{-}\) solutions, and the rate of passive F\(^{-}\) efflux is followed electrochemically. To eliminate the aromatic quadrupole, the conserved phenylalanine residues were mutated individually to isoleucine. For F85I and F82I, efflux of F\(^{-}\) is two and three orders of magnitude slower, respectively, than for wild type (\(\sim 3 \times 10^{-4}\) s\(^{-1}\); ref. 15) (Extended Data Table 2). These mutations preserve the integrity of the channel, as F\(^{-}\) efflux is 50–80% blocked by 6 μM of monobody (Extended Data Fig. 6). Strong selectivity against Cl\(^{-}\) is observed in parallel experiments with this haloide.

The conserved asparagine was substituted to alter or remove H-bonding capability (N43S and N43A), or to place an isoteric carboxylate at this position (N43D). The first two mutants were biochemically intractable, but N43D produced stable protein. Under standard conditions at pH 7, N43D supports robust F\(^{-}\)-selective efflux (Fig. 3d and Extended Data Table 2). We had envisioned that an anionic carboxylate at this position would prevent F\(^{-}\) entry into the channel. It is possible, however, that the pK\(_{a}\) of this group is perturbed upwards by its local environment, so that at neutral pH conditions the carboxyl acts as a protonated surrogate for the N43 amide. The N43D mutant was therefore examined at several pH values. In stark contrast to the pH-independent activity of wild-type Bpe and the F85I mutant (Extended Data Fig. 5), F\(^{-}\) efflux in N43D falls with increasing pH and is extinguished at pH 9 (Fig. 3d), verifying a key role of the H-bond-donating N43 side chain in F\(^{-}\) conduction.

These mutagenic manipulations of F\(^{-}\) permeation add mechanistic evidence for assigning F\(^{-}\) ions to the densities in question. This inference points to the conclusion that Flucs are double-barrelled F\(^{-}\) channels, with the observed F\(^{-}\) ions marking the ion-selective pathways. The two pathways are not segregated to each subunit as in CLC channels\(^3\); instead, each pore comprises side chains from TM2, TM3b, and TM4 of one subunit plus the TM3-break phenylalanine from the opposing subunit. Although unexpected, this idea does not clash with any electrophysiological experiments, and double-pore assembly was cited previously as a possible, albeit unlikely, architecture consistent with the functional behaviour of the channel\(^3\).

Two-pore assembly neatly accounts for evolutionary drift in eukaryotic Fluc channels, all of which consist of an inverted repeat of two homologous Fluc domains fused into a single polypeptide\(^{4,5}\). Alignments (Extended Data Fig. 7) show that ‘pore 2’, where most residues arise from the carboxy-terminal domain, retains the strict sequence conservation typical of the homodimeric bacterial Flucs, whereas the equivalent residues of ‘pore 1’, mostly in the amino-terminal domain, are far less conserved, notably along the TM4 polar track. This pattern is further confirmed for the two conserved phenylalanines that, from the same domain, contribute to alternate pores. Thus, in pore 2, the strongly conserved equivalents of F82 project from the N-terminal domain, and those of F85 from the C-terminal domain, in contrast to their poorly conserved residue-counterparts contributing to pore 1. These sequence-based considerations suggest that pore 2 alone fulfils the F\(^{-}\)-export function in eukaryotic Flucs. Recent experiments reinforce this idea by showing that mutations of several C-terminal domain residues in pore 2 produce F\(^{-}\) hypersensitivity in yeast, whereas mutations of the equivalent residues of N-terminal domain, pore 1 are relatively harmless\(^4\). These features chronicle an evolutionary lineage of gene duplication, fusion, and finally functional degradation of a redundant pore by genetic drift in the eukaryotic homologues.

Other inferences emerging from these structures will require further testing to confirm or refine. First, a buried Na\(^{+}\) ion occupying a unique position on the two-fold axis invites us to view this cation as an intrinsic structural element stabilizing the dimer interface. Second, all
four $F^-$ ions observed in Bpe probably occupy the channel simultaneously, given their high occupancies (>80%) in the 2.1 Å structure, with B-factors matched to those in their coordination shells. The channel might therefore display multi-ion conduction phenomena akin to those long-known in K$^+$ channels\cite{1, 2}. Third, the strong F$^-$ selectivity of the channel may arise from the narrow bore of the permeation pathway, which would exclude Cl$^-$ ions while permitting the smaller F$^-$ ions to enter. However, it is unclear why F$^-$ would enter this confined space, and how the protein compensates for the high energy of dehydrating F$^-$. We note that many of the coordinating groups are H-bond donors, able to satisfy the H-bonding proclivity of the F$^-$ ion. While the unusual edge-on coordination by conserved phenylalanine rings is chemically intriguing, the energetic contributions of these interactions have not been established; nevertheless, in light of the conservation of these residues, we speculate that this short-range quadrupolar interaction contributes to F$^-$ recognition and permeation in an essential way. The pore, although narrow, is lined with H-bonding residues and so could provide a polar conduit for transport of the ion across the membrane span.

A final point concerns the mechanism of electrodiffusive F$^-$ transport through these oddly fashioned pores. The crucial role of N43 in permeation in Bpe, and the confined crevice in which it resides, lead us to conjecture that F$^-$ moves along the pore concomitant with a rotameric switch of this side chain, such that the amide nitrogen remains H-bonded as the anion moves along the pore (Fig. 4). Thus, the conduction mechanism we propose here would be subtly distinct from classic diffusion through a fixed, water-filled channel. Instead, it would incorporate a central feature of membrane transporters: substrate transport coupled to concerted movement of the protein. An asparagine side-chain rotation could easily occur on the conduction timescale of microseconds, but formally this picture is a nuanced mix of electrodiffusion and configurational change, and so can be termed a ‘channspor’ mechanism.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Information Atomic coordinate files have been uploaded to the Protein Data Bank (PDB) with accession codes 5A40, 5A41 and 5A43 for the Bpe–S7, Bpe–L2 and Ec2–S9 complexes, respectively. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests.

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METHODS

No statistical methods were used to predetermine sample size.

Preparation of crystals. Expression, purification, and reconstitution of Fluc channels were performed as previously described8,10. In the final purification step, Fluc protein was collected from a S200 size-exclusion column equilibrated in 100 mM NaF (or NaCl for zero-F preparations), 10 mM HEPES, pH 7.0, and 5 mM n-decyl-β-D-maltoside (DDM). Bpe constructs carried two functionally neutral mutations to enhance expression (R29K and E94C), and, for selenomethionine incorporation, an additional methionine was introduced (A51M) to enhance phasing power. The C-terminal His tag was removed from Bpe by treatment with lysine endoprotease C (Roche) but was left on Ec2. Fluc protein was typically reconstituted into liposomes at low density (0.1–0.2 μg of protein per mg of lipid). For single-channel recording, liposomes were fused into planar lipid bilayers in symmetrical solutions of 300 mM NaF, 15 mM MOPS, pH 7.0, and channels were recorded at 200 mV holding voltage.5. Monobodies were expressed in Escherichia coli and purified as previously described. N-terminal His tags were removed while bound to talon beads by 16-h treatment with tobacco etch virus (TEV) protease also carrying a His tag; monobodies with cleaved His tags were eluted from the affinity column with 150 mM NaCl and 40 mM Tris-HCl, pH 7.5. For the final purification step, the preparation was passed over a 575 size-exclusion column in 100 mM NaF (or NaCl) and 10 mM HEPES, pH 7. Monobodies were used immediately for crystallization or stored in frozen aliquots for channel-blocking experiments. For cryocrystallization from detergent micelles, Fluc protein in solution containing 5 mM DDM was concentrated to 10 mg ml−1, a step that concentrates the detergent 5–10-fold. Monobody solution (10 mg ml−1) was supplemented with 4 mM DDM immediately before mixing with channel in a 1:2.1 molar ratio. This protein solution was then mixed with an equal volume of crystallization solutions (0.5 μl for sitting drops in 96-well plates or 1 μl for hanging drops in 24-well plates). Bpe–S7 crystals grew in 3–5 days in crystallization solutions of 36–41% (w/v) polyethylene glycol monomethyl ether 550, 0.2 M MgCl2 or CaCl2, and 0.1 M N-(2-acetamido)imidodiacetic acid, pH 6.0–6.7. Crystals were frozen in liquid nitrogen for data collection. For lipicubic phase crystallization, Fluc protein concentrated to 10 mg ml−1 as above was dialysed overnight to reduce the DDM concentration to 10 mM. This was then mixed with monobody solution (10 mg ml−1, with 4 mM DDM) in a 1:1.2 molar ratio. The protein-laden mesophase was prepared by homogenizing 9.9 monoaacglycerol (monoolein) lipid with protein solution (10 mg ml−1) at a weight ratio of 1:1.5 (protein:lipid) using a coupled syringe mixing device at 20 °C (ref. 27). Crystallization trials were carried out in 96-well glass sandwich plates with 50 mM sodium mesophase (NaCl) and 0.8 μl precipitant solution using an in meso robot. Crystallization solutions contained 22–26% (v/v) polyethylene glycol dimethyl ether 550, and 0.1 M Na-citrate, pH 5.5, with or without 10 mM NaF. Surfaced-shaped crystals grew to a maximum size of 100 × 50 × 5 μm in 5–10 days. Wells were opened using a tungsten–carbide glasscutter, and the crystals were collected using 50–100 μm micromounts (MiTeGen). Crystals were snap-cooled in liquid nitrogen before data collection on the Diamond Light Source beamlines I24 or I04.

Anion efflux from liposomes. Efflux of F− or Cl− out of liposomes was followed electrochemically as described11,12. Liposomes (10 mg ml−1 lipid, 0.2–1 μg protein per mg of lipid) were loaded with 300 mM KF or KC1 solutions were freeze-thawed for 3 cycles and then extruded 21 times through a 400-nm filter. Immediately before the assay, a 100 μl sample was centrifuged through a 1.5-ml Sephacryl column equilibrated with flux buffer (300 mM K-isonethionate, 1 mM KF or KC1, and 25 mM HEPES, pH 7) and was diluted 20-fold into a stirred chamber containing 3.8 ml flux buffer. Halide concentration in the suspension was continuously monitored with a F− or Cl− electrode amplified through a pH meter and digitized at 5 Hz sampling frequency. Efflux was initiated by adding 1 μl valinomycin, and after several minutes 30 mM octylglycoside was added to obtain the 100% efflux level. Efflux rates were calculated after calibration with 25 μM additions of NaF or NaCl. For experiments with the Bpe N430 mutant, the flux buffer contained an additional 100 mM Na-isonethionate and 25 mM CHES (N-cyclohexyl-2-aminoethanesulfonic acid) buffer. Single-channel block by monobodies was recorded in planar phospholipid bilayers exactly as described.

Structure determination. Diffraction data for Bpe–S7 were processed by the Xia2 pipeline13 to XDS14 and scaled using Aimless15. The space group was determined to be P212121 with two Bpe dimers and four S7 monobodies in the asymmetric unit (Extended Data Fig. 1). A phasing strategy was devised that used pre-derivation of Bpe mutated with a single cysteine residue (E94C) with Hg(t) acetate before crystallization (see above). None of the native crystals were isomorphous with the Hg-derivatized crystals (Rmerge > 40%), despite having similar cell dimensions. Indeed, Hg-derivatized crystals were observed to diffract X-rays to slightly higher resolution than native crystals, therefore effort was directed at these samples for phasing and refinement. The four Hg sites were located using the SAD method as implemented in SHEXL20 with the positions further refined and initial phases calculated using a solvent flattening in SOLVE-OMON21. To improve the phases, a second and third data set were also collected at the Se edge using both Hg- and seleno-methionine-derivatized protein and another Hg-derivatized data set, respectively (Extended Data Table 1). All 16 Se plus 4 Hg sites were located using SHEXL and this data set was combined with the initial 3.6 Å Hg-derivatized data. Phases were substantially improved using SIRAS combining the three data sets in SHARP. We did not observe higher resolution data in the native crystals, which typically gave diffraction between 3.6–3.8 Å. Our highest resolution data set with optimal scaling statistics was one of the Hg-derivatized crystals; we therefore used this data set for subsequent refinement of the model built into the experimental electron density maps calculated from SHARP (see below). For the Bpe–L2 crystals, data were similarly processed and scaled in space group P1. Phases were calculated using molecular replacement as implemented in Phaser24, using the experimentally determined Bpe model and a homology model of the L2 monobody based on a previously determined structure of a loop-library monobody (PDB code: 3RZW). The unambiguous solution showed two Bpe homodimers and four L2 monobodies. The electron density maps clearly showed major differences in the selected variable regions of the monobody. For the Ec2–S9 crystals, the data were processed as above with space group P41. Phases were calculated using Se-SAD with eight Se sites, and processed as above. The experimental electron density maps were of high quality following phase extension to the highest resolution shell of 2.58 Å. Data were collected at Advanced Light Source beamlines 8.2.1 and 8.2.2, and Diamond Light Source beamlines I24 and I04.

Model building and refinement. For the Bpe–S7 complex structure, a model for the channel was built into the experimental electron density maps calculated from SHARP using O2O with a σA-weighted Fo−Fm value, and Fm−Fo electron density maps. The S7 monobodies were initially built using a homology model based on a previously determined structure of a side-library monobody (PDB code: 4EGQ). These models were placed into the experimental electron density maps using MolRep25. The partial models were further cycled back into phase calculation in SHARP to improve the initial solvent envelope used for the solvent flattening procedure. The amino acid side chains were then built using the Se and Hg sites to determine the correct register. Refinement of the Bpe–S7 model was carried out in Refmac5 (refs 37, 38) against the highest resolution data set for these crystals, 3.6 Å, which came from one of the Hg-derivatized crystals used for phasing (Extended Data Table 1). No previous phase information was used during the refinement; however, refinement was improved following anisotropic truncation of the structure factors. To avoid biasing the model, non-cryohyalinoglyph symmetry was not used except at the final round of refinement to improve model geometry. Model validation was carried out using the Molprobity server26. The Ec2–S9 model was built directly into the experimental maps, using Se sites to ensure the correct register, and then monobodies were placed by molecular replacement using Phaser with a homology model based on S7. The Bpe–L2 model was built into the electron density maps calculated from Phaser following iterative rounds of structure refinement in PHENIX27 and Refmac5. The structural model was revised in real space with Coot28.

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Extended Data Figure 1 | Crystal lattices for the Bpe–S7, Bpe–L2 and Ec2–S9 crystal structures. The asymmetric unit is shown in green and red (channel and monobody, respectively), and symmetry mates are shown in black and blue.
Extended Data Figure 2 | Bpe–L2 complex. Left, cartoon schematic of Bpe crystal structure, coloured as in Fig. 1b. The variable regions of monobody L2 are coloured cyan. Mesh-rendering is shown for the lower monobody. Right, single-channel recording of Bpe in the presence of 200 nM L2. Zero-current level is indicated by the dashed line.
Extended Data Figure 3 | Stereo images of Bpe-L2. a–d, Stereo images corresponding to the structures shown in Fig. 2a–d.
Extended Data Figure 4 | Single channel trace of Bpe in Na\(^+\)-free recording solution, with addition of 200 nM blocking monobody L3. Channels were recorded in the presence of 300 mM N-methyl-glucamine-fluoride, from which all small cations were rigorously excluded. The zero-current level is indicated by the dashed line.
Extended Data Figure 5 | Experimental electron density for the Ec2–S9 crystal structure. Left, cartoon schematic of Ec2 with S9 monobodies bound, coloured as in Bpe in Fig. 1b. Variable sequences of the monobodies (cyan) with ribbon or mesh representation. Right, cartoon view of TM4 from Ec2, with the solvent-flattened electron density map calculated from SHARP contoured at 1.8σ (blue), and anomalous difference density from seleno-L-methionine contoured at 5σ (magenta).
Extended Data Figure 6 | Liposome flux assays of Bpe variants. Top three panels: F⁻ transport from liposomes by Bpe mutants F82I, F85I, and N43D, in the presence and absence of 6 μM blocking monobody. F⁻ efflux from proteoliposomes (0.2 μg protein per mg lipid for Phe mutants; 1 μg protein per mg lipid for N43D) was monitored with a F⁻ electrode and normalized against total trapped F⁻. Bottom panel: F⁻ dump by F85I measured at pH 7 and pH 9. Rates are summarized in Extended Data Table 2.
Extended Data Figure 7 | Sequence alignment of eukaryotic N- and C-terminal Fluc domain sequences, with bacterial homodimer sequences below. Highly conserved residues are shaded in grey. For the eukaryotic sequences, residues expected to line ‘pore 2’, the pore mostly encompassed by the C-terminal domain, are coloured red.
| Extended Data Table 1 | Data collection, phasing and refinement statistics |
|-----------------------|-----------------------------------------------|
|                       | Bpe-S7-Hg* (PDB 5a40) | Bpe-S7-Se+Hg | Bpe-S7- Hg (PDB 5a41) | Bpe-L2 (PDB 5a41) | Eg2-S9-Se (PDB 5a43) |
| **Data collection**    |                               |               |                        |                   |                       |
| Space group            | P2,2,2                        | P2,2,2        | P2,2,2                  | P1                | P4₁                   |
| Cell dimensions        |                               |               |                         |                   |                       |
| \( a, b, c \) (Å)      | 146.8, 183.7, 72.8           | 146.9, 184.0, 72.3 | 145.2, 185.0, 72.5     | 40.7, 83.9, 86.9₁ | 87.4, 87.4, 146.8     |
| \( \alpha, \beta, \gamma \) (°) | 90, 90, 90          | 90, 90, 90    | 90, 90, 90              | 108.9, 96.9, 97.6 | 90, 90, 90             |
| Resolution (Å)         | 48 – 3.6 (3.7 – 3.6)         | 48 – 3.6 (3.7 – 3.6) | 57 – 4.7 (4.8 – 4.7)   | 41 – 2.1 (2.5 – 2.1) | 25 - 2.6 (2.7 – 2.6)  |
| \( R_{\text{merge}} \) | 8.8 (65.2)                   | 15.2 (141)    | 12.7 (92.2)             | 8.8 (82.7)        | 17.4 (165)            |
| Mn I / αf              | 10.6 (2.6)                   | 9.2 (1.7)     | 8.7 (2.9)               | 7.6 (1.3)         | 9.0 (1.9)             |
| CC(1/2)¹               | 99.8 (91.9)                  | 99.8 (90.0)   | 99.9 (84.8)             | 99.4 (43.8)       | --                    |
| Completeness (%)       | 99.6 (75.0)                  | 98.7 (96.7)   | 99.7 (99.8)             | 96.8 (96.3)       | 99.9 (100)            |
| Redundancy             | 6.3 (6.7)                    | 6.4 (6.2)     | 6.0 (6.1)               | 3.4 (3.4)         | 14.3 (14.3)           |
| \( R_{\text{crys}} \) (%) | -- / 96.0               | 45.8 / 96.0   | 93.0 / 99.0             | 87.9              |                       |
| Isomorphous / Anomalous | -- / 0.323                  | 1.032 / 0.501 | 0.338 / 0.073           | 0.825             |                       |
| **Refinement**         |                               |               |                         |                   |                       |
| Resolution (Å)         | 47.3 – 3.6                   | 40.9 – 2.1    | 24.2 – 2.6              |                   |                       |
| No. reflections        | 21,085                       | 51,555        | 34,593                  |                   |                       |
| \( R_{\text{work}} / R_{\text{free}} \) | 23.6 / 26.9               | 20.5 / 24.0   | 22.4 / 26.4             |                   |                       |
| Ramachandran Favored   | 85.4                         | 96.5          | 92.6                    |                   |                       |
| Ramachandran Outliers  | 4.02                         | 3.04          | 2.53                    |                   |                       |
| R.m.s. deviations      | 0.011                        | 0.009         | 0.009                   | 1.26              | 1.50                  |
| Bond lengths (Å)       | 1.53                         |               |                         |                   |                       |

*For details on derivatization see Methods.

¹Mn(I) half-set correlation as reported by Aimless.

²Phasing power = r.m.s. \((\langle F_u \rangle \langle F_d \rangle - \langle F_{null} \rangle)^2\).
Extended Data Table 2 | F⁻ turnover rate for Bpe mutants

| Mutant               | rate (s⁻¹)  |
|----------------------|-------------|
| WT                   | ~3x10⁵      |
| N43D, pH 6.5         | 4330 ± 440  |
| N43D, pH 7           | 1860 ± 210  |
| N43D, pH 7 + Mb      | 210 ± 30    |
| N43D, pH 9           | undetectable|
| F82I                 | 200 ± 12    |
| F82I + Mb            | 45 ± 7      |
| F85I                 | 1950 ± 200  |
| F85I + Mb            | 300 ± 25    |
| F85I, pH 9           | 1680 ± 110  |

Analogous experiments in which Cl⁻ efflux was measured gave no detectable activity in any samples. Wild-type rate estimated based on single channel currents. F⁻ turnover by wild type exceeds response time of the electrode. Each value represents mean ± s.e.m. of three determinations calculated from initial efflux rate.