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Synthesis, X-ray absorption spectroscopy and purification of the seleno-bis (*S*-glutathionyl) arsinium anion from selenide, arsenite and glutathione

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Abstract

We report a new synthesis of the seleno-bis (*S*-glutathionyl) arsinium anion, $[(GS)_2AsSe]^-$. An aqueous solution of bis-glutathionylarsenous acid, $(GS)_2As–OH$, prepared from stoichiometric glutathione and arsenite, was reacted in situ with a solution of sodium hydrogen selenide, prepared from elemental selenium and sodium borohydride. Analysis of the arsenic and selenium K-edge X-ray absorption spectra indicated virtually quantitative formation of $[(GS)_2AsSe]^-$, with As–Se and As–S distances of 2.31 and 2.25 Å, respectively, and the concentrated sample allowed a definitive X-ray spectroscopic characterization. Size-exclusion chromatography was used to separate $[(GS)_2AsSe]^-$ from residual borate in the reaction mixture.

Keywords: X-ray absorption spectroscopy; Size-exclusion chromatography; *S*-glutathionyl; Arsinium anion

1. Introduction

Arsenite and selenite both have a very high tendency to react with thiols in aqueous solution, and this affinity is important in determining the biological chemistry of these metalloid species. Arsenite, which is present predominantly as $As(OH)_3$ at physiological pH, reacts with the biologically endogenous thiol glutathione (GSH) to form species of the type $(GS)_xAs(OH)_{3-x}$ [1,2]. In contrast, selenite undergoes reduction by six mole-equivalents of GSH to yield selenide (HSe^- at physiological pH) and oxidized glutathione (GSSG) [3]. Both arsenite and selenite are highly toxic to mammals, but surprisingly, when co-administered a mutual detoxification occurs [see [4] and refs. therein]. We have recently shown that this is due to the formation in vivo, and excretion in the bile, of a novel arsenic–selenium-containing compound, spectroscopically identified as the seleno-bis (*S*-glutathionyl) arsinium ion, $[(GS)_2AsSe]^-$ (neglecting charges on the amine and carboxylic acids of the glutathiones) [4].

In vitro, equimolar arsenite and selenite ($HSeO_3^-$ at physiological pH) react with eight or more mole-equivalents GSH to yield $[(GS)_2AsSe]^-$ as shown in Eq. (1) [4].

$$As(OH)_3 + HSeO_3^- + 8GSH \rightarrow [(GS)_2AsSe]^- + 3GSSG + 6H_2O \quad (1)$$

The metalloid-based negative charge on $[(GS)_2AsSe]^-$ was confirmed previously by micellar size-exclusion chromatography, using arsenic, selenium and sulfur-specific detection by inductively-coupled plasma atomic emission spectrometry (ICP-AES) [5]. This study additionally confirmed that two glutathionyl entities are bound to the ion [5]. While the formal

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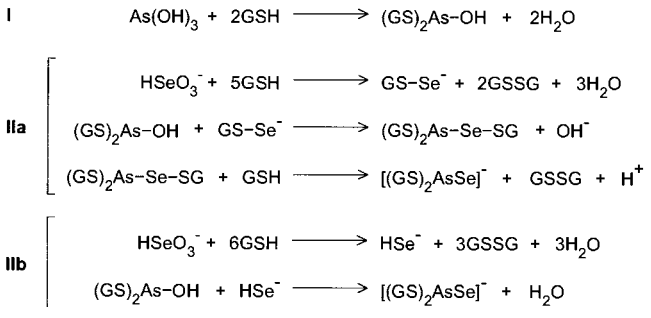
oxidation state of the arsenic atom remains unchanged during the reaction, selenite is reduced from a formal oxidation state of +IV to −II, with concomitant oxidation of six mole-equivalents of GSH to GSSG. The mechanism of the reaction is currently unknown, but by chemical analogy a two-part mechanism appears reasonable. In the first part arsenite would react with two GSH to form $(GS)_2As–OH$ [1] (Scheme 1), which would then react with a product of the reduction between $HSeO_3^-$ and GSH. Two alternative mechanisms for this are feasible, as shown in Scheme 1.

According to mechanism IIa, selenite would react with 5 GSH to form $GSSe^-$ (as proposed by Seko et al. [6]), which could then react with $(GS)_2As–OH$ to form $(GS)_2As–Se–SG$. The latter would then be reduced with one additional GSH to form $[(GS)_2AsSe]^-$. Alternatively, according to mechanism IIb, $HSeO_3^-$ would react with 6 GSH to yield HSe^- (also proposed by Seko et al. [6]), which would then react directly with $(GS)_2As–OH$ to produce $[(GS)_2AsSe]^-$. If the overall reaction proceeds via mechanism IIb (Scheme 1), then it should be possible to synthesize $[(GS)_2AsSe]^-$ from selenide and $(GS)_2As–OH$. In the present study, we have investigated this possibility and analyzed the product(s) by X-ray absorption spectroscopy (XAS) and size exclusion chromatography.

The in vivo formation of $[(GS)_2AsSe]^-$ links for the first time the biogeochemical cycles of arsenic and selenium with that of sulfur [4]. In addition, the formation and biliary excretion of $[(GS)_2AsSe]^-$ may be one of the first detoxification mechanisms by which ingested arsenite is removed from the mammalian body [7]. However, the metabolism, toxicity, and fate of $[(GS)_2AsSe]^-$ are currently unknown. Studies to determine toxicity require chemically pure $[(GS)_2AsSe]^-$. This is difficult to achieve with the previously reported synthetic route [4], because, the threefold excess of GSSG produced cannot be easily separated [5]. In contrast, the new synthetic route described herein gives a high yield of $[(GS)_2AsSe]^-$ and a byproduct that is easily separated chromatographically.



Scheme 1.

2. Experimental

2.1. Chemicals

Selenium powder (gray powdered form, 100 mesh, 99.999%) and sodium borohydride (99%) were purchased from Aldrich (Milwaukee, WI, USA) and $NaAsO_2$ (>99%) from GFS Chemicals (Columbus, OH, USA). Sodium sulfate (anhydrous, 99.9%) was obtained from J. T. Baker (Phillipsburg, NJ, USA), glutathione (>98%) from Sigma (St. Louis, MO, USA), sodium hydroxide from MCB Reagents (Cincinnati, OH, USA), tris(hydroxymethyl)aminomethane (Tris) from Eastman Kodak Company (Rochester, NY, USA) and sodium selenite pentahydrate (>97%) from Fluka (Buchs, Switzerland).

2.2. Synthesis of the seleno-bis (S-glutathionyl) arsinium ion, $[(GS)_2AsSe]^-$

All reactions were performed under an N_2 atmosphere and at room temperature (r.t.). Colorless sodium hydrogen selenide solution was prepared as reported [8] by addition of solid selenium to aqueous sodium borohydride. A solution containing $(GS)_2As–OH$ was prepared by mixing aqueous GSH (1.882 g, 6.0 mmol) and aqueous sodium arsenite (0.378 g, 2.9 mmol), and adjusting the pH to 7.6 by dropwise addition of 4.0 M NaOH. An aliquot of the selenide solution (5.0 ml; 2.9 mmol sodium hydrogen selenide) was mixed with 10 ml of the $(GS)_2As–OH$ solution, yielding a solution of final pH 8.55. A 100 μl aliquot was immediately transferred to a Lucite sample holder and frozen in liquid nitrogen for analysis by XAS.

2.3. X-ray absorption spectroscopy

X-ray absorption spectra were recorded on beamline 7–3 at the Stanford Synchrotron Radiation Laboratory (SSRL) with a Si(220) double crystal monochromator, an upstream vertical aperture of 1 mm, and no specular optics. Harmonic rejection was accomplished by detuning one monochromator crystal by approximately 50%, and X-ray absorption was monitored in transmittance using N_2-filled ionization chambers. The sample was maintained at 10 K during data acquisition using an Oxford Instruments liquid helium flow cryostat. Four 30-min scans of the sample were averaged at both the As and the Se K-edges, and the spectrum of an elemental foil (α-As or hexagonal Se) was recorded simultaneously with each scan. X-ray energy calibration was by reference to the lowest energy K-edge inflection of the foil standard which was assumed to be 11 867.0 and 12 658.0 eV for As and Se, respectively. Data were analyzed with the EXAFSPAK program suite [9], employing ab initio phase and amplitude functions calculated with the program FEFF (V 8.2) [10].

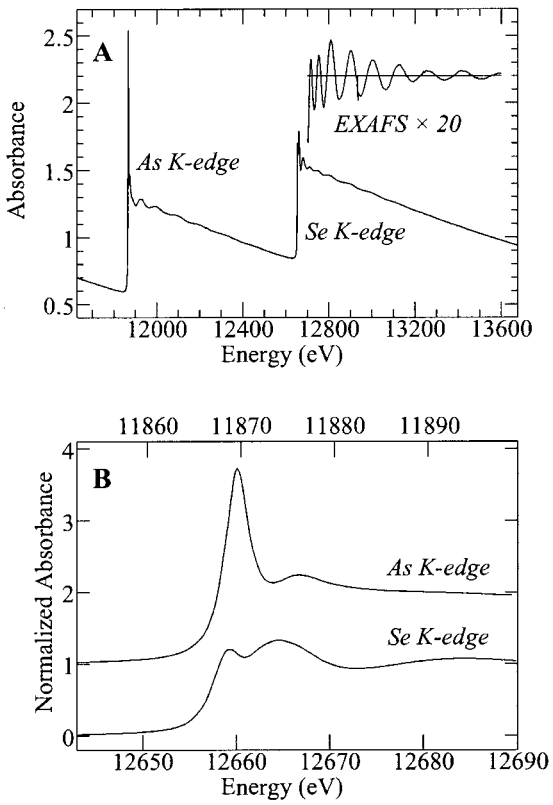


Fig. 1. (A) The total X-ray absorption spectrum of the reaction product, with the As and Se K-edges indicated. Inset shows the background-subtracted raw (un-weighted) Se K-edge EXAFS oscillations. (B) The As and Se K near-edge spectra of the reaction product. The top and bottom abscissas refer to the As and Se spectra, respectively.

2.4. Chromatography

A Beckman 110 B Solvent Delivery Module HPLC pump in conjunction with a Rheodyne six-port injection valve (200-μl loop) was used. A Pharmacia HR10/30 column (i.d. 1.0 cm) was packed with Sephadex G-10 to a height of 29.0 cm and was maintained at 4 °C. After equilibration of the column with 60 ml of 0.1 M tris-buffer (pH 8.0), 200 μl of the obtained reaction mixture (and aqueous sodium borate) was injected at 1.0 ml min^{-1}.

2.5. Inductively coupled plasma-atomic emission spectroscopy

As, Se, S and B-specific detection was achieved with a Thermo Jarrel Ash (Franklin, MA, USA) IRIS HR radial view ICP-AES at 189.042, 203.985, 180.731 and 249.678 nm (order 178, 165, 186 and 135), respectively. Time-scans were performed using THERMO-SPEC/CID software (version 2.10.09), and the multitasking controller allowed the processing of one atomic line every 0.02 s. The nebulization gas was maintained at a flow of 1.5 dm^3 min^{-1}. All other instrumental parameters were the same as previously reported [5]. Four minutes after the injection of the analyte onto the column, monitoring of the emission lines was initiated. In addition to the obtained mixture, a solution containing aqueous sodium borate was chromatographed.

3. Results and discussion

The product of the reaction of aqueous $(GS)_2As$–OH with a solution of selenide was investigated using XAS. XAS provides a tool for investigating the coordination chemistry of a wide range of elements both in vivo and in vitro. Fig. 1A shows the X-ray absorption spectrum of the reaction product, with both the As and Se K-edges clearly apparent. The XAS spectrum can be separated into two regions, the near-edge spectrum and the extended X-ray absorption fine structure (EXAFS) oscillations. While the division between these two regions is indistinct, the near-edge is usually taken as the region within about 50 eV of the absorption edge, and is dominated by dipole-allowed ($\Delta l = \pm 1$) transitions to vacant frontier molecular orbitals. Interpretation of the near-edge is often complex, but it can provide a unique fingerprint of a particular chemical species. The EXAFS oscillations extend to high energies above the edge (Fig. 1A, inset) and arise from backscattering of emitted photoelectrons by nearby atoms. Unlike the near-edge, the EXAFS are comparatively simple to interpret since accurate theoretical treatments and well-established analysis procedures exist. Curve-fitting analysis of the EXAFS provides very accurate local interatomic distances together with information on the number and type of nearby atoms and their relative thermal and static displacements.

The As and Se near-edge spectra of the reaction product are shown in Fig. 1B, and are identical (within the noise) to those previously reported for $[(GS)_2AsSe]^-$ [4]. The As near-edge spectrum is at a position corresponding to a formal As(III) oxidation state [4], and has an intense absorption at 11 869.6 eV, which is due to a dipole-allowed transition to unoccupied orbitals with substantial 4p character. The Se near-edge spectrum lacks the intense resonance observed at As, which is consistent with a formal oxidation state of Se(−II) (which has no 4p vacancies), and the pre-edge peak at 12 659.2 eV is attributable to transitions to unoccupied levels associated with covalent bonding with arsenic.

The As and Se K-edge EXAFS oscillations, together with best-fits, are shown in Fig. 2. These are of superior signal to noise relative to those previously reported [4] due to the concentrated nature of the sample, but the As analysis is still of limited resolution, because, the Se K-edge at about 12 658 eV inevitably truncates the As

EXAFS at about $k = 13$ Å^{-1} (Fig. 1A, Fig. 2A). The Se EXAFS is dominated by backscattering from a single As at 2.31 Å, with indications of longer-range backscattering from two S atoms at about 3.1 Å. For transmittance data (Figs. 1 and 2), the proximity of the As and Se absorption edges means that As EXAFS will protrude into the Se EXAFS region. This will result in a low-frequency background in the Se data that might interfere with data analysis, especially at low k. However, the Se K-edge EXAFS spectrum measured by monitoring the Se–K_α X-ray fluorescence (not illustrated), while showing amplitude reduction due to concentration effects, is otherwise identical to the transmittance, and we conclude that any residual As EXAFS is removed during background subtraction. The putative long-range Se–S interaction has a very large Debye–Waller factor (Fig. 2), indicating the presence of significant disorder in the Se–As–S bond angle. The As EXAFS shows both As–Se and As–S interactions at 2.31 and 2.26 Å, respectively. While both these interactions are needed to reproduce the experimental EXAFS, they are not resolved as separate peaks in the Fourier transform (Fig. 2B), due to the aforementioned truncation of the As EXAFS. These structural parameters, and the near-edge spectra, are identical within experimental error to those previously reported for $[(GS)_2AsSe]^-$ [4], and unambiguously identify the product as the $[(GS)_2AsSe]^-$, with no other As or Se species present in detectible quantities. Thus, the reaction shown in Eq. (2) occurs.

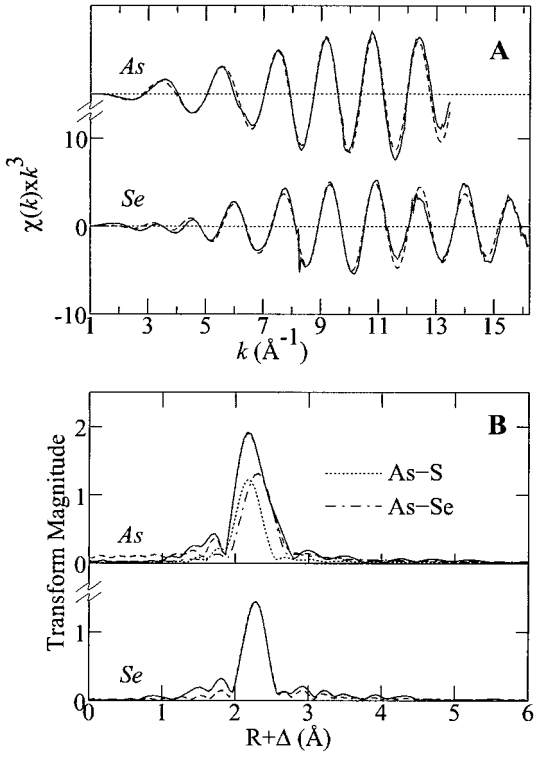
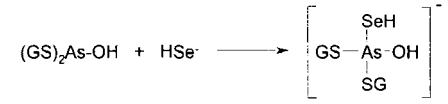


Fig. 2. (A) The As and Se K-edge EXAFS oscillations of the reaction product (solid lines), plus the best fits from EXAFS curve-fitting analysis (broken lines). (B) Fourier transforms of the As and Se EXAFS (solid line), phase corrected for S and As backscatterers, respectively, together with best fits (broken lines). The transforms of the incompletely resolved As–S and As–Se components of the fit are shown. Best fits were obtained with the following parameters: As EXAFS, two As–S at 2.255(3) Å with $\sigma^2 = 0.0034(1)$ Å^2, and one As–Se at 2.307(3) Å with $\sigma^2 = 0.0027(3)$ Å^2; Se EXAFS, one Se–As at 2.308(1) Å with $\sigma^2 = 0.0023(1)$ Å^2, and two Se–S at 3.14(2) with $\sigma^2 = 0.021(2)$ Å^2. σ^2 is the mean-square deviation of the interatomic distance. Values in parentheses are the estimated standard deviations (precessions) obtained from the diagonal elements of the covariance matrix (we note that the accuracies will always be somewhat larger than the precessions). The long-distance 3.1 Å Se–S interaction is required for adequate fit below $k \approx 5$ Å^{-1}.

$$(GS)_2As\text{–}OH + HSe^- \rightarrow [(GS)_2AsSe]^- + H_2O \quad (2)$$

This reaction involves nucleophilic attack of the hydrogen selenide ion on the arsenic atom, with the displacement of the OH group. It is similar to the nucleophilic displacement by GSH on arsenous acid to give $(GS)_2As$–OH. The mechanism for these displacements is probably analogous to the mechanism of ^{17}O exchange between arsenite ion and solvent water [11] and the hydrolysis of alkylarsenites [12]. In these reactions, nucleophilic addition to arsenic to give a four-coordinate intermediate is suggested, as illustrated in Eq. (3).



(3)

Collapse of this intermediate to the product requires proton transfer from selenium to oxygen. For the ^{17}O exchange between arsenous acid and solvent water, the required proton transfer is suggested [11] to occur in the transition state via a second water molecule (the first acts as the nucleophile). In addition to this possibility, proton transfer prior to nucleophilic attack, or a four-center reaction combining nucleophilic attack with proton-transfer, has been suggested for the reaction of alcohols, thiols and selenols with aminodialkylarsines [13]. Under biological conditions, in which GSH is present in excess, $(GS)_3As$ rather than $(GS)_2As$–OH may be formed, and then may react with a selenium donor species [14].

While the reaction shown in Eq. (2) is an attractive method for synthesizing $[(GS)_2AsSe]^-$, residual Na^+ and borate $[B(OH)_3$ at the final pH of the reaction], left over from the synthesis of sodium hydrogen selenide, remain in the product solution. Fortunately, the substantial size difference between these species and $[(GS)_2AsSe]^-$ allowed separation by size-exclusion chromatography. Fig. 3 shows the results of chromatography using a Sephadex G-10 column, simultaneously monitoring the arsenic, selenium, sulfur and boron emission lines. A slight orange–red coloration

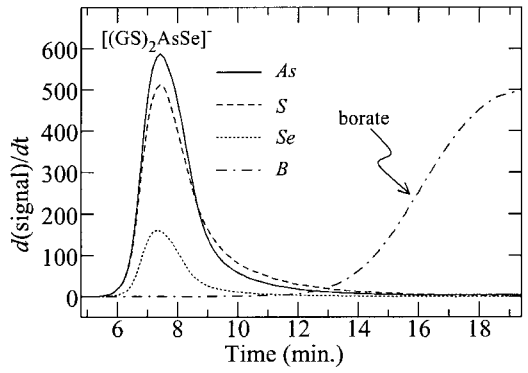


Fig. 3. Size exclusion chromatography of the reaction product using arsenic, sulfur, selenium and boron-specific detection by ICP-AES at wavelengths of 189.042, 180.731, 203.985 and 249.678 nm for As, S, Se and B, respectively. A sharp coincident peak for As, Se and S was observed, followed by a broader B peak.

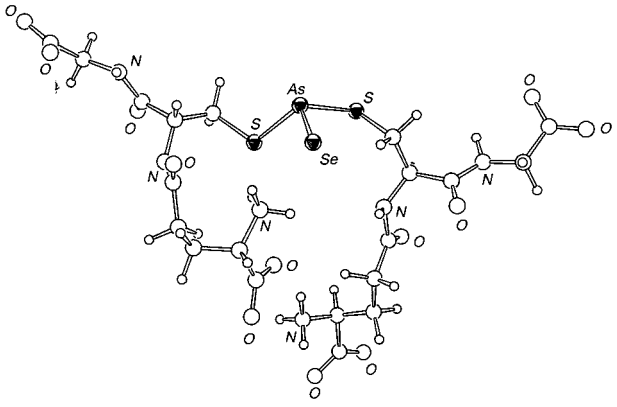


Fig. 4. Calculated structure of $[(GS)_2AsSe]^-$ obtained with MOPAC-2000 using the PM3 Hamiltonian. C and H atom labels have been omitted for clarity.

on the column head following loading suggested that marginal decomposition of $[(GS)_2AsSe]^-$ had occurred, possibly due to small amounts of residual O_2 in the mobile phase, with the red color most likely arising from α-Se. A single peak containing arsenic, selenium and sulfur eluted in the void volume of the column, followed by a very broad boron peak, which was identified as borate after calibration with aqueous sodium borate. The intensities of the As, S and Se peaks were calibrated with reference to standard solutions of mixtures of sodium arsenite, selenite, and sulfate. This gave an As:S:Se stoichiometry for the peak of 1.1:1.9:1.1, which is in excellent accord with the 1:2:1 stoichiometry expected for $[(GS)_2AsSe]^-$.

Fig. 4 shows the calculated structure for $[(GS)_2AsSe]^-$ obtained using the semi-empirical MOPAC-2000 software [15] and employing the PM3 Hamiltonian [16]. The amines were assumed to be protonated and the carboxylic acid groups deprotonated, resulting in a total charge on the molecule of −3. Due to the flexibility of the glutathione moiety, $[(GS)_2AsSe]^-$ can adopt a large number of different conformations and the computed structure will not be a unique solution. Furthermore, the computation does not take into account the effects of solvent, which will be important in determining the conformation of the glutathione groups in solution. Nevertheless, the computed structure of the $[(RS)_2AsSe]^-$ core, which is of primary interest to us, gave bond-lengths in good agreement with those determined from the EXAFS, suggesting that it is relatively insensitive to solvent effects. Thus, the computed structure gave As–Se and As–S bond-lengths of 2.33 and 2.27 Å, respectively, which compare very well with the experimental values derived from EXAFS of 2.31 and 2.26 Å, respectively. Bond angles of 102.7 and 108.9° were obtained for S–As–Se and S–As–S, respectively. One of the glutathione $-NH_3^+$ groups is associated with the As–Se moiety in the calculated structure, with the nitrogen only 2.9 Å from Se and 4.0 Å from As, and this type of interaction may be important in stabilizing $[(GS)_2AsSe]^-$. Previous studies using micellar chromatography indicated that the negative charge on $[(GS)_2AsSe]^-$ was accessible to solvent [5]. Accessibility of the charged As–Se core may be important in the mechanisms for recognition and transport of $[(GS)_2AsSe]^-$ in vivo. We have previously discussed the two possible resonance forms of $[(GS)_2AsSe]^-$, $(GS)_2As–Se^- \leftrightarrow (GS)_2As^- = Se$ [4]. Typical ranges for As–Se bond-lengths are 2.26–2.33 Å for a formal double, and 2.39–2.47 Å for single bonds. Thus, the As–Se bond-length for $[(GS)_2AsSe]^-$ is in the range for a double bond, and in agreement with this, our calculated structure indicates a bond-order of 1.6 for the As–Se bond.

In conclusion, we report an efficient aqueous solution synthesis and chromatographic purification of $[(GS)_2AsSe]^-$. Our results provide evidence for the mechanism of formation of this novel and biochemically very important compound by the previously reported route [4].

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