Similarities between the abiotic reduction of selenite with glutathione and the dissimilatory reaction mediated by *Rhodospirillum rubrum* and *Escherichia coli*

Janine Kessi and Kurt W. Hanselmann

*Microbial Ecology Group, Institute of Plant Biology, University of Zürich, Zollikerstrasse 107, CH-8008, Zürich, Switzerland.*

Corresponding author: Janine Kessi

Mailing address: Institute of Plant Biology, University of Zürich, Zollikerstrasse 107, CH 8008, Zürich, Switzerland.

Phone: ++41 01 634 82 11

Fax: ++41 01 634 82 04

Email address: Janine.kessi@access.unizh.ch
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Abstract

Various mechanisms were proposed to explain the biological dissimilatory reduction of selenite ($\text{SeO}_3^{2-}$) to elemental selenium ($\text{Se}^0$), although none are without controversy. Glutathione, the most abundant thiol in the eukaryotic cells, the cyanobacteria, and the alpha, beta, and gamma groups of the proteobacteria, has long been suspected to be involved in selenium metabolism. Experiments with the phototrophic alpha proteobacterium *Rhodospirillum rubrum* showed that the rate of selenite reduction was decreased when bacteria synthesized lower than normal levels of glutathione, and in *Rhodobacter sphaeroides* and *Escherichia coli* the reaction was reported to induce glutathione reductase. In the latter organism superoxide dismutase (SOD) was also induced in cells grown in the presence of selenite, indicating that superoxide anions ($\text{O}_2^-$) were produced. These observations led us to investigate the abiotic (chemical) reduction of selenite by glutathione and to compare the features of this reaction with those of the reaction mediated by *R. rubrum* and *E. coli*. Our findings imply that selenite was first reduced to selenodiglutathione, which reached its maximum concentration within the first minute of the reaction. Formation of selenodiglutathione was paralleled by a rapid reduction of cytochrome c (cyt c), a known oxidant for superoxide anions. Cyt c reduction was inhibited by superoxide dismutase (SOD), indicating that O$_2^-$ was the source of electrons for the reduction. These results demonstrated that superoxide was produced in the abiotic (chemical) reduction of selenite with glutathione, thus lending support to the hypothesis that glutathione may be involved in the reaction mediated by *R. rubrum* and *E. coli*. The second phase of the reaction, which led to the formation of elemental selenium ($\text{Se}^0$), developed more slowly. Se$^0$ precipitation reached a maximum within two hours after the beginning of the reaction. Secondary reactions leading to the degradation of
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the superoxide significantly decreased the yield of Se° in the abiotic reaction as
compared to that of the bacterially-mediated selenite reduction.

Abiotically formed selenium particles showed the same characteristic orange-red
color, spherical structure and size as particles produced by *Rodospirillum rubrum*,
again providing support for the hypothesis that glutathione is involved in the reduction
of selenite to elemental selenium in this organism.

Introduction

Selenium is an essential trace element in the nutrition of many organisms, but it can
be highly toxic depending on its concentration and speciation. High selenium
concentrations may cause severe abnormalities in the development of various animals
and plants (1,2,3). Deformation and structural modifications have been noted,
especially in creatine-formed tissues (i.e, hooves, horns, hair, feather, beaks, and
nails) in which appreciable quantities of selenium may accumulate. In these cases,
selenium toxicity has been attributed to its ability to replace sulfur in proteins or other
sulfur-containing biomolecules. In their investigations of selenite toxicity in
prokaryotes, Kramer and Ames (4) did not observe any non-specific incorporation of
selenium into proteins. They demonstrated that a mutant strain of *Salmonella
typhimurium*, which is able to overexpress oxidative stress proteins like catalase and
superoxide dismutase, is significantly more resistant to selenite toxicity than the wild
type. Their results suggest that free radical formation might be involved. They also
considered the high reactivity of selenite with sulfhydryl groups and the formation of
oxygen radicals when selenium reacted with cysteine or glutathione and concluded
that selenite toxicity in bacteria might be the result of oxidative damage. Consistent
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with these results Bébien et al. (5) observed that two types of superoxide dismutase are induced in cultures of *E. coli* exposed to selenite, thus confirming the involvement of free radicals in selenium toxicity. In addition, glutathione reductase was induced in cultures of *Rhodobacter sphaeroides* (6) and *E. coli* (5) amended with selenite. In the bacterial domain glutathione is present in the cyanobacteria and the alpha, beta, and gamma groups of the proteobacteria (7). Considering these data we investigated the chemical reduction of selenite with glutathione and compared the features of this reaction with those of the dissimilatory selenite reduction in *R. rubrum* and *E. coli*.

In a chemical approach, Painter (8) observed the high reactivity of selenite with thiol groups. He was the first to demonstrate the formation of selenotrisulfides (RS – Se - SR), according to the following equation:

$$4 \text{RSH} + \text{H}_2\text{SeO}_3 \rightarrow \text{RS} – \text{Se} – \text{SR} + \text{RSSR} + 3 \text{H}_2\text{O} \quad (1)$$

Ganther (9) studied the reaction of selenite with glutathione (GSH), the most abundant thiol found in the eukaryotic cells, the cyanobacteria, and the alpha, beta and gamma groups of the proteobacteria (7). He showed that the selenotrisulfide of glutathione (GS – Se – SG), which was later renamed selenodiglutathione, is a very good substrate for glutathione reductase with $K_m$ and $V_{max}$ values comparable to those of glutathione itself. He described the reaction with the equation 2:

$$\text{Glutathione reductase} \quad \begin{array}{c}
\text{GS} – \text{Se} – \text{SG} + \text{NADPH} \\
\searrow \quad \nearrow
\text{GSH} + \text{GS} – \text{Se} – + \text{NADP}^+ \quad (2)
\end{array}$$
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Ganther (9) also proposed that the unstable selenopersulfide of glutathione (GS-Se⁻) dismutates into elemental selenium (Se°) and reduced glutathione according to the following stoichiometry:

\[
\text{GS – Se}^- + \text{H}^+ \rightarrow \text{GSH} + \text{Se}^0
\]  

(3)

In experiments about the kinetics of selenite reduction in cultures of \textit{Rhodospirillum rubrum} we observed that the rate of the reaction is significantly decreased when the organism synthesizes low levels of glutathione (10). In the present study, we investigate the kinetics of formation of selenodiglutathione, superoxide anions, and elemental selenium during abiotic (chemical) reduction of selenite by glutathione and we compare the features of this reaction with those of the reaction mediated by \textit{R. rubrum} and \textit{E. coli}. We also compare the properties of the abiotically formed Se° particles with those produced during the bacterial process.

Material and Methods

Selenite reduction

Chemical reactions were performed in 50 mM Tris-HCl buffer (pH 7.0) at room temperature in tubes kept anoxic (Hungate, Bellco, Vineland, NJ, USA) under a nitrogen atmosphere. The buffer was degassed with an aspirator pump for about an hour before use. Anoxic stock solutions of selenite, glutathione and cytochrome c were also prepared in Hungate tubes under nitrogen and with degassed buffer. Addition of reactants and sampling was performed using syringes purged with nitrogen.
Selenite

$\text{SeO}_3^{2-}$ was determined using a colorimetric method based on the formation of a piazselenol complex with diaminonaphthalene (11,12).

Selenodiglutathione

GS-Se-SG was detected by monitoring its absorbance at 260 nm (9). Its appearance and disappearance was followed in a spectrophotometer (Uvikon 860, Kontron, Zürich, Switzerland). All measurements were performed in quartz cuvettes purged with nitrogen.

Superoxide anions

Since superoxide anions are known to reduce cytochrome c (cyt c) (13), relative superoxide levels can be determined by measuring the rate of cyt c reduction at various times during the reaction. The essays were performed in a thermostat-controlled compartment of the spectrophotometer at 25°C. Samples were prepared in glass cuvettes purged with nitrogen and tightly closed with silicon stoppers. The reaction mixture was allowed to equilibrate to the indicated temperature for 20 min before Cyt c was added with a syringe purged with nitrogen at various reaction times. The rate of reduction was recorded at 550 nm. Cyt c concentration in the samples varied between 40 $\mu$M and 160 $\mu$M, depending on the initial selenite concentration. A high concentration of 1mg/ml or about 25 $\mu$M superoxide dismutase (SOD) was required to markedly inhibit cyt c reduction under the conditions described in this work.
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Oxygen

Oxygen (O₂) concentration was monitored using a Clark cell (oxygen membrane polarographic detector; Rank Brothers Ltd, Cambridge, England). The reaction mixture was quickly transferred to the cell after the start of the reaction and monitored for 20-30 min. The Clark cell and syringe were purged with nitrogen before use.

Hydrogen peroxide

Hydrogen peroxide (H₂O₂) was determined using the horseradish peroxidase-coupled reaction described in Frew et al., (14). Measurements were performed 20 to 30 min after the start of the reaction in order to avoid inhibition of the peroxidase with superoxide anions.

Elemental selenium

Elemental selenium (Se⁰) was determined after oxidation to selenite with concentrated nitric acid. Samples containing 20-200 nmoles of Se⁰ were centrifuged in 1.5 ml Eppendorf tubes for 10 min at 15’000 × g. The supernatant was discarded and 50–60 µl of concentrated nitric acid were added to the pellet. The tubes were carefully closed and incubated in a boiling water bath until the orange-red color of Se⁰ disappeared (about 2–4 min). Double-distilled water was added to the tubes for a final volume corresponding to the initial sample volume, and the samples were mixed well. The Se⁰ transformed to selenite was determined using the method described above for selenite. The presence of Se⁰ in the reaction mixture could also be observed qualitatively due to its turbidity visible at 400 nm, where GS – Se – SG does not absorb.
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Preparation and purification of chemically-produced Se° particles

The Se° particles were prepared in 50 mM Tris•HCl buffer (pH 7.0) at room temperature under a nitrogen atmosphere. The chemically well-defined detergent diheptanoyl–phosphatidylcholine (DHPC) (15) was added to the buffer for a final concentration of 5.0 mM, to avoid aggregation and stacking of the particles on the walls of the vials. The reaction was performed with 0.5 mM selenite and 2.0 mM glutathione (GSH to selenite ratio of 4:1). The reaction was allowed to develop for 2 h before the Se° particles were centrifuged for 40 min at 130’000 x g at room temperature. The supernatant fluid was discarded, and the particles were washed three times with 50 mM Tris•HCl buffer (pH 7.0) containing 2 mM DHPC (=2 mM DHPC–Tris•HCl buffer). The DHPC concentration used is slightly higher than its critical micellar concentration (1.4 mM).

Isolation of biologically-produced Se°- particles

Phototrophically grown cultures of R. rubrum amended with 0.5 mM selenite were harvested two days after they entered stationary phase and processed immediately. Following centrifugation at room temperature for 5 min at 5’000 x g, the cell pellet was discarded. The supernatant, containing Se° particles together with bilayer vesicles (16), was centrifuged for 40 min at 130’000 x g. The resulting small pellet was resuspended in a volume of 50 mM Tris•HCl buffer (pH 7.0) corresponding to 1/10 of the initial volume of the culture. The contaminating bilayer vesicles were solubilized by adding the detergent DHPC to a final concentration of 15 mM (15); solubilization was achieved at room temperature. Membrane debris were removed by centrifugation at 12’000 x g for 10 min immediately after solubilization. The pellet was discarded, and the supernatant containing the Se° particles was centrifuged again at 130’000 x g.
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for 40 min. The small pellet was homogenized in a volume of 2 mM DHPC–Tris·HCl buffer corresponding to about 1/10 of the solubilization volume. This three-stage washing procedure (centrifugation of membrane debris and subsequent centrifugation and homogenization of particles), was repeated three times.

Electron microscopy

For transmission electron microscopy cells were fixed in 2.5% glutaraldehyde for 60 min (samples were diluted with 5% aqueous glutaraldehyde), washed with running water, and embedded in low-melting-point agarose. Agar blocks (approximately 1 by 1 by 1 mm were fixed in 1% OsO₄, washed in running water for 60 min, dehydrated in ethanol and acetone, and embedded in Epon-Araldit. Sections cut from the Epon-Araldit preparation were contrasted with uranyl acetate and lead citrate as described by Hess (17).

For transmission electron microscopy 10 µl of Se° particles suspension with a selenium concentration of about 1 mM were transferred via pipette onto a grid of cellulose nitrate and observed without any additional treatment. For energy dispersive X-ray analysis (EDAX) three to four portions of 5 µl – 10 µl of Se°-particle suspension with a Se°-concentration of about 10 mM were deposited on a carbon plate and the Se°-layer was dried at room temperature after each addition of particles. Spectra were recorded with an EDAX-DX4 microanalysis system (Philips, Eindhoven, The Netherland).

Results

Transformation of selenite by glutathione. Selenite was rapidly transformed in the presence of glutathione at room temperature. The yield of the reaction was largely
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improved by increasing the GSH to selenite ratio from 2:1 to 4:1. Approximately
40%-50% and 80%-95% of the initial selenite concentrations disappeared from the
reaction mixtures in samples containing GSH to selenite ratios of 2:1 and 4:1,
respectively (Fig. 1A and 1B). The half-life of selenite decreased when the initial
selenite and GSH concentrations were increased (Table 1).

Formation of selenodiglutathione (GS – Se – SG). The concentration of
selenodiglutathione increased rapidly within the first minute of the reaction. It
attained a maximum within 30-60 sec and decreased over the next 10-20 min. Higher
rates of appearance and disappearance of selenodiglutathione were correlated with
higher initial selenite concentrations and increased GSH to selenite ratios (Fig. 2). In
samples with a starting GSH to selenite ratio higher than 2, turbidity was visible in the
absorption spectra within a few min after initiation of the reaction (Fig. 2B).
Appearance of turbidity paralleled the formation of orange-red Se°-particles. Higher
turbidity correlated with higher initial selenite concentration and higher GSH to
selenite ratios.

Inhibition of GS – Se – SG degradation by SOD. Addition of SOD to the reaction
mixture greatly diminished the decrease in selenodiglutathione during the first
minutes of the reaction (absorption at 260 nm) (data not shown), suggeating that the
superoxide anions participated to the degradation of the selenodiglutathione.

Formation of superoxide anions. Figure 3 shows that maximal superoxide
concentrations were reached within the first minute of the reaction for all conditions
tested. In the samples containing the lowest selenite concentration (0.125 mM) and a
GSH to selenite ratio of 2:1, redox equilibrium was observed for about 10 min before
the cyt c reduction rate began to decrease again. The superoxide anions disappeared
Reduction of selenite with glutathione completely about 25 min after the reaction was started. Degradation of the superoxide was faster in reaction mixtures containing the GSH to selenite ratio of 4:1. Under this condition degradation of the superoxide proceeded in two steps: A rapid step, that took place within the first min of the reaction, was followed by a significantly slower step that extended up to 25 min after initiation of the reaction (Fig. 3). Higher initial concentrations of GSH and selenite correlated with a faster degradation of the superoxide (Table 1). Cyt c reduction, which reveals the presence of superoxide, was inhibited by superoxide dismutase (SOD); the inhibition was proportional to the SOD concentration (data not shown).

**Oxygen.** O$_2$ did not accumulate in the reaction mixtures; a constant low O$_2$ concentration of 9.0 μM (±1.5 μM) was determined using the Clark cell.

**Hydrogen peroxide (H$_2$O$_2$).** Hydrogen peroxide concentrations were determined in reaction mixtures containing 0.5 mM or 1.0 mM selenite and a GSH to selenite ratios of 4:1. In the 0.5 mM selenite mixture, low H$_2$O$_2$ concentrations of 9.05 ± 1.15 μM and 8.30 ± 0.056 μM were attained after 20 and 50 min, respectively. In assays with 1.0 mM selenite H$_2$O$_2$ concentrations of 19.2 ± 0.17 μM and 9.94 ± 0.47 μM were measured 20 min and 50 min, respectively, after the start of the reaction. H$_2$O$_2$ rapidly reacted with GSH. A decrease in hydrogen peroxide concentration in the presence of 0.5 mM glutathione was investigated for glutathione/hydrogen peroxide ratios ranging from 4:1 to 1:1. The reaction reached equilibrium within a few seconds, with 55%-70% of the H$_2$O$_2$ disappearing from the reaction mixtures (data not shown).

H$_2$O$_2$ also reacted with Se°. A decrease of Se° of 30% was measured after 15 min in a reaction mixture containing 0.125 mM H$_2$O$_2$ and 0.150 mM Se°.
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**Formation of Se° particles.** Kinetics of the abiotic formation of Se° are presented in Fig. 4A. Turbidity measurements (400 nm) of the reaction mixtures at different times showed that precipitation of Se° particles started a few minutes after the initiation of the reaction. Shorter precipitation lag times correlated with higher initial concentration of selenite and glutathione (Table 1). According to both spectrophotometric and chemical determinations Se° formation was slow and leveled off after 20-90 min. In some cases, Se° concentrations decreased slowly after having reached a maximum value (Fig. 4A). The final yield of Se° was relatively low (45–80%) as compared to that of the bacterially-mediated reaction (see for example:6,18,12,19), and was in most cases only slightly improved by increasing the ratio of GSH to selenite (Fig. 4B).

For the GSH to selenite ratio of 2:1, as estimated from turbidity measurements, yields of Se° were low as compared to those obtained in reaction mixtures with GSH to selenite ratios of 4:1 or higher. They increased with increasing initial selenite concentrations and varied between 4.5% and 27.5% in samples containing initial selenite concentrations between 0.125 mM and 1.00 mM (data not shown).

The selenium particles formed during chemical reduction of selenite by glutathione showed the same characteristic orange-red color as the particles which are produced in bacterial cultures amended with selenite (12). The chemically produced particles, washed in buffer containing 2 mM DHPC (2 mM DHPC-Tris·HCl buffer), also had the same spherical morphology and diameter (35 – 45 nm) as the particles isolated from bacterial cultures (Fig. 5). Omission of the detergent in the reaction and/or the washing buffer led to the formation of large aggregates of both artificial and
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biological particles. It must be noted that the small bacterially-produced selenium particles isolated from the culture medium after removing the cells by centrifugation, (Fig. 5A), only represent about 3% of the total particle content of the culture. Most particles present in the culture medium had diameters varying between 250 and 350 nm (data not shown) and they sedimented during centrifugation of the cells. However, in cells of *R. rubrum* observed under the electron microscope the diameter of the particles varied between 35 and 45 nm, which corresponds to the size of the smallest particles isolated from the culture medium (Fig 5C).

Energy dispersive X-ray analysis (EDAX) of both chemically and biologically produced Se°-particles shows the expected emission lines for selenium at 1.37 keV, 11,22 keV, and 12.49 keV corresponding to the SeLα, SeKα, and SeKβ transitions, respectively (Fig. 6). No additional sharp emission lines were present. A slightly larger background was observed for the biological particles (Fig. 6A) than for the abiotically produced particles (Fig. 6B).

**Discussion**

In the chemical reduction of selenite with glutathione much more selenite was reduced with a GSH to selenite ratio of 4:1, as compared to a ratio of 2:1. This strongly supports the process outlined in equation 1 (Introduction). However, the rapid increase in the production of O₂⁻ within the first minute of the reaction (as determined by cyt c reduction), and the concomitant formation of selenodiglutathione, infer that the formation of oxygen radicals occurs during the first step of the reaction. The observed proportionality between inhibition of cyt c reduction and SOD concentration in the reaction mixture gives evidence that cyt c was reduced by the
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superoxide anion. These results suggest that equation 1 proposed by Painter (8) and Ganther (9) must be modified to take into account the formation of \( \text{O}_2^- \). We propose to describe the first step of the abiotic reduction of selenite with glutathione by equation 1A:

\[
6 \text{GSH} + 3 \text{SeO}_3^{2-} + 4 \text{H}^+ \rightarrow 3 \text{GS} -\text{Se} - \text{SG} + 2 \text{O}_2^- + 5 \text{H}_2\text{O}
\]

(1A)

According to Bébien et al., (5), who observed a large induction of two types of superoxide dismutase in \( \text{E. coli} \) grown in the presence of selenite, we propose that reaction 1A also takes place in this organism, and that it may constitute the first step of the dissimilatory reduction of selenite in all cells containing high levels of glutathione and performing intracellular selenite reduction.

In the chemical reaction the superoxide spontaneously dismutates into oxygen and hydrogen peroxide (13) according to equation 1B:

\[
\text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{O}_2 + \text{H}_2\text{O}_2
\]

(1B)

The fate of the hydrogen peroxide formed in this reaction is described below.

In the biological reduction (Fig. 7) the highly reactive superoxide produced during the first step of the reaction undergoes a cascade of degradation reactions catalyzed by enzymes which are induced during oxidative stress (superoxide dismutase, catalase, peroxidase, and probably also cytochrome(s)). These enzymes catalyze the rapid disappearance of oxygen radicals, thus preventing oxidative damage and preserving
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metabolic functions and cell integrity. In a second step selenodiglutathione (GS-Se-SG) is converted to selenopersulfide (GS-Se\textsuperscript{-}) and reduced glutathione (GSH) by the glutathione reductase as described by Ganther (9) and outlined in equation 2 (Introduction). In a last step, the unstable selenopersulfide converts into the reduced glutathione and elemental selenium according to equation 3 (Introduction). It is not known whether this reaction is enzymatically catalyzed or not. A comprehensive view of the entire process is summarized in figure 7.

In cultures of many species of alpha, beta or gamma proteobacteria amended with up to 1 mM or 2 mM selenite the reaction is performed to completion (see for example:5,20,12,19). The threshold of selenite concentration that the cells are able to reduce may reflect, at least in part, the large requirement of oxidative stress enzymes for this process. Another challenge the cells are confronted with in the presence of selenite, is the uptake of the oxyanions into the cytoplasm. Unregulated uptake would give rise to fast reactions of selenite with –SH groups of proteins, which would affect metabolic functions. Damages observed in keratine rich tissues by higher organisms (1,2,3) may be due to the absence of an efficient protective mechanism able to prevent the penetration of selenite into the cell.

The abiotic degradation of selenodiglutathione (Fig. 8) may be described by equation 2A:

\[
\text{GS – Se – SG} + \text{GSH} \rightleftharpoons \text{GS – Se}^- + \text{H}^+ + \text{GSSG} \quad (2A)
\]

In contrast to the biological reaction (reaction 2), in which the reduced glutathione is continually regenerated by the action of the glutathione reductase, the abiotic reaction
Reduction of selenite with glutathione leads to an accumulation of oxidized glutathione (GSSG), which slows down the reaction rate.

The last step of the abiotic reaction, the dismutation of GS-Se\(^{-}\) to GSH and Se\(^{0}\), does probably not differ from that of the biological process.

Another important difference between the abiotic and the biotic process is the persistence of the highly reactive peroxide anion in the reaction mixture, which gives rise to a cascade of secondary reactions that are summarized in figure 8. Since Se\(^{0}\) can be reoxidized by H\(_2\)O\(_2\) (reaction 6) the yield of the abiotic reduction of selenite to elemental selenium largely depends on the relative rate of formation and degradation of H\(_2\)O\(_2\) (reactions 4 and 5, respectively). Since reactions 2A and 4 proceed competitively (Fig. 8), the rate of formation of H\(_2\)O\(_2\) also depends on the rate of reaction 2A, i.e. the supply of GSH.

Reaction 4 is supported by the observed inhibition of selenodiglutathione (GS-Se-SG) degradation in the presence of superoxide dismutase (SOD), i.e. the removal of the superoxide anion (O\(_2\)\(^{-}\)). This observation indicates that the superoxide anion participates in the transformation of selenodiglutathione to selenopersulfide (GS – Se\(^{-}\)), according to equation 4:

\[
\text{GS} – \text{Se} – \text{SG} + 2 \text{O}_2^{-} + 4 \text{H}_2\text{O} + \text{H}^+ \rightarrow \text{GS} – \text{Se}^{-} + \text{GSH} + 4 \text{H}_2\text{O}_2
\]

(4)

Inhibition of the degradation of selenodiglutathione by SOD combined with the rapid degradation of the superoxide anion during the first minutes of the reaction, as shown in figure 3, suggests that the fast degradations of superoxide and selenodiglutathione proceed according to reaction 4. Spontaneous dismutation of the superoxide anion...
Reduction of selenite with glutathione proceeds significantly slower (13). The kinetics of this last reaction possibly corresponds to the second, slower step of the superoxide degradation shown in figure 3.

The abiotic degradation of hydrogen peroxide takes place in two ways. 1) The fast decrease of H₂O₂ concentrations in the presence of GSH (see Results section and 21) indicates that hydrogen peroxide can be detoxified through reduction by GSH according to equation 5:

\[
2 \text{GSH} + \text{H}_2\text{O}_2 \rightleftharpoons \text{GSSG} + 2 \text{H}_2\text{O} \quad (5)
\]

2) In agreement with the observed decrease of Se⁰ in the presence of hydrogen peroxide (Figure 8) we propose reaction 6:

\[
\text{Se}^0 + 4 \text{H}_2\text{O}_2 \rightarrow \text{SeO}_3^{2-} + 3 \text{H}_2\text{O} + \text{O}_2 + 2\text{H}^+ \quad (6)
\]

Considering that selenodiglutathione is a moderately stable intermediate that can be isolated from the reaction mixture (9,22), and that the oxidized glutathione accumulates in the reaction mixture, we propose that reaction 2A develops more slowly than reaction 4 (Fig. 8). This would promote the formation of hydrogen peroxide and the subsequent loss of Se⁰ (reaction 6) as shown in the abiotic reduction of selenite with glutathione (Fig. 4).

Close correlations between the half-life of selenite, the rate of superoxide degradation and the initiation of Se⁰ particle formation at various initial selenite concentrations strongly support equations 1A, 3, and 4 (Table 1).
In addition to reactions 4 and 5, which describe the fast degradation of the superoxide anion shown in figure 3, we have to consider the spontaneous dismutation of O$_2^-$ (Equation 1B), which may represent the slower step of the reaction (Fig. 3). Since elemental oxygen (O$_2$) was only detected in micromolar amounts we propose that the oxygen produced by this reaction was consumed in the radical chain of reactions involving O$_2^-$, O$_2$, and GSH, as described by Winterbourn and Metodiewa (21).

Through these reactions GSH and O$_2$ are consumed and O$_2^-$ is regenerated, maintaining the consumption of reduced glutathione as long as O$_2^-$ and O$_2$ are present.

The entire process is summarized in equations 7:

\[
O_2^- + 4 \text{GSH} + O_2 \rightarrow 2 \text{GSSG} + 2\text{H}_2\text{O} + O_2^- \quad (7)
\]

The efficiency of reaction 7 depends on pH, pO$_2$, and the superoxide generation rate. The reaction is very fast at neutral pH, in air, thiol concentration of about 0.5 mM and superoxide production rates in the micromoles per min range. Estimates of the rate constants for GSH under these conditions are in the range of $10^3$ M$^{-1}$ sec$^{-1}$ (21).

We therefore propose that GSH disappearance according to reaction 7 will proceed slowly in the presence of the low O$_2$ concentrations (9 µM) and the O$_2^-$ generation rates (10-180 µmoles · ml$^{-1}$ · min$^{-1}$) measured in our experiments.

Consumption of GSH and production of GSSG according to reaction 7 will contribute to lower the rate and the yield of the processes described by reactions 2A and 5 (Fig. 8). This explains the weak enhancement of the yield of Se° in the abiotic reduction of selenite with glutathione when the glutathione to selenite ratio is increased from 4/1 to 8/1 (Fig. 4).
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Considering i) the high GSH levels (1-10 mM) in the cytoplasm of various alpha, beta or gamma proteobacteria (23,7), ii) the high efficiency of the glutathione reductase in catalyzing the degradation of the selenodiglutathione to selenopersulfide and oxidized glutathione (9) (reaction 2), iii) the high reaction rate observed for selenite reduction by GSH under abiotic conditions (reaction 1A), iv) the decrease of the selenite reduction rate in *R. rubrum* with decreased glutathione levels (10), and v) the production of oxygen radicals in both, the abiotic reduction of selenite with glutathione and the reaction mediated by *E. coli*, we suggest that glutathione may be the electron donor in the dissimilatory reduction of selenite in organisms performing this reaction intracellularly and containing high GSH levels in their cytoplasm. Other biomolecules containing –SH groups may also be involved in the reduction. The reduction of selenite and selenodiglutathione by the thioredoxin – thioredoxin reductase system of *E. coli* was described by (24). Involvement of this enzymatic system in the biological dissimilatory reduction of selenite is supported by the observed induction of thioredoxin and thioredoxin reductase in cultures of *E. coli* amended with millimolar levels of selenite (5).

In the bacterial domain only the cyanobacteria and representatives of the alpha, beta and the gamma groups of the proteobacteria are able to synthesize glutathione (7). Consistent with the involvement of glutathione in the dissimilatory reduction of selenite, various proteobacteria belonging to these groups have shown to tolerate millimolar levels of selenite (6, 20, 12, 19). In contrast, a large survey of selenite tolerance in bacteria (63 species) demonstrated that most species (81%) are not able to grow in the presence of 0.2 mM selenite (25). Since the proposed mechanism of selenite reduction releases highly reactive oxygen species it cannot take place in strict anaerobe, which do not synthesize oxidative stress enzymes.
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A different mechanism of selenite reduction may take place in gram- positive bacteria, which are not able to synthesize glutathione (7), but which can tolerate high levels of selenite. *Bacillus subtilis*, for example, has been shown to grow in the presence of up to 5 mM selenite (26). As for proteobacteria, however, a significant induction of thioredoxin and thioredoxin reductase has been observed in *Bacillus subtilis* exposed to millimolar concentrations of selenite (27) and, generally, gram-positive bacteria accumulate Coenzyme A and other organic compounds containing disulfide groups at millimolar levels (7). Additionally, a disulfide reductase capable of reducing Coenzyme A disulfide and other related disulfides is produced by Bacilli (28). This suggests that the mechanism of selenite reduction in these bacteria may be similar to that proposed for the proteobacteria with high glutathione level. It must also be mentioned that reduction of disulfides can be catalyzed by selenols, which may be present in certain cells as secondary products of the selenium metabolism (29). A prerequisite for a high tolerance toward selenite may therefore be a high cytoplasmic level of disulfide containing molecules, catalysts for the reduction of disulfides, and a functional oxidative stress protection system.

The small sizes of the Se° particles (35 nm to 45 nm) present in cells of *R. rubrum* grown in the presence of selenite (Fig. 5C) suggest that the smallest particles purified from the culture medium represent the original size of biologically produced Se°-particles. The larger particles (250 - 300 nm), which sediment during centrifugation of the cells are likely produced by aggregation of the 35 nm to 45 nm particles (Fig. 5A). Energy dispersive X-ray analysis provides evidence that Se° particles purified from both, the bacterial cultures and from the chemical reaction mixtures contain nearly pure selenium. The larger background observed in the biological particles is possibly
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due to the presence of small amounts of organic material present in the biological particles. We are presently analyzing these particles in more details.

Conclusion

The evidence for the formation of superoxide anions during the abiotic reduction of selenite with glutathione provided in this work is consistent with the observed production of superoxide in *E. coli* grown in the presence of selenite (5). It lends support to the hypothesis that glutathione may be involved in the dissimilatory reduction of selenite in organisms containing high levels of glutathione. Involvement of glutathione in the dissimilatory reduction of selenite in proteobacteria is also consistent with the observed decrease of the selenite reduction rate in *R. rubrum* containing low levels of glutathione (10).

The most important difference between abiotic and biotic reduction of selenite is the fate of the superoxide anion. In living cells the superoxide is rapidly transformed by oxidative stress enzymes. In the abiotic process it is involved in a cascade of secondary reactions, which leads to a significant decrease in the yield of elemental selenium.

Among the bacterial domain the proposed mechanism of selenite reduction can only take place in the cyanobacteria and the alpha, beta, and gamma groups of the proteobacteria, which are able to synthesize glutathione, and it is incompatible with strict anaerobic organisms, which are not able to synthesize oxidative stress enzymes. The similarities between the Se°-particles obtained in the abiotic and the bacterial processes, with regard to size, color, and spherical structure provide additional support for the role of glutathione in the dissimilatory reduction of selenite in living
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cells. Other reductants such as ascorbate or H$_2$S did not yield particles of the same small size and with a similar spherical morphology (30). Abiotic synthesis of small Se° particles employing glutathione in the presence of DHPC might be of technological interest, e. g. for the production of selenium nanowires used in the electronic industry (31).

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References

1. Ohlendorf, H. M. 1989. Bioaccumulation and effects of selenium in wildlife. In: Selenium in Agriculture and the Environment. Jacobs, L. W., ed., Amer. Soc. Agron., Madison, Wisconsin, pp. 133-177.

2. Läuchli, A. 1993. Selenium in plants: Uptake, functions, and environmental toxicity. Bot. Acta 106:455-468.
Reduction of selenite with glutathione

3. O’Toole, D., and M. F. Raisbeck. 1998. Magic numbers, elusive lesions: Comparative pathology and toxicology of selenosis in waterfowl and mammalian species. In: Environmental Chemistry of Selenium. W. T. Frankenberger and R. A. Engberg, eds., Marcel-Dekker, New York, NY, pp. 355-395.

4. Kramer, G. F., and B. N. Ames. 1988. Mechanisms of mutagenicity and toxicity of sodium selenite (Na₂SeO₃) in Salmonella typhimurium. Mutation Res. 201:169-180.

5. Bébien, M., G. Lagniel, J. Garin, D. Touati, A. Verméglio, and J. Labarre. 2002. Involvement of superoxide dismutases in the response of Escherichia coli to selenium oxides. J. Bacteriol. 184:1556-1564.

6. Bébien, M., J.-P. Chauvin, J.-M. Adriano, S. Grosse, and A. Verméglio. 2001. Effect of selenite on growth and protein synthesis in the phototrophic bacterium Rhodobacter sphaeroides. Appl. Env. Microbiol. 67:4440-4447.

7. Newton, G. L. and R. C. Fahey. 1989. Glutathione in prokaryotes in: Glutathione: Metabolism and physiological functions, José Viña Ed., CRC Press, Boca Raton. Pp. 69-77.

8. Painter, E. P. 1941. The chemistry and toxicity of selenium compounds with special reference to the selenium problem. Chem. Rev. 28:179-213.
9. Ganther, H. E. 1971. Reduction of the selenotrisulfide derivative of glutathione to a persulfide analog by glutathione reductase. Biochemistry 10:4089-4098.

10. Kessi, J. Glutathione is a key molecule in the dissimilatory reduction of selenite by purple non-sulfur bacteria. (In preparation).

11. Watkinson, J. H. 1966. Fluorometric determination of selenium in biological material with 2,3-diaminonaphtalene. Anal. Chem. 38:92-97.

12. Kessi, J., M. Ramuz, E. Wehrli, M. Spycher, and R. Bachofen. 1999. Reduction of selenite and detoxification of elemental selenium by the phototrophic bacterium *Rhodospirillum rubrum*. Appl. Env. Microbiol. 65:4734-4740.

13. McCord, J. M., and I. Fridovich. 1969. Superoxide dismutase. An enzymic function for erythrocuprein (Hemocuprein). J. Biol. Chem. 244:6049-6055.

14. Frew, J. E., P. Jones, and G. Scholes. 1983. Spectrophotometric determination of hydrogen peroxide and organic hydroperoxides at low concentrations in aqueous solution. Anal. Chim. Acta 155:139-150.

15. Kessi, J., J.-C. Poirée, E. Wehrli, R. Bachofen, G. Semenza, and H. Hauser. 1994. Short-chain phosphatidylcholines as superior detergents in solubilizing membrane proteins and preserving biological activity. Biochemistry. 33:10825-10836.
Reduction of selenite with glutathione

16. Beveridge, T. J. 1999. Structures of gram-negative cell walls and their derived membrane vesicles. J. Bacteriol. 181:4725-4733.

17. Hess, W. M. 1966. Fixation and staining of fungus hyphae and host plant root tissues for electron microscopy. Stain Technol. 41:27-35.

18. Dungan, R. S., S. R. Yates, and W. T. Frankenberger, Jr. 2003. Transformations of selenate and selenite by Stenotrophomonas maltophilia isolated from a seleniferous agricultural drainage pond sediment. Environ. Microbiol. 5:287-294.

19. Roux, M., G. Sarret, I. Pignot-Paintrand, M. Fontecave, and J. Coves. 2001. Mobilization of selenite by Ralstonia metallidurans CH34. Appl. Env. Microbiol. 67:769-773.

20. Lortie, L., W. D. Gould, S. Rajan, R. G. L. McCready, and K.-J. Cheng. 1992. Reduction of selenate and selenite to elemental selenium by a Pseudomonas stutzeri isolate. Appl. Env. Microbiol. 58:4042-4044.

21. Winterbourn, C. C., and D. Metodiewa. 1995. Reaction of superoxide with glutathione and other thiols. Meth. Enzym. 251:81-86.

22. Björnstedt, M., S. Kumar, and A. Holmgren. 1995. Selenite and selenodiglutathione: Reactions with thioredoxin systems. Meth. Enzymol. 252:209-219.
Reduction of selenite with glutathione

23. Apontoweil, P., and W. Berends. 1975. Glutathione biosynthesis in Escherichia coli K12. Properties of the enzymes and regulation. Biochim.Biophys.Acta. 399:1-9.

24. Björnstedt, M., S. Kumar, and A. Holmgren. 1992. Selenodiglutathione is a highly efficient oxidant of reduced thioredoxin and a substrate for mammalian thioredoxin reductase. J. Biol. Chem. 267:8030-8034.

25. Lapage, S. P., and S. Bascomb. 1968. Use of selenite reduction in bacterial classification. J. appl. Bact. 31:568-580.

26. Garbisu, C., S. Gonzalez, W.-H. Yang, B. C. Yee, D. L. Carlson, A. Yee, N. R. Smith, R. Otero, B. B. Buchanan, and T. Leighton. 1995. Physiological mechanisms regulating the conversion of selenite to elemental selenium by Bacillus subtilis. BioFactors 5:29-37.

27. Garbisu, C., D. Carlson, M. Adamkiewicz, B. C. Yee, J. H. Wong, E. Resto, T. Leighton, and B. B. Buchanan. 1999. Morphological and biochemical responses of Bacillus subtilis to selenite stress. BioFactors 10:311-319.

28. Swerdlow, R. and P. Setlow. 1983. Purification and characterization of a bacillus megaterium disulfide reductase specific for disulfides containing pantethine 4’, 4’’-diphosphate. J. bacteriol. 153:475-484.
Reduction of selenite with glutathione

29. Singh, R. and G. M. Whitesides. 1991. Selenols catalyze the interchange reactions of dithiols and disulfides in water. J. Org. Chem. 56:6931-6933.

30. Oremland, R. S., M. J. Herbel, J. S. Blum, S. Langley, T. J. Beveridge, P. M. Ajayan, T. Sutto, A. V. Ellis, and S. Curran. 2004. Structural and spectral features of selenium nanospheres produced by Se-respiring bacteria. Appl. Env. Microbiol. 70:52-60.

31. Gates, B., B. Mayers, B. Cattle, and Y. Xia. 2002. Synthesis and characterization of uniform nanowires of trigonal selenium. Adv. Funct. Mater. 12:219-227.

Figure Legends

Table 1. Correlations between half-life of selenite, rate of disappearance of superoxide and appearance of Se° particles in the abiotic reduction of selenite with glutathione. The reaction was performed with the GSH to selenite ratio of 4:1 under the condition described in Methods.

a The half-life was graphically determined from the curves presented on Figure 1B.
b The time necessary for the disappearance of 80% of the maximal superoxide concentration was graphically determined from the curves presented on Figure 3.
c The lag time of appearance of the Se° particles was determined from the spectra of the reaction mixtures recorded at increasing times after initiation of the reaction. The values represent a mean of 2 experiments. The error was lower than 5% in each case.
Figure 1. Disappearance of selenite at various selenite concentrations and ratios of glutathione to selenite. (A) GSH to selenite = 2:1. (B) GSH to selenite = 4:1. Symbols: ● 0.125 mM; ■ 0.250 mM; ▲ 0.500 mM; ● 1.00 mM. Error bars represent the standard deviation of triplicate experiments.

Figure 2. Representative absorption spectra of reaction mixtures illustrating the appearance and disappearance of selenodiglutathione during the reaction. Starting selenite concentration = 0.125 mM. (A) GSH to selenite = 2:1. (B) GSH to selenite = 4:1. Experiments were conducted in triplicate. Formation of Se°, which results in a visible increase in sample turbidity, can be seen at 400 nm where selenodiglutathione does not absorb.

Figure 3. Time course of the appearance of the superoxide anion at various initial selenite concentrations. GSH to selenite ratios 4:1 (dark symbols), 2:1 (open symbols). Symbols as in Figure 1. Error bars represent the standard deviation of three experiments.

Figure 4. (A) Time course of the appearance of Se° for various initial selenite concentrations and the GSH to selenite ratio of 4:1. (B) Yields of Se° for various initial selenite concentrations and GSH to selenite ratios 120 min after the start of the reactions. Symbols as in Figure 1. Error bars represent the standard deviation of two experiments.

Figure 5. TEM of Se° particles. (A) Bacterially-produced particles isolated as described in the Methods section. (B) Abiotically produced particles. The initial selenite concentration was 0.5 mM in each case. A 4:1 glutathione to selenite ratio
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was used in the chemical reaction. (C) Particles present in the cytoplasm of *R. rubrum* grown in the presence of 0.5 mM selenite. The cells were earned and prepared for TEM as soon as reduction was complete.

Figure 6. Energy Dispersive Analysis of Se°-particles. A) Particles produced by the bacterial reduction of selenite. (B) Particles produced by the abiotic reduction of selenite with glutathione. Energy levels in keV are indicated on the x axis. The emission lines for selenium are at 1.37 keV (peak SeLα), 11.22 keV (peak SeKα), and 12.49 keV (peak SeKβ).

Figure 7. Hypothesis for the biological reduction of selenite. Glutathione is proposed to function as electron donor in the reduction. The first intermediary product is selenodiglutathione (GS-Se-SG), which is a substrate for the glutathione reductase (GR) (Ganther, 1971) and the bacterial thioredoxin (Björnstedt et al., 1992). The oxidized thioredoxin is regenerated by the thioredoxin reductase (TR). Reduction of selenodiglutathione by glutathione reductase leads to the formation of the selenopersulfide of glutathione (GS-Se⁻), which dismutates into reduced glutathione (GSH) and elemental selenium (Se°). In the biological reaction degradation of the superoxide is catalized by enzymes which are induced under oxidative stress. The electron source for the regeneration of glutathione is NADPH. The numbers enclosed in circles refer to the equation numbers indicated in the text.

Figure 8. Proposed fate of the superoxide anion in the abiotic reduction of selenite with glutathione, and formation of Se° particles. Since reaction 4 is fast (Fig. 3), and if H₂O₂ is not completely reduced as proposed by reaction 5, part of the H₂O₂ formed reoxidizes Se° and oxidizes glutathione. This explains the relatively low yield of Se°
Reduction of selenite with glutathione obtained in the abiotic process. The numbers enclosed in circles refer to the equation numbers indicated in the text.
Table 1

| Initial SeO₃ conc. (mM) | Half-life of SeO₃ (min)a | Time for 80% O₂⁻ to disappear (min)b | Lag time for Se⁰ to appear (min)c |
|------------------------|--------------------------|-------------------------------------|----------------------------------|
| 0.125                  | 4.90                     | 11.0                                | 7.0                              |
| 0.250                  | 2.35                     | 6.0                                 | 4.2                              |
| 0.500                  | 0.93                     | 4.3                                 | 2.3                              |
| 1.00                   | 0.40                     | N.d.                                | 1.8                              |
Figure 1

A. GSH/selenite 2/1

B. GSH/selenite 4/1

Selenite (% of initial conc.)

Time (min)

Selenite (% of initial conc.)

Time (min)
Figure 3

Graph showing the reduction rate of Cyt c (umoles/ml x min) over time (min) with different markers and error bars.
Figure 4

A

Se° formed (% of initial selenite) vs. Time (min)

B

Se° formed (% of initial selenite) vs. GSH/selenite
Figure 7

```
3 GS-Se-SG

1A
3 SeO₃²⁻
6 GSH
2 O₂⁻
Degradation by enzymes
of oxidative stress

3 NADPH + 3H⁺
3 NADP⁺
3 GSH
GR, TR

2
3 GS-Se⁻

3
3 GSH

3 Se⁰
```

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Figure 8

GS-Se-SG

GSH

2 $O_2^-$

GSSG

GS-Se$^-$

4 $H_2O_2$

4 $H_2O_2$

8 GSH

4 GSSG + 2 $H_2O$

Se$^0$

$SeO_3^{2-} + 3 H_2O + O_2 + H^+$

3

5

6
Similarities between the abiotic reduction of selenite with glutathione and the dissimilatory reaction mediated by Rhodospirillum rubrum and Escherichia coli
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