Semisynthetic Nanoreactor for Reversible Single-Molecule Covalent Chemistry

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ABSTRACT: Protein engineering has been used to remodel pores for applications in biotechnology. For example, the heptameric α-hemolysin pore (αHL) 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 αHL pores obtained by coupling either two or three fragments by native chemical ligation (NCL). The synthetic αHL 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.

RESULTS

General Approach to Two-Fragment Ligation. Two-fragment ligations involved the reaction of an N-terminal fragment (NTF) containing a C-terminal “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). After expression of the protein in Escherichia coli, the NTF–thioester was cleaved from the intein–CBD with sodium 2-mercaptoethanesulfonate (MESNa), while the rest of the chimera remained bound to chitin beads. A CTF with an N-Cys can be generated by cleavage of a precursor fusion protein with a site-specific protease. However, this method may not work 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.

α-Hemolysin (αHL) is a pore-forming toxin secreted by Staphylococcus aureus. The pore contains seven subunits, and each subunit comprises 293 amino acids. Use of the heptameric αHL protein pore as a nanoreactor has proved profitable in studies of covalent chemistry at the single-molecule level. 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 αHL 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. Recently, complex reaction networks and the motion of individual molecular walkers have been examined by this means. However, the chemistry carried out within engineered αHL nanoreactors has until recently been confined to the reactions of thiolates and derivatives of the side chains of cysteine residues. Lately, we expanded the range of chemistry that can be approached by introducing unnatural amino acid side chains into the αHL polypeptide by using native chemical ligation (NCL). By this means, an irreversible reaction of a side chain bearing a terminal alkyne was detected at the ensemble 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 αHL pores obtained by coupling either two or three fragments by native chemical ligation (NCL). The synthetic αHL 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

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Cys is unmasked with hydroxylamine or a hydroxylamine bodies is subsequently purified from the non-denaturant (8 M urea) present during the purification of the synthetic monomers (SM). (B–D) Characterization of the synthetic αHL monomers by LC-MS. (B) SM12 [M + H]+ = 34 983 (observed mass, obs), 34 981 (calculated mass, calcld). (C) SMα6-113M [M + H]+ = 33 908 (obs), 33 907 (calcld). (D) SMα6-113F [M + H]+ = 33 924 (obs), 33 923 (calcld). (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 αHL monomer (row 1) lysed rRBCs, whereas TBMΔ6 (row 3) did not due to its truncated β barrel. Similarly, the full-length synthetic αHL monomer SM12 (5.9 μg mL⁻¹, in well 1) lysed rRBCs, whereas SMα6-113M (7.4 μg mL⁻¹) and SMα6-113F (7.8 μg mL⁻¹) did not. WT and TBMΔ6 monomers were produced by IVTT. (F) SDS-PAGE gel analysis of WT and synthetic αHL (SM). Lane 1: molecular markers. Lane 2: radiolabeled αHL monomer (mon) produced by IVTT. Lane 3: radiolabeled WT pores (hep) produced in the presence of DPhPC liposomes (7 mg mL⁻¹). Lane 4: (SM)7 pores assembled with purified SM12 in the presence of DPhPC liposomes under the same conditions comigrate with the WT pore. An autoradiogram is superimposed on the Coomassie Blue-stained gel. (G) Heteroheptameric pores. WT αHL (radiolabeled protein) and SM were mixed in various ratios in the presence of rRBCm to yield heteromeric WT6...SMn (n = 0–7) pores. The heptameric pores with different numbers of SM12 were separated by SDS-PAGE based on the different electrophoretic mobilities produced by D8 tails at the C-terminus of SM12. (H) Homohexameric pores formed with SMα6-113M (left) and SMα6-113F (right). Homomorphic pores were prepared in the presence of DPhPC liposomes (10 mg mL⁻¹).

Figure 1. Preparation of αHL pores. (A) αHL 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 αHL monomers by LC-MS. (B) SM12 [M + H]+ = 34 983 (observed mass, obs), 34 981 (calculated mass, calcld). (C) SMα6-113M [M + H]+ = 33 908 (obs), 33 907 (calcld). (D) SMα6-113F [M + H]+ = 33 924 (obs), 33 923 (calcld). (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 αHL monomer (row 1) lysed rRBCs, whereas TBMΔ6 (row 3) did not due to its truncated β barrel. Similarly, the full-length synthetic αHL monomer SM12 (5.9 μg mL⁻¹, in well 1) lysed rRBCs, whereas SMα6-113M (7.4 μg mL⁻¹) and SMα6-113F (7.8 μg mL⁻¹) did not. WT and TBMΔ6 monomers were produced by IVTT. (F) SDS-PAGE gel analysis of WT and synthetic αHL (SM). Lane 1: molecular markers. Lane 2: radiolabeled αHL monomer (mon) produced by IVTT. Lane 3: radiolabeled WT pores (hep) produced in the presence of DPhPC liposomes (7 mg mL⁻¹). Lane 4: (SM)7 pores assembled with purified SM12 in the presence of DPhPC liposomes under the same conditions comigrate with the WT pore. An autoradiogram is superimposed on the Coomassie Blue-stained gel. (G) Heteroheptameric pores. WT αHL (radiolabeled protein) and SM were mixed in various ratios in the presence of rRBCm to yield heteromeric WT6...SMn (n = 0–7) pores. The heptameric pores with different numbers of SM12 were separated by SDS-PAGE based on the different electrophoretic mobilities produced by D8 tails at the C-terminus of SM12. (H) Homohexameric pores formed with SMα6-113M (left) and SMα6-113F (right). Homomorphic pores were prepared in the presence of DPhPC liposomes (10 mg mL⁻¹).
Figure 2. Reactivity of the Cys-127 residue in WT6SMf1 αHL. (A) WT6SMf1 was eluted from a gel (Figure 1G), and a single WT6SMf1 pore was established in a planar bilayer. Me-PEG-OPSS (5 mM, 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 WT6SMf1 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-CTFΔ114-DH was prepared by two successive homologous recombinations21 from pT7-TBMΔ6, which encodes TBMΔ6 (Figure S5). The codons for residues 1–113 were removed (retaining the initiator Met), and codons for a DβH6 (DH) tag were added in the first and second rounds, respectively. The two different C-terminal fragments (CTF127 [Cys127–Asn293]–DβH6, and CTFΔ114 [Cys114–Asn293, ΔPhe120–Thr125, ΔGly133, Ala136–DβH6] 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 Met 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]+ = 20974 (obs), 20974 Da (calcd); CTFΔ114: [M + H]+ = 21214 (obs), 21212 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 αHL 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 αHL synthetic monomer (SM6). NTF113M (~0.6 mM) and NTF113F (~0.7 mM) were separately coupled with CTFΔ114 (~0.8 mM) to yield two different truncated monomer mutants (~SMΔ6–113M and SMΔ6–113F). The rate of ligation is highly dependent on the steric properties of the C-terminal amino acid residue of an NTF22,23 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 ligation 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 SM6, SMΔ6–113M, and SMΔ6–113F (Figures S8 and S9) in 48, 46, and 50% yields, respectively.

Purification, Folding, and Functional Properties of α-Hemolysin Polypeptides. We purified the SMs (SM6, SMΔ6–113M, and SMΔ6–113F) by gel filtration in 8 M urea (Figure S8) and characterized them by LC-MS (Figure 1B–D and Figure S8, SM6: [M + H]+ = 34983 (obs), 34981 Da (calcd); SMΔ6–113M: [M + H]+ = 33908 (obs), 33907 Da (calcd); SMΔ6–113F: [M + H]+ = 33924 (obs), 33923 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 αHL monomers produced either in E. coli or by in vitro transcription and translation (IVTT). As expected, only SM6 exhibited hemolytic activity toward rRBCs. The specific hemolytic activity of SM6 was HC50 = 92 ng mL−1, which is in the same range as that of WT αHL (HC50 = 31 ng mL−1).24 To visualize the formation of αHL heptamers, we incubated SM6 at 37 °C in the presence of liposomes (10 mg mL−1, diphytanoylphosphatidylcholine, 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 SM6 in the presence of rabbit red blood cell membranes (rRBCm) in different ratios with radiolabeled WT αHL produced by IVTT. The SM6 oligomerized to form heteroheptamers with...
different stoichiometries (WT7−nSMf, n = 0–7) (Figure 1F, right). SMΔ6−113M and SMΔ6−113F were incubated with DPhPC liposomes (10 mg mL−1) and oligomerized to form homomeric structures (Figure 1G).

Electrical Properties of Two-Fragment Pores and Binding of Cyclodextrins. To examine the electrical properties of the various heptameric αHL pores containing synthetic subunits [WT6SMf1, (SMΔ6−113M)7, and (SMΔ6−113F)7], we determined the unitary conductance values under defined conditions and measured I−V curves (Figure S10a,b). The conductance values for the αHL pores containing synthetic monomers were similar to that of pores comprising WT αHL 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 (βCD and am−βCD (heptakis(6-deoxy-6-amino)-β-cyclodextrin)) with the WT6SMf1, (SMΔ6−113M)7, and (SMΔ6−113F)7 pores. It was already known that the homohexameric (SMΔ6/M113F) binds am−βCD very tightly. We determined the association and dissociation rate constants (k_on and k_off) of βCD for the three different protein pores. At least three measurements were made for each construct. βCD blocks the ionic current transiently when it is lodged within the lumen of the αHL pore. The dissociation constants (K_D = k_off/k_on, Figure S11) of βCD for WT6SMf1 (K_D = 14.5 ± 0.4 × 10−5 M, k_on = 10.0 ± 0.2 × 106 M−1s−1, and k_off = 14.5 ± 0.2 × 104 s−1) and (SMΔ6−113M)7 (K_D = 6.5 ± 0.2 × 10−2 M, k_on = 2.4 ± 0.1 × 107 M−1s−1, and k_off = 15.7 ± 0.3 × 103 s−1) were similar to the values obtained in our previous studies for WT6 and (TMBΔ6/M113F). The K_D (6.1 ± 1.3 × 10−5 M) of βCD for (SMΔ6−113F)7 (Figure S12A) was smaller by 3 orders of magnitude than the K_D for the (SMΔ6−113M)7 pore, which makes sense as it is known that βCD 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. We also analyzed the binding of am−βCD to the (SMΔ6−113F)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. The binding kinetics of βCD and am−βCD 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 αHL 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 SMf, we carried out thiolate chemistry on single WT6SMf1 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 WT6SMf1 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 αHL Polypeptide. With NTF113M and CTF127, and a synthetic central peptide, we next carried out three-fragment coupling to construct a full-length αHL 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. However, reactions of a ketone have not been observed yet at the single-molecule level. The synthetic...
methods used to obtain an unnatural amino acid containing a ketone group are not very efficient, and the techniques 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: Thz114 ThrLeu KetTyrGlyPheAsnGlyAsnVal-ThrGly126-Nbz), such that the Ket side chain would project into the transmembrane β barrel of an αHL pore. CSP was prepared with a C-terminal acylurea (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 αHL bearing the ketone group with two sequential NCL reactions (Figure S15). The final product (SMket) was purified (Figure S16) and characterized by LC-MS (Figure 3B and Figure S9D; [M + H]+ = 35 109 (obs), 35 107 Da (calcld)). A hemolysis assay showed that folded SMket (HC50 = 47 ng mL−1) had similar activity to the WT αHL monomer (Figure 3C). SMket 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 WTαSMket 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 WTαSMket (0.93 ± 0.10 nS, n = 9) at +100 mV was similar to that of the WTγ 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 βCD binding from the values of the mean dwell times (τ_on and τ_off).

Figure 4. Single-molecule reactions of the WTαSMket pore. (A) A WTαSMket 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 WTαSMket 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 (WTαSMket). 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, IRES = 91%). Subsequently, a positive potential (+75 mV, c) was applied, and the pore closed. The pore became fully open (violet arrow) with IRES = 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).
\[ \text{WT}_{\text{O}}; \quad K_{D} = 6.1 \pm 0.2 \times 10^{-2} \text{ M} \ (n = 3), \quad k_{\text{on}} = 71.7 \pm 0.1 \times 10^{5} \text{ M}^{-1} \cdot \text{s}^{-1}, \quad \text{and} \quad k_{\text{off}} = 4.4 \pm 0.2 \times 10^{5} \text{ s}^{-1}; \quad \text{WT}_{\text{T}} \text{SM}_{\text{ket}}; \quad K_{D} = 8.6 \pm 0.7 \times 10^{-2} \text{ M} \ (n = 3), \quad k_{\text{on}} = 83.7 \pm 6.8 \times 10^{5} \text{ M}^{-1} \cdot \text{s}^{-1}, \quad \text{and} \quad k_{\text{off}} = 7.2 \pm 0.1 \times 10^{5} \text{ s}^{-1} \] (Figure S17). The ketone residue presented by the SM subunit affects neither the electrical properties of the \( \text{OHL} \) pore nor its ability to bind the \( \beta CD \) adaptors.

**Single-Molecule Covalent Chemistry with a Ketone-Containing \( \alpha HL \) Pore.** We then carried out imine chemistry with single WT\( _{\text{T}} \text{SM}_{\text{ket}} \) pores. We first examined the interaction of the WT\( _{\text{T}} \) 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 (~100 \( \mu \)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 WT\( _{\text{T}} \) pore during 2 h of monitoring. We then added 2 mM MPHA to the trans side of the WT\( _{\text{T}} \text{SM}_{\text{ket}} \) 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 WT\( _{\text{T}} \text{SM}_{\text{ket}} \) 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 mV), the pore was almost closed (\( I_{\text{RES}} = 3.2\% \), Figure S18B) with very short openings (<50 ms).

It follows that the current–voltage (\( I–V \)) characteristics of WT\( _{\text{T}} \text{SM}_{\text{ket}} \)-ime-PEG show virtually complete current rectification (Figure S18B). Previously, we developed a diode-like \( \alpha HL \) pore (7R-\( \alpha HL \)) with positively charged Arg side chains projecting into the lumen of the transmembrane \( \beta \) barrel,\(^{33}\) allowing ions to flow only at positive potentials. We used 7R-\( \alpha HL \) to construct a bridge rectifier circuit from droplet interface bilayers. Therefore, the WT\( _{\text{T}} \text{SM}_{\text{ket}} \)-oxime-PEG pore, which shows the opposite electrical properties to 7R-\( \alpha HL \) (Figure 4A), might be used in related applications. To confirm that the attachment was through imine formation, we added 20 mM NH\( _{2} \)OH to both compartments to cleave the linkage. In ~15 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 WT\( _{\text{T}} \text{SM}_{\text{ket}} \) pore to return its initial conductance level led us to investigate an oxime–oxide exchange reaction on the ketone side chain at the single-molecule level. In the presence of 2 mM MPHA, in the trans compartment, and 10 mM of HONH\( _{2} \) 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 HONH\( _{2} \), 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 transamination with HONH\( _{2} \), the current increased from ~56 to ~67 pA at ~75 mV (violet arrow in Figure 4D) and an open-level current of ~78 pA was observed during the subsequent application of a positive potential (~75 mV).

The mean dwell time of the open pore (\( t_{\text{on}} \)) is the mean lifetime of the oxime formed by HONH\( _{2} \) (\( \alpha \)), which is the mean reaction time for O-alkyloxime (ao) formation with MPHA. Similarly, the mean dwell width of the closed pore (\( t_{\text{off}} \)) is the mean lifetime of the O-alkyloxime, which is the mean reaction time for oxime formation with HONH\( _{2} \). The measured mean lifetimes the O-alkyloxime and the oxime were 52 ± 2 s (n = 79) and 51 ± 2 s (n = 78) (Figure S19), which yield rate constants for transamination of \( k_{\text{on}} = 10 \text{ M}^{-1} \cdot \text{s}^{-1} \) and \( k_{\text{off}} = 2 \text{ M}^{-1} \cdot \text{s}^{-1} \), respectively, in 1 M KCl, Na acetate buffer (pH 3.4), at +75 mV, where \( k_{\text{on}} = k_{\text{on}} = 1/t_{\text{on}} \cdot \text{[MPHA]} \) and \( k_{\text{off}} = k_{\text{off}} = 1/t_{\text{off}} \cdot \text{[NH}_{2}\text{OH]} \).

**CONCLUSIONS**

The ability to introduce unnatural amino acids into the \( \alpha HL \) 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 \( \alpha HL \) 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 \( \alpha HL \) 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 \( \alpha HL \) pore (WT\( _{6} \text{SM}_{\text{ket}} \)), 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. Transamination also proceeds reversibly through a tetrahedral intermediate that subsequently breaks down to form a new oxime and a hydroxylamine.\(^{44}\) In our work, no intermediates were observed in both the O-alkyloxime formation by MPHA and the transamination 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 (\( k_{\text{on}} = 10 \text{ M}^{-1} \cdot \text{s}^{-1} \) and \( k_{\text{off}} = 2 \text{ M}^{-1} \cdot \text{s}^{-1} \)). 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.\(^{41,43}\)

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 mM), from which pyruvate had been removed, was mixed with 50 \( \mu \)L of NTF (>0.5 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-mercapto-2-hexan-1-ol (MPAA)] and concentrated to 100 μL using a centrifugal filter (Amicon, MWCO 3k) at 14,000 g 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-Nlbz (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-4thioester (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).

**REFERENCES**


