

Semisynthetic Nanoreactor for Reversible Single-Molecule Covalent Chemistry

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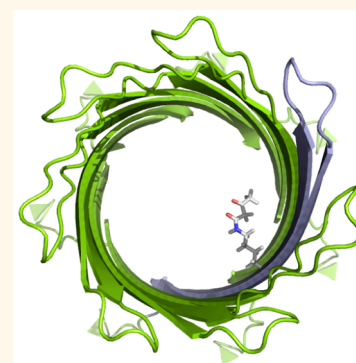
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S Supporting Information

ABSTRACT: Protein engineering has been used to remodel pores for applications in biotechnology. For example, the heptameric α -hemolysin pore (α HL) has been engineered to form a nanoreactor to study covalent chemistry at the single-molecule level. Previous work has been confined largely to the chemistry of cysteine side chains or, in one instance, to an irreversible reaction of an unnatural amino acid side chain bearing a terminal alkyne. Here, we present four different α HL pores obtained by coupling either two or three fragments by native chemical ligation (NCL). The synthetic α HL monomers were folded and incorporated into heptameric pores. The functionality of the pores was validated by hemolysis assays and by single-channel current recording. By using NCL to introduce a ketone amino acid, the nanoreactor approach was extended to an investigation of reversible covalent chemistry on an unnatural side chain at the single-molecule level.



KEYWORDS: nanoreactor, single-molecule chemistry, membrane protein, native chemical ligation, unnatural amino acid, protein semisynthesis

α -Hemolysin (α HL) is a pore-forming toxin secreted by *Staphylococcus aureus*. The pore contains seven subunits, and each subunit comprises 293 amino acids.¹ Use of the heptameric α HL protein pore as a nanoreactor has proved profitable in studies of covalent chemistry at the single-molecule level.^{2–5} For example, the nanoreactor approach is advantageous because large, potentially interfering, fluorescent probes are not required. When a molecule undergoes a chemical reaction on the inner wall of the transmembrane β barrel of the α HL pore, the current carried by ions flowing through the pore is perturbed. Hence, individual reaction steps, including those that are not rate-limiting and therefore not detectable at the ensemble level, are visualized in the microsecond time domain, and the kinetics of each step can be determined.^{2–5} Recently, complex reaction networks⁴ and the motion of individual molecular walkers⁵ have been examined by this means. However, the chemistry carried out within engineered α HL nanoreactors has until recently been confined to the reactions of thiolates^{2–8} and derivatives of the side chains of cysteine residues.^{9,10} Lately, we expanded the range of chemistry that can be approached by introducing unnatural amino acid side chains into the α HL polypeptide by using native chemical ligation (NCL).¹¹ By this means, an irreversible reaction of a side chain bearing a terminal alkyne

was examined. In the present work, we advance the unnatural amino acid approach by introducing a ketone side chain which allows for observation of reversible chemistry. We also describe truncated pores made by the NCL approach.

RESULTS

General Approach to Two-Fragment Ligation. Two-fragment ligations involved the reaction of an N-terminal fragment (NTF) containing a C-terminal α -thioester with a C-terminal fragment (CTF) bearing an N-terminal cysteine (N-Cys). The NTF coding sequence was fused in-frame with DNA encoding a *Mycobacterium xenopi* DNA gyrase A (Mxe GyrA) intein–chitin binding domain (CBD), that is, NTF–intein–CBD, in the pTXB3 plasmid (New England Biolabs).¹² After expression of the protein in *Escherichia coli*, the NTF- α -thioester was cleaved from the intein–CBD with sodium 2-mercaptoethanesulfonate (MESNa), while the rest of the chimera remained bound to chitin beads.^{13,14} A CTF with an N-Cys can be generated by cleavage of a precursor fusion protein with a site-specific protease.^{15,16} However, this method may not work

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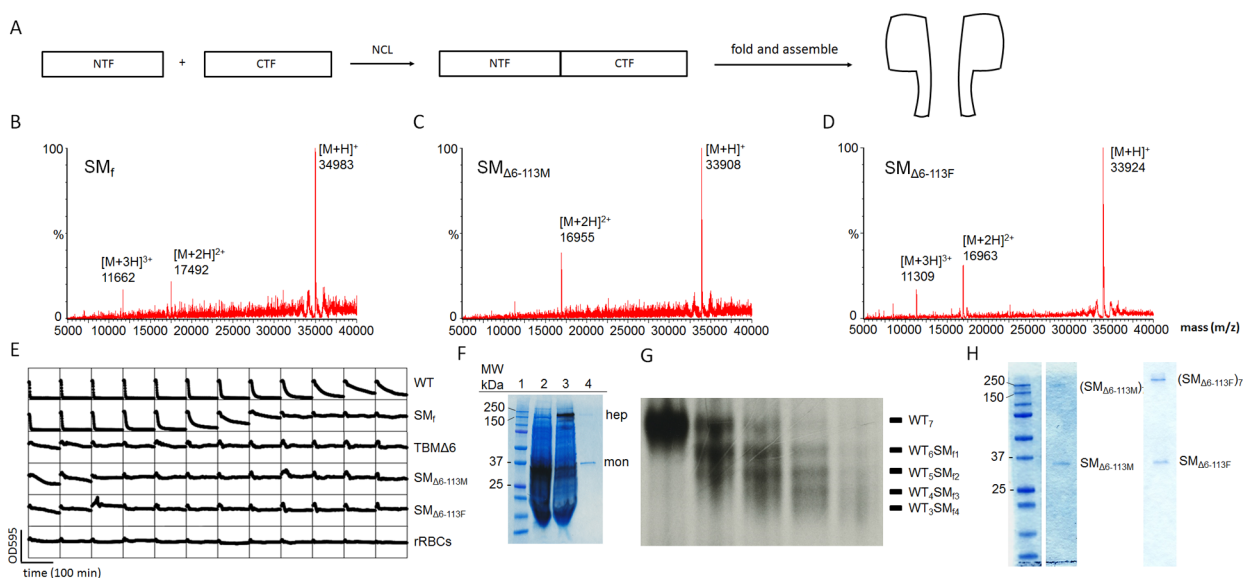


Figure 1. Preparation of α HL pores. (A) α HL monomers were synthesized by native chemical ligation from two fragments (NTF and CTF) expressed in *E. coli*. Folding was performed by reducing the concentration of the denaturant (8 M urea) present during the purification of the synthetic monomers (SM). (B–D) Characterization of the synthetic α HL monomers by LC-MS. (B) SM_f : $[M + H]^+ = 34\,983$ (observed mass, obs), $34\,981$ (calculated mass, calcd). (C) $SM_{\Delta 6-113M}$: $[M + H]^+ = 33\,908$ (obs), $33\,907$ (calcd). (D) $SM_{\Delta 6-113F}$: $[M + H]^+ = 33\,924$ (obs), $33\,923$ (calcd). (E) Hemolysis assays (see Supporting Information, Experimental procedures). The decrease in light scattering over time was recorded in a microplate reader at 595 nm. WT α HL monomer (row 1) lysed rRBCs, whereas TBM Δ 6 (row 3) did not due to its truncated β barrel. Similarly, the full-length synthetic α HL monomer SM_f ($5.9\ \mu\text{g mL}^{-1}$, in well 1) lysed rRBCs, whereas $SM_{\Delta 6-113M}$ ($7.4\ \mu\text{g mL}^{-1}$) and $SM_{\Delta 6-113F}$ ($7.8\ \mu\text{g mL}^{-1}$) did not. WT and TBM Δ 6 monomers were produced by IVTT. (F) SDS-PAGE gel analysis of WT and synthetic α HL (SM_f). Lane 1: molecular markers. Lane 2: radiolabeled α HL monomer (mon) produced by IVTT. Lane 3: radiolabeled WT_7 pores (hep) produced in the presence of DPhPC liposomes ($7\ \text{mg mL}^{-1}$). Lane 4: $(SM_f)_7$ pores assembled with purified SM_f in the presence of DPhPC liposomes under the same conditions comigrate with the WT_7 pore. An autoradiogram is superimposed on the Coomassie Blue-stained gel. (G) Heteroheptameric pores. WT α HL (radiolabeled protein) and SM were mixed in various ratios in the presence of rRBCm to yield heteromeric $WT_{7-n}SM_n$ ($n = 0-7$) pores. The heptameric pores with different numbers of SM_f were separated by SDS-PAGE based on the different electrophoretic mobilities produced by D_8 tails at the C-terminus of SM_f . (H) Homoheptameric pores formed with $SM_{\Delta 6-113M}$ (left) and $SM_{\Delta 6-113F}$ (right). Homomeric pores were prepared in the presence of DPhPC liposomes ($10\ \text{mg mL}^{-1}$).

efficiently with proteins of poor solubility or proteins from inclusion bodies. In the latter case, the protease can be inactivated by denaturants used to solubilize a target protein, and even if cleavage is successful, an additional purification step may be required to separate the product from the protease and unwanted fragments. An alternative approach to generate N-Cys, which was taken here, is to express the polypeptide initiated by fMet-Cys. Following expression, formylmethionine (fMet) is removed by endogenous deformylation and methionine aminopeptidase activity, and the N-Cys residue undergoes condensation with pyruvic acid, an abundant metabolite, to form a thiazolidine.¹⁷ This strategy is protease-free and allows rapid overexpression of the target polypeptide (4 h) at 37 °C. The polypeptide accumulated in inclusion bodies is subsequently purified under denaturing conditions (8 M urea or 6 M guanidine hydrochloride (Gu·HCl)), and the N-Cys is unmasked with hydroxylamine or a hydroxylamine derivative.¹⁸ The use of denaturants enables the purification of polypeptides containing transmembrane regions, which are often insoluble and obtained in very low yields when processed under nondenaturing conditions (unpublished work).

Preparation of Polypeptides for Two-Fragment Ligation. We targeted three α HL polypeptides for semisynthesis by two-fragment coupling (Figure 1A): a full-length α HL monomer and two different truncated barrel mutants (TBM Δ 6) (Figure S1A–C). TBM Δ 6 forms an α HL heptamer in which amino acids have been removed in pairwise fashion from both of the two β strands contributed by each subunit,

resulting in a β barrel shortened by 6 amino acids.¹⁹ TBM Δ 6 forms conductive pores despite the short length of the barrel, presumably by inducing the formation of toroidal lipid pores that span the bilayer.¹⁹ Two TBM Δ 6s bearing a different amino acid at residue 113 (Met, Phe) were chosen, as it has been shown that the mutation Met-113→Phe significantly alters the binding kinetics of cyclodextrin adaptors to the pore.²⁰

Three different NTFs (NTF₁₂₆ [Ala¹-Gly¹²⁶], NTF_{113M} [Ala¹-Met¹¹³], and NTF_{113F} [Ala¹-Phe¹¹³]) each bearing an α thioester at the C-terminus were obtained by thiolysis of the corresponding intein–CBD fusion proteins after expression in *E. coli*. The pTXB3–NTF₁₂₆ and pTXB3–NTF_{113M} plasmids were prepared by cloning PCR-amplified DNA, encoding residues 1–126 and 1–113 of α HL, upstream of the intein–CBD codons (Figure S2A,B). pTXB3–NTF_{113F} was prepared by mutagenesis of pTXB3–NTF_{113M} by homologous recombination (Figure S2C).²¹ Fusion proteins were produced in *E. coli* (BL21(DE3), NEB) and solubilized from inclusion bodies under denaturing conditions (8 M urea). The NTF- α thioesters were obtained by on-column thiolysis of the fusion proteins bound to chitin columns with MESNa (Figure S3). The purified NTF- α thioesters were characterized by LC-MS (Figure S4, NTF₁₂₆: $[M + H]^+ = 14\,150$ (obs), $14\,150$ Da (calcd); NTF_{113M}: $[M + H]^+ = 12\,838$ (obs), $12\,838$ Da (calcd); NTF_{113F}: $[M + H]^+ = 12\,853$ (obs), $12\,854$ Da (calcd)).

The pT7-SC1-CTF₁₂₇ plasmid was prepared by replacing plasmid DNA encoding residues 1–127 with the codons for Met-Cys as previously reported.¹¹ To produce CTF Δ ₁₁₄, pT7-

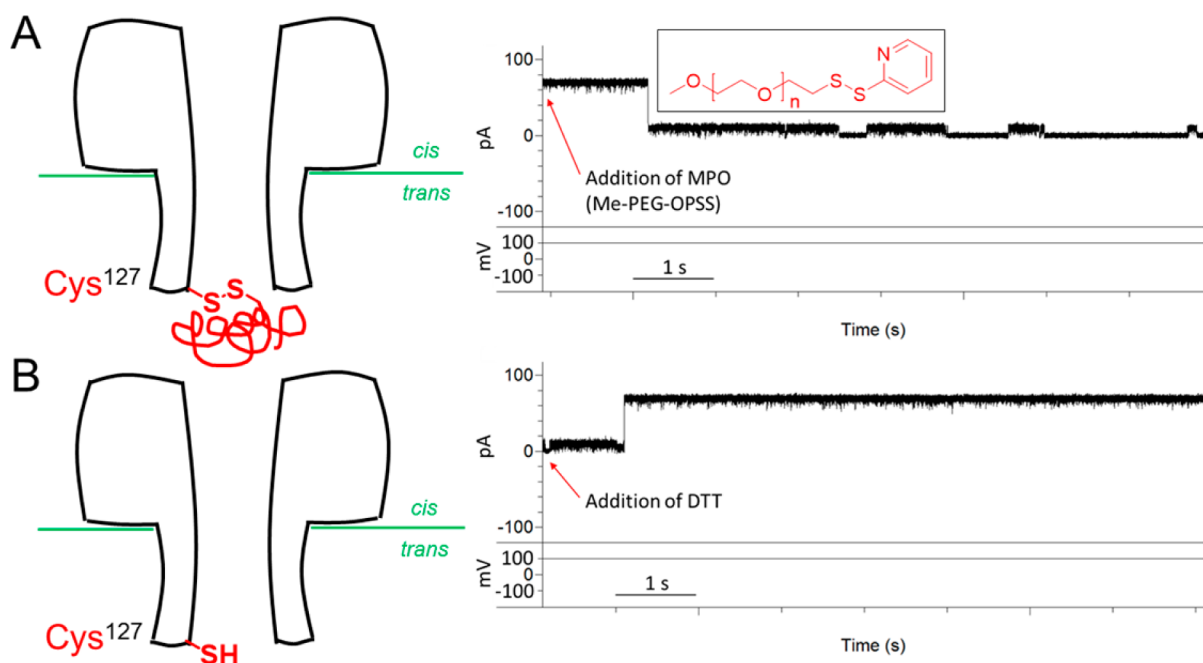


Figure 2. Reactivity of the Cys-127 residue in WT₆SM_{f1} α HL. (A) WT₆SM_{f1} was eluted from a gel (Figure 1G), and a single WT₆SM_{f1} pore was established in a planar bilayer. Me-PEG-OPSS (5 kDa, 0.1 mM, inset, $n = 3$) was added to the *trans* compartment. The current drop indicates a blockade caused by reaction of the PEG derivative with the pore through the formation of a disulfide bond with the side chain of Cys-127. (B) Addition of 5 mM DTT to both compartments cleaved the PEG chain from the WT₆SM_{f1} pore. The buffer was 1 M KCl, 20 mM Tris·HCl (pH 8.5). The currents in (A) and (B) were filtered and sampled at 2 and 10 kHz, respectively.

SC1-CTF _{Δ 114}-DH was prepared by two successive homologous recombinations²¹ from pT7-TBM Δ 6, which encodes TBM Δ 6 (Figure S5). The codons for residues 1–113 were removed (retaining the initiator Met), and codons for a D₈H₆ (DH) tag were added in the first and second rounds, respectively. The two different C-terminal fragments (CTF₁₂₇ [Cys¹²⁷-Asn²⁹³]-D₈H₆, and CTF _{Δ 114} [Cys¹¹⁴-Asn²⁹³, Δ Phe¹²⁰-Thr¹²⁵, Δ Gly¹³³-Ala¹³⁸]-D₈H₆) were overexpressed in *E. coli*. Like the NTFs, the CTFs were obtained from inclusion bodies and, in this case, purified under denaturing conditions (6 M Gu·HCl) by FPLC (AKTA purifier, GE Healthcare Life Sciences) at room temperature by use of the His₆ tag at the C-terminus (Figure S6). The N-terminal fMet was found to be absent, and the thiazolidine produced by condensation of the N-Cys with pyruvic acid was removed with 0.4 M HONH₂·HCl for 4 h at room temperature. The purified CTFs were characterized by LC-MS (Figure S7, CTF₁₂₇: [M + H]⁺ = 20 974 (obs), 20 974 Da (calcd); CTF _{Δ 114}: [M + H]⁺ = 21 214 (obs), 21 212 Da (calcd)).

α -Hemolysin Polypeptides by Two-Fragment Ligation. We ligated NTFs and CTFs (Figure S1) in NCL buffer [200 mM NaH₂PO₄ (pH 6.9), 6 M Gu·HCl, 200 mM 4-mercaptophenylacetic acid (MPAA), and 50 mM tris(2-carboxyethyl)phosphine (TCEP)] to make three different α HL constructs. In each case, an NTF and a CTF were mixed and the buffer was exchanged by dilution–concentration cycles with a centrifugal filter. NTF₁₂₆ (~0.5 mM) and CTF₁₂₇ (~0.5 mM) were coupled to produce the full-length α HL synthetic monomer (SM_f). NTF_{113M} (~0.6 mM) and NTF_{113F} (~0.7 mM) were separately coupled with CTF _{Δ 114} (~0.8 mM) to yield two different truncated mutant monomers¹⁹ (SM _{Δ 6–113M} and SM _{Δ 6–113F}). The rate of ligation is highly dependent on the steric properties of the C-terminal amino acid residue of an NTF^{22,23} and the concentration of reactants.

The ligation reactions were carried out for >12 h at a final concentration of ~1 mM as previous work²² had suggested that ligations of NTFs containing C-terminal Ala, Val, Ile, Met, and Phe are completed within 9 h at a final peptide concentration of 1–3 mM. The two-fragment couplings gave SM_f, SM _{Δ 6–113M}, and SM _{Δ 6–113F} (Figures S8 and S9) in 48, 46, and 50% yields, respectively.

Purification, Folding, and Functional Properties of α -Hemolysin Polypeptides. We purified the SMs (SM_f, SM _{Δ 6–113M}, and SM _{Δ 6–113F}) by gel filtration in 8 M urea (Figure S8) and characterized them by LC-MS (Figure 1B–D and Figure S8, SM_f: [M + H]⁺ = 34 983 (obs), 34 981 Da (calcd); SM _{Δ 6–113M}: [M + H]⁺ = 33 908 (obs), 33 907 Da (calcd); SM _{Δ 6–113M}: [M + H]⁺ = 33 924 (obs), 33 923 Da (calcd)). We then folded the purified SMs by diluting the 8 M urea in the purification buffer to ~60 mM and concentrating the proteins using a centrifugal filter (MWCO 3k). The folded monomers were examined for hemolytic activity toward rabbit red blood cells (rRBCs) (Figure 1E). We observed similarities between the synthetic monomers and the WT or truncated α HL monomers produced either in *E. coli* or by *in vitro* transcription and translation (IVTT). As expected, only SM_f exhibited hemolytic activity toward rRBCs.¹⁹ The specific hemolytic activity of SM_f was HC₅₀ = 92 ng mL⁻¹, which is in the same range as that of WT α HL (HC₅₀ = 31 ng mL⁻¹).²⁴ To visualize the formation of α HL heptamers, we incubated SM_f at 37 °C in the presence of liposomes (10 mg mL⁻¹, diphyanoylphosphatidylcholine, DPhPC), which produced a new band upon sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) corresponding to the size (~240 kDa) of the heptamer (Figure 1F, left). We also incubated SM_f in the presence of rabbit red blood cell membranes (rRBCm) in different ratios with radiolabeled WT α HL produced by IVTT. The SM_f oligomerized to form heteroheptamers with

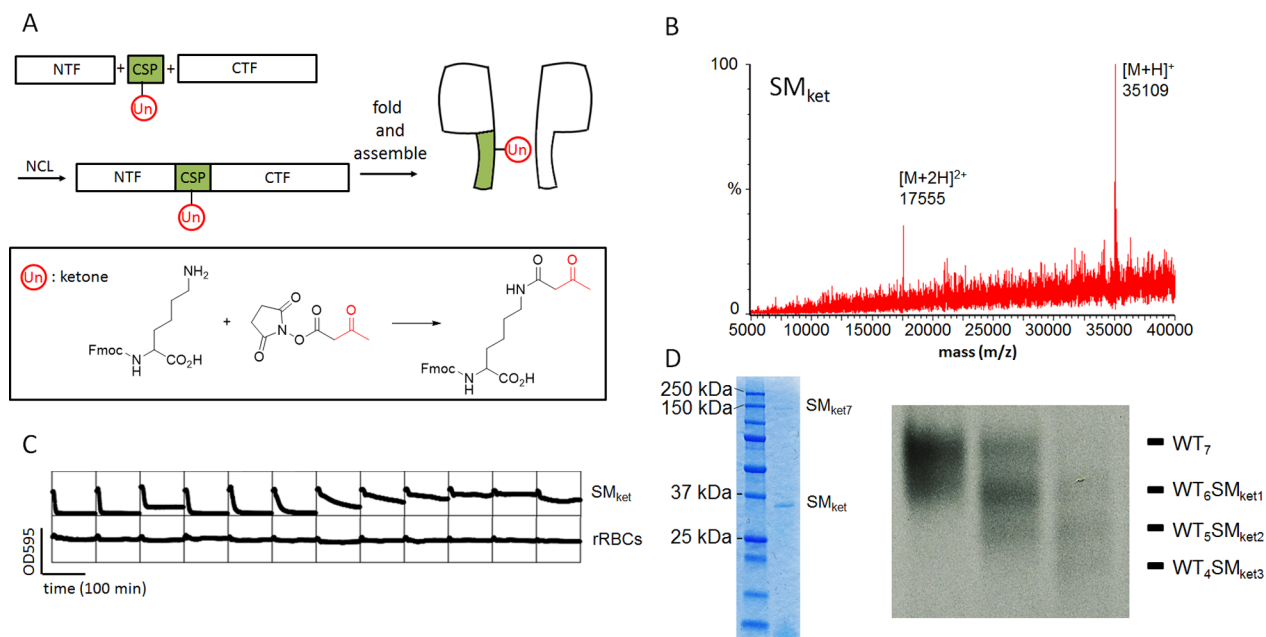


Figure 3. Preparation of α HL pores containing an unnatural amino acid. (A) An α HL monomer was synthesized from three fragments with a central segment (CSP) bearing an unnatural amino acid with a side chain bearing a ketone group (inset: Un, Fmoc-N⁶-(3-oxobutanoyl)lysine, Fmoc-Ket-OH). (B) Characterization of the synthetic α HL monomer (SM_{ket}) by LC-MS. $[M + H]^+ = 35\ 109$ (obs), 35 107 (calcd). (C) Hemolysis assay for SM_{ket} . The full-length synthetic α HL monomer [SM_{ket} (0.31 mg mL⁻¹)] lysed rRBCs. rRBCs alone (row 6 in Figure 2E) are displayed for comparison. (D) Homo- (left) and hetero- (right) heptameric pores formed with SM_{ket} . The pores were prepared by the same methods described in Figure 1F,G.

different stoichiometries (WT_{7-n}SM_n, $n = 0-7$) (Figure 1F, right). $SM_{\Delta 6-113M}$ and $SM_{\Delta 6-113F}$ were incubated with DPhPC liposomes (10 mg mL⁻¹) and oligomerized to form homomeric structures¹¹ (Figure 1G).

Electrical Properties of Two-Fragment Pores and Binding of Cyclodextrins. To examine the electrical properties of the various heptameric α HL pores containing synthetic subunits [WT_6SM_{f1} , ($SM_{\Delta 6-113M}$)₇, and ($SM_{\Delta 6-113F}$)₇], we determined the mean unitary conductance values under defined conditions and measured $I-V$ curves (Figure S10a,b). The conductance values for the α HL pores containing synthetic monomers were similar to that of pores comprising WT α HL subunits produced by IVTT.

To confirm that the transmembrane β barrels of the semisynthetic pores were intact, we evaluated the binding kinetics at the single-molecule level of cyclodextrin molecular adapters (β CD and α - β CD (heptakis(6-deoxy-6-amino)- β -cyclodextrin)) with the WT₆(SM_f)₁, ($SM_{\Delta 6-113M}$)₇, and ($SM_{\Delta 6-113F}$)₇ pores.^{25,26} It was already known that the homoheptamer formed from TBM Δ 6/M113F binds α - β CD very tightly.^{19,20} We determined the association and dissociation rate constants (k_{on} and k_{off}) of β CD for the three different protein pores. At least three measurements were made for each construct. β CD blocks the ionic current transiently when it is lodged within the lumen of the α HL pore. The dissociation constants ($K_D = k_{off}/k_{on}$, Figure S11) of β CD for WT₆SM_{f1} ($K_D = 14.5 \pm 0.4 \times 10^{-3}$ M, $k_{on} = 10.0 \pm 0.2 \times 10^4$ M⁻¹·s⁻¹, and $k_{off} = 14.5 \pm 0.2 \times 10^2$ s⁻¹) and ($SM_{\Delta 6-113M}$)₇ ($K_D = 6.5 \pm 0.2 \times 10^{-2}$ M, $k_{on} = 2.4 \pm 0.1 \times 10^4$ M⁻¹·s⁻¹, and $k_{off} = 15.7 \pm 0.3 \times 10^2$ s⁻¹) were similar to the values obtained in our previous studies for WT₇²⁵ and (TBM Δ 6-113M)₇.¹⁹ The K_D ($6.1 \pm 1.3 \times 10^{-5}$ M) of β CD for ($SM_{\Delta 6-113F}$)₇ (Figure S12A) was smaller by 3 orders of magnitude than the K_D for the ($SM_{\Delta 6-113M}$)₇ pore, which makes sense as it is known that β CD binds more

tightly by 3–4 orders of magnitude to a pore formed by the full-length M113F subunit than it does to the WT pore.²⁰ We also analyzed the binding of α - β CD to the ($SM_{\Delta 6-113F}$)₇ pore (Figure S12B) and found that it remained bound to the pore “permanently” as previously reported for the same truncated pore produced by conventional means.¹⁹ The binding kinetics of β CD and α - β CD suggest that the semisynthetic protein pores produced by two-fragment coupling, and thereby containing a Cys mutation (S114C), are very similar to the protein pores derived from WT α HL produced directly by IVTT from the corresponding genes.

Two-Fragment Ligation Forms a Native Amide Bond.

To verify the existence of a native amide bond formed between Gly126 and Cys127 in SM_f, we carried out thiolate chemistry on single WT₆SM_{f1} pores by using the side chain of Cys-127 generated by NCL. In the absence of methyl-PEG-OPSS (MPO, 5.0 kDa), the open state of WT₆SM_{f1} had a long duration (>30 min). The addition of MPO (0.1 mM) to the *trans* compartment at +100 mV generated an irreversible current drop (Figure 2A), due to the formation of a disulfide bond between MPO and the side chain of Cys-127. The pore remained blocked over a range of potentials (−100 to +100 mV), indicating that the current drop is not due to simple clogging of the pore. In the presence DTT (5 mM, both compartments), the open current level was restored (Figure 2B) because the PEG chain was cleaved from the pore.

Three-Fragment Ligation To Form a Ketone-Containing α HL Polypeptide. With NTF_{113M} and CTF₁₂₇, and a synthetic central peptide, we next carried out three-fragment coupling to construct a full-length α HL monomer containing a ketone group (Figure 3A). The ketone is a versatile functional group in organic chemistry and participates in a large number of reactions.^{27–30} However, reactions of a ketone have not been observed yet at the single-molecule level. The synthetic

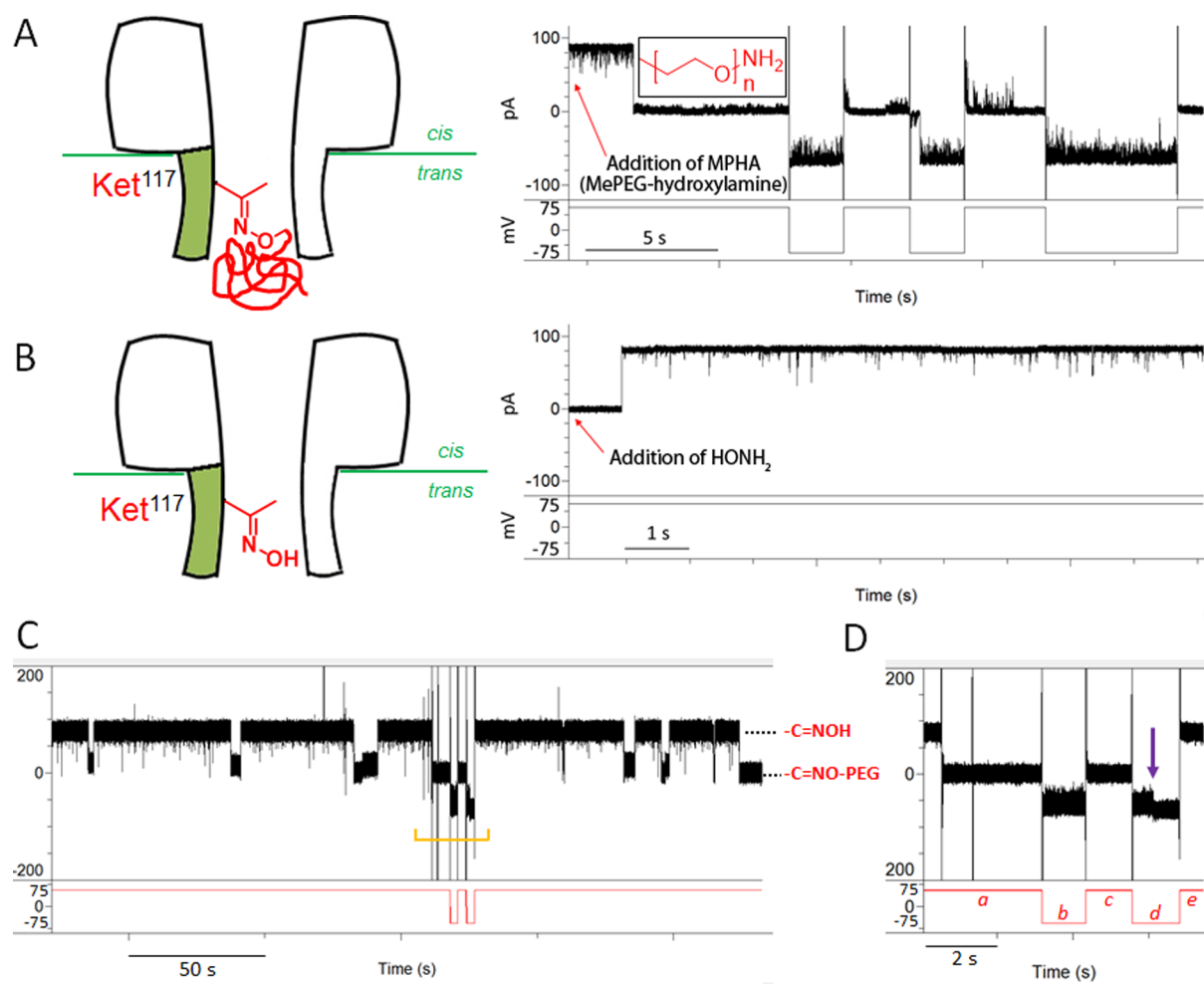


Figure 4. Single-molecule reactions of the WT₆SM_{ket1} pore. (A) A WT₆SM_{ket1} pore was reacted with MePEG-hydroxylamine (MPHA, 1.1 kDa, 2 mM, inset) added to the *trans* compartment. Reaction occurred at a positive potential (+75 mV) and led to a permanent current blockade of the WT₆SM_{ket1} pore. The modified pore only opened at negative applied potentials. (B) The pore was restored to an open state when 20 mM HONH₂·HCl was added to both compartments to release the PEG chain. The currents in (A) and (B) were filtered at 5 kHz and sampled at 25 kHz. For display, further digital filtering was carried out at 2 kHz with an 8-pole low-pass Bessel filter. The buffer was 1 M KCl, 50 mM Na acetate (pH 3.4). (C) Reversible oxime formation in a single synthetic pore containing a ketone (WT₆SM_{ket1}). Oxime formation with MPHA leads to a current drop, while reversal with HONH₂ returns the current to its initial level. The section defined by the orange bracket is magnified in panel D. (D) Negative potential (−75 mV, *b*) was applied during the PEG-oxime state (*a*), which opened the pore (residual current, $I_{RES} = 91\%$). Subsequently, a positive potential (+75 mV, *c*) was applied, and the pore closed. The pore became fully open (violet arrow) with $I_{RES} = 100\%$ at negative potential (−75 mV, *d*), presumably when the formation of an oxime with HONH₂ led to release of the pore-bound polymer. The pore was restored to an open state at a positive potential (+75 mV, *e*).

methods used to obtain an unnatural amino acid containing a ketone group^{28,29} are not very efficient, and the techniques^{27,30} used to incorporate a ketone amino acid into the middle of a polypeptide chain are often arduous. We made an unnatural amino acid bearing a ketone (Fmoc-Ket-OH; Fmoc-N⁶-(3-oxobutanoyl)lysine), Figure 3A, inset) from Fmoc-Lys-OH and *N*-hydroxysuccinimidyl acetoacetate (NHA). Fmoc-Ket-OH was used for SPPS of a central segment of the polypeptide chain (CSP: Thz¹¹⁴ThrLeuKetTyrGlyPheAsnGlyAsnVal-ThrGly¹²⁶-Nbz), such that the Ket side chain would project into the transmembrane β barrel of an α HL pore. CSP was prepared with a C-terminal acylurea³¹ (Figures S13 and S14), which yields a peptide arylthioester with 4-mercaptophenylacetic acid (MPAA), accelerating the NCL reaction. We then proceeded to assemble a full-length α HL bearing the ketone group with two sequential NCL reactions (Figure S15). The final product (SM_{ket}) was purified (Figure S16) and

characterized by LC-MS (Figure 3B and Figure S9D; $[M + H]^+ = 35\,109$ (obs), 35 107 Da (calcd)). A hemolysis assay showed that folded SM_{ket} ($HC_{50} = 47$ ng mL⁻¹) had similar activity to the WT α HL monomer (Figure 3C). SM_{ket} also formed homo- and heteroheptameric pores in the presence of liposomes and rRBCm, respectively (Figure 3D).

We determined the mean unitary conductance values for individual WT₆SM_{1ket} pores in 1 M KCl and 50 mM Na acetate buffer over a range of applied potentials (−100 to +100 mV) (Figure S18A). The buffer was adjusted to pH 3.4 in anticipation of an acid-catalyzed addition reaction (imine formation) on the ketone side chain (see below). The conductance of WT₆SM_{ket1} (0.93 ± 0.10 nS, $n = 9$) at +100 mV was similar to that of the WT₇ pore (1.07 ± 0.02 nS, $n = 9$) under the same conditions. We also determined the association and dissociation rate constants at pH 3.4 (k_{on} and k_{off}) for β CD binding from the values of the mean dwell times (τ_{on} and τ_{off})

[WT₇: $K_D = 6.1 \pm 0.2 \times 10^{-2} \text{ M}$ ($n = 3$), $k_{\text{on}} = 71.7 \pm 0.1 \times 10^2 \text{ M}^{-1}\cdot\text{s}^{-1}$, and $k_{\text{off}} = 4.4 \pm 0.2 \times 10^2 \text{ s}^{-1}$; WT₆SM_{ket1}: $K_D = 8.6 \pm 0.7 \times 10^{-2} \text{ M}$ ($n = 3$), $k_{\text{on}} = 83.7 \pm 6.8 \times 10^2 \text{ M}^{-1}\cdot\text{s}^{-1}$, and $k_{\text{off}} = 7.2 \pm 0.1 \times 10^2 \text{ s}^{-1}$] (Figure S17). The ketone residue presented by the SM subunit affects neither the electrical properties of the α HL pore nor its ability to bind the β CD adaptor.

Single-Molecule Covalent Chemistry with a Ketone-Containing α HL Pore. We then carried out imine chemistry with single WT₆SM_{1ket} pores. We first examined the interaction of the WT₇ pore with 1.1 kDa MePEG-hydroxylamine (MPHA). The addition of 2 mM MPHA to the *trans* compartment at +75 mV in the presence of 1 M KCl, 50 mM Na acetate buffer (pH 3.4), produced short blockades ($\sim 100 \mu\text{s}$), which arise from the entry of MPHA into the lumen of the pore without covalent attachment.³² No prolonged current decrease was observed with the WT₇ pore during 2 h of monitoring. We then added 2 mM MPHA to the *trans* side of the WT₆SM_{ket1} pore, under the same conditions, which led to a permanent current blockade at +75 mV within 10 min, presumably due to the covalent attachment of MPHA to the ketone group within the pore through imine formation. Interestingly, the modified WT₆SM_{ket1} pore opened at negative applied potentials (-100 to 0 mV) with $I_{\text{RES}} = 91\%$ (residual current), compared with the original open state. By contrast, at positive potentials (0 to $+100 \text{ mV}$), the pore was almost closed ($I_{\text{RES}} = 3.2\%$, Figure S18B) with very short openings ($< 50 \text{ ms}$). It follows that the current–voltage (I – V) characteristics of WT₆SM_{ket1}-imine-PEG show virtually complete current rectification (Figure S18B). Previously, we developed a diode-like α HL pore (7R- α HL) with positively charged Arg side chains projecting into the lumen of the transmembrane β barrel,³³ allowing ions to flow only at positive potentials. We used 7R- α HL to construct a bridge rectifier circuit from droplet interface bilayers. Therefore, the WT₆SM_{ket1}-oxime-PEG pore, which shows the opposite electrical properties to 7R- α HL (Figure 4A), might be used in related applications. To confirm that the attachment was through imine formation, we added 20 mM NH₂OH to both compartments to cleave the linkage. In $\sim 15 \text{ min}$, the current returned to its initial level (Figure 4B), suggesting that the PEG chain had been cleaved from the pore.

The observation of the ability of the WT₆SM_{ket1} pore to return its initial conductance level led us to investigate an oxime–oxime exchange reaction on the ketone side chain at the single-molecule level. In the presence of 2 mM MPA, in the *trans* compartment, and 10 mM of HONH₂, in both the *cis* and *trans* compartment, a reversible reaction was observed (Figure 4C). During the formation of the *O*-alkyloxime by MPHA, the current was greatly reduced to levels in the range of 1.5 to 17 pA (Figure 4C). Subsequent transitions between this “closed” level and the open level were apparent. The open level represents the formation of an oxime with HONH₂, and release of the PEG chain from the pore. The formation of an *O*-alkyloxime within the pore with MPHA only allowed substantial ion flow at a negative potential (-75 mV) (orange bracket in Figure 4C and a–d in Figure 4D). After transoximation with HONH₂,³⁴ the current increased from -56 to -67 pA at -75 mV (violet arrow in Figure 4D) and an open-level current of $+78 \text{ pA}$ was observed during the subsequent application of a positive potential ($+75 \text{ mV}$).

The mean dwell time of the open pore (τ_{on}) is the mean lifetime of the oxime formed by HONH₂ (o), which is the mean reaction time for *O*-alkyloxime (ao) formation with

MPHA. Similarly, the mean dwell time of the closed pore (τ_{off}) is the mean lifetime of the *O*-alkyloxime, which is the mean reaction time for oxime formation with HONH₂. The measured mean lifetimes of the *O*-alkyloxime and the oxime were $52 \pm 2 \text{ s}$ ($n = 79$) and $51 \pm 2 \text{ s}$ ($n = 78$) (Figure S19), which yield rate constants for transoximation of $k_{\text{f,ao}} = 10 \text{ M}^{-1}\cdot\text{s}^{-1}$ and $k_{\text{f,o}} = 2 \text{ M}^{-1}\cdot\text{s}^{-1}$, respectively, in 1 M KCl, Na acetate buffer (pH 3.4), at $+75 \text{ mV}$, where $k_{\text{f,ao}} = k_{\text{on}} = 1/\tau_{\text{on}} \cdot [\text{MPHA}]$ and $k_{\text{f,o}} = k_{\text{off}} = 1/\tau_{\text{off}} \cdot [\text{NH}_2\text{OH}]$.

CONCLUSIONS

The ability to introduce unnatural amino acids into the α HL pore has the potential to provide a large variety of reactive side chains for the investigation of single-molecule covalent chemistry. We have previously produced α HL polypeptides with unnatural alkyl and aryl amino acids by using *in vitro* chemically acylated tRNAs.³⁵ However, this approach is demanding and often gives poor results when more than one amino acid is introduced. The coupling of polypeptide segments by NCL has been used extensively to produce proteins^{36–38} and is a favorable alternative means to incorporate unnatural amino acids. In the present work, we demonstrate a variety of synthetic protein pores using α HL polypeptides and use the synthetic pore containing a ketone as single-molecule nanoreactor.

Oxime chemistry was examined in an aqueous environment at the single-molecule level with a ketone-containing α HL pore (WT₆SM_{ket1}), and the work described here is the first observation of reversible covalent chemistry using an unnatural amino acid side-chain in a nanoreactor. Oxime formation from a ketone proceeds *via* nucleophilic addition to form a tetrahedral intermediate,^{39,40} followed by the elimination of water. Transoximation also proceeds reversibly through a tetrahedral intermediate that subsequently breaks down to form a new oxime and a hydroxylamine.³⁴ In our work, no intermediates were observed in both the *O*-alkyloxime formation by MPHA and the transoximation reaction. Presumably, the lifetimes of the tetrahedral intermediates are too short or the current changes too small to observe under our recording conditions. We obtained rate constants for oxime and *O*-alkyloxime formation within the pore ($k_{\text{f,ao}} = 10 \text{ M}^{-1}\cdot\text{s}^{-1}$ and $k_{\text{f,o}} = 2 \text{ M}^{-1}\cdot\text{s}^{-1}$). Earlier determinations in bulk solution^{41,42} are in the range ~ 1 to $10^4 \text{ M}^{-1}\cdot\text{s}^{-1}$ and depend strongly on substituents, solvent, pH, and temperature. In our case, the partitioning of the polymer reactant, MPHA, into the pore must be considered.^{11,43}

We have observed oxime formation by using a semisynthetic pore containing an unnatural amino acid as a nanoreactor. The ketone-containing pore expands the range of covalent chemistry that can be studied by the nanoreactor approach to reversible reactions for which statistically significant data can be acquired rapidly without tedious repeats. Taken together with our recent demonstration of alkyne chemistry,¹¹ the versatility of the nanoreactor approach is apparent, and we look forward to developing even more ambitious possibilities, such as single-molecule catalysis, which may require the placement of several different unnatural amino acids within a single polypeptide chain.

METHODS

Native Chemical Ligation. Two-Fragment Coupling. Fifty microliters of CTF ($>0.5 \text{ mM}$), from which pyruvate had been removed, was mixed with $50 \mu\text{L}$ of NTF ($>0.5 \text{ mM}$) in 0.4 mL of NCL

buffer [200 mM NaH₂PO₄ (pH 6.9) containing 6 M Gu·HCl, 50 mM tris(2-carboxyethyl)phosphine (TCEP), and 200 mM 4-mercaptophenylacetic acid (MPAA)] and concentrated to 100 μ L using a centrifugal filter (Amicon, MWCO 3k) at 14 000g for 20 min. The buffer containing NTF and CTF was replaced with NCL buffer by repeated (5 times) dilution and concentration with the same filter. The reaction was allowed to proceed overnight at room temperature.

Three-Fragment Coupling. CTF (0.5 mM), from which pyruvate had been removed, was mixed with CSP-Nbz (5 mM) in 0.5 mL of NCL buffer [200 mM NaH₂PO₄ (pH 6.9) containing 6 M Gu·HCl, 50 mM tris(2-carboxyethyl)phosphine (TCEP), and 50 mM MPAA]. After overnight reaction at room temperature, the unreacted peptide was removed by passing the mixture through a size-exclusion column (Superdex 200 10/300 GL). The product was analyzed by LC-MS.

The N-terminal Thz group was subsequently cleaved by treatment with 0.4 M HONH₂·HCl in 200 mM NaH₂PO₄ buffer (pH adjusted to 4.0) containing 6 M Gu·HCl and 50 mM TCEP for 4 h at room temperature. For the next round of ligation, the buffer was replaced with NCL buffer containing 200 mM MPAA by repeated dilution and concentration with a centrifugal filter (Amicon, MWCO 3k). NTF-^athioester (0.6 mM) was mixed with the first ligation product (0.3 mM), and the reaction was allowed to proceed overnight. The final ligation product was purified by gel filtration followed by ion-exchange chromatography. The reaction yield was determined by quantifying the intensity of the polypeptide bands after SDS-PAGE by using ImageJ (NIH).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.6b04663.

Experimental procedures and supplementary figures for analytical and spectral characterization data (PDF)

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Author Contributions

J. Lee, A.J.B., M.A.B., S.C., O.D., and H.B. designed the research. A.J.B., M.A.B., S.C., and O.D. carried out early synthetic work. J. Lee performed recent synthetic work and characterization of the synthetic pores. J. Li and H.T. assisted with solid-phase peptide synthesis. J. Lee and H.B. wrote the manuscript.

Notes

The authors declare no competing financial interest.

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