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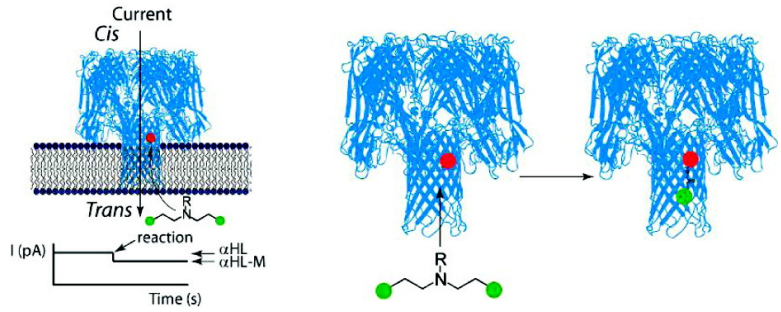

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Single-Molecule Detection of Nitrogen Mustards by Covalent Reaction within a Protein Nanopore

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Abstract: Mustards, including sulfur mustards and nitrogen mustards, form a class of cytotoxic, vesicant chemical warfare agents. Mustards have also been used to treat cancer and played a vital role in the development of chemotherapy. Additionally, because of their destructive properties, ease of synthesis, and the lack of effective antidotes, mustards are unquestionably terrorist threats. Therefore, quick and convenient detection of mustards is a critical issue. In the present study, we achieved detection of various mustards on the basis of their chemical reactivity by using engineered α-hemolysin (αHL) protein pores as sensor elements. We describe four classes of reactions for detecting mustards. These reactions occur between mustards and thiol groups contributed by cysteine side-chains within the lumen of the αHL pore or on an internal molecular adapter. The approach is quick and straightforward. It can confirm the existence of mustards in as little as 10 min at 50 μM or lower.

Introduction

Mustard gas is the common name given to 1,1-thiobis(2-chloroethane), a chemical warfare agent that was used in World War I and World War II.[1] It is a vesicant and can cause damage to the skin, eyes, and respiratory system. Although it is referred to as mustard gas, it is a colorless, odorless, viscous liquid at room temperature. But, when impure, it has an odor similar to that of mustard, garlic, or horseradish.[2] Mustard gas is a member of the sulfur mustards [$ClCH_2CH_2SCH_2CH_2Cl$].[3] The nitrogen mustards are a second group of toxic mustard compounds, which differ from the sulfur mustards by having a nitrogen atom at the position of the sulfur atom [$ClCH_2CH_2N(R)CH_2CH_2Cl$]. Although they have never been used in combat, nitrogen mustards are also powerful and persistent blister agents. Mustards have also been used to treat cancer.[4] It was accidentally found, during World War I, that mustard gas can kill rapidly growing white blood cells.[5] A later clinical trial proved that mustard gas was useful for the treatment of lymphomas. Therefore, in the 1940s, researchers began to search for substances that might have similar effects against other forms of cancer. As a result, many other drugs (notably nitrogen mustards) have been developed to treat cancer,[6] and chemotherapy has since then developed into a multibillion dollar industry.[7]

Mustard gas has been determined by the International Agency for Research on Cancer (IARC) to be a class I human carcinogen and an experimental teratogen.[8] Further, once mustard gas disperses in the environment, it can remain active for periods varying from several hours to a few weeks, depending on the environmental conditions.[9] Despite the ease of hydrolysis, mustard gas has been found to persist as encrusted balls in the soil or even under water for decades,[10,11] and this has led to lethal accidents.[12] Therefore, the detection of mustard gas and its analogues has been a very important endeavor, ever since mustard gas was first used during World War I. Early detection methods relied on the color changes associated with reactions between mustard gas and various metal salts.[13–16] A recent Swedish patent employed a similar strategy by reacting mustard gas with an analytical reagent containing 4-(4-nitrophenylmethyl)pyridine and a salt, preferably a nitrate salt of a rare earth metal. The color that develops indicates the presence of mustard gas.[17] Another early approach for the detection of mustard gas and other chemical warfare agents, such as Lewisite and ethyldichloroarsine, was with trained dogs and rats.[18] However, the ability of the animals to detect these materials at

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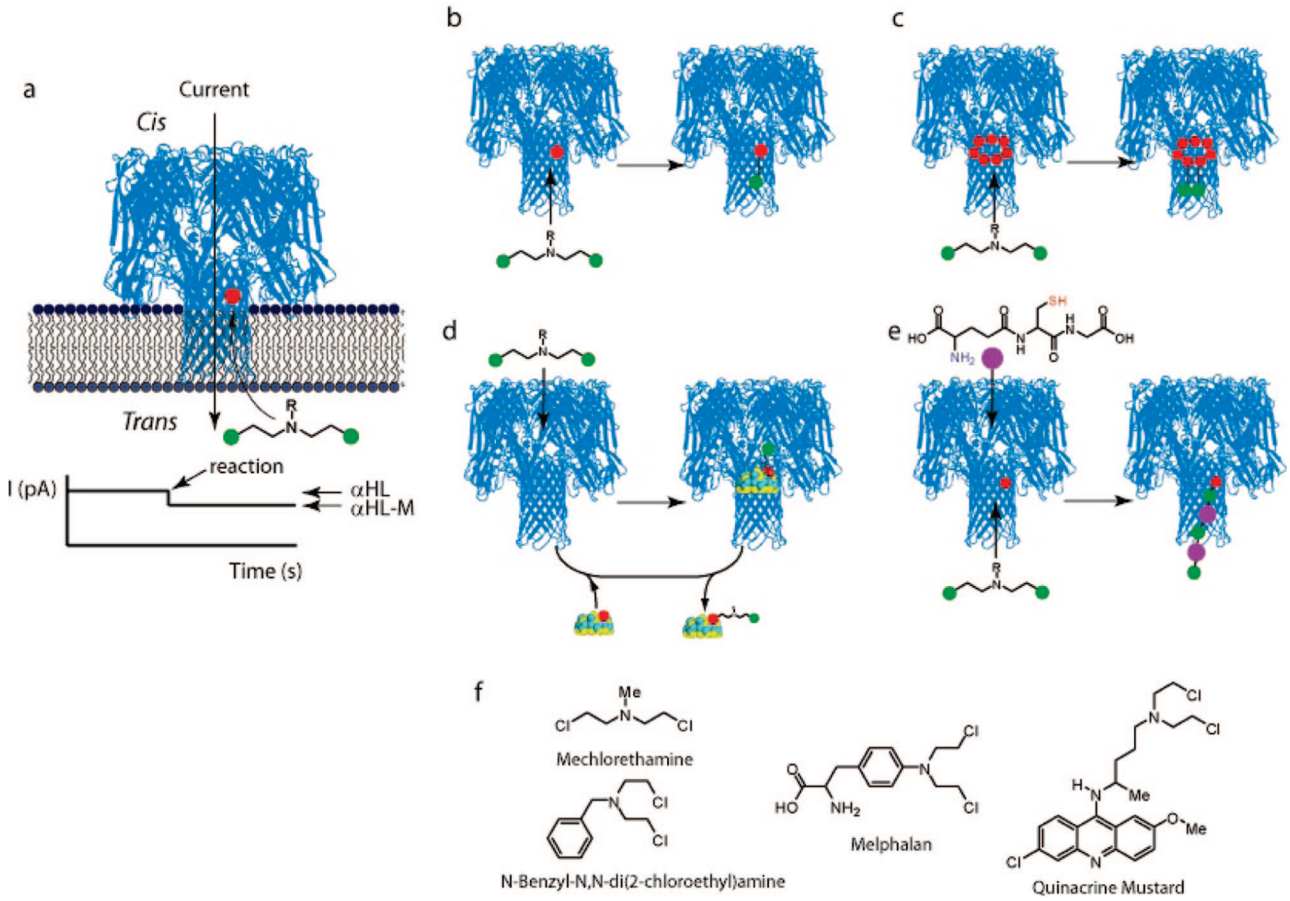

Figure 1. Strategies employed in the detection of mustard gas analogues with engineered αHL pores. Thiol groups are represented as red spots. Chemical structures in A−E are not to scale: (A) Illustration of the principle for the detection of mustards through covalent reaction with thiol groups within the lumen of αHL nanopores. After reaction, the mustard produces a reduction in current as illustrated (αHL-M). (B) Detection of mustards with an engineered αHL pore containing a single thiol group contributed by a cysteine residue (class I reaction). (C) Detection of mustards with an engineered αHL pore containing multiple thiol groups contributed by cysteine residues (class II reaction). (D) Detection of mustards with an engineered αHL pore containing the noncovalent molecular adapter 6-monodeoxy-6-monothio-β-cyclodextrin (βCD-SH), which contributes the reactive thiol group represented as a red spot on the βCD-SH molecule (class III reaction). After reaction, the adduct βCD-S-M dissociates and is replaced by an unreacted βCD-SH molecule. (E) Stepwise growth of a single polymer chain built from mustard and glutathione molecules within an engineered αHL pore containing a single thiol group contributed by a cysteine residue (class IV reaction). (F) Chemical structures of mustard molecules used in the present study.

above the threshold value (0.2 μg/L air) only lasted for about two months. Paper chromatography has also been reported for the detection of nitrogen mustard, and the minimum detectable concentration was (2 μg/L air).[19] As both chromatographic technologies and mass spectrometry evolved in the 1970s to 1980s, they were combined to develop into the key method for detecting mustards in the modern era.[20–23] The detection limit of GC−MS has reached 1.0 ppb. Therefore, it can be used to detect mustards in contaminated biological materials in which the mustard concentration is usually very low. The detection of mustard gas with a surface acoustic wave sensor[24] and the observation of biomarkers (e.g., adducts to macromolecules, such as hemoglobin)[25] provide complementary methods.

Stochastic detection is an approach that relies on the observation of individual binding events between analyte molecules and a receptor. When protein nanopores are used as the sensor element, these binding events can be detected by observing the ionic current carried by a single pore under an applied potential. The frequency of occurrence of current steps reveals the concentration of an analyte. The identity of an analyte is revealed through its characteristic current signature, especially the extent of current block and its mean duration (Figure 1a).[26,27] Engineered versions of the bacterial pore-forming toxin α-hemolysin (αHL) have been used in our group for stochastic sensing of many classes of molecules.[27–35] In the course of this work,

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we found that attempts to engineer αHL to bind small organic analytes directly are rarely successful.[32] Different strategies are required to circumvent this problem. One such strategy is to utilize noncovalently attached molecular adapters, notably cyclodextrins,[36] but also cyclic peptides[37] and cucurbiturils.[31] In the present work, we developed another approach, on the basis of our previous studies of covalent chemistry at the single molecule level,[34,38–43] and detected mustards by their covalent reactions with thiols inside the β barrel of the αHL pore.

There are four different classes of reactions described in the present paper (Figure 1B−E): class I, reactions between mustards and single thiol groups inside the β barrel of the αHL pore, provided by the side chains of cysteine residues; class II, reactions between mustards and multiple thiol groups inside the β barrel of the αHL pore; class III, reactions between mustards and the thiol group of the noncovalent molecular adapter 6-monodeoxy-6-monothio-β-cyclodextrin (βCD-SH); class IV, stepwise growth of a single polymer chain built from mustards and glutathione within an αHL pore containing a single thiol.

Materials and Methods

αHL Pores. The α-hemolysin mutant pores (T117C-D8RL3)$_1$(WT)$_6$, (T117C-D8RL3)$_7$ and (M113F)$_7$ (WT background) were expressed, assembled, and purified as previously described.[44] Cysteine-containing proteins were stored in 2 mM DTT, which was highly diluted when the protein was added to the bilayer apparatus (see below). RL3 is identical to the WT αHL polypeptide at the amino acid level, but the gene contains six silent mutations that aid cassette mutagenesis in the region encoding the stem domain (Cheley, S. unpublished work).

Chemicals. Reagents were obtained as follows: 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids), pentane (JT Baker), hexadecane (99+%, Sigma-Aldrich), 3-(cyclohexylamino)-1-propanesulfonic acid, CAPS (99%, Aldrich), 6-monodeoxy-6-monothio-β-cyclodextrin (99%, Cyclolabs Budapest, Hungary), concentrated HCl (analytical reagent grade, Fisher Scientific), sodium chloride (99%, Sigma-Aldrich), quinacrine mustard dihydrochloride (90%, Sigma-Aldrich), mechlorethamine hydrochloride (98%, Sigma-Aldrich), melphalan (Sigma-Aldrich), *N*-benzyl-*N,N*-di(2-chloroethyl)amine (95%, Apollo Scientific), reduced L-glutathione (>99%, Sigma-Aldrich).

Single-Channel Current Recording. A bilayer of 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids) was formed on an aperture 100−150 μm in diameter in a polycarbonate film (20-μm thickness from Goodfellow, Malvern, PA) that divided a planar bilayer chamber into two compartments, cis and trans.[26] Both compartments contained 1 mL of buffer. Engineered heptameric αHL pores were added to the cis compartment, which was connected to ground. Mustards dissolved in methanol were added to the trans compartment, which was connected to the head-stage of the amplifier. All experiments were carried out at ± 100 mV, in 20 mM CAPS, 2 M NaCl, pH 10.5 (titrated with 1.0 M NaOH), at 22.5 ± 2 °C, unless otherwise stated. Freshly thawed aliquots of protein and fresh aliquots of mustard solutions in methanol were used each day. Currents were recorded with a patch clamp amplifier (Axopatch 200B; Axon instruments, Foster City, CA), low pass filtered with a built-in 4-pole Bessel filter at 10 kHz, and sampled at 20 kHz by a computer equipped with a Digidata 1200 A/D converter (Axon instruments).

Data Analysis. Current traces were analyzed with pClamp 9.0 software (Axon Instruments). Events were detected using the Event Detection feature, and used to construct amplitude and dwell time histograms. Excel (Microsoft) was used for curve fitting and graph presentation.

Results

Detection of Mustards with an αHL Pore Containing a Single Thiol (Class I Reaction). The chloroethyl group of mustards reacts with thiol groups under basic conditions at room temperature.[45,46] In the present study, we limited our experiments to nitrogen mustards. When a single pore, (T117C-D8RL3)$_1$(WT)$_6$, containing a single cysteine side-chain projecting into the lumen at position 117, was introduced into the lipid bilayer from the cis chamber, we measured an ionic current of 125 ± 10 pA (+100 mV, 20 mM CAPS, pH 10.5, 2 M NaCl; number of bilayers examined, $n = 12$). To observe a reaction with the pore, mustards in methanol solution were added into the trans chamber (final concentration, 50 μM) while the potential was held at +100 mV. After a period ranging from 2 to 10 min, a current reduction was observed for all the four mustards tested (Figure 1F). In the case of the small molecule mechlorethamine, a current reduction of 3.0 ± 0.5 pA ($n = 6$) was observed at the reaction point and the current level became permanently locked into this lower conductance state (Figure 2A). No recovery of the current from the lower value to that of the unreacted pore was observed during a total recording period of 6 h (over six experiments). In the case of the larger molecule melphalan, a larger current reduction (36 ± 4 pA, $n = 3$) was observed when the reaction occurred and the current again remained permanently at the reduced level (Supporting Information, Figure S1). Interestingly, for *N*-benzyl-*N,N*-di(2-chloroethyl)amine and quinacrine mustard, after the reaction occurred, we observed characteristic substates at both positive and negative potentials (Figures 2B−D, S2). These signals are presumably due to the movement of the mustards attached to the cysteine side chains inside the lumen of the αHL pore. These distinctive current signatures are useful in distinguishing different mustards.

Detection of Mustards with an αHL Pore Containing Multiple Thiols (Class II Reaction). To observe multiple reactions of an analyte inside a single protein pore, and thereby ensure more secure detection, we made a homoheptameric αHL pore (T117C-D8RL3)$_7$ with seven cysteine residues at position 117. When a single (T117C-D8RL3)$_7$ pore was introduced into

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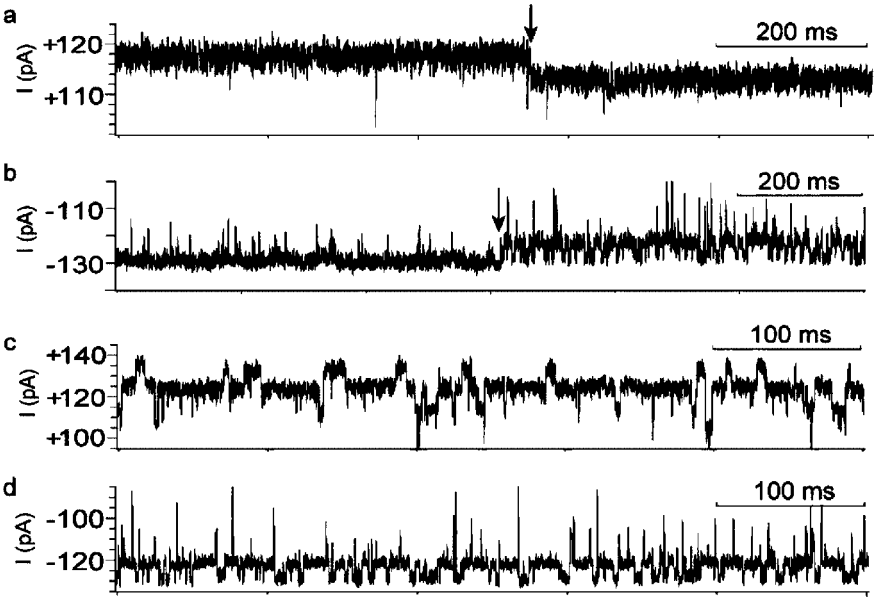

Figure 2. Detection of mustards with an engineered αHL pore containing a single cysteine residue. (A) Single-channel current trace of $(T117C\text{-}D8RL3)_1(WT)_6$ with mechlorethamine (50 μM) in the trans chamber in 20 mM CAPS, pH 10.5, 2 M NaCl, at +100 mV. The reaction point is marked by an arrow and was at 3.0 min in this case. The mean time to reaction was 2.5 ± 0.5 min (n = 3). (B) Single-channel current trace of $(T117C\text{-}D8RL3)_1(WT)_6$ with *N*-benzyl-*N,N*-di(2-chloroethyl)amine (50 μM) in the trans chamber in 20 mM CAPS, pH 10.5, 2 M NaCl, at −100 mV. The reaction point is marked by an arrow and was at 10.0 min in this case. The mean time to reaction was 10 ± 3 min (n = 3). (C) Characteristic current trace at +100 mV of the $(T117C\text{-}D8RL3)_1(WT)_6$ pore after reaction with *N*-benzyl-*N,N*-di(2-chloroethyl)amine. (D) Characteristic current trace at −100 mV of the $(T117C\text{-}D8RL3)_1(WT)_6$ pore after reaction with *N*-benzyl-*N,N*-di(2-chloroethyl)amine.

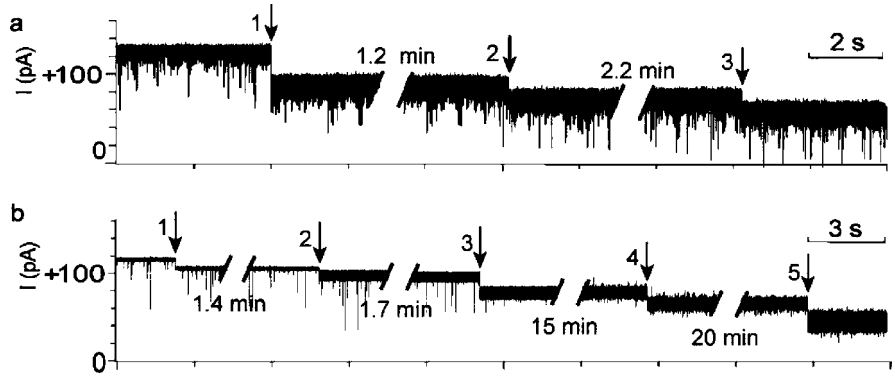

Figure 3. Detection of mustards with an engineered αHL pore containing multiple cysteine residues. (A) Single-channel current trace of $(T117C\text{-}D8RL3)_7$ with *N*-benzyl-*N,N*-di(2-chloroethyl)amine (50 μM) in the trans chamber in 20 mM CAPS, pH 10.5, 2 M NaCl, at +100 mV. The reaction points are marked by arrows. (B) Single-channel current trace of $(T117C\text{-}D8RL3)_7$ with mechlorethamine (50 μM) in the trans chamber in 20 mM CAPS, pH 10.5, 2 M NaCl, at +100 mV. The reaction points are marked by arrows.

the lipid bilayer from the cis chamber, we measured an ionic current of 120 ± 8 pA (+100 mV, 20 mM CAPS, pH 10.5, 2 M NaCl, n = 6). Mustards in methanol solution were added into the trans chamber (final concentration, 50 μM) and the potential was held at +100 mV. For *N*-benzyl-*N,N*-di(2-chloroethyl)amine, the first reaction occurred within 10 min of the addition (8 ± 2 min, n = 3). A second reaction occurred 1.2 ± 0.3 min (n = 3) after the first one, and 2.0 ± 0.5 min (n = 3) later a third reaction occurred (Figure 3A). The recording was continued for another 30 min, and no more reaction steps were observed (n = 3). For mechlorethamine, the first reaction occurred 3.5 ± 1.0 min (n = 3) after addition. A second reaction occurred 1.5 ± 0.5 min (n = 3) later, and then at 1.9 ± 0.5 min a third reaction occurred. There was a relatively long time gap (15 ± 3 min, n = 3) before a fourth reaction was observed. After another long gap (18 ± 5 min, n = 3), a fifth reaction occurred (Figure 3B). The recordings were continued for another 35 min, and no further reactions were observed (n = 3).

Detection of Mustards with an αHL Pore Containing the Noncovalent Molecular Adapter βCD-SH (Class III Reaction). When a single $(M113F)_7$ pore was introduced into the lipid bilayer from the cis chamber, we measured an ionic current of 105 ± 10 pA (+100 mV, 20 mM CAPS, pH 10.5, 2 M NaCl, n = 3) (level 1, Figure 4A). After the noncovalent molecular adapter 6-monodeoxy-6-monothio-β-cyclodextrin (βCD-SH) was introduced into the trans chamber (50 μM), we observed reversible binding events (τ_{off} = 8.5 ± 2.5 s) during which the current level was 25 ± 2 pA (level 2, Figure 4A). Mechlorethamine in methanol (50 μM) was then added into the cis chamber and an even lower current level was observed (level 3, Figure 4A). The initial transient events (τ_{off} = 6.0 ± 0.3 ms) were attributed to noncovalent binding of mechlorethamine to βCD-SH lodged inside the lumen of the $(M113F)_7$ pore. Once mechlorethamine reacted with the cyclodextrin, the current level dropped from level 2 to level 3 and remained at this level until the covalent βCD adduct (βCD-S-M) dissociated from the αHL

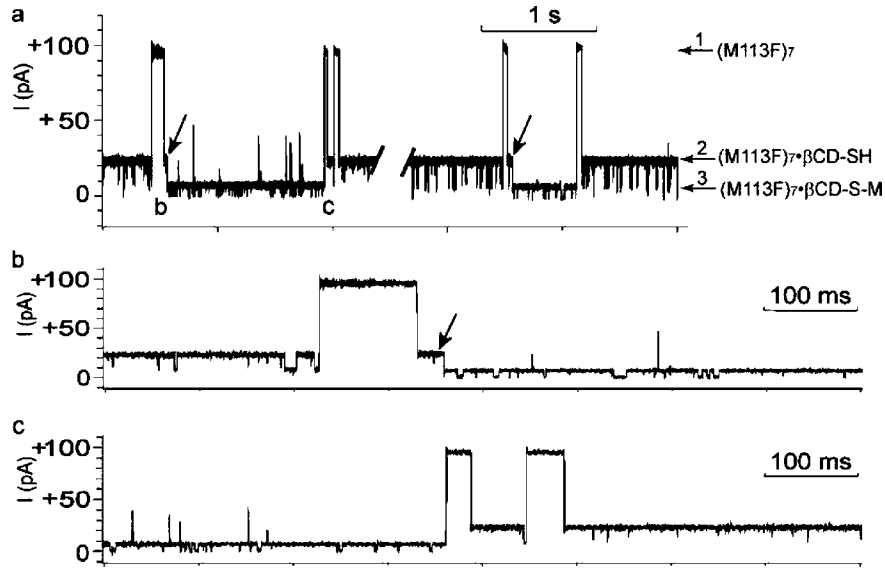

Figure 4. Detection of mustards with an engineered αHL pore containing the noncovalent molecular adapter 6-monodeoxy-6-monothio-β-cyclodextrin (βCD-SH), which contributes a reactive thiol group. (A) Single-channel current trace of $(M113F)_7$ and βCD-SH with mechlorethamine (50 μM) in the cis chamber in 20 mM CAPS, pH 10.5, 2 M NaCl, at +100 mV. Level 1 represents the open pore current level. Level 2 represents the current level when βCD-SH is bound. Level 3 represents $(M113F)_7$•βCD-SH after mechlorethamine has bound or reacted. The reaction points are marked by arrows. The beginning and end of the first reaction cycle are marked as "b" and "c", respectively, and are reproduced with expanded timescales in panels B and C. (B) Binding of βCD-SH to $(M113F)_7$ and its subsequent reaction in situ with mechlorethamine to form the adduct βCD-S-M. (C) Dissociation of the adduct βCD-S-M from $(M113F)_7$.

pore (Figures 4B,C, S3). After the dissociation of βCD-S-M, the current level jumped directly to that of the empty $(M113F)_7$ pore and the reaction cycle continued.

Stepwise Growth of a Single Polymer Chain Built from Mustard Molecules and Glutathione within an αHL Pore Containing a Single Thiol (Class IV Reaction). Mustards have two reactive chloroethyl groups. After one of the two chloroethyl groups has reacted with a thiol group within the αHL pore, the other is still available to react with free thiol groups or reactive primary amino groups. A single $(T117C\text{-}D8RL3)_1(WT)_6$ pore was introduced into the lipid bilayer from the cis chamber, and mustards in methanol were added to the trans chamber (50 μM) and glutathione to the cis chamber (50 μM). For mechlorethamine, the first reaction, with the thiol group at position 117 within the αHL pore, occurred after 2.5 ± 0.5 min with a current reduction of 4.0 ± 0.5 pA (n = 3). Very soon afterward (0.4 ± 0.1 min), a second reaction was observed with a further current reduction of 15.0 ± 1.0 pA (n = 3). Given the fact that thiol groups are much more reactive than amino groups under these conditions, this reaction was very likely between the remaining chloroethyl group on the attached mechlorethamine molecule and the thiol group of glutathione. After 1.2 ± 0.4 min, a third reaction occurred with a current reduction of 12.0 ± 1.0 pA (n = 3). This reaction was most likely between the primary amino group of the glutathione and one of the two chloroethyl groups of a second mechlorethamine molecule. Subsequently, the same chemistry was repeated and three more reactions steps were recorded (Figure 5). After the sixth reaction step, we continued to record for another 30 min and no more steps were observed. For *N*-benzyl-*N,N*-di(2-chloroethyl)amine, similar current traces were recorded with only five reaction steps (Figure S4). We also carried out control experiments without adding glutathione (cis) (Figure 5B) or mustard (trans) (Figure 5C). When no glutathione was added in the cis chamber, only one reaction step between the mustard and the protein pore was observed. When no mustard was added in the trans chamber, we did not observe any reaction.

Control Experiment with $(WT)_7$. To confirm that the reactions observed were between mustards and the thiol groups within the lumen of the αHL pore, we carried out control experiments with $(WT)_7$ under the same conditions. When a single $(WT)_7$ pore was introduced into the lipid bilayer from the cis chamber, we measured an ionic current of 125 ± 10 pA (+100 mV, 20 mM CAPS, pH 10.5, 2 M NaCl, n = 3). Mustards in methanol solution were added to the trans chamber (final concentration, 50 μM) and the potential was held at +100 mV. No reaction was observed for all four mustards during a 40 min recording for each (data not shown). These results rule out the possibility of reactions between mustards and the amino acid side chains inside the β barrel of αHL.

Discussion

Because of the destructive properties of mustards and the lack of effective antidotes, some experts have classified mustards as the "most significant of chemical warfare agents".[47] Although mustard agents are regulated under the 1993 Chemical Weapons Convention, they remain potential threats. For example, mustards might be used in terrorist attacks, because of their ready availability. Therefore, the quick and convenient detection of mustards is a critical issue.

In the present work, we detected various mustards on the basis of their reactivity by using engineered αHL protein pores as sensor elements. We describe four classes of reactions (I−IV) for detecting mustards. In the class I reaction, the mustard reacts with a single thiol group within the lumen of the αHL pore, which in the example examined is at position-117. The magnitude of the current reduction upon reaction is related to the size of the mustard molecule (for mechlorethamine and melphalan, Figures 2A, S1). For *N*-benzyl-*N,N*-di(2-chloroethyl)amine and quinacrine mustard, as well as the current drop, distinctive current signatures were observed after the mustards

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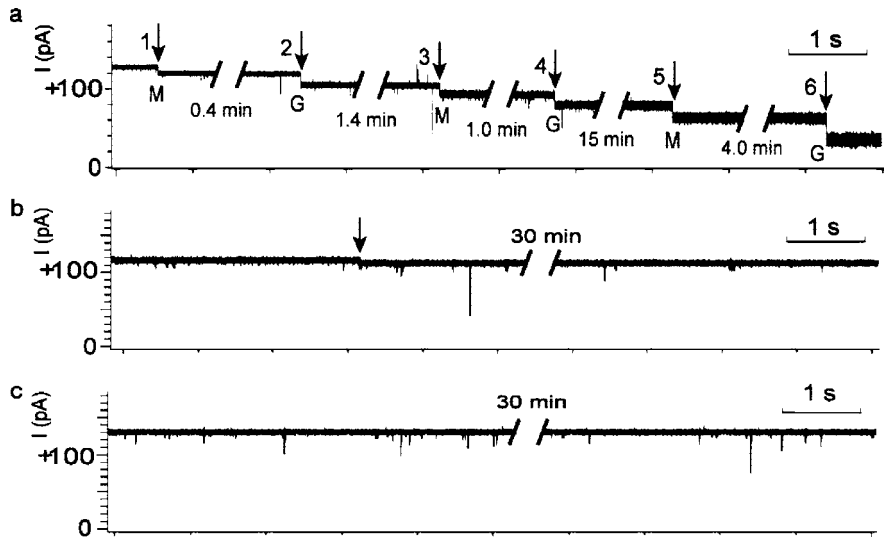

Figure 5. Stepwise growth of a single polymer chain incorporating mechlorethamine and glutathione within an αHL pore containing a single cysteine residue. (A) Single-channel recording of the reaction between mechlorethamine (50 μM, trans) and $(T117C\text{-}D8RL3)_1(WT)_6$ and subsequent chain elongation reactions by sequential reactions with glutathione (100 μM, cis) and additional mechlorethamine molecules (trans) in 20 mM CAPS, pH 10.5, 2 M NaCl, at +100 mV. The reaction points are marked by arrows and numbered in order. M and G show where mustard molecules and glutathione, respectively, are proposed to react with the elongating chain. (B) Single-channel current trace of $(T117C\text{-}D8RL3)_1(WT)_6$ with mechlorethamine (50 μM) in the trans chamber under the same conditions but without glutathione in the cis chamber. The reaction point is marked by an arrow. (C) Single-channel current trace of $(T117C\text{-}D8RL3)_1(WT)_6$ with glutathione (100 μM) in the cis chamber under the same conditions but without mechlorethamine in the trans chamber.

reacted with the thiol group (Figures 2B−D, S2). However, the disadvantage of the class I reaction is that there is only one reaction step and therefore detection might not be considered conclusive, especially if the reacted mustard does not provide a distinctive current signature.

In the class II reaction, cysteine side chains provide multiple reactive thiols within the lumen of the pore. In the example described, there are potentially seven reaction steps. In the case of *N*-benzyl-*N,N*-di(2-chloroethyl)amine, three steps were observed during a 50-min recording period (Figure 3A). For mechlorethamine, five reactions were observed in 60 min, suggesting that there is steric hindrance to reaction in the case of the larger molecule (Figure 3B). In both cases, the current trace became noisier as the reaction proceeded, presumably because the reacted mustards undergo conformational changes within the β barrel. The limitation of the class II reaction is that large mustards, such as quinacrine mustard, produce complex but irreproducible signals after reaction (data not shown). This could be due to the large number of permutations of reaction sites. Therefore, this approach is more suitable for the detection of small mustards.

We designed the class III reaction as a different means to observe multiple reactions of mustards with a single pore. In the example described here, a membrane containing a single $(M113F)_7$ pore was used. The noncovalent molecular adapter 6-monodeoxy-6-monothio-β-cyclodextrin (βCD-SH) was added to the trans chamber and mechlorethamine was added to the cis chamber. The reaction of mechlorethamine with βCD-SH could be observed while the cyclodextrin was bound to the pore (Figure 4A). However, in this scheme, the derivatized βCD can dissociate to be replaced with an unreacted βCD, which can in turn react with a mechlorethamine molecule. The reaction cycle continues allowing the observation of multiple events, giving increased confidence to the identification of the analyte. The class III reaction worked well with mechlorethamine and βCD-SH. However, for a larger molecule, *N*-benzyl-*N,N*-di(2-chloroethyl)amine, no reaction was observed under the same conditions.

In the class IV reaction, the stepwise growth of a single polymer chain built from mustard molecules and glutathione takes place within an αHL pore. In this case, multiple mustard molecules are again detected one at a time. After one of the two chloroethyl groups of a mustard has reacted with a thiol group within an αHL pore, the other one is still available to react with free thiol groups or reactive primary amino groups. Shin and colleagues had reported the growth of a single polymer chain when 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and (mercaptoethyl)ether (MEE) were presented from opposite chambers to an αHL pore with a single internal thiol.[40] We tested whether dithiol compounds, such as dithiothreitol (DTT) and MEE, would participate in polymer growth reactions with mustards. Unfortunately, bilayers become very fragile in the presence of these dithiols and mustards, and the reactions were difficult to reproduce. Therefore, we tested glutathione as a constituent of the polymer chain. In this case, the first reaction is between a mustard molecule, presented from the trans chamber, and the thiol at position-117 inside the β barrel of the pore. The reacted mustard molecule has a second chloroethyl group and therefore glutathione in the cis chamber can enter the pore and react with the immobilized mustard molecule. Because thiol groups are much more reactive than amino groups, under the conditions of the experiment, this reaction is very likely with the thiol of glutathione. The pK_a value of the 2-amino group of glutathione is ~9.46[48] and is expected to react readily with the chloroethyl groups of mustards under the prevailing conditions (20 mM CAPS, pH 10.5, 2 M NaCl).[49] So, the third reaction in the sequence is most likely between the terminal amino group of the attached glutathione molecule and a second mustard, which enters from the trans side. Subsequently, the same chemistry is repeated and more reaction steps are recorded (Figures 5, S4). For mechlorethamine, we observed up to six reaction steps in one experiment. But for *N*-benzyl-*N,N*-di(2-

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chloroethyl)amine, presumably due to its larger size, the current level dropped to only 4 pA after the fifth step and no further reaction occurred after that. In some experiments, we recorded fewer reaction steps for both mustards, presumably because a hydrolyzed product, a "hemimustard" $HOCH_2CH_2N(R)$-CH_2CH_2Cl, reacted with the growing polymer chain, terminating growth (or because the terminal chloroethyl group in the growing polymer hydrolyzed in situ). Importantly, the class IV reaction provides a means of detecting mustards that identifies them as bifunctional molecules.

In the present study, we achieved single-molecule detection of various mustards through their reactions with thiols inside the β barrel of an engineered αHL pore. Although there are several existing methods for detection of mustards, they have various limitations. Some methods, such as metal salt detection, have low sensitivity (0.75 g of mustard gas/m^2).[50] Other methods have a limited effective duration, such as detection with trained dogs and rats. Although the combined use of chromatography and mass spectrometry can detect mustards at as low as 1.0 ppb,[20] sophisticated and expensive instruments are required. The method we have developed is quick and direct. It can confirm the existence of mustards in as little as 10 min at 50 μM or lower, without capture and concentration of the sample. The sensitivity would be far greater if analyte capture were employed. With the development of new approaches for building bilayer chips[51] and for ruggedizing lipid bilayers by encapsulation,[52] it is highly likely that a robust and stable detecting system can be built in the near future.

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Supporting Information Available: Figures S1−S4. This material is available free of charge via the Internet at http://pubs.acs.org.

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