SUPPORTING INFORMATION

Multiple base-recognition sites in a biological nanopore– two heads are better than one

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Protein preparation.

αHL was produced as described in detail elsewhere.[1] In brief, the protein was expressed in the presence of [^{35}S]methionine in an *E. coli in vitro* transcription and translation (IVTT) system (*E. coli* T7 S30 Extract System for Circular DNA, Cat. #L1130, Promega). The IVTT product (100 μL) was incubated with rabbit red blood cell membranes for 1 h at 37°C to form αHL heptamers. The membrane suspension was centrifuged at 25,000 x g and the pellet containing the heptamers was solubilized in loading buffer without heating and run on a 5% SDS-polyacrylamide gel for 4 h at 100 V. The gel was then vacuum dried without heating onto Whatman 3M filter paper, which required 3 to 4 h. The dried gel was exposed to photographic film for 2 h and the developed film was used to locate the position of the heptameric protein in the gel. This region of the gel was

excised, rehydrated and crushed in 400 μL of 10 mM Tris.HCl, pH 8.0, containing 100 μM EDTA. After 20 min at room temperature, the polyacrylamide was removed by centrifuging the suspension at 25,000 x g for 7 min at room temperature through a cellulose micro spin column (Microfilterfuge tubes, Cat. #7016-024, Rainin). Aliquots of the purified protein were stored at -80°C.

The mutant αHL gene (NNY) was prepared from αHL NN by using a kit for site-directed mutagenesis (QuickChange II XL, Cat. #200522-5, Stratagene). The DNA sequence of the new gene was verified.

Planar bilayer recordings.

Electrical recordings were carried out with a planar lipid bilayer apparatus with a bilayer of 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC, Avanti Polar Lipids) formed across an aperture (~100 μm in diameter) in a 25-μm thick polytetrafluoroethylene (Teflon) film (Goodfellow, Cambridge, Cat. #FP301200/10), which separated the apparatus into *cis* and *trans* compartment.[2] Bilayers were formed by first pre-treating the aperture with hexadecane in n-pentane (10 mg mL^{-1}). Electrolyte solution (0.5 mL: 1 M KCl, 25 mM Tris.HCl, 0.1 mM EDTA, pH 8.0) was added to both compartments. Then, DPhPC in n-pentane (10 mg mL^{-1}) was added to both compartments. The solvent was allowed to evaporate and the bilayer was formed by lowering and raising the electrolyte level past the aperture.

Under the conditions of the experiments, all of the pores exhibited a stable open-pore current. NNY and NN pores have a similar I_O values (at +160 mV): 171 ± 7 pA, and 167 ± 7 pA, respectively. Both compartments of the recording chamber contained 0.5 mL of 1 M KCl, 25 mM Tris.HCl, pH 8.0, with 0.1 mM EDTA. Planar bilayer current recordings were performed with a patch clamp amplifier (Axopatch 200B, Molecular Devices) with the *cis* compartment connected to ground. The αHL pores and the DNA were added to the *cis* compartment. ssDNA molecules, with a biotinyl group covalently attached to the 3' end through a linker (Figure S1), were obtained from Sigma-Aldrich (UK). Solutions of the biotinylated ssDNAs, at 100 μM in 10 mM Tris.HCl, pH 8.0, 0.1 mM EDTA, were pre-incubated with equal volumes of 25 μM streptavidin (Sigma-Aldrich) in the same buffer for at least five minutes. Each oligonucleotide was added to the *cis* compartment to a final concentration of 400 nM. Initially, +160 mV was applied to the *trans* side for 900 ms to drive the negatively charged, biotinylated DNA into the pore. The capture of a ssDNA strand by an αHL pore is observed as a stepwise decrease in the open pore current level (I_O) to a lower, but stable, current level (I_B). A voltage of -140 mV was then applied for 50 ms to eject the immobilized DNA from the pore. The applied potential was then stepped to 0 mV for 50 ms. This one-second sequence was repeated for at least 100 cycles for each ssDNA species added. The amplified signal (arising from the ionic current passing through the pore) was low-pass filtered at 1 kHz and sampled at 5 kHz with a computer equipped with a Digidata 1440A digitizer (Molecular Devices).

Data analysis.

Data were analyzed and prepared for presentation with pClamp software (version 10.1, Molecular Devices). Event searches were performed to obtain the average current level for each ssDNA blockade event (I_B). The mean I_B value for each oligonucleotide was determined by a performing a Gaussian fit to a histogram of the I_B values. The current blockade for each oligonucleotide was also expressed as the residual current (I_{RES}), in which the average current level for a DNA blockade (I_B) is expressed as a percentage of the open pore current (I_O): $I_{RES} = (I_B/I_O) \times 100$. In general, when comparing several oligonucleotide species (as seen in Figure 3), a single oligonucleotide species was first added to the *cis* chamber and the current trace required for the determination of I_B and I_{RES} was recorded. Subsequently, a second, third and fourth oligonucleotide were added and additional currents recorded. The experiment displayed in Figure 4, which involves the probing of pores with 16 different sequences, was obtained by adding sets of 4 oligonucleotides at a time rather than adding individual oligonucleotides. Each of the 4 oligonucleotides within a set (N_9X_{14}) differed in the base at R_1, but had the same base at R_2, and each set had a different base at R_2. The peaks in the derived histograms were assigned based on previous experiments with the separate sets of 4 oligonucleotides.

When such experiments were repeated, the oligonucleotides were added to the chamber in a different order, and in the case of the 16 oligonucleotide experiment (Figure 4), the sets of oligonucleotides were added in a different order.

REFERENCES

[1] S. Cheley, O. Braha, X. Lu, S. Conlan, H. Bayley, *Protein Sci.* **1999**, *8*, 1257.

[2] M. Montal, P. Mueller, *Proc.Natl.Acad.Sci.USA* **1972**, *69*, 3561.

Table S1. Residual currents (I_{RES}) for poly(dC) and oligonucleotides that contain a single nucleotide substitution at position 14 (to probe R_2). The position of the substituted nucleotide is given relative to the 3' biotin tag. I_{RES} and ΔI_{RES} values are shown for three experiments. ΔI_{RES} is defined as the difference in residual current between a poly(dC) oligonucleotide that contains either a T, G or A at position 14 and a pure poly(dC) oligonucleotide (C at position 14, $\Delta I_{RES} = I_{RES}^{N} - I_{RES}^{C}$). Mean I_{RES} and ΔI_{RES} values (± s.d.) are given.

Experiment number	Nucleotide at R_2 (position 14)						
	C	T		A		G	
	I_{RES} (%)	I_{RES} (%)	ΔI_{RES}^{T-C} (%)	I_{RES} (%)	ΔI_{RES}^{A-C} (%)	I_{RES} (%)	ΔI_{RES}^{G-C} (%)
1[1]	31.8	32.8	+1.0	33.2	+1.4	34.6	+2.8
2	30.9	32.0	+1.1	32.4	+1.4	33.8	+2.9
3	31.2	32.3	+1.1	32.6	+1.4	34.1	+2.9
Mean	31.3	32.4	+1.1	32.7	+1.4	34.2	+2.9
S.D.	0.4	0.4	0.0	0.4	0.0	0.4	0.1

[1] The histogram corresponding to this experiment is displayed in Figure 3a

Table S2. Residual currents (I_{RES}) for poly(dC) and oligonucleotides that contain a single nucleotide substitution at position 9 (to probe R_1). I_{RES}, and ΔI_{RES} values are shown for five experiments. ΔI_{RES} is defined as the difference in residual current between a poly(dC) oligonucleotide that contains either a T, G or A at position 9 and a pure poly(dC) oligonucleotide (C at position 9, $\Delta I_{RES} = I_{RES}^{N} - I_{RES}^{C}$). Mean I_{RES} and ΔI_{RES} values (± s.d.) are given.

Experiment number	Nucleotide at R_1 (position 9)						
	C	T		A		G	
	I_{RES} (%)	I_{RES} (%)	ΔI_{RES}^{T-C} (%)	I_{RES} (%)	ΔI_{RES}^{A-C} (%)	I_{RES} (%)	ΔI_{RES}^{G-C} (%)
1	32.3	29.3	−2.9	30.7	−1.5	30.1	−2.2
2[1]	32.2	29.3	−2.9	30.7	−1.5	30.0	−2.1
3	30.7	27.8	−2.9	29.2	−1.4	28.6	−2.1
4	29.0	26.5	−2.5	27.8	−1.3	27.2	−1.8
5	30.4	27.6	−2.8	29.0	−1.4	28.4	−2.0
Mean	30.9	28.1	−2.8	29.5	−1.4	28.9	−2.0
S.D.	1.3	1.2	0.2	1.2	0.1	1.2	0.1

[1] The histogram corresponding to this experiment is displayed in Figure 3b

Table S3. Residual currents (I_{RES}) for poly(dC) and oligonucleotides that contain a single adenine at position 14 (corresponding to R_2) and a single nucleotide substitution at position 9 (to probe R_1). I_{RES} and ΔI_{RES} values are shown for four experiments. ΔI_{RES} is defined as the difference in residual current between a poly(dC) oligonucleotide (with an A at position 14) that contains either a T, G or A at position 9 and a poly(dC) oligonucleotide (with an A at position 14) that contains a C at position 9 ($\Delta I_{RES} = I_{RES}^{N9A14} - I_{RES}^{C9A14}$). Mean I_{RES} and ΔI_{RES} values (± s.d.) are given.

Experiment number	Nucleotide at R_1 (position 9)						
	C	T		A		G	
	I_{RES} (%)	I_{RES} (%)	ΔI_{RES}^{T-C} (%)	I_{RES} (%)	ΔI_{RES}^{A-C} (%)	I_{RES} (%)	ΔI_{RES}^{G-C} (%)
1	33.2	29.9	−3.2	31.6	−1.6	31.0	−2.2
2	34.0	30.8	−3.2	32.6	−1.4	31.7	−2.3
3[1]	32.9	29.8	−3.1	31.5	−1.5	30.9	−2.0
4	34.1	31.0	−3.1	32.6	−1.5	32.1	−2.0
Mean	33.5	30.4	−3.2	32.0	−1.5	31.4	−2.1
S.D.	0.6	0.6	0.1	0.6	0.1	0.6	0.1

[1] The histogram corresponding to this experiment is displayed in Figure 3c

Table S4. Residual currents (I_{RES}) for poly(dC) and oligonucleotides that contain a single thymine at position 14 (corresponding to R_2) and a single nucleotide substitution at position 9 (to probe R_1). I_{RES}, and ΔI_{RES} values are shown for three experiments. ΔI_{RES} is defined as the difference in residual current between a poly(dC) oligonucleotide (with a T at position 14) that contains either a T, G or A at position 9 and a poly(dC) oligonucleotide (with a T at position 14) that contains a C at position 9 ($\Delta I_{RES} = I_{RES}^{N9T14} - I_{RES}^{C9T14}$). Mean I_{RES} and ΔI_{RES} values (± s.d.) are given.

Experiment number	Nucleotide at R_1 (position 9)						
	C	T		A		G	
	I_{RES} (%)	I_{RES} (%)	ΔI_{RES}^{T-C} (%)	I_{RES} (%)	ΔI_{RES}^{A-C} (%)	I_{RES} (%)	ΔI_{RES}^{G-C} (%)
1	32.8	29.9	−2.9	31.3	−1.5	30.7	−2.1
2	33.0	30.0	−3.0	31.3	−1.8	30.8	−2.2
3[1]	33.2	30.3	−2.9	31.6	−1.6	31.2	−2.1
Mean	33.0	30.1	−2.9	31.4	−1.6	30.9	−2.1
S.D.	0.2	0.2	0.1	0.2	0.1	0.2	0.1

[1] The histogram corresponding to this experiment is displayed in Figure 3d

Table S5. Residual currents (I_{RES}) for poly(dC) and oligonucleotides that contain a single guanine at position 14 (corresponding to R_2) and a single nucleotide substitution at position 9 (to probe R_1). I_{RES}, and ΔI_{RES} values are shown for three experiments. ΔI_{RES} is defined as the difference in residual current between a poly(dC) oligonucleotide (with a G at position 14) that contains either a T, G or A at position 9 and a poly(dC) oligonucleotide (with a G at position 14) that contains a C at position 9 ($\Delta I_{RES} = I_{RES}^{N9G14} - I_{RES}^{C9G14}$). Mean I_{RES} and ΔI_{RES} values (± s.d.) are given.

Experiment number	Nucleotide at R_1 (position 9)						
	C	T		A		G	
	I_{RES} (%)	I_{RES} (%)	ΔI_{RES}^{T-C} (%)	I_{RES} (%)	ΔI_{RES}^{A-C} (%)	I_{RES} (%)	ΔI_{RES}^{G-C} (%)
1	33.2	30.4	−2.7	32.0	−1.1	31.4	−1.8
2[1]	35.2	32.3	−2.9	34.0	−1.1	33.3	−1.9
3	32.8	30.1	−2.7	31.8	−1.0	31.2	−1.5
Mean	33.7	30.9	−2.8	32.6	−1.1	32.0	−1.7
S.D.	1.3	1.2	0.1	1.2	0.1	1.1	0.2

[1] The histogram corresponding to this experiment is displayed in Figure 3e

Table S6. Mean residual current differences (ΔI_{RES}) between poly(dC) oligonucleotides that contain nucleotide substitutions at position 9 (to probe R_1) and/or position 14 (to probe R_2). The positions of the substitutions are relative to the 3' biotin tag (Figure S1). The sequence of each oligonucleotide is abbreviated as X_9X_{14} (Table S8). The mean ΔI_{RES} value (± s.d.) is for at least three experiments. ΔI_{RES} is directly measured as the difference between the residual current levels of two specified oligonucleotides (Figure 3). In the uppermost row (oligonucleotide set C_9N_{14}), $\Delta I_{RES} = I_{RES}^{C9N14} - I_{RES}^{C9C14}$ (Figure 3a). In the other four rows, $\Delta I_{RES} = I_{RES}^{N9X14} - I_{RES}^{C9X14}$ (Figure 3b-e).

Oligonucleotide set	Residual Current difference (%)		
C_9N_{14}	$\Delta I_{RES}^{C9A14-C9C14}$	$\Delta I_{RES}^{C9T14-C9C14}$	$\Delta I_{RES}^{C9G14-C9C14}$
	+1.4 ± 0.0	+1.1 ± 0.0	+2.9 ± 0.1
N_9C_{14}	$\Delta I_{RES}^{A9C14-C9C14}$	$\Delta I_{RES}^{T9C14-C9C14}$	$\Delta I_{RES}^{G9C14-C9C14}$
	−1.4 ± 0.1	−2.8 ± 0.2	−2.0 ± 0.1
N_9A_{14}	$\Delta I_{RES}^{A9A14-C9A14}$	$\Delta I_{RES}^{T9A14-C9A14}$	$\Delta I_{RES}^{G9A14-C9A14}$
	−1.5 ± 0.1	−3.2 ± 0.1	−2.1 ± 0.1
N_9T_{14}	$\Delta I_{RES}^{A9T14-C9T14}$	$\Delta I_{RES}^{T9T14-C9T14}$	$\Delta I_{RES}^{G9T14-C9T14}$
	−1.6 ± 0.1	−2.9 ± 0.1	−2.1 ± 0.1
N_9G_{14}	$\Delta I_{RES}^{A9G14-C9G14}$	$\Delta I_{RES}^{T9G14-C9G14}$	$\Delta I_{RES}^{G9G14-C9G14}$
	−1.1 ± 0.1	−2.8 ± 0.1	−1.7 ± 0.2

Table S7. Predicted and measured residual currents differences (ΔI_{RES}) between poly(dC) oligonucleotides that contain single nucleotide substitutions at position 9 (to probe R_1) and/or position 14 (to probe R_2) and poly(dC). The positions of the substitutions are relative to the 3' biotin tag (Figure S1). The sequence of each oligonucleotide is abbreviated as X_9X_{14} (Table S8). A measured ΔI_{RES} value is the mean of at least three experiments (± s.d.). The predicted ΔI_{RES} values are calculated from $\Delta I_{RES}^{X9X14-C9C14} = \Delta I_{RES}^{X9X14-C9X14} + \Delta I_{RES}^{C9X14-C9C14}$ (using the values in Table S6) and the quoted error is the square root of the sum of the squares of the standard deviations for the two ΔI_{RES} values used to determine the predicted value.

	$\Delta I_{RES}^{Oligo-C9C14}$ (%)														
Oligo	T_9C_{14}	G_9C_{14}	T_9T_{14}	T_9A_{14}	A_9C_{14}	G_9T_{14}	G_9A_{14}	A_9T_{14}	A_9A_{14}	T_9G_{14}	C_9T_{14}	G_9G_{14}	C_9A_{14}	A_9G_{14}	C_9G_{14}
Predicted	−2.8 ± 0.2	−2.0 ± 0.1	−1.8 ± 0.1	−1.8 ± 0.1	−1.4 ± 0.1	−1.0 ± 0.1	−0.7 ± 0.1	−0.5 ± 0.1	−0.1 ± 0.1	+0.1 ± 0.1	+1.1 ± 0.1	+1.2 ± 0.2	+1.4 ± 0.1	+1.8 ± 0.1	+2.9 ± 0.1
Measured	−2.8 ± 0.0	−1.9 ± 0.1	−1.8 ± 0.1	−1.8 ± 0.1	−1.4 ± 0.1	−1.0 ± 0.0	−0.6 ± 0.0	−0.6 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	+1.1 ± 0.1	+1.1 ± 0.1	+1.4 ± 0.0	+1.7 ± 0.0	+2.8 ± 0.1

Table S8. Sequences of the oligonucleotides used in this study. B represents the 3' biotin-TEG tag and linker (Figure S1). Each oligo X_9X_{14} is a member of the set N_9N_{14}.

Oligonucleotide name	Oligonucleotide sequence (5'→3')
C_9C_{14}	CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCB
C_9T_{14}	CCCCCCCCCCCCCCCCCCCCCCCCCC**T**CCCCCCCCCCCCCB
C_9A_{14}	CCCCCCCCCCCCCCCCCCCCCCCCCC**A**CCCCCCCCCCCCCB
C_9G_{14}	CCCCCCCCCCCCCCCCCCCCCCCCCC**G**CCCCCCCCCCCCCB
T_9C_{14}	CCCCCCCCCCCCCCCCCCCCCCCCCCCCCC**T**CCCCCCCCB
A_9C_{14}	CCCCCCCCCCCCCCCCCCCCCCCCCCCCCC**A**CCCCCCCCB
G_9C_{14}	CCCCCCCCCCCCCCCCCCCCCCCCCCCCCC**G**CCCCCCCCB
T_9A_{14}	CCCCCCCCCCCCCCCCCCCCCCCCCC**A**CCCC**T**CCCCCCCCB
A_9A_{14}	CCCCCCCCCCCCCCCCCCCCCCCCCC**A**CCCC**A**CCCCCCCCB
G_9A_{14}	CCCCCCCCCCCCCCCCCCCCCCCCCC**A**CCCC**G**CCCCCCCCB
T_9G_{14}	CCCCCCCCCCCCCCCCCCCCCCCCCC**G**CCCC**T**CCCCCCCCB
A_9G_{14}	CCCCCCCCCCCCCCCCCCCCCCCCCC**G**CCCC**A**CCCCCCCCB
G_9G_{14}	CCCCCCCCCCCCCCCCCCCCCCCCCC**G**CCCC**G**CCCCCCCCB
T_9T_{14}	CCCCCCCCCCCCCCCCCCCCCCCCCC**T**CCCC**T**CCCCCCCCB
A_9T_{14}	CCCCCCCCCCCCCCCCCCCCCCCCCC**T**CCCC**A**CCCCCCCCB
G_9T_{14}	CCCCCCCCCCCCCCCCCCCCCCCCCC**T**CCCC**G**CCCCCCCCB

Table S9. Residual currents levels (I_{RES}) and differences (ΔI_{RES}) for poly(dC) and oligonucleotides that contain nucleotide substitutions at positions 9 (to probe R_1) and 14 (to probe R_2). I_{RES}, and ΔI_{RES} values are shown for three experiments. ΔI_{RES} is defined as the difference in residual current between a poly(dC) oligonucleotide containing substituted nucleotides and poly(dC) itself ($\Delta I_{RES} = I_{RES}^{Oligo} - I_{RES}^{C9C14}$). Mean I_{RES} and ΔI_{RES} values (± s.d.) are given.

Oligo	1		2		3[1]		I_{RES} (%)		$\Delta I_{RES}^{Oligo-C1C2}$ (%)	
	I_{RES} (%)	$\Delta I_{RES}^{Oligo-C1C2}$ (%)	I_{RES} (%)	$\Delta I_{RES}^{Oligo-C1C2}$ (%)	I_{RES} (%)	$\Delta I_{RES}^{Oligo-C1C2}$ (%)	Mean (%)	SD (%)	Mean (%)	SD (%)
T_9C_{14}	27.3	−2.7	28.3	−2.8	29.4	−2.7	28.3	1.1	−2.8	0.0
G_9C_{14}	28.1	−1.9	29.3	−1.9	30.1	−1.9	29.2	1.0	−1.9	0.1
T_9A_{14}	28.3	−1.8	29.3	−1.9	30.4	−1.7	29.3	1.1	−1.8	0.1
T_9T_{14}	28.3	−1.8	29.3	−1.9	30.4	−1.7	29.3	1.1	−1.8	0.1
A_9C_{14}	28.6	−1.4	29.6	−1.5	30.7	−1.4	29.6	1.0	−1.4	0.1
G_9T_{14}	29.0	−1.0	30.1	−1.0	31.0	−1.1	30.0	1.0	−1.0	0.0
G_9A_{14}	29.4	−0.6	30.5	−0.6	31.5	−0.6	30.5	1.0	−0.6	0.0
A_9T_{14}	29.6	−0.4	30.5	−0.6	31.5	−0.6	30.5	0.9	−0.6	0.1
A_9A_{14}	30.0	0.0	31.1	−0.1	32.1	0.0	31.0	1.0	0.0	0.0
C_9C_{14}	30.0	0.0	31.1	0.0	32.1	0.0	31.1	1.0	0.0	0.0
T_9G_{14}	30.0	0.0	31.1	0.0	32.1	0.0	31.1	1.0	0.0	0.0
C_9T_{14}	31.1	+1.1	32.3	+1.2	33.1	+1.0	32.2	1.0	+1.1	0.1
G_9G_{14}	31.1	+1.1	32.3	+1.2	33.1	+1.0	32.2	1.0	+1.1	0.1
C_9A_{14}	31.4	+1.4	32.5	+1.4	33.5	+1.4	32.5	1.0	+1.4	0.0
A_9G_{14}	31.7	+1.7	32.8	+1.7	33.7	+1.7	32.8	1.0	+1.7	0.0
C_9G_{14}	32.9	+2.9	34.0	+2.8	34.9	+2.8	33.9	1.0	+2.8	0.1

[1] The histogram corresponding to this experiment is displayed in Figure 4

Fig S1. The chemical structure of the biotin-TEG linker used to biotinylate the 3' terminus of the DNA oligonucleotides. The structure was produced with ChemBioDraw Ultra 11.