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Continuous observation of the stochastic motion of an individual small-molecule walker

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Abstract

Motion - be it the ability to change shape, rotate or translate - is an important potential asset for functional nanostructures. For translational motion, a variety of DNA-based and small-molecule walkers have been created, but observing the translational motion of individual molecules in real time remains a significant challenge. Here, we show that the movement of a small-molecule walker along a 5-foothold track can be monitored continuously within a protein nanoreactor. The walker is an organoarsenic(III) molecule with exchangeable thiol ligands, and the track a line of cysteine residues 6 Å apart within an α -haemolysin protein pore that acts as the nanoreactor. Changes in the flow of ionic current through the pore reflect the individual steps of a single walker, which require the making and breaking of As-S bonds, and occur in aqueous solution at neutral pH and room temperature. The walker moves considerably faster (~ 0.7 s per step) than previous walkers based on covalent chemistry and is weakly processive (6 ± 1 steps per outing). It shows weak net directional movement, which can be described by a thermodynamic sink arising from the different environments of the cysteines that constitute the track.

Creating nanoscale objects that can change shape¹, rotate^{2,3} or translate⁴ is an important goal in nanotechnology. Examples of translational motion include chemotactic droplets⁵, chemically propelled objects⁶, and DNA walkers⁷, some of which offer the possibility of cargo delivery^{8–10}. The development of small-molecule walkers is also under intense investigation¹¹. The motion of such walkers can be random, or biased directionally through asymmetry of the track or the input of "fuel". Examples of non-covalent small-molecule walkers embrace various rotaxanes and related structures¹². Walkers that employ covalent chemistry afford optimal spatial precision^{13, 14}. For example, Leigh and co-workers observed the slow random motion of a functionally symmetric covalent walker, α -methylene-4-nitrostyrene, with a step-time of hours on a 9-foothold oligoethylenimine track^{15, 16}. The walker lingered in a thermodynamic well at a terminal track site, thereby

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exhibiting movement with a net directional bias¹⁶. The Leigh group have also prepared walkers for which each individual step has a directional bias, requiring the expenditure of chemical fuel. For example, a hetero-bifunctional walker migrates along a four-foothold platform in a direction that is controlled by cyclical manipulation of light, pH or redox conditions^{17, 18}. The latter effectively alter the walker and/ or track to promote forward motion and prevent backward motion in a ratchet-like mechanism.

The visualization of the translational motion of individual molecules in real time is experimentally demanding. The movement of various small molecules^{19, 20} and the triggered motion of a synthetic nanocar⁴ have been visualized by STM in a vacuum at low temperatures. Individual DNA walkers have been monitored by high-speed AFM⁹, FRET²¹ or optical microscopy²². However, individual small-molecule covalent walkers, which take far smaller steps, have not been monitored continuously, in real time. Here, we observe the step-wise stochastic motion of an organoarsenic(III) molecule along a linear (one-dimensional) track of thiols within a protein nanoreactor. We watched chemistry at the single-molecule level by monitoring the ionic current that flows through a single α -hemolysin (α HL) protein pore²³. The chemistry of reactants tethered within the α HL pore is reflected by step changes in the current associated with individual bond making and breaking events, or even isomerizations. The α HL nanoreactor has been used to monitor a variety of chemistries with millisecond temporal resolution²³: for example, photodeprotection²⁴ and photoisomerization²⁵, thiol-disulfide chemistry²⁶, polymer chain growth²⁷, metal chelation^{28, 29}, a kinetic isotope effect³⁰ and S-nitrosothiol chemistry³¹. The molecular walker used here features As(III)-thiol chemistry. We have already explored several aspects of As(III)-thiol chemistry in the nanoreactor, including the formation and breakdown of As(III)-thiol adducts³² and the stereochemical inversion of the adducts³³.

Overview of approach

The walker, SPAA-MEET₂ (Fig. 1a), was formed in situ by the reaction of SPAA (4-sulfophenylarsonous acid) with excess MEET (2-(2-methoxyethoxy)ethanethiol) (Fig. S1, S2, S3). Thiol ligands of the walker are readily displaced by exchange with free thiols, a reaction that is the basis of the walking mechanism (Fig. 1b). The track comprised five cysteine (Cys) residues (“footholds”) spaced 6 Å apart (C α -C α) on a β strand within an α HL pore (Fig. 1c). The α HL subunit containing the five Cys residues (at positions 113, 115, 117, 119 and 121) was assembled with Cys-free subunits and heteroheptameric pores containing a single track were purified by SDS-polyacrylamide gel electrophoresis (Fig. S4). All five thiol side-chains pointed into the lumen of the transmembrane β barrel and constituted footholds 1 to 5, respectively (Fig. 1c). To understand the movement of the walker, we first analyzed the reactions of SPAA-MEET₂ with single- and double-Cys mutant α HL pores. We then conducted experiments with a triple-Cys mutant to document the walker’s ability to make two steps. Finally, we demonstrated walking along the full five-Cys track.

The walker at single footholds

Single-Cys mutants were made at all five positions (113, 115, 117, 119 and 121) and a heteroheptamer containing one single-Cys subunit was made with each mutant. The five

single-Cys pores afforded similar electrical traces in the presence of SPAA-MEET₂, displaying two major current levels arising from the unreacted pore (P₀) and the As(III) adduct (P_I) (Fig. 2). The measurements were made at room temperature at an applied potential of -50 mV, with SPAA in the trans compartment and MEET in the cis compartment. The current blockade in the P_I state ($I_n = I_{P_0} - I_{P_I}$, where *n* is the foothold position, Fig. 1c) differed in value depending upon the location (Table 1). Footholds 1 and 5 each showed two blockade levels, which might represent the two enantiomers of each adduct³². The enantiomers, which are diastereomeric when the attached protein is considered, could not be distinguished at footholds 2, 3, and 4 (Table 1). The two current levels at footholds 1 and 5 were treated as single states in subsequent mechanistic and kinetic analyses.

Mono-adduct formation was analyzed according to a two-state model (Fig. 2b). The reaction rates of SPAA-MEET₂ with each of the five single-Cys mutants were directly proportional to the concentration of SPAA-MEET₂ (taken to be the bulk concentration of SPAA) with second-order association rate constants of $>10^4 \text{ M}^{-1}\text{s}^{-1}$ [Cys-115: $k_{+2} = (9 \pm 1) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$; Cys-117: $k_{+3} = (2.0 \pm 0.7) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$; Cys-119: $k_{+4} = (15 \pm 2) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$; Cys-121: $k_{+5} = (5 \pm 1) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$], with the exception of Cys-113 (foothold 1) for which the second-order rate constant was an order of magnitude lower [$k_{+1} = (3 \pm 1) \times 10^3 \text{ M}^{-1}\text{s}^{-1}$] (Table 1). Dissociation rates depended on the concentration of MEET (Fig. 2c); that is, cleavage of the mono-adduct at As(III) by thiol interchange outcompeted hydrolysis³³ under the prevailing conditions. The second-order rate constants for dissociation were all $>10^2 \text{ M}^{-1}\text{s}^{-1}$ [Cys-115: $k_{-2} = (3.7 \pm 0.8) \times 10^2 \text{ M}^{-1}\text{s}^{-1}$; Cys-117: $k_{-3} = (2.8 \pm 0.2) \times 10^2 \text{ M}^{-1}\text{s}^{-1}$; Cys-119: $k_{-4} = (4 \pm 1) \times 10^2 \text{ M}^{-1}\text{s}^{-1}$; Cys-121: $k_{-5} = (3.4 \pm 1.7) \times 10^2 \text{ M}^{-1}\text{s}^{-1}$], again with the exception of Cys-113, in which case breakdown of the adduct occurred an order of magnitude more slowly ($k_{-1} = (0.4 \pm 0.1) \times 10^2 \text{ M}^{-1}\text{s}^{-1}$) (Table 1).

Behaviour with two footholds

Next, we examined displacement of the second ligand of the walker in a cyclization reaction with a neighbouring Cys residue by using double-Cys mutants. The excess MEET in solution subsequently opened the cyclic-adduct yielding a mono-adduct on one of the two Cys residues, followed by subsequent closures and re-openings, which were terminated when MEET cleaved a mono-adduct (Fig. 3). The movement of the mono-adduct from one foothold to its neighbor constituted a single step. Five double-Cys mutants were examined: 113C/115C, 115C/117C, 117C/119C, 119C/121C and 115C/137C (Table S1). For the first four of these mutants, we observed three current levels in the presence of SPAA-MEET₂: one level corresponded to P₀ and another to the P_I states observed with the single-Cys mutants, as deduced from the residual current values (Tables 1 and 2), while a new intermediate current level, P_{II}, was taken to represent the cyclic-adduct (Fig. 3). Importantly, the P_{II} level was not observed with 115C/137C, in which the two Cys residues are 21 Å apart (Ca-Ca). The current block in the P_{II} state ($I_{nm} = I_{P_0} - I_{P_{II}}$, where *n* and *m* are the foothold positions) became lower as the trans entrance of the pore was approached (e.g. I₁₂: $5.9 \pm 0.7 \text{ pA}$; I₄₅: $3.9 \pm 0.2 \text{ pA}$, *n* = 3; Table 2). The P_{II} level was later found to be especially useful for following movement along the 5-Cys track.

The transitions between the P_o , P_I and P_{II} states were modelled in three ways: (i) a six-state model for 113C/115C; (ii) a three-state model for 115C/117C and 117C/119C; and (iii) a four-state model for 119C/121C (Fig. S5, S6). In all cases, transition rates from P_o to the mono-adduct state P_I increased linearly with increasing concentrations of SPAA-MEET₂ (taken to be the bulk concentration of SPAA), but were independent of the concentration of MEET, which was in excess over SPAA, as exemplified for 119C/121C (Fig. 4b; Table 2). In contrast, the rates at which the cyclic-adducts opened ($P_{II} \rightarrow P_I$) and the rates at which the walker was released from the track ($P_I \rightarrow P_o$) were both dependent on the concentration of MEET, but independent of the concentration of SPAA-MEET₂ (Fig. 4b; Table 2). Finally, the cyclization ($P_I \rightarrow P_{II}$) was independent of the concentrations of both reagents (Fig. 4b, Fig. S6), confirming the unimolecular nature of the reaction (Fig. 1b(ii)).

The predominant event cycle observed for the double-Cys mutants comprised transitions from the open pore to a mono-adduct to the cyclic-adduct, back to a mono-adduct, and finally to the open pore (i.e. $P_o \rightarrow [P_I \rightarrow P_{II} \rightarrow P_I]_N \rightarrow P_o$). Importantly, P_{II} rarely preceded (<0.5% of events), nor was it followed by P_o . The mean duration (τ_{cycle}) of the event cycle and the frequencies with which cyclic-adducts formed and opened during the cycles depended on the site of the reaction. For example, in the presence of 15 mM MEET and at pH 8.0, the mean duration of the event cycle varied from $\tau_{\text{cycle}} = 1.0 \pm 0.1$ s ($n = 3$) for 113C/115C to $\tau_{\text{cycle}} = 7 \pm 3$ s for 115C/117C ($n = 3$). The remaining values were $\tau_{\text{cycle}} = 5 \pm 2$ s ($n = 3$) for 117/119C and $\tau_{\text{cycle}} = 3.5 \pm 0.4$ s ($n = 2$) for 119/121C. The number of openings and closings of the cyclic-adducts per event cycle was greater near the trans entrance of the pore (21 ± 0.9 openings and closings at 119C/121C) than at other locations (113C/115C, 1.1 ± 0.1 ($n = 3$) openings and closings; 115C/117C, 4.6 ± 1.9 ($n = 3$); 117C/119C, 1.9 ± 0.4 ($n = 3$)).

A track with three footholds

Features that might be used to monitor a walk on a track could be discerned from the data on the single-Cys and double-Cys mutants, notably the current levels of the mono-adducts (P_I) and the cyclic-adducts (P_{II}) at different locations within the lumen of the α HL pore, and, again with values that depend on the location, the rate constants for association of the walker (k_{+n} : $P_o \rightarrow P_I$), the formation and opening of cyclic-adducts (k_{nm} , for $P_I \rightarrow P_{II}$; $k_{mn \rightarrow m}$, $P_{II} \rightarrow P_I$), and release from the pore (k_{-n} , $P_I \rightarrow P_o$). Armed with this knowledge, we examined an α HL pore containing one subunit with three Cys residues: 113C/115C/117C. Again, we observed three principal current levels P_o , P_I and P_{II} (Fig. 5), for which the current amplitudes corresponded well with our previous observations with single- and double-Cys mutants: $I_2 = 8.4 \pm 0.5$ pA (mean value for positions 115 and 117, $n = 2$, from 113C/115C/117C), 8.8 ± 0.2 ($n = 3$, from 115C), 7.8 ± 0.6 ($n = 5$, from 117C) (Table 1); $I_{12} = 5.7 \pm 0.2$ pA ($n = 3$, from 113C/115C/117C), $I_{12} = 5.9 \pm 0.7$ pA ($n = 3$, from 113C/115C) (Table 2); $I_{23} = 4.7 \pm 0.4$ pA ($n = 2$, from 113C/115C/117C), $I_{23} = 4.8 \pm 0.2$ pA ($n = 3$, from 115C/117C) (Table 2). The two current levels for the cyclic-adduct P_{II} , which were assigned to cyclic-adduct 12 and cyclic-adduct 23 (P_{12} and P_{23} , Fig. 5), were nearly always separated by a signal for the mono-adduct P_2 (e.g. $\dots \rightarrow P_{I(2)} \rightarrow P_{II(12)} \rightarrow P_{I(2)} \rightarrow P_{II(23)} \rightarrow P_{I(2)} \rightarrow P_{II(12)} \rightarrow P_{I(3)} \rightarrow P_{II(23)} \rightarrow P_{I(3)} \rightarrow \dots$) demonstrating that the walker moves between the two cyclic-adducts with a mono-adduct as an intermediate. Based on the P_{II} current

amplitudes, when the walker was a cyclic-adduct it resided for 97% of the time on 115C/117C and for only 3% of the time on 113C/115C, in keeping with simulations based on rate constants obtained with the double-Cys mutants (see below). Our findings with the triple mutant revealed that the walker could step back and forth on a linear track consisting of three footholds (Fig. 5). Movement of the mono-adduct from foothold 1, to foothold 2, to foothold 3 constituted two steps.

Complete walk on the 5-foothold track

We then examined the motion of the walker on the complete 5-site track by using α HL pores containing one mutant subunit with the substitutions 113C/115C/117C/119C/121C (Fig. 1c, Fig. 6). Based on the previous experiments, which had revealed the characteristics of the current levels observed with single- and double-Cys mutants, we were able to monitor the direction of walking based on the current values of the P_{II} levels and the intervening P_I levels, and to determine when the walker detached from the track (P_o). The four cyclic-adducts, represented by the current levels $P_{II(12)}$, $P_{II(23)}$, $P_{II(34)}$ and $P_{II(45)}$, were especially important in following the walker. For example, a current trace reflecting a series of 11 steps along the 5-site track is displayed (Fig. 6b), along with current traces for the double-Cys mutants corresponding to each of the steps (Fig. 6a). The walking motion (Fig. 6b), which is summarized in a movie (Supplementary Movie), can be described as follows. The track is empty (a) until the walker engages at foothold 2 (i.e. 115C) to form the mono-adduct (b). A cyclic-adduct then forms at footholds 12 (i.e. 113C/115C (step 1: (c), Fig. 6c), which then opens to reform the mono-adduct at foothold 2 (d). The cyclic-adduct then forms again at footholds 12 (step 2: (e)), and again opens to the mono-adduct at foothold 2 (f). The walker then moves along the track by forming a cyclic-adduct at footholds 23 (115C/117C) with a lifetime of ~ 10 ms (step 3: (g)), which opens again to form a mono-adduct at foothold 2 (115C, (h)). Following this, a cyclic-adduct once more forms at footholds 23 (step 4, (i)), but this time it opens to the mono-adduct at foothold 3 (117C) (j), which forms a short-lived (~ 10 ms) cyclic-adduct (step 5: (k)) again opening back to foothold 3 (117C) (l), before movement along the track by the formation of a cyclic-adduct at footholds 34 (117C/119C, step 6: (m)). After opening to foothold 4 (119C, (n)), a cyclic-adduct forms at footholds 45 (119C/121C, step 7: (o)), which then opens and closes 7 times at the bottom of the track. Eventually, the mono-adduct at foothold 4 (119C, (p)), moves back up the track to form a cyclic-adduct at footholds 34 (step 8: (q)). From here the walker returns to foothold 3 or 4 (117C or 119C, (r)) as a mono-adduct, again forms a cyclic-adduct at 23 or 34 (115C/117C or 117C/119C, step 9: (s)), which opens to a mono-adduct, (t). A cyclic-adduct with a lifetime of 13 ms (u), leads to a mono-adduct at foothold 5 (121C; (w)) followed by the formation of the cyclic-adduct at footholds 45 (119C/121C, step 10: (x)). The cyclic-adduct (x) at this level opens and closes 4 times before the appearance of a mono-adduct at foothold 4 ((119C): (y)) which generates the final short lived cyclic-adduct at 34 (117C/119C, step 11: (z)). The following mono-adduct at foothold 4 (119C, (aa)), is finally cleaved and falls off the track (bb).

Only seven (7.4%) of the walks we observed ($n = 94$, performed at pH 8.0 with 7.5 mM MEET), were complete (i.e. movement was observed from the bottom to the top of the track, or vice versa, Figs 6b, e). These walks were completed with 6 ± 1 steps in 5.3 ± 1.2 s. The

mean step duration between cyclic-adducts during these complete walks was 0.7 s, which given the 6 Å-spacing of the footholds equates to a speed of approximately 1 nm s⁻¹, substantially faster than a previous stochastic chemical walker¹³.

Simulations of 5-foothold walks

To support our experimental observations, we modeled molecular walking with QuB software³⁴, which was developed for the statistical analysis of single-molecule kinetics, by inputting the experimental current levels and kinetic constants obtained with the double-Cys mutants. Simulated current traces derived with a ten-state model composed of states representing the mono-adducts at each of the five footholds, the four cyclic-adducts and one open pore state showed good correspondence with experiments that examined the complete 5-foothold track (Fig. 6f and Fig. S10). The mean total duration of a simulated walk on the track was 7.2 ± 0.9 s, with a mean step duration of 1.0 s, compared to the experimental values of 5.3 ± 1.2 s and 0.7 s, respectively. The processivity derived from the simulations (6 ± 2 steps per walk) was also similar to values obtained by experiment (6 ± 1 steps per walk). In 92.5% of the simulated data traces, the walker patrolled only the bottom of the track (footholds 3, 4 and 5), and only 6.5% of the walks were complete, again in accord with experiment (see SI for full details). In addition, we tested the directional bias of the walker (see Fig. S10c); simulations revealed that there is net directional movement (85%) towards foothold 5 (121C).

Conclusion

In conclusion, our study demonstrates continuous real time monitoring of the movement of a small-molecule walker at the single-molecule level, at room temperature, in water, at neutral pH. The direction and duration of individual steps have been observed with millisecond time resolution and atomic precision. The As(III) walker undergoes stochastic motion with a weak bias towards a well at foothold 5 at the end of the track. The net movement is most probably towards a thermodynamic sink arising from the different environments of the five cysteine residues in the β barrel, although it should be noted that a system under an applied potential is not at equilibrium³⁵. This motion should be distinguished from strongly biased motion requiring the expenditure of chemical fuel, which is an asset of synthetic molecular motors^{17, 18}. The loosely bound walker moves more quickly than existing small-molecule walkers at the expense of robust processivity. Interestingly, modest processivity has been shown to enhance the rate at which a target is reached in a one-dimensional search by diffusive motion, while strong processivity reduces the rate of target encounters on long tracks^{36, 37} (see SI; and Supplementary Data Table 3 and 4, and Fig. S11). Further, the speed of the As(III) walker can be manipulated by the use of different thiol ligands, and changes in the pH and temperature. The walker can be stopped on the track by washing out the MEET and the small step size of 6 Å permits more precise movement than is possible with DNA walkers or motor proteins. Significantly, the free arm of our walker (presently a sulfophenyl group) might be elaborated for cargo attachment and delivery^{8–10}. In this regard, an immediate goal is better control of directionality, which within a pore might be driven by a gradient such as the applied potential³⁵. Finally, while the nanoreactor has

proved useful for characterizing SPAA-MEET₂, similar walkers might be readily adapted to move on a variety of modified surfaces.

Methods

4-Sulfophenylarsonous acid (SPAA) was prepared as described³². MEET (2-(2-methoxyethoxy)ethanethiol) was from Sigma Aldrich (no. 632295). Heteroheptameric staphylococcal α -hemolysin (α HL) pores with a single cysteine, with two cysteines, with three cysteines and five cysteines (Table S1) were prepared as described earlier³⁸ (Fig. S4). Single-channel current recordings were performed as described previously³². Both compartments contained 2 M KCl, 10 mM Bis-tris propane, 100 μ M EDTA, pH 8.0, at -50 mV and at 20°C. To analyze the current traces, we used Clampfit (version 10.0, Axon Instruments) and QuB software³⁴, which are normally used for the analysis of ion channel activity. (For the nomenclature of the current levels and rate constants refer to Table S2). The simulations were carried out with QuB software by using a 10-state model (Fig. S10a), representing the open pore (P_O), the five mono-adducts at the 5 different Cys footholds 1 to 5 (residues 113, 115, 117, 119, 121, levels P_I), and the four cyclic-adducts (footholds 12, 23, 34 and 45, levels P_{II}). The transition rates were calculated based on 200 nM SPAA and 7.5 mM MEET, by using the experimental rate constants for the double-Cys mutants (Table 2). Full details are in Fig. S10.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. The walker and its track.

a, Formation of the organoarsenic(III) molecule SPAA(MEET)₂ from 4-sulfophenylarsonous acid (SPAA) and 2-(2-methoxyethoxy)ethanethiol (MEET). **b,** Chemistry of walking by SPAA(MEET)₂ on a five-cysteine track. **(i)** At the starting point of the walk, the walker engages with the track as a mono-adduct when one of the MEET ligands is displaced by a cysteine side-chain in a thiol interchange reaction. **(ii)** The second MEET ligand is displaced by a neighboring cysteine side-chain to form a cyclic-adduct **(iii)** MEET in solution attacks at As(III) and displaces one of the cysteines to generate a mono-adduct one foothold further

along the track, completing the first step of the walk. **(iv)** A second cyclic-adduct is formed at the new foothold. The walker can proceed up and down the track in stochastic motion. **c**, Section through the heteroheptameric α HL pore showing the five-cysteine track. The cysteine residues are: 113C (red, foothold 1), 115C (orange, foothold 2), 117C (green, foothold 3), 119C (blue, foothold 4) and 121C (purple, foothold 5).

Figure 2. Electrical recordings of α HL pores containing a single-Cys residue in the presence of SPAA(MEET)₂.

a, (i) 113C; (ii) 115C; (iii) 117C; (iv) 119C; (v) 121C. Representative portions of current traces recorded with 20 μ M SPAA (trans) and 15 mM MEET (cis) are shown. **b,** The formation of an As(III) mono-adduct by thiol interchange at foothold 'n'. The two states are represented by current levels P_o and P_I . **c.** Kinetic analysis of mono-adduct formation at 115C at various concentrations of (i) SPAA and (ii) MEET. Transition rates are shown from the open pore to the mono-adduct ($P_o \rightarrow P_I$, $\blacksquare$) and from the mono-adduct to the open pore ($P_I \rightarrow P_o$, $\bullet$).

Figure 3. Comparison of the reaction of SPAA(MEET)₂ with single-Cys and double-Cys mutant α HL pores.

Current traces for 115C, 113C/115C and 115C/137C are shown. Current levels: open pore, P_0 , black; mono-adduct, P_I , blue; cyclic-adduct, P_{II} , red; both Cys of a double mutant occupied, P_{double} , green. The two P_I levels for 115C/137C represent the two diastereomers of the As(III) adduct on Cys-137. This trace also illustrates a rare double-occupancy event (P_{double}). Representative portions of current traces recorded with 20 μ M SPAA (trans) and 15 mM MEET (cis) are shown. 137C is designated foothold 6 (white).

Figure 4. Electrical recordings of αHL pores containing double-Cys mutations in the presence of SPAA(MEET)₂.

a, (i) 113C/115C; (ii) 115C/117C; (iii) 117C/119C; (iv) 119C/121C. The three current levels, P_0 , P_I and P_{II} , are defined in Fig. 3 (legend). The four double mutants exhibit different current blockades (I), noise and spike behaviour, and reaction kinetics. In the case of 113C/115C, two additional current levels were observed, arising from P_I : P_{Ex1} , a possible enantiomer of the mono-adduct at 113C; P_{Ex2} , characteristic spikes associated with P_I (see Supplementary Fig. 5). Data were collected in the presence of 10 μM SPAA (trans) and 15 mM MEET (cis). **b**, Kinetic analyses of the four-state model showing the dependence of transition rates for 119C/121C on the concentrations of SPAA (**i**, **iii**) and MEET (**ii**, **iv**). In **i**

and **ii**: $P_O \rightarrow P_{I(4)}$, ●; $P_O \rightarrow P_{I(5)}$, ■; $P_I \rightarrow P_{II(4)}$, ▼; $P_I \rightarrow P_{II(5)}$, ▲. In **iii** and **iv**: $P_{II} \rightarrow P_{I(4)}$, ▽; $P_{II} \rightarrow P_{I(5)}$, ▲; $P_{I(4)} \rightarrow P_O$, ○; $P_{I(5)} \rightarrow P_O$, □. $P_{I(4)}$ and $P_{I(5)}$ represent current levels for mono-adducts at the two different cysteines (119C and 121C). Data for other double-Cys mutants are in the Fig. S5, S6. **c**, The four-state model used to fit the current recording data for 119C/121C. **d**, Molecular representation of the four-state model.

Figure 5. Electrical recordings of an α HL pore containing a triple-Cys mutation in the presence of SPAA(MEET)₂.

a, (i) 113C/115C; **(ii)** 115C/117C. The three current levels, P_0 , P_I and P_{II} , are defined in Fig. 3 (legend). **b**, 113C/115C/117C. Four current levels (P_0 , P_I and $P_{II(12)}$ and $P_{II(23)}$) are shown, where P_{II} has two levels representing the cyclic-adducts at 113/115C ($P_{II(12)}$) and 115/117C ($P_{II(23)}$). Data were collected in the presence of 10 μ M SPAA (trans) and 15 mM MEET (cis).

Figure 6. Electrical recordings of the α HL pore containing a five-Cys track in the presence of SPAA(MEET)₂.

a, (i) 113C/115C; (ii) 115C/117C; (iii) 117C/119C and (iv) 119C/121C. The three current levels, P₀, P_I and P_{II}, are defined in Fig. 3 (legend). **b,** 113C/115C/117C/119C/121C. The experiments were performed with 200 nM SPAA (trans) and 7.5 mM MEET (cis). The trace shows a downward walk from foothold 1 to foothold 5, followed by a “patrol” of the final three footholds 3 to 5. The location of the walker is assigned at each cyclic-adduct level (12, 23, 34 and 45). Note that 23 and 34 are not readily distinguished, but can be assigned from

the preceding and following states. Individual movements are labeled with lower case letters and described in detail in the text. **c**, Expanded sections of the electrical trace in 6b. Red dashed lines indicate the cyclic-adducts. **d**, Trace showing a “patrol” of the three footholds 3 to 5 on the 5-Cys track. **e**, A trace showing an upward walk from foothold 5 to foothold 1. **f**, Simulated data traces for walks along the five-cysteine track. Traces (i), (ii) and (iii) can be compared with the traces in Fig. 6b, d, e, respectively (see Fig. S10).

Table 1
Current blockades (I) and kinetic constants (k) for single-Cys mutants.

Single-Cys α HL pores were examined with 10 μ M to 100 μ M SPAA at MEET concentrations from 7.5 mM to 30 mM. The buffer was 10 mM Bis-tris propane, 2 M KCl, 100 μ M EDTA, pH 8.0, at 20°C. The applied potential was -50 mV. The errors are the standard deviations of the means for 'n' experiments.

	P_I I (pA)	$P_o \rightarrow P_I$ (k_{PI} (10^4) $\times M^{-1}s^{-1}$)	$P_I \rightarrow P_o$ (k_{PI} (10^2) $\times M^{-1}s^{-1}$)
(113C) _I (WT) ₆ n = 4	12.8 $\pm$ 0.5 13.5 $\pm$ 0.4	0.3 $\pm$ 0.1	0.4 $\pm$ 0.1
(115C) _I (WT) ₆ n = 3	8.8 $\pm$ 0.2	9.0 $\pm$ 1.0	3.7 $\pm$ 0.8
(117C) _I (WT) ₆ n = 5	7.8 $\pm$ 0.6	2.0 $\pm$ 0.7	2.8 $\pm$ 0.2
(119C) _I (WT) ₆ n = 3	7.0 $\pm$ 0.4	15 $\pm$ 2	4.0 $\pm$ 1.0
(121C) _I (WT) ₆ n = 3	7.9 $\pm$ 0.1 7.1 $\pm$ 0.2	5.0 $\pm$ 1.0	3.4 $\pm$ 1.7

Table 2
Current blockades (I) and kinetic constants (k) for double-Cys mutants.

Double-Cys α HL pores were examined with 10 μ M to 24 μ M SPAA at MEET concentrations from 7.5 mM to 22.5 mM. The buffer contained 10 mM Bis-tris propane, 2 M KCl, 100 μ M EDTA, pH 8.0, at 20°C. The applied potential was -50 mV. The errors are the standard deviations of the means for 'n' experiments.

	P_I	P_{II}	$P_O \rightarrow P_I$	$P_I \rightarrow P_O$	$P_I \rightarrow P_{II}$	$P_{II} \rightarrow P_I$
	I (pA)	I_{nm} (pA)	$(k_p (10^4) \times [M^{-1}s^{-1}])$	$(k_{-p} (10^2) \times [M^{-1}s^{-1}])$	$(k_{nm}[s^{-1}])$	$(k_{nm} n (10^2) \times [M^{-1}s^{-1}])$
(113/115)_I(WT)₆ n = 3	9.7 $\pm$ 1.1 10.3 $\pm$ 0.8	5.9 $\pm$ 0.7	5.0 $\pm$ 0.1 2.0 $\pm$ 0.1	0.7 $\pm$ 0.3 2.8 $\pm$ 0.2	6 $\pm$ 1 0.3 $\pm$ 0.1	0.6 $\pm$ 0.2 0.4 $\pm$ 0.3
(115/117)_I(WT)₆ n = 3	7.3 $\pm$ 0.4 7.8 $\pm$ 0.2	4.8 $\pm$ 0.2	20 $\pm$ 5	3.8 $\pm$ 0.7	20 $\pm$ 6	0.6 $\pm$ 0.2
(117/119)_I(WT)₆ n = 3	7.3 $\pm$ 0.3	4.7 $\pm$ 0.2	12 $\pm$ 1	3.0 $\pm$ 0.5	10 $\pm$ 1	0.6 $\pm$ 0.1
(119/121)_I(WT)₆ n = 3	6.8 $\pm$ 0.3 6.3 $\pm$ 0.3	3.9 $\pm$ 0.2	11 $\pm$ 4 6 $\pm$ 4	1.8 $\pm$ 0.6 1.5 $\pm$ 0.3	7 $\pm$ 2 120 $\pm$ 20	6 $\pm$ 1 3 $\pm$ 0.7