

**Single-Molecule Covalent Chemistry****Single-Molecule Covalent Chemistry with Spatially Separated Reactants\*\****Tudor Luchian,\* Seong-Ho Shin, and Hagan Bayley\**

The investigation of covalent chemistry at the single-molecule level offers several advantages including the ability to observe short-lived intermediates and the means to untangle complex reaction pathways. The available techniques of single-molecule detection have been brought to bear on single-molecule

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[\*\*] This work was supported by the Office of Naval Research, the U.S. Department of Energy, the Multidisciplinary University Research Initiative (ONR-1999), and the National Institutes of Health. We thank Sean Conlan for help with molecular modeling.

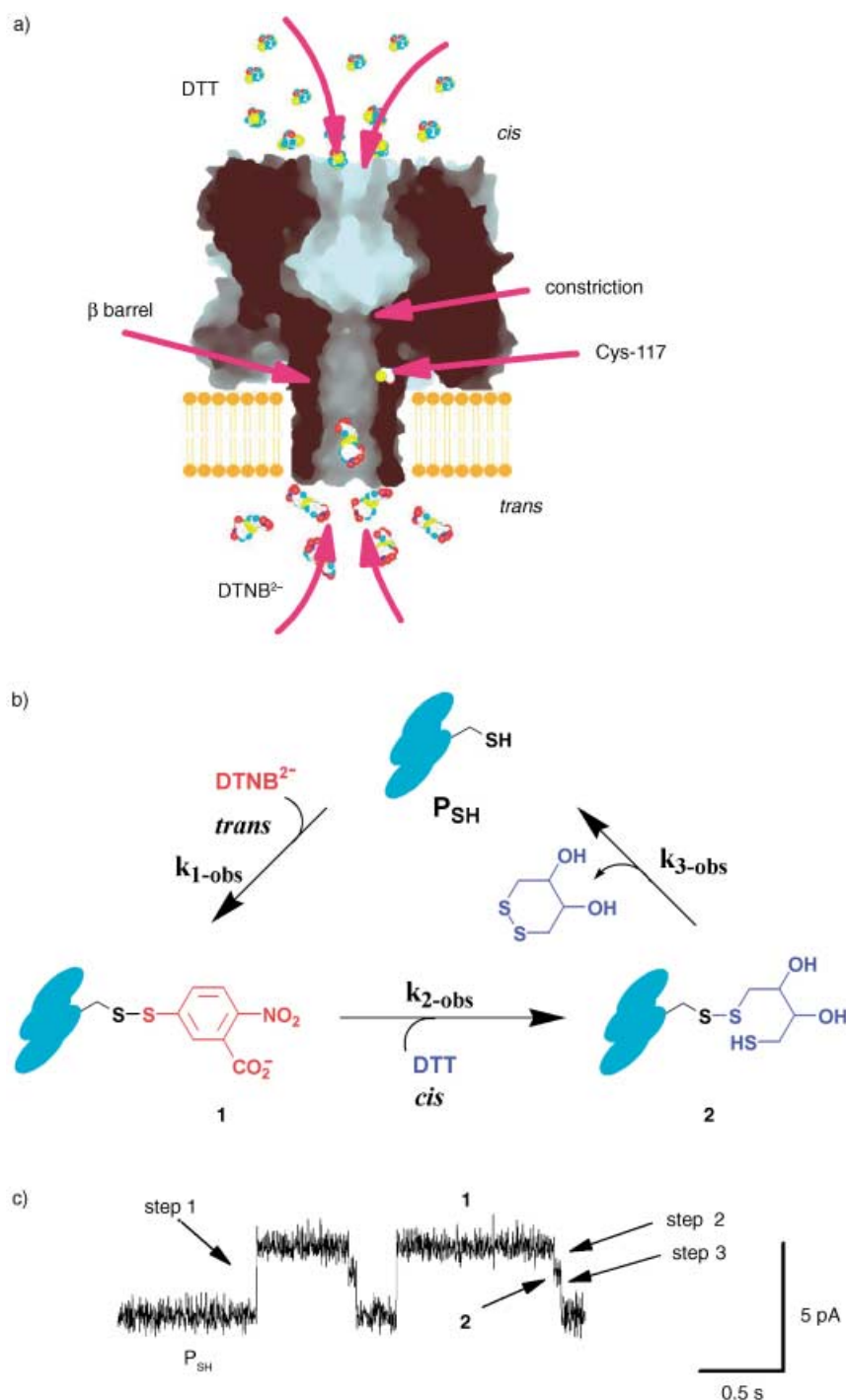
chemistry. For example, the forces required to break individual covalent bonds between polymers and surfaces have been determined,<sup>[1]</sup> and optical methods have been used to investigate single turnovers of enzymes and ribozymes by using fluorescent substrates and probes.<sup>[2–5]</sup> Herein, we report the use of electrical measurements to observe the covalent chemistry of small untagged molecules inside a protein pore, which acts as a nanoreactor. Multiple turnovers were achieved through separating two reactants by the membrane containing the pore and allowing them to diffuse into the reaction zone. We demonstrate the utility of the approach by measuring the rate constant for breakdown of the short-lived intermediate ( $\text{RSSCH}_2\text{CH}(\text{OH})\text{CH}(\text{OH})\text{CH}_2\text{SH}$ ,  $k = 250 \text{ s}^{-1}$  as the thiolate) that is generated during disulfide reduction with dithiothreitol, a reagent commonly used in protein chemistry. Our approach is applicable to much of the covalent chemistry that occurs in aqueous solution. Further, it permits the development of sensors<sup>[6]</sup> for reactive molecules: for example, those found in chemical weapons, pesticides, and various pharmaceuticals and foodstuffs.

Force measurements and optical techniques are not well adapted for examining the covalent chemistry of individual unmodified small molecules. Scanning probe techniques can be applied to molecules lying on surfaces,<sup>[7]</sup> but not to molecules in solution. An alternative approach is to use electrical measurements to observe the chemistry of molecules tethered inside or near the mouth of a protein pore. The ionic current that is carried by the pore in an applied potential is highly sensitive to the size, shape, and polarity of the reactants. Hence, molecular rearrangements and bond cleavage can be monitored. The approach works with molecules of a few hundred daltons, does not require a tag (such as a fluorescent probe) or very low reagent concentrations, and has a time resolution of a few tens of microseconds under optimal conditions. The potential of the technique was recognized by Finkelstein and co-workers, who measured the rates of single-step modifications of cysteine residues in diphtheria toxin channels by observing multiple individual reactions.<sup>[8]</sup> We recently used the approach to investigate more complex chemistry. Two short-lived intermediates, a nitronate and a carbamate, were observed after the photolysis of an amine protected with a 4,5-dimethoxy-2-nitrobenzyloxy-carbonyl group tethered inside the  $\alpha$ -hemolysin ( $\alpha$ HL) pore.<sup>[9]</sup> However, to obtain data of useful statistical significance, it was essential to perform many individual experiments. Multiple events in a single experiment were observed by Woolley and Jaikaran, who examined the reversible thermal isomerization of a carbamate group attached at the mouth of a gramicidin channel.<sup>[10]</sup> More recently, we observed reversible covalent-bond formation and cleavage between a single cysteine residue on the wall of an engineered  $\alpha$ HL pore and an organoarsenic(III) compound in solution.<sup>[11]</sup> We now show that the versatility of this single-molecule approach can be enhanced by bringing about multiple turnovers of what, in solution, would be a rapid irreversible reaction. This is achieved by separating two reactants by the membrane containing the nanoreactor.

We measured the kinetics of the reaction of a cysteine residue on the luminal wall of the  $\alpha$ HL pore with 5,5'-

dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent) to form a disulfide bond, and cleavage of that bond with dithiothreitol (DTT, threo-1,4-dimercapto-2,3-butandiol) to regenerate the cysteine residue. The short-lived intermediate in the DTT cleavage reaction, which is invisible to optical spectroscopy, was observed for the first time and the dependence of its lifetime on pH was measured. We used a heteroheptameric  $\alpha$ HL pore,  $P_{\text{SH}}$ , in which one of the seven subunits contains a cysteine residue at position 117 and the other six are cysteine-free (Figure 1a).<sup>[11]</sup> Cys 117 in the lumen of the pore reacts with DTNB in a bimolecular reaction (step 1) with rate constant  $k_{1-\text{obs}}$  to form a mixed disulfide **1**, in which the sulfur atom proximal to the protein wall is activated towards attack by free thiolate groups because the aromatic thiolate is a good leaving group (Figure 1b). Disulfide **1** reacts with DTT in a bimolecular reaction (step 2) with rate constant  $k_{2-\text{obs}}$  to form the unstable disulfide **2**, which breaks down in a unimolecular reaction (step 3) in which  $P_{\text{SH}}$  is regenerated with rate constant  $k_{3-\text{obs}}$  (Figure 1b). In this way,  $P_{\text{SH}}$  is able to undergo multiple reaction cycles. In the first configuration we tested, DTNB was present in the *trans* chamber and DTT in the *cis* chamber. In single-channel recordings, three separate current levels representing  $P_{\text{SH}}$ , **1**, and **2** were observed as predicted (Figure 1c). Intermediate **2** was assigned the structure depicted because thiols other than DTT react with **1** to form intermediates or relatively stable molecules that are distinguished by distinct current levels, and also because the breakdown of **2** has the characteristics of a unimolecular reaction with the anticipated pH-dependence (see below).

The rate of reaction of DTNB with the cysteine residue of  $P_{\text{SH}}$  was measured in the DTNB(*trans*)/DTT(*cis*) configuration. Assuming that DTT(*cis*) reverses, but does not alter the rate of reaction of  $P_{\text{SH}}$  with DTNB (see below), the rate constant can be determined by analyzing the single-channel current trace. By using  $k_{1-\text{obs}} = 1/(\tau_1[\text{DTNB}])$ , where  $\tau_1$  is the lifetime of  $P_{\text{SH}}$  (Figure 2a), we calculated  $k_{1-\text{obs}} = 4.9 \pm 0.5 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$  (based on five independent experiments) in 2 M KCl, 30 mM 3-(4-morpholinyl)-1-propanesulfonic acid (MOPS), 100  $\mu\text{M}$  ethylenediaminetetraacetic acid (EDTA), pH 8.5, at an applied potential of  $-50 \text{ mV}$ , with 50  $\mu\text{M}$  DTT(*cis*). The analysis assumes that the effective concentration of DTNB in the  $\beta$  barrel is the same as the concentration in the *trans* chamber. The analysis also assumes that although DTT(*cis*) can reverse the reaction with DTNB(*trans*) leading to multiple turnovers, it does not compete with the internal thiol (Cys117) by reacting with DTNB in the barrel.<sup>[12]</sup> This lack of an effect of low concentrations of DTT(*cis*) on the rate of reaction with DTNB(*trans*) was demonstrated experimentally (Figure 2b).<sup>[13]</sup> Because the rate of reaction of **1** with DTT might have been affected by a reduced rate of diffusion of DTT(*cis*) through the central constriction of the pore (Figure 1a), we used the configuration DTNB(*cis*)/DTT(*trans*) to determine  $k_{2-\text{obs}}$ . By using  $k_{2-\text{obs}} = 1/(\tau_2[\text{DTT}])$ , we found  $k_{2-\text{obs}} = 1.1 \pm 0.1 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$  in 2 M KCl, 30 mM MOPS, 100  $\mu\text{M}$  EDTA, pH 8.5, at an applied potential of  $-50 \text{ mV}$ , in the presence of 50  $\mu\text{M}$  DTNB(*cis*) (Figure 2c). As required, we demonstrated that low concentrations of DTNB(*cis*) do not affect the value of  $k_{2-\text{obs}}$  (Figure 2d).



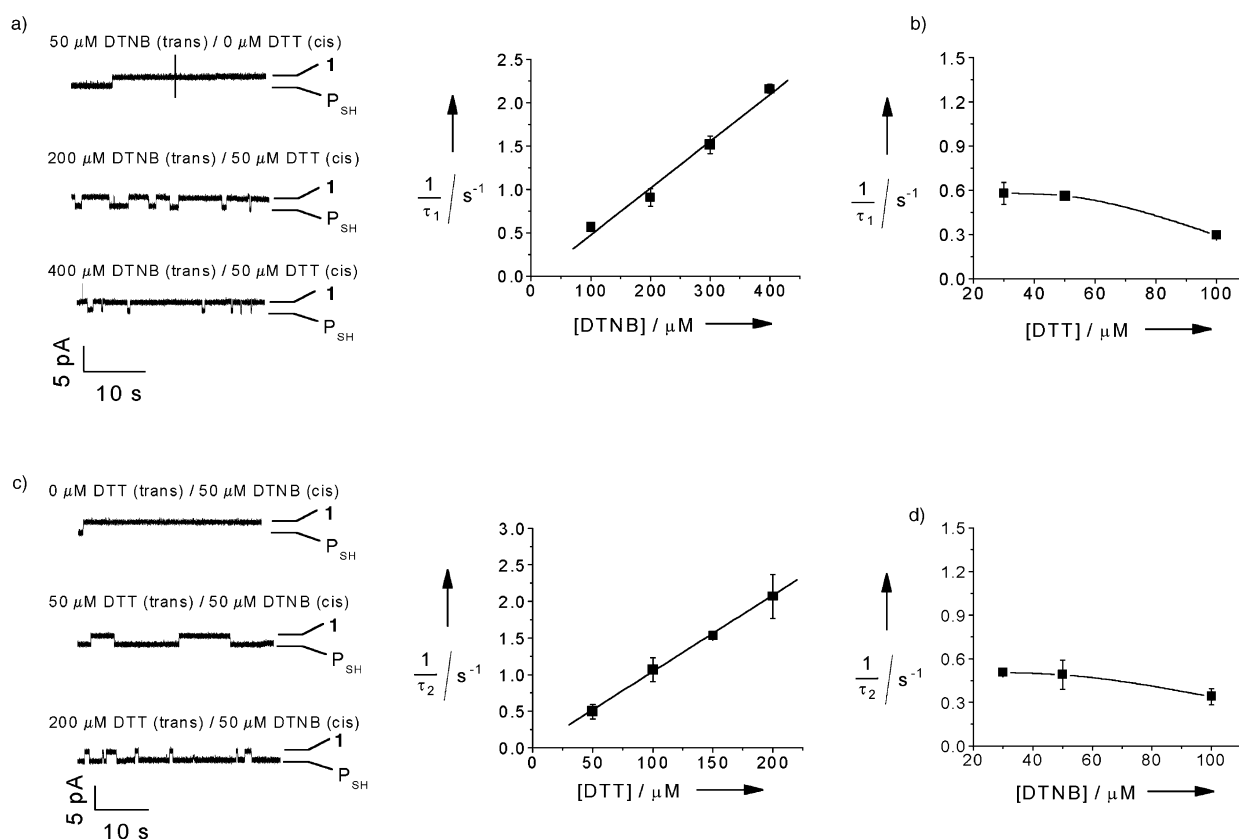
**Figure 1.** Single-molecule chemistry with spatially separated reactants. a) The orientation of the  $\alpha$ HL pore ( $P_{SH}$ ) and disposition of the reactants with respect to the bilayer are shown. The position of Cys 117 in the  $\beta$  barrel is indicated; this residue is present in one of the seven subunits of the  $P_{SH}$  heteromer. Small molecules are drawn to scale. The *cis* chamber of the bilayer apparatus was at zero potential. All experiments were conducted at an applied potential of  $-50$  mV (*trans*) and positive current represents the flow of positive charge from *trans* to *cis*. The orientation of the pore was the same in all the experiments described here, while the *cis* or *trans* disposition of the reactants was varied. b) Details of the chemical cycle. Cys 117 reacts with DTNB in a bimolecular reaction, with rate constant  $k_{1-obs}$ , to form the mixed disulfide **1**. Disulfide **1** then reacts with DTT in a second bimolecular reaction (rate constant  $k_{2-obs}$ ) to form the unstable disulfide **2**, which breaks down in a unimolecular reaction (rate constant  $k_{3-obs}$ ) in which  $P_{SH}$  is regenerated. D,L-DTT was used throughout this work. c) Single-channel current recording. Two successive chemical cycles are shown; the levels representing  $P_{SH}$ , **1**, and **2** are indicated. See text for reaction conditions.

The breakdown of the unstable mixed disulfide **2** is a unimolecular reaction and therefore  $k_{3-obs} = 1/\tau_3$ , where  $\tau_3$  is the lifetime of the intermediate determined from current recordings.<sup>[14]</sup> By using the DTNB(*trans*)/DTT(*cis*) configuration, we found that  $k_{3-obs} = 23 \pm 1 \text{ s}^{-1}$  (six experiments) in 2 M KCl, 30 mM MOPS, 100  $\mu$ M EDTA, pH 8.5, at an applied potential of  $-50$  mV. Because the reaction is unimolecular,  $k_3$  should be independent of the concentrations of DTT and DTNB, and whether or not they are applied from the *trans* or *cis* sides of the bilayer; this was found to be the case (e.g., Figure 3 a).

Intermediates with structures similar to **2** have not been observed directly in the reactions of disulfides with DTT. We examined the lifetime of the intermediate at various pH values and thereby derived  $k_{3-obs}$  as a function of pH value (Figure 3 b). The data could be fitted (Figure 3 b) to the equation:  $k_{3-obs} = (k_3 K_a)/(K_a + [H^+])$ . This result suggests that, as expected, the reactive form of **2** is the thiolate. The data yield a  $pK_a$  value of  $9.5 \pm 0.2$  and  $k_3 = 250 \pm 80 \text{ s}^{-1}$  in the deprotonated form. By comparison, the first  $pK_a$  value of DTT is 9.2, while that of mercaptoethanol is 9.5.<sup>[15]</sup>

Relatively little covalent chemistry of small molecules has been examined at the single-molecule level. The use of engineered protein pores to provide a nanoreactor for this purpose is a promising approach. Here, we have advanced previous efforts by using a system in which two reactants are separated by the lipid bilayer containing the engineered pore. In this way, multiple turnovers can be observed for a reaction that would be "short-circuited" in bulk solution by direct reaction of the two components. Vectorial chemistry in bilayer systems has been observed previously, and can be traced back to the ideas of Mitchell.<sup>[16,17]</sup> Despite this and related work, we know of no previous examples of single-molecule covalent chemistry utilizing spatially separated reactants.

The rates of reaction of a wide variety of thiols with DTNB have been investigated in solution.<sup>[15,18]</sup> The reactive species in both the initial reaction and the subsequent reaction of the intermediate mixed disulfide (equivalent to  $k_1$  and  $k_2$  in our case) are thiolates. Therefore, the observed rates



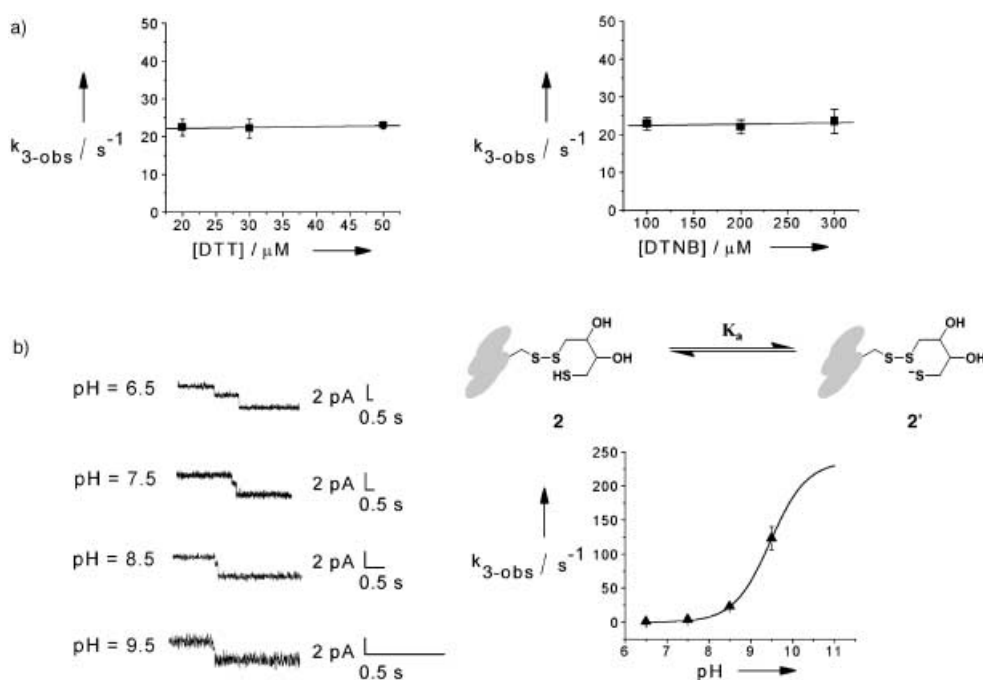
**Figure 2.** Bimolecular reactions within the P<sub>SH</sub> pore. a) Dependence of  $1/\tau_1$  on [DTNB]<sub>trans</sub>, where  $\tau_1$  is the interval between step 3 and step 1, that is, the interval before the reaction of P<sub>SH</sub> with DTNB.  $k_{1-obs}$  was obtained from the slope of the straight-line fit. The conditions were 2 M KCl, 30 mM MOPS, 100 μM EDTA, pH 8.5, at -50 mV, with 50 μM DTT (cis). Typical traces are shown on the left. The current level corresponding to the single unmodified pore (P<sub>SH</sub>) was  $-82.2 \pm 2.1$  pA. b) Low concentrations of DTT (cis) do not affect  $1/\tau_1$  for the reaction of P<sub>SH</sub> with DTNB (trans). The conditions were as in a) with [DTNB]<sub>trans</sub> = 100 μM. c) Dependence of  $1/\tau_2$  on [DTT]<sub>trans</sub>, where  $\tau_2$  is the interval between step 1 and step 2, that is, the interval before the reaction of 1 with DTT.  $k_{2-obs}$  was obtained from the slope of the straight-line fit. The conditions were 2 M KCl, 30 mM MOPS, 100 μM EDTA, pH 8.5, at -50 mV, with 50 μM DTNB (cis). Typical traces are shown on the left. d) Low concentrations of DTNB (cis) do not affect  $1/\tau_2$  for the reaction of 1 with DTT (trans). The conditions were as in c) with [DTT]<sub>trans</sub> = 50 μM.

depend on pH value according to the relationship  $k_{obs} = (kK_a)/(K_a + [H^+])$ , where  $k$  is the bimolecular rate constant for attack of the thiolate on the disulfide. For reactions with DTNB, values of  $k_1$  are correlated with the pK<sub>a</sub> values of the thiols with a Brønsted coefficient,  $\beta_{nuc}$ , of  $\approx 0.5$ , which means that the thiolates formed from thiols with higher pK<sub>a</sub> values are more reactive.<sup>[15,18]</sup> According to these studies, the value of  $k_{1-obs}$  for an unperturbed cysteine residue in a peptide (pK<sub>a</sub> = 8.7 for glutathione)<sup>[19]</sup> should be  $k_{1-obs} \approx 4 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$  at pH 8.5 (calculated from  $k_1 = 10^5 \text{ M}^{-1} \text{ s}^{-1}$ ).<sup>[15,18,20]</sup> The value we obtain is about ten times lower. DTNB carries a charge of  $z = -2$ ; it is expected to be driven into the pore at -50 mV and react more rapidly than it does in bulk solution. Therefore, the observed rate probably reflects several small perturbations of the kinetics, which will require additional work to resolve. For example, steric hindrance near the protein surface may occur, or Cys117 may have a higher than expected pK<sub>a</sub> value (although the residue has no charged neighbors). The dependence of  $k_2$  on the structure of the thiol has been less thoroughly investigated. Nevertheless, it is clear that the values of  $k_2$  are lower than those for  $k_1$  and in the range  $k_2 = 10^4 \text{ M}^{-1} \text{ s}^{-1}$ .<sup>[15,18]</sup> Therefore, in our case, we predict

$k_{2-obs} \approx 1.7 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ , by using  $k_{2-obs} = (k_2 K_a)/(K_a + [H^+])$ , pK<sub>a</sub> = 9.2 (for DTT) and pH 8.5. The observed value of  $1.1 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$  is about six times higher.

No kinetic constants for a reaction related to step 3 ( $k_3$ ) are available from the literature, although the reaction is clearly fast.<sup>[15]</sup> According to our data, the rate of breakdown of the deprotonated intermediate is  $250 \text{ s}^{-1}$ . By comparison, the bimolecular rate constant for the reaction of the thiolate of 2-mercaptoethanol with oxidized glutathione is  $57 \text{ M}^{-1} \text{ s}^{-1}$ .<sup>[19]</sup> Therefore the effective concentration of the thiolate in **2** is about 4 M. These findings demonstrate how short-lived intermediates that would be invisible kinetically in an ensemble experiment can be characterized by using single-molecule chemistry.

The present approach rivals other advanced single-molecule methodologies.<sup>[5]</sup> For example, high reagent concentrations are tolerated and a bulky fluorophore is not required. We believe the approach can be expanded to examine much of the varied chemistry that occurs in aqueous solutions.<sup>[21,22]</sup> From one point of view, we might mimic, as closely as possible, reaction conditions in solution. Our results indicate that the environment inside the  $\beta$  barrel of the  $\alpha$ HL



**Figure 3.** Rate of breakdown of the intermediate **2**. a) The lifetime of **2** is independent of the concentrations of the reactants DTT and DTNB. Left:  $k_{3-obs}$  as a function of  $[DTT]_{cis}$ . The conditions were as described in the legend of Figure 2 with  $[DTNB]_{trans} = 50 \mu M$ ; right:  $k_{3-obs}$  as a function of  $[DTNB]_{trans}$ . The conditions were as described in the legend of Figure 2 with  $[DTT]_{cis} = 50 \mu M$ . b) pH-Dependence of the rate of breakdown of the intermediate **2**. Left: typical current traces at different pH values. The conditions were as before with  $[DTNB]_{trans} = 50 \mu M$  and  $[DTT]_{cis} = 50 \mu M$ ; right:  $k_{3-obs}$  as a function of pH value. The data were fitted to  $k_{3-obs} = (k_3 K_a) / (K_a + [H^+])$ .

pore at least approximates the conditions in solution. More might be done in this regard by replacing charged and hydrophobic side chains in the barrel with uncharged hydrophilic side chains such as Ser, Thr, Asn, and Gln. Surface charge effects can also be minimized by working at high salt concentrations, as we have done here. Greater attention might be paid to the applied potential, which can attract or repel charged and uncharged substrates, the latter through electro-osmotic flow.<sup>[23]</sup> From a second viewpoint, the approach can be used to study the effect of the protein environment on chemistry. In this case, residues that bind substrates or take part in the chemistry can be introduced onto the wall of the  $\beta$  barrel by mutagenesis, targeted chemical modification, or non-natural amino acid substitution. Catalysis in a synthetic barrel lined with histidine residues has been achieved by Matile and co-workers.<sup>[24,25]</sup> With the precise and diverse substitutions made feasible by protein engineering, it may be possible to improve turnover and even couple catalysis to transmembrane transport.

As with most other single-molecule techniques, a potential weakness of the approach described here is the inability to assign structures to intermediates by using spectroscopy. It would be dangerous to attribute the subtle changes in pore conductance to specific alterations in structure, such as the loss of a bulky group, and we have avoided doing so. Until now, our studies have involved chemistry where there is little doubt about the general reaction pathway. However, if unknown chemistry is to be investigated, it will be necessary to resort to non-spectroscopic approaches, well known in mechanistic organic chemistry to clarify the nature of reaction

intermediates. They include the generation of intermediates by alternative routes, the trapping or diversion of intermediates,<sup>[14]</sup> the dependence of the lifetimes of intermediates on pH value (Figure 3b), the effects of temperature on reaction kinetics,<sup>[10,26]</sup> the use of isotope effects, and the investigation of substituents, for example, through linear free-energy relationships. In many cases, it may be necessary to supplement single-molecule studies with ensemble measurements. Indeed, the two approaches can be seen as complementary.

### Experimental Section

$P_{SH}$  is a heteromeric  $\alpha HL$  pore,  $(RL2)_6(T117C-D8)_1$ , containing a single cysteine residue at position 117 of one of the seven subunits.  $P_{SH}$  was prepared as described,<sup>[11]</sup> except for the buffer that was used to elute the protein after preparative SDS-polyacrylamide gel electrophoresis, which was changed to 10 mM tris(hydroxymethyl)-aminoethane hydrochloride (Tris-HCl), 5 mM DTT, 1 mM EDTA, pH 7.5. The high concentration of DTT ensured that Cys117 in  $P_{SH}$  remained reduced during storage.

Single-channel recordings were carried out in a planar bilayer apparatus as previously described.<sup>[11]</sup> Both chambers contained 2 M KCl, 30 mM MOPS, 100  $\mu M$  EDTA, titrated to pH 8.5 with aqueous KOH. For experiments at pH 9.5, MOPS was replaced by 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS). A single pore was allowed to insert into the bilayer after the addition of a dilute solution of the  $P_{SH}$  to the *cis* chamber to give a final concentration of  $\approx 0.5$  ng protein per mL. The DTT from the storage buffer was diluted more than 10000 times in the chamber. The electrical current was filtered with a low-pass Bessel filter (80 dB/decade) with a corner frequency of 1 kHz and then digitized with a DigiData 1320 A/D converter (Axon Instruments) at a sampling frequency of 5 kHz. Data

samples were stored on the hard disk of a computer. For further analysis and display, current traces were filtered digitally at 100 Hz. When fast transitions were observed (e.g., step 3 at high pH values), the filter was set at 5 kHz and the sampling frequency to 10 kHz. Event data files used to determine reaction rates were constructed by manual inspection of the experimental traces with Clampfit 9.0 software (Axon Instruments). The mean lifetime ( $\tau_1$ ,  $\tau_2$ ,  $\tau_3$ ) of each species ( $P_{SH}$ , **1**, or **2**) was used to calculate rate constants ( $k_1$ ,  $k_2$ ,  $k_3$ ) as described in the text. At least 40 events were used to calculate each lifetime under each set of conditions. With the exception of some events used to determine  $k_3$ , single-channel recordings were used exclusively. Because  $k_3$  is the rate constant of a unimolecular reaction, events observed in membranes containing two or three channels could be added to the data. The processed data were plotted using Origin 7.0 (Microcal Software). Individual values are presented as the mean  $\pm$  standard deviation.

Prewashed samples of solid D,L-DTT (Gold Biotechnology) were prepared and stored at  $-20^\circ\text{C}$ . At the time of the experiment, buffer was added to the tube to generate a stock of 1M DTT, which was kept on ice. Fresh stock solutions of DTT were prepared every two hours. DTNB (Aldrich) was dissolved in 200 mM sodium phosphate, pH 8.5, at 100 mM. Fresh DTNB stocks were made daily as it decomposes in basic solution.

Received: March 3, 2003

Revised: June 12, 2003 [Z51313]

**Keywords:** kinetics · nanoreactors · protein pores · single-molecule studies · thiols

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- [12] This is reasonable because the effective concentration of the thiol provided by Cys117 in the barrel (volume  $\approx 14000 \text{ \AA}^3$ , as determined with the program HOLE)<sup>[27]</sup> is  $\approx 0.12 \text{ M}$ . In single-molecule terms, the probability of DTT being in the barrel, at a time when DTNB is also in the barrel, is  $p \approx 3.6 \times 10^{-4}$  ( $p \approx V_{\text{barrel}}/V_{\text{DTT}}$ ; where  $V_{\text{barrel}}$  is the volume inside the barrel and  $V_{\text{DTT}}$  is the volume of solution occupied by one DTT molecule, based on the external concentration).
- [13] At high concentrations of DTT(*cis*), the reduction in rate most likely arises from depletion of DTNB(*trans*) by reaction with DTT that has reached the *trans* unstirred layer.
- [14] To check whether the intermediate **2** was indeed formed from DTT, we carried out several controls. For example, in the absence of DTT, the reaction proceeded through the first step, but remained there indefinitely ( $> 60 \text{ s}$ ; Figure 2a, upper trace). Further, when DTT was replaced with  $50 \text{ \mu M}$   $\beta$ -mercaptoethanol ( $\beta\text{ME}$ , *cis*), a monothiol, the reaction took a different course. The first step with DTNB ( $50 \text{ \mu M}$ , *trans*) was observed as before, but it was followed by a step to a level where the system remained for a prolonged period ( $> 60 \text{ s}$ ), which was distinct from the level representing **2**. We interpret the sequence as cleavage of **1** with  $\beta\text{ME}$  to generate a mixed disulfide that reacts relatively slowly with a second molecule of  $\beta\text{ME}$ .
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