Shedding light on selenium biomineralization: proteins associated with bionanominerals

Running title: Identification of proteins on selenium bionanominerals

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ABSTRACT

Selenium reducing microorganisms produce elemental selenium nanoparticles with particular physico-chemical properties being due to an associated organic fraction. This study identified high affinity proteins being associated with such bionanominerals and with non-biogenic elemental selenium. Proteins with an anticipated functional role in selenium reduction, such as a metalloid reductase, were found associated with nanoparticles formed by one selenium respirer, *Sulfurospirillum barnesii*. 

KEYWORDS: selenium nanoparticles; bionanominerals; bioreduction; dissimilatory selenium reduction; nanoparticle-protein association

Certain anaerobic microorganisms utilize selenite and selenate as terminal electron acceptors for respiration and growth (=dissimilatory reduction), producing elemental selenium, either as internal accumulations that can be released to the medium or extracellularly [5, 11, 12]. Despite these differences in mechanism of formation, biogenic elemental selenium generally does not form large crystals but rather spherical nanoparticles [7, 8, 12]. It has been hypothesized that biogenic selenium nanoparticles can be stabilized against crystallization due to the presence of proteins [5], yet hitherto no proteins have been identified associated with nanoparticles of respiratory selenium reducers. This study investigated such proteins using two dissimilatory selenium reducers, *Bacillus selenatarsenatis* (DSMZ No. 18680) and *Sulfurospirillum barnesii*. 
(DSMZ No. 10660). For comparison, Rhodospirillum rubrum (DSMZ No. 467) was investigated, which can induce selenium precipitation, yet not in a dissimilatory manner.

Microorganisms were grown anaerobically in media according to the culture collection (DSMZ) under addition of 20 mM selenate (B. selenatarsenatis, S. barnesii) or 1 mM selenite (R. rubrum). B. selenatarsenatis was first pre-grown aerobically without addition of selenium and transferred to anaerobic medium during exponential growth. S. barnesii was grown using a N₂:CO₂ (80:20 v/v) headspace instead of N₂. R. rubrum was grown under light. Selenium nanoparticles were harvested in all batches in the late stationary phase, since formation in R. rubrum occurs during the latter [5]. As a control, lysed cells and culture medium were incubated with conventionally synthesized elemental selenium (from now on referred to as "non-biogenic"; Sigma Aldrich, Buchs, Switzerland). For this, pure cultures were grown in the absence of selenium oxyanions and subsequently lysed by means of an ultrasonic probe (Labsonic M, Sartorius, Tagelswangen, Switzerland).

High affinity proteins associated with elemental selenium (biogenic, non-biogenic) were isolated from low affinity / not associated proteins and residual biomass using density-based centrifugation in sodium polytungstate solution (SPTS) modified after [10, 13]. Batch medium (50 mL) was centrifuged (30 min; 5,000 g), pellets washed twice with tris-buffer (50 mM, pH 7.2) and transferred to the top of SPTS (density 3 g/mL) (TC Tungsten Compounds, Grub, Germany). Samples were then subjected to low-speed centrifugation (30 min, 2,000 g), the supernatants containing low affinity / non associated proteins and biomass were discarded and the pellets transferred into tubes containing fresh SPTS. Controls using biomass grown without selenium received the same treatment. Since no
visible pellet was formed in the latter, 200 µL of SPTS was transferred further, instead.
Centrifugation (5 repetitions) was followed by washing with tris-buffer (7 repetitions) to
exhaustively remove remaining SPTS.
Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to
separate proteins following standard protocols \[^{[6]}\]. SYPRO® Orange (LOD 1 to 2 ng /
band) (Figure 1) and Colloidal Blue staining (LOD <10 ng / band) revealed a number of
protein bands for all microbial species used. Most abundant protein bands were found
between 23-60 kDa (\textit{B. selenatarsenatis}, Figure 1A), 61-69 kDa (\textit{R. rubrum}, Figure 1B)
and 52-80 kDa (\textit{S. barnesi}, Figure 1B) apparent molecular weights.
For mass spectrometry (MS) analysis, Colloidal Blue stained bands were excised,
reduced with dithiothreitol (10 mM, 2 h, 37°C), alkylated with iodoacetamide (50 mM,
15 min, room temperature, darkness), trypsin-digested (125 ng trypsin, sequencing grade,
18 h, 37°C), and concentrated in SpeedVac concentrator. Capillary Liquid
Chromatography-Electro Spray Ionization-tandem Mass Spectrometry (LC-ESI-MS/MS)
was conducted on an Orbitrap FT hybrid instrument ((Thermo Finnigan, San Jose, CA,
USA) \[^{[17]}\]. The MS/MS spectra were searched against the NCBI data bank (using the
http://www.matrixscience.com portal) applying the following constraints:
carbamidomethyl cysteine as fixed, N-acetylation and oxidized methionine as variable
modifications; tryptic specificity allowing two missed cleavages; peptide tolerance 10
ppm; MS/MS tolerance 0.6 Da; taxonomy: bacteria. The significance threshold was set to
0.05 .The search output was filtered in a stringent manner, omitting all proteins with less
than 4 peptides matched and proteins with molecular weights outside 100% +/- 20% of
the apparent molecular weight determined by SDS.
Our results allowed for the first time to qualitatively characterize the high affinity protein fraction associated with selenium bionanominerals of different microbial origin. For all microorganisms used here, a plenitude of proteins with diverse cellular functions were identified (Table 1). Furthermore, we could firstly demonstrate that non-biogenic selenium as well associates with a number of proteins produced by dissimilatory selenium reducers. Taking into account the total number of proteins expressed in the studied organisms, it is striking that several proteins were found on both biogenic and non-biogenic particles. For silver nanoparticles contacted with *E. coli* cell free extracts, it has been shown that proteins associated did not simply reflect the most abundant proteins, yet are due to higher affinities of some proteins to the particles in contrast to others [18]. In this regards, it is most striking that the protein with the highest number of peptides matched in this study was the so called "metalloid reductase RarA" found on both biogenic (*S. barnesii*) and non-biogenic selenium nanoparticles of (Table 1).

Numerous peptide motifs, so called aptamers, have to date been described to specifically bind inorganic nanomaterials, amongst others chalcogen based (CdS, PbS, ZnS) and metallic (Au, Ag, Si, etc.) nanomaterials, yet not elemental selenium ([16] and references therein). It may thus well be that the metalloid reductase RarA found in this study, contains a natural peptide motif conferring its high affinity to elemental selenium. Such peptide motifs are of high interest, since they can be used to bind elemental selenium surfaces in particular applications - e.g. in bioremediation. Here, a current technically unresolved challenge is to remove the nanosized biogenic selenium from the aqueous phase [7]. Although its name suggests a selenate reducing enzymatic activity, unfortunately at present no further information is available regarding the enzymatic
characterization of this protein. A role as outer membrane porin can be anticipated from sequence similarities with porins from closely related *Sulfurospirillum deleyianum* (59% identity) and *Geobacter lovleyi* (49% identity, NCBI blast). It is thus possible that the metalloid reductase found here is involved in the initial uptake of selenate to the periplasm, where other selenate reductases have been found [15].

Furthermore, we were able to identify proteins with additional anticipated functional roles in selenium reduction in spatial association with the formed bionanominerals. Such proteins include, next to the metalloid reductase RarA, proteins involved in electron transport during microbial respiration, peptides with reactive thiol functional groups and enzymes involved in reactive oxygen species degradation (Table 1).

For *S. barnesii*, we first observed respiratory electron transport chain proteins associated with the bionanominerals, i.e. a nickel-dependent hydrogenase and an aldehyde ferredoxin oxidoreductase. This can be interpreted in the way that electrons necessary for reduction of selenate can be supplied by the hydrogenase. This has been described for microbial reduction of other toxic oxyanions [1]. Then, electrons can be transferred via ferredoxin, like that shown for selenate reduction in *Synechocystis* [9] and selenite reduction in *Clostridium* [19].

Selenite can be reduced to elemental selenium by reaction with reactive thiol groups of proteins / peptides in the so called "Painter-type" reaction, which has been suggested as a general microbial detoxification reaction to oxyanions [3]. The latter idea might be further supported by this study, since we were able to identify peroxiredoxins in *B. selenatarsenatis*, which can contain such catalytic cysteine-thiols [2, 14]. Next to the reaction of their reactive thiols, the peroxiredoxins matched (peroxiredoxin, alkyl
hydroperoxide reductase) can have a further general role in reaction to toxic selenium oxyanions, since are involved in degradation of reactive oxygen species. These are, amongst others, generated during the latter Painter-type reactions \[^{[3, 4]}\]. The same general detoxification role applies to NADH peroxidase found in the non respiratory selenium reducer, *R. rubrum*.

This study shows that selenium nanoparticles can be associated with a plenitude of high affinity proteins, despite their microbial origin and/or biogenic character. It is important to note that identification of proteins associated with selenium nanoparticles critically depends on the successful isolation of proteins associated intrinsically from adventitiously associated proteins that are potentially co-purified in the density-based centrifugation in SPTS. In this regards, the modification of the centrifugation procedure as applied in this study allowed firstly to definitively distinguish between these protein fractions, since selenium free controls did not show any protein bands in the SDS gels (Figure 1). Indisputably, the protein modification on the selenium particles will change the physico-chemical properties of the selenium solid to some extent and in consequence influence the environmental fate of selenium, like demonstrated for other bionanominerals \[^{[10]}\]. Consequently, this study represents a very first step only to understand the mechanisms underlying the formation of such associations. The high affinity proteins identified here might be used to design specific probes for e.g. immunofluorescence or immunoelectron microscopy to further study and visualize the mechanisms of intracellular nanoparticle formation. On the one hand, owing to the high importance of selenium in animal and human health, there is a clear need to further study the association of the protein fraction to nanoparticles, e.g. by proteomic analysis.
focusing on expression of proteins / enzymes found here. On the other hand, future studies should also consider the dissociation or biodegradation of the proteic fraction on selenium (or other bionanominerals), hence opening a fascinating yet challenging future field of interdisciplinary biogeochemical research.

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Figure 1. SDS-page gel images (SyproOrange stain) of proteins associated with biogenic selenium nanoparticles (+Se) and controls (-Se) produced by bacillus selenatarsenatis (A), Rhodospirillum rubrum (B) and Sulfurospirillum barnesi (C) after density based centrifugation. Molecular weight standards (L) in kDa.

Table 1. Proteins identified by LC-ESI-MS/MS associated to biogenic and non-biogenic elemental selenium. For matches relating to homologous proteins in different organisms, all accession numbers (GI) yet only the organism with the highest ion score is mentioned.

Proteins with anticipated role in selenium reduction are highlighted in grey color.
Table 1. Proteins identified by LC-ESI-MS/MS associated to biogenic and non-biogenic elemental selenium. For matches relating to homologous proteins in different organisms, all accession numbers (GI) yet only the organism name is given.

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<th>Organism</th>
<th>Protein Name</th>
<th>Match Score</th>
<th>E-value</th>
<th>Protein ID</th>
<th>Accession Numbers</th>
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**Notes:**
- A protein score greater than 42 is considered significant (p < 0.05).
- The identity and coverage are calculated based on the match score and E-value.
- Accession numbers and organism names are provided for each protein match.