Chiral Organoarsenic Compounds

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Formation of a Chiral Center and Pyrimidal Inversion at the Single-Molecule Level**

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The recent development of single-molecule techniques has been largely targeted at solving biological problems;[1–5] for example, protein folding has been examined by means of force spectroscopy, and enzyme kinetics have been investigated by single-molecule fluorescence. These approaches provide insight into individual trajectories that might be obscured in ensemble measurements. In contrast, bond-forming and bond-breaking reactions of small molecules in solution have rarely been observed at the single-molecule level. We have developed an approach through which the covalent chemistry of individual molecules can be monitored in an aqueous environment inside a "nanoreactor", that is, the transmembrane protein pore formed by α-hemolysin.[6–9] By monitoring an ionic current driven through the pore by a transmembrane potential, subtle changes in the structures of individual reactants tethered within the lumen can be detected. We now use this approach to follow both the formation of a chiral center at As^{III} (through the making of an As–S bond) and the inversion taking place at that center. The nanoreactor also serves to isolate the relatively short-lived As^{III} adduct and thereby prevent additional reaction steps that would complicate the chemistry. As–S bond making and breaking are important reactions in pharmacology, toxicology,[10–13] and experimental cell biology.[14,15]

We earlier reported reversible covalent As–S bond formation between the side chain of a cysteine residue at position 117 in the α-hemolysin pore and 4-sulfophenylarsonous acid (Figure 1a).[9] Herein, we examine the same reaction at a different position within the nanoreactor (residue 137) and observed two distinct conductance levels, which corresponded to the formation of two As–S adducts at the wall of the protein (see Figure 1b). Re-examination of the earlier data from the reaction at position 117 also revealed two current levels, but they were very closely spaced. The unitary conductance of $P_{137\text{-}SH}$—a heteromeric pore with just one of the seven subunits containing a cysteine residue at position 137—in 2 M KCl at −50 mV is (1.71 ± 0.04) nS (this is

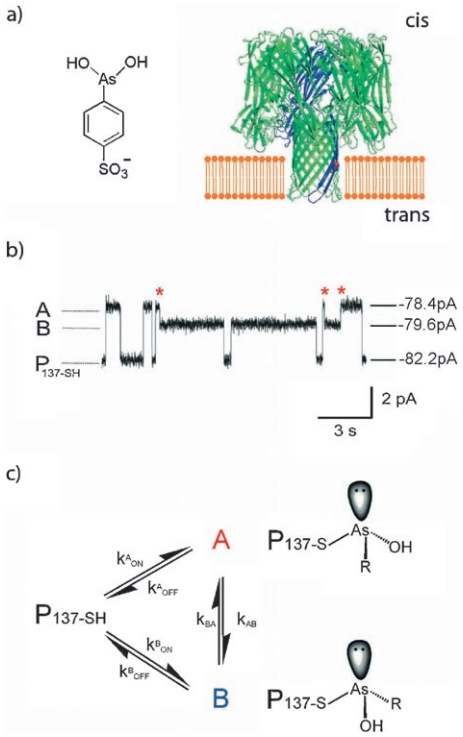

Figure 1. Two adducts are formed when 4-sulfophenylarsonous acid reacts with the side chain of Cys-137 in the $P_{137\text{-}SH}$ pore. a) Structure of 4-sulfophenylarsonous acid and schematic of the $P_{137\text{-}SH}$ pore. In aqueous solution, the dehydrated form, that is, 4-sulfophenylarsane oxide, may exist in equilibrium with 4-sulfophenylarsonous acid. The $P_{137\text{-}SH}$ pore consists of six cysteine-free wild-type subunits (green) and one mutated subunit (blue) in which Gly137 is substituted by Cys. The sulfur atom of the Cys residue is shown in yellow, the carboxy oxygen of residue 137 in red. b) Single-channel recording at −50 mV and 23 °C with a buffer containing 2 M KCl, 80 mM 3-(4-morpholinyl)-1-propanesulfonic acid (MOPS), 100 μM ethylenediaminetetraacetate (EDTA) (pH 8.4) in both chambers. 4-Sulfophenylarsonous acid (100 μM) was in the trans chamber. The current levels corresponding to the free pore, $P_{137\text{-}SH}$, and the two adducts, A and B, are shown for the depicted experiment. The assignment of the diastereomeric adducts as A or B is arbitrary. The asterisks represent inversion events; these events are over-represented in the trace shown here. c) Kinetic scheme describing the observed chemistry (R = 4-sulfophenyl).

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the mean value obtained from $n = 10$ separate experiments). The two adducts formed by 4-sulfophenylarsonous acid reduce the single-pore current (mean value 85.5 pA) by (3.7 ± 0.2) pA ($n = 10$, adduct A) and (2.5 ± 0.2) pA ($n = 10$, adduct B). The two adducts, A and B, interconvert, although these interconversion events are rare relative to the rates of association and dissociation of 4-sulfophenylarsonous acid. The interconversion events represent $(7.8 \pm 1.7)\%$ of all other current steps, that is, $100 \times (n_{AB} + n_{BA})/(n_{PA} + n_{AP} + n_{PB} + n_{BP}$; see the Supporting Information), and are over-represented in the selected trace (Figure 1b). Based on known[16–21] and supporting ensemble chemistry, and on the kinetic analysis presented below, we believe that the As–S adducts A and B are the diastereomers[22–24] formed at the surface of the chiral protein by substitution of one or the other of the enantiotopic hydroxy groups of 4-sulfophenylarsonous acid. Because our measurements were carried out under conditions of dynamic equilibrium, the situation can be represented with a simple kinetic scheme (Figure 1c). The kinetic analysis outlined below and the fact that A and B interconvert eliminate the possibility that A and B are two distinct reaction products, one formed, for example, by an impurity in the arsonous acid. Additional experiments showed that buffer components did not take part in the reaction and that the formation of an –O–As–S– adduct did not occur, involving the hydroxy group of the proximal Thr-125 on the neighboring subunit of the α-hemolysin heptamer (see the Supporting Information).

The six rate constants in the proposed scheme were determined by the analysis of mean inter-event intervals (τ_{ON}) and mean adduct lifetimes (τ_{OFF}) (Table 1; for details, see also the Supporting Information). Plots of $1/\tau_{ON}$ versus the concentration of 4-sulfophenylarsonous acid (Figure 2a) were of the form $1/\tau = k[A]$, which shows that the formation of the proposed adducts involves bimolecular reactions. As expected, analogous plots for the reversal of the adduct formation and the interconversion of the adducts were of the form $1/\tau = k$, thus confirming that these are unimolecular reactions (Figure 2b,c). As a further test of the kinetic scheme, we note that at equilibrium, the product of the rate constants for a clockwise movement around the triangle (Figure 1c) must equal the product for the anticlockwise movement.[25] In keeping with this, we found $k_{on}^{A} k_{AB} k_{off}^{B} = (0.39 \pm 0.05) \times 10^3\,\text{M}^{-1}\text{s}^{-3}$ and $k_{on}^{B} k_{BA} k_{off}^{A} = (0.43 \pm 0.08) \times 10^3\,\text{M}^{-1}\text{s}^{-3}$. We also found that the formation constants of A and B ($K_f^A = k_{on}^A/k_{off}^A = (9.3 \pm 0.9) \times 10^3\,\text{M}^{-1}$, $K_f^B = k_{on}^B/k_{off}^B = (15 \pm 2) \times 10^3\,\text{M}^{-1}$) are similar to that of the adduct between

Table 1: Rate constants[a] for the formation (k_{ON}) of As–S adducts within the $P_{137\text{-SH}}$ pore and their interconversion (k_{INV}) and dissociation (k_{OFF}).[b]

	A	B
k_{ON} [$\text{M}^{-1}\text{s}^{-1}$]	$(14 \pm 1) \times 10^3$	$(5.9 \pm 0.8) \times 10^3$
k_{OFF} [s^{-1}]	1.5 ± 0.1	0.40 ± 0.02
K_f [M^{-1}]	$(9.3 \pm 0.9) \times 10^3$	$(15 \pm 2) \times 10^3$
k_{INV} [s^{-1}]	$k_{AB} = 0.071 \pm 0.007$	$k_{BA} = 0.049 \pm 0.007$

[a] For conditions see Figure 1 (legend). [b] K_f ($= k_{ON}/k_{OFF}$) is the formation constant for each adduct.

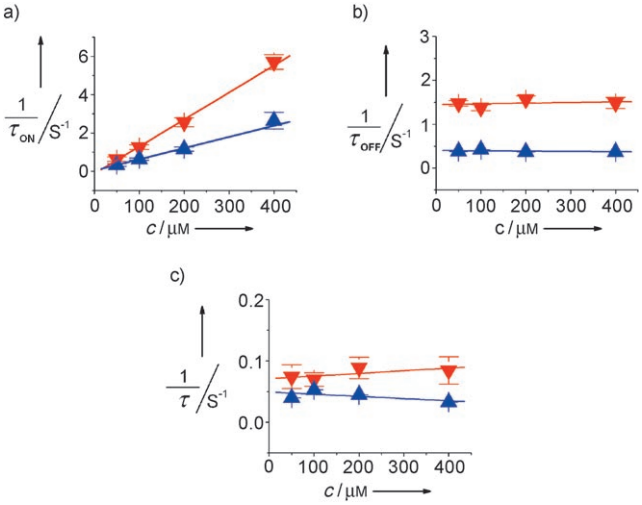

Figure 2. Reciprocals of the mean interevent intervals and the dwell times (τ values) versus the concentration of 4-sulfophenylarsonous acid. a) Reciprocals of the mean interevent intervals (▼ $\tau_{on}^{A\,-1}$; ▲ $\tau_{on}^{B\,-1}$). τ_{on}^{A} is the total time in the unmodified $P_{137\text{-SH}}$ state divided by the number of exits from $P_{137\text{-SH}}$ to adduct A (n_{PA}) and τ_{on}^{B} is the total time in the unmodified $P_{137\text{-SH}}$ state divided by the number of exits from $P_{137\text{-SH}}$ to adduct B (n_{PB}). b) Reciprocals of the mean dwell times in states A or B before dissociation (▼ $\tau_{off}^{A\,-1}$; ▲ $\tau_{off}^{B\,-1}$). τ_{off}^{A} is the total time in state A divided by the number of exits from A to $P_{137\text{-SH}}$ (n_{AP}) and τ_{off}^{B} is the total time in state B divided by the number of exits from B to $P_{137\text{-SH}}$ (n_{BP}). c) Reciprocals of the mean dwell times in states A or B before inversion (▼ τ_{AB}^{-1}; ▲ τ_{BA}^{-1}). τ_{AB} is the total time in state A divided by the number of exits from A to B (n_{AB}) and τ_{BA} is the total time in state B divided by the number of exits from B to A (n_{BA}). For further details see the Supporting Information.

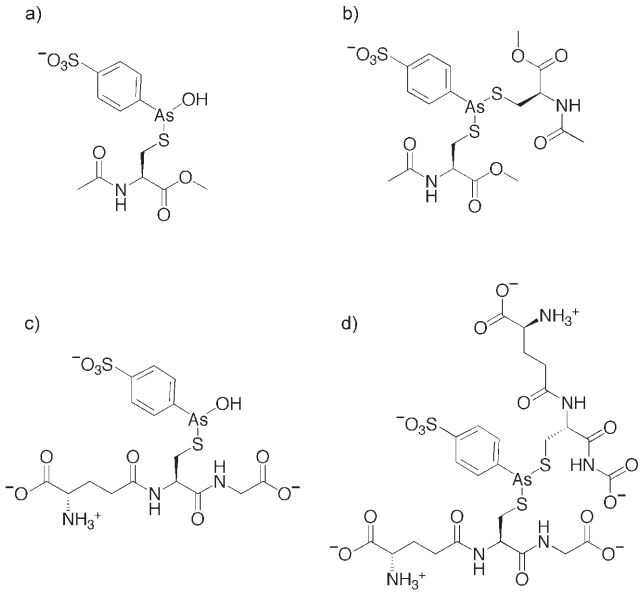

Figure 3. Structure of various S-adducts of 4-sulfophenylarsonous acid. a) 1:1 adduct with *N*-acetyl-L-cysteine methyl ester; b) 2:1 adduct with *N*-acetyl-L-cysteine methyl ester; c) 1:1 adduct with glutathione (GSH); d) 2:1 adduct with GSH. All molecules are tetrahedral at the As center, but the configuration is not defined at the stereogenic As in compounds (a) and (c).

4-sulfophenylarsonous acid and Cys117, namely, $K_f = (14 \pm 3) \times 10^3\ \text{M}^{-1}$.[9] Therefore, the assignment of the current levels and transitions makes sense in terms of the kinetic scheme. Furthermore, if it is assumed that the "concentration" of 4-sulfophenylarsonous acid in the lumen of the pore (that is, the probability of finding the reactant in a unit volume of solution) is equal to that in free solution—an assumption that has been borne out by experiment[8]—the bimolecular rate constants found herein should approximate the solution rate constants for the reaction of thiols and arsonous acids.

Electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) and ^{1}H NMR experiments provided information about the formation of covalent thiol adducts by 4-sulfophenylarsonous acid and their chirality. The monothiol *N*-acetyl-L-cysteine methyl ester was mixed with 4-sulfophenylarsonous acid in water. The solution was allowed to stand (at 23 °C) for 5 min before performing the ESI-TOF-MS experiments in the negative-ion mode. At a thiol/As^{III} ratio of 1:2, a peak at *m*/*z* 423.83 appeared in the spectrum, which was assigned to the 1:1 adduct (Figures 3a and 4a). At a higher thiol/As^{III} ratio (of 4:1), the major peak was observed at *m*/*z* 582.82 and assigned to the 2:1 adduct (Figure 3b, theoretical 582.99). The two remaining prominent peaks at this ratio were that of the 1:1 adduct (*m*/*z* 423.84, theoretical 423.95) and that of the dehydrated monoanionic form of the arsonous acid (namely, 4-sulfophenylarsane oxide *m*/*z* 246.86, theoretical 246.91).

The peaks at *m*/*z* 583 and 424 were examined by tandem mass spectrometry (MS–MS, Figure 4b,c). In both cases, elimination to form *N*-acetyl-L-dehydrocysteine[26] yielded peaks at *m*/*z* 262.83 (corresponding to the mono-anion of 4-sulfophenylarsane sulfide, theoretical 262.88). In the case of the peak at *m*/*z* 424, breakdown to form a species with *m*/*z* 246.87 (that is, the mono-anion of 4-sulfophenylarsane oxide) also occurred (Figure 4b). These observations strengthen the assignments of the peaks at *m*/*z* 583 and 424 as being derived from the 2:1 and 1:1 adducts of *N*-acetyl-L-cysteine methyl ester with 4-sulfophenylarsonous acid.

^{1}H NMR spectroscopy was used to investigate the interaction of 4-sulfophenylarsonous acid with L-glutathione (GSH, which is the tripeptide γ-Glu–Cys–Gly). We titrated GSH with 4-sulfophenylarsonous acid (and vice versa) in a 100 mM sodium phosphate solution (pD 7.8). With the aid of

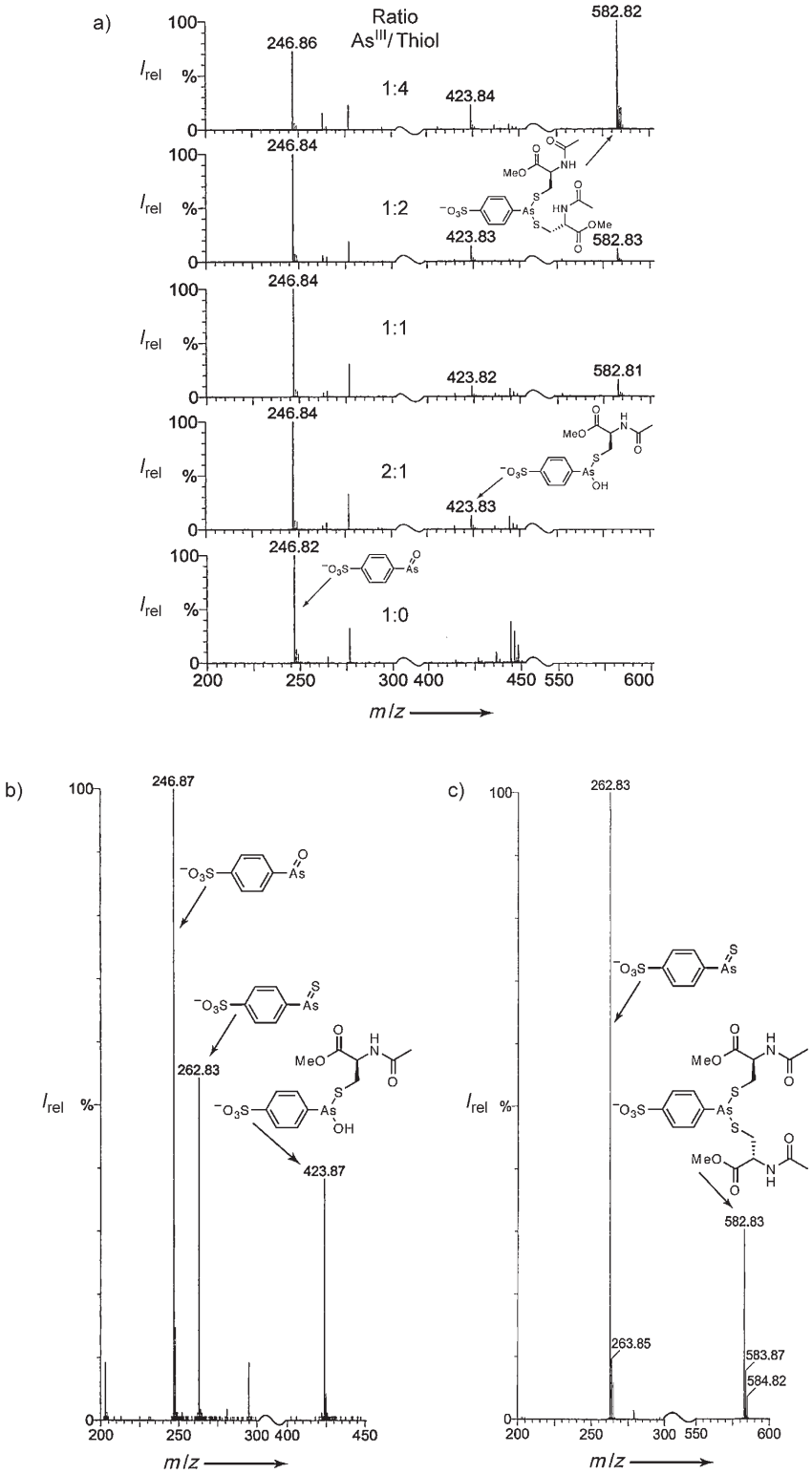

Figure 4. MS data supporting the interpretation of the single-molecule experiments. a) Negative-ion ESI-TOF-MS of mixtures (in various ratios) of 4-sulfophenylarsonous acid and the monothiol *N*-acetyl-L-cysteine methyl ester in water. b) MS–MS of the ion at *m*/*z* 424. c) MS–MS of the ion at *m*/*z* 583.

2D correlation spectroscopy (COSY), we then assigned all the ^{1}H resonances to individual molecular species (see the Supporting Information). The titrations suggested that the GSH–4-sulfophenylarsonous acid 2:1 adduct (Figure 3d) was more stable than the 1:1 adduct (Figure 3c)—as found elsewhere for related compounds.[19] Peaks that were assigned to the 1:1 adduct were apparent in the presence of an excess of arsonous acid (Figure 5a), but were of low intensity when

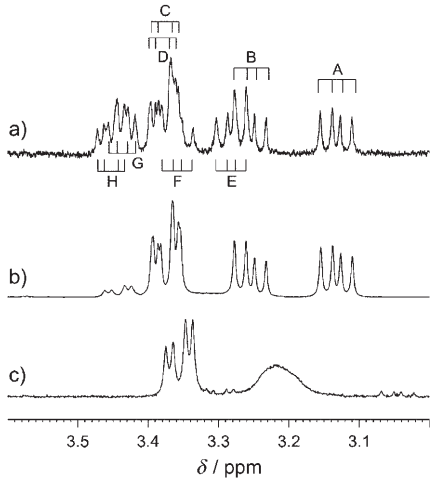

Figure 5. ^{1}H NMR spectra illustrating the cysteine methylene ($-SCH_2-$) resonances in 1:1 and 2:1 adducts of glutathione (GSH) and 4-sulfophenylarsonous acid. a) A mixture containing 1:1 and 2:1 adducts (2.3 mM GSH: 30 mM 4-sulfophenylarsonous acid, 25 °C). Labeled are the eight double-doublet resonances observed for the 2:1 complex [upper labels A–D; see also trace (b)] and the two diastereomeric 1:1 complexes (lower labels E–H). b) A mixture in which the 2:1 adduct is dominant (28.2 mM GSH: 30 mM 4-sulfophenylarsonous acid, 25 °C). c) Spectrum of the 2:1 adduct at 80 °C (18 mM GSH: 21 mM 4-sulfophenylarsonous acid). The NMR spectra were recorded in a 100 mM sodium phosphate solution [pD 7.8 in (a) and (b), and pD 8.8 in (c)].

both reagents were present at a concentration of about 30 mM (Figure 5b). The cysteine methylene ($-SCH_2-$) resonances (Figure 5b) in the 2:1 adduct can be ascribed to four chemically non-equivalent protons, which is consistent with the fact that this adduct is tetrahedral at the arsenic center and contains two identical ligands of the same chirality[22,23] (see the Supporting Information). In contrast, the 1:1 adducts comprise a pair of diastereomers, each of which is stereogenic at As and exhibits diastereotopic methylene protons. The ^{1}H NMR resonances for the $-SCH_2-$ group of each diastereomer appear as a pair of double doublets, thus giving a total of sixteen lines. All of these peaks could be identified in the ^{1}H NMR spectrum of a mixture of the 2:1 and 1:1 adducts by analyzing the titration data (see Figure 5a and the Supporting Information). It is worth emphasizing that a 1:1 species is isolated within the nanoreactor in our single-molecule approach.

To shed light on the kinetics of As–S bond formation and dissociation, we examined the temperature dependence of the ^{1}H NMR spectrum and performed a magnetization-transfer experiment (see the Supporting Information). At 80 °C in a 100 mM sodium phosphate solution (pD 8.8), two of the $-SCH_2-$ resonances of the 2:1 complex, which arise from protons on different cysteine Cβ atoms (according to the 2D COSY experiment), coalesced into a single broad peak, whilst the two remaining $-SCH_2-$ resonances of near-identical chemical shifts fully coalesced and sharpened to a single double doublet (see Figure 5c and the Supporting Information). Simulation of the spectra from a temperature series yielded a rate constant for the exchange process of approximately 8 s^{-1} at 30 °C (see the Supporting Information). The exchange process for the same two $-SCH_2-$ resonances was also examined in greater detail in a 1D magnetization-transfer experiment (in 100 mM sodium phosphate, pD 8.8), which yielded a rate constant of 4.7 s^{-1} at 30 °C (see the Supporting Information). These experiments demonstrate that sulfur ligands at the As center of the 2:1 adduct with GSH exchange at approximately the same rate as that observed for As–S bond cleavage in the 1:1 adduct seen in the single-molecule experiments.

Pyrimidal inversion at an arsenic center with three carbon substituents is presumed to occur without bond breaking and is extremely slow;[27–29] for example, ethylmethylphenylarsine has a half-life for racemization in decalin of about 6 days at 218 °C.[27] In contrast, As^{III} compounds with Si substituents isomerize faster; for example, $C_6H_5As[SiH(CH_3)_2]_2$ has a solvent-independent barrier to inversion of 74 kJ mol^{-1}, which corresponds to a half-life for racemization of about 1 s at 25 °C.[30] Although detailed kinetic studies are lacking, As^{III} compounds with S substituents also isomerize faster; for example, stereoisomers of the cyclic 1:1 adducts of phenyldichloroarsine with 1,3-dimercapto-2-propanol and 1,2-dimercaptopropane interconvert with rate constants in the range of 0.42 to 3.1 s^{-1} at about 25 °C in acidic CD_3OD,[20] in contrast with earlier measurements in neutral apolar solvents.[31] The cyclic diastereomeric adducts between phenylarsonous acid and a dicysteine peptide isomerized in acetonitrile–water ($pH \approx 2$) with $t_{1/2} \approx 40$ h.[32] The diastereomeric adducts between methylphenylarsinous acid and glutathione could be separated by means of high-performance liquid chromatography (HPLC), but were interconverted upon removal of the solvent.[22]

While these reactions may proceed without bond breaking, as suggested for ethylmethylphenylarsine,[27] an alternative is a dissociative mechanism, in which one of the bonds to As is broken and reformed.[20,24,32] The case for such a mechanism is bolstered by the existence of arsenium cations,[33,34] which would be intermediates in the process. Interestingly, a "thiaarsahydroxy" species was observed in the active site of an arsenate reductase,[35] and $[RAsOH]^+$ (where R = 3-nitro-4-hydroxyphenyl) was found in the ESI-MS of the oxidized form of Ehrlich's organoarsenic therapeutic salvarsan.[11] Herein, we did not observe any intermediate that might be ascribed to an arsenium cation at the time resolution of the experiments (that is, 50 μs). Finally, in the presence of excess ligand (in this case water), an associative mechanism for ligand exchange and racemization is a possibility.

The inversion is slow relative to the breakdown of the As–S adducts, and it is instructive to note that this infrequent

pathway would not be observed in most ensemble measurements, such as a dynamic NMR spectroscopy experiments where dissociation and re-association of the sulfur ligand would dominate the signal. Furthermore, in bulk solution, the initial adduct would react with a second thiol, thereby complicating the chemistry.[16–19] In the α-hemolysin nanoreactor, the initial adduct is isolated at the reaction site. Finally, the surface of the protein on which inversion occurs has a weak preference for one of the two diastereomers ($k_{AB}/k_{BA} = 1.4$). It would be interesting to attempt to strengthen that preference by the appropriate positioning of neighboring groups.

Experimental Section

Procedures for MS and NMR spectroscopy, additional MS and NMR data, procedures for site-directed mutagenesis and the preparation of protein pores, the kinetic analysis, and control experiments that eliminate the participation in the chemistry of buffer components and neighboring groups on the nanoreactor surface are available in the Supporting Information.

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