Mobilization of sulfane sulfur from cysteine desulfurases to the Azotobacter vinelandii sulfurtransferase RhdA

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Abstract Mobilization of the l-cysteine sulfur for the persulfuration of the rhodanese of Azotobacter vinelandii, RhdA, can be mediated by the A. vinelandii cysteine desulfurases, IscS and NifS. The amount of cysteine was higher in mutant strains lacking rhdA (MV474) than in wild type. The diazotrophic growth of MV474 was impaired. Taking into account the functional results about rhodanese-like proteins and RhdA itself suggests that RhdA-dependent mobilization of l-cysteine levels must deal with a redox-related process.

Keywords RhdA · Sulfurtransferase · Cysteine desulfurase · l-Cysteine · Thiosulfate · Azotobacter vinelandii

Introduction

Numerous cellular processes require the mobilization of elemental sulfur from l-cysteine. Among those are included the assembly of Fe–S cluster (Johnson et al. 2005) as well as the synthesis of molybdoenzyme, thiamine, biotin and thionucleosides in tRNA (Mihara and Esaki 2002). The initial step in sulfur mobilization is catalyzed by a cysteine desulfurase (CD) that converts l-cysteine to sulfane sulfur and l-alanine in a process that uses pyridoxal-5’-phosphate as a cofactor (Zheng et al. 1993). The sulfane sulfur, present as persulfide intermediate on the active site cysteine of the desulfurase, is then transferred to sulfur acceptors on various physiological pathways. The work by Dean et al. defined the mechanism for l-cysteine desulphuration by Azotobacter vinelandii NifS, a prototype for CDs (Zheng et al. 1994). In this mechanism the formation of the persulfide intermediate is also recognized in all CDs.

In addition to NifS, A. vinelandii contains also another CD, IscS, which is involved in Fe–S cluster assembly in Fe–S enzymes other than nitrogenase (Zheng et al. 1998). Among the CDs, IscS is highly conserved among Gram-negative bacteria and cyanobacteria and it is the major cellular catalyst for the mobilization and distribution of sulfur from cysteine to a number of different biosynthetic pathways (Kambampati and Lahou 1999; Kate et al. 2002; Ikeuchi et al. 2006). It is generally recognized that mobilization of sulfur necessary for the synthesis and modification of these fundamental biomolecules is a process that requires strict control to protect cells from the formation of toxic sulfur species. The S0 bound on the CD could, in principle, be reductively released as sulfide prior to incorporation into the cofactors or could undergo direct covalent transfer to accessory proteins. It has been demonstrated that NifS may transfer the persulfide sulfur to NiU (Smith et al. 2001), and IscS to IscU and Thl proteins (Kambampati and Lahou 2000; Urbina et al. 2001). The exact pathway and mechanism of sulfur transfer for the eventual incorporation of sulfur into biomolecules still remains unclear. However, trafficking sulfur as persulfide intermediates has been recognized as a cellular strategy to bypass the toxicity of free...
sulfide (Beinert 2000; Mueller 2006). Specifically, sulfur
incorporation systems that rely on persulfide chemistry
often involve rhodanese homology domain proteins. It was,
indeed, shown that the essential catalytic Cy8456 in the
rhodanese homology domain of Escherichia coli ThiI was
a recipient of the terminal sulfid of the persulfide form
of IscS in the biosynthesis of 4-thiouridine in tRNA
(Palenchar et al. 2000), and that the rhodanese homology
domain of the human enzyme MCO3 transiently bears a
persulfide group on the pathway of sulfur incorporation
into molybdopterin (Matthies et al. 2004, 2005).

Azotobacter vinelandii expresses a unique rhodanese
called RhdA (Colnaghi et al. 1996). The RhdA active site
cysteine (Cy3230) is located at a structural loop (Bordo et
al. 2000, 2001; Pagani et al. 2000) that appears to be properly
designed to stabilize persulfide bond on its catalytic resi-
due. Moreover, in E. coli, recombinant RhdA is expressed
either in the persulfurated form (RhdA-SH) or in the
sulfane sulfur-deprived form (RhdA-SH), depending on the
sulfur source present during growth. We reported that
L-cysteine was the effective sulfur source of RhdA per-
sulfuration, and that the CD IscS present in E. coli pro-
moted the production of RhdA in the persulfurated form
(Forlani et al. 2005).

Considering that RhdA’s active site favors the stabil-
ization of its persulfurated form, we sought to determine if
RhdA-SH could function as “escort” protein during sul-
fur mobilization processes. In order to frame RhdA-SH
functions in a cellular context, we first investigated direct
transfer of S\(^2\) from the A. vinelandii CD NiS, and IscS to
RhdA. In the present study, we report the effect of RhdA
inactivation on the levels of cysteine and on diazotrophic
growth of A. vinelandii.

Materials and methods

Bacterial strains and growth conditions

The A. vinelandii strains used in this study were UW136, a
derivative of UW136 (MV474) in which disruption of the
rhdA gene was achieved by the insertion of a KIXX cas-
tette, following deletion of 584 bp as described in Coln-
aghi et al. (1996). Cells were grown aerobically in Burk’s
medium for 24 h at 30°C, supplemented with 2.5 mM
ammonium acetate and 1% sucrose. For diazotrophic
growth, ammonium acetate was omitted and culture time
was 48 h. Cells were spun down at 3,800g for 10 min, and
stored at -80°C. For protein expression, the E. coli strains,
BL21[pRep4] (for RhdA and RhdA\(_{C230A}\)) and BL21(DE3)
(for E. coli IscS, A. vinelandii IscS and NiS) were grown
in Luria–Bertani medium (containing 100 µg/ml ampicillin
and, only for BL21[pRep4], 25 µg/ml kanamycin) at 37°C
and, when absorbance at 600 nm was 0.4, 1 mM IPTG was
added. After 4 h of growth cells were collected and used
for protein purification.

Protein preparations

In the RhdA mutant (RhdA\(_{C230A}\)) the catalytic Cy3230
residue was replaced by an alanine residue. Site-directed
mutagenesis of rhda generated the plasmid pQER1MP and
was performed using pQER1 plasmid as template (Pagani
et al. 2000), and the mutagenic primers 5’-CCG GAC AAG
GAA ATC GTG ACg CAt ggc CAG ACC CAT CAC GCG
TCC-3’ (FMP1) and 5’-GGA GCC GTG ATG GGT CTG
cgc aTG eGT GAC GAT TTC CTT GTC CGG-3’ (RMP1)
where lower case letters indicate the mutated bases and
italic letters indicate the SphI restriction site introduced for
quick verification of mutagenesis. The accuracy of muta-
genesis was checked by DNA sequencing. His-tagged
RhdA and RhdA\(_{C230A}\) were expressed in E. coli strains
(BL21[pRep4]) harboring pQER1 and pQER1MP, respect-
ively. Expressed proteins were purified by Ni–NTA
affinity chromatography (Forlani et al. 2003), and gel-
filtered using a G25 column equilibrated in 50 mM Tris–HCl,
100 mM NaCl (pH 8). Sulfane sulfur-deprived RhdA and
recombinant E. coli IscS were prepared as described in
Forlani et al. (2005). Cy3230-blocked RhdA (RhdA-mBBR)
was obtained by incubation of 156 µM sulfane sulfur-
deprived RhdA in 50 mM Tris–HCl, 100 mM NaCl buffer
(pH 8) with 5 mM monobromobimane (mBBR, thiolate,
Calbiochem, Darmstadt, Germany; diluted from a 100 mM
stock solution in acetonitrile). After 2 h in the dark at room
temperature with continuous stirring, the reaction mixture
was gel-filtered on a G-25 column to remove reagent exces.
RhdA-bound mBBR was determined by measuring the
fluorescence (λ\(_{exc}\) = 345 nm; λ\(_{em}\) = 465 nm)(Kosow-
er and Kosower 1987). E. coli IscS\(_{C232A}\) was obtained
courtesy of Prof. L. E. Vickery (UC Irvine, CA, USA).

The plasmids pDB943 (Zheng et al. 1998) and pDB551
(Zheng et al. 1993) were used for E. coli heterologous
expression of A. vinelandii IscS and NiS, respectively.

Purification of recombinant A. vinelandii IscS and NiS
were performed as described previously (Zheng et al.
1993), except that after the ammonium-sulfate-fractiona-
tion the material was solubilized in 25 mM Tris–HCl (pH
7.5) and desalted by gel-filtration chromatography on G25-
Sephadex. Gel-filtered protein fraction was submitted to
anion exchange chromatography that was carried out in the
same buffer on Mono Q Sepharose (5/50 GL; Tricorn™),
in the case of IscS, or on DEAE 15 h (16/100 Protein-
Pak™, Water™), in the case of NiS, in a HPLC system
(Waters™ 600S). Protein elution was achieved with a
0–1 M NaCl gradient (A. vinelandii IscS and NiS eluted at
~0.3 M NaCl). To complete NiS purification, the eluted

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protein was concentrated using ultrafiltration devices (Amicon® Ultra 5 K) and submitted to Superose 12 (10/300 GL, Tricorn™) HPLC chromatography in 25 mM Tris–HCl 0.1 M NaCl (pH 8). Purification of A. vinelandii IscS and NiFS was continuously monitored by determining the ratio OD425/OD280 and assessed by SDS-PAGE analyses. Purified proteins were fractionated and stored at −30°C until used.

Analytical procedures

Cell-free extracts of A. vinelandii were prepared by sonicating (five 30 s pulses with intermitted 1 min cooling periods in Sonoprep 150; UK) in 10 mM Tris–HCl, 100 mM NaCl (pH 8), and cell debris was removed by centrifugation (30 min at 10,000 g). Protein concentration was determined by the Bradford assay (Bradford 1976) using bovine serum albumin as standard. Thiocyanate-cyanide sulfurtransferase (TST) activity was tested by the discontinuous method described in Söörbo (1953) that quantifies the product thiocyanate. One unit (U) of TST activity is defined as the amount of enzyme that produces 1 μmol thiocyanate/min at 37°C. Persulfide formation in CDs was tested as cyanolizable sulfur that was revealed as thiocyanate (Sörbo 1953). In particular, for thiocyanate quantification, to 0.65 ml of assay mixtures, 0.1 ml 37% formaldehyde and 0.25 ml Sörbo’s reagent (10% ferric nitrate in 13% nitric acid) were added sequentially before the spectrophotometric determination of the absorbance of the ferric-thiocyanate complex at 460 nm (ε = 2,890 M⁻¹ cm⁻¹). Detection of cysteine and thiolsulfate was carried out by a monobromobimane HPLC method (Riemenschneider et al. 2005) that was applied to 50 mg (f. wt) cell samples.

Spectroscopic determinations

Fluorescence measurements were carried out in a Perkin-Elmer LS-50 instrument, and data were analyzed as previously described (Pagani et al. 2000). Time-course fluorescence experiments were carried out at 25°C in 50 mM Tris–HCl, 100 mM NaCl (pH 8) in the presence of 4 mM l-cysteine, 10 μM pyridoxal phosphate and 0.5–3 μM sulfane-sulfur deprived RhdA (RhdA-SH). For the evaluation of the formation rates of RhdA-SSH, the intrinsic fluorescence (λex = 280 nm, λem = 340 nm) change, obtained after the addition of CD (0.4 μM), was converted in RhdA-SSH concentration. For conversion, the value of total fluorescence change obtained after the final addition of 0.5 mM Na2S2O3 was used as quantitative standard. Time-course data of RhdA-SSH concentration were fitted to an exponential-rise function in order to approximate the initial rate.

Results

L-Cysteine:RhdA sulfurtransferase activity of E. coli IscS

In our previous studies (Forlani et al. 2005), we identified the distinct fluorescence of both RhdA forms (RhdA-SH and RhdA-SSH). In this work, we developed a time-scale fluorescence assay for monitoring the production of RhDA-SSH. The formation of the stable RhdA-SSH form is an intrinsic property of this rhodanese-like protein due to the particular environment of its catalytic loop (Bordo et al. 2000). To gain insight into the molecular mechanism of sulfur transfer, we developed a kinetic fluorescence assay to monitor the formation of RhDA-SSH via CD activity. Typical runs of fluorescence changes are shown in Fig. 1 (panel a). These experiments showed the effects of l-cysteine (i.e. the substrate of CD) and the importance of the intermediate persulfurated form of the CD for effective sulfur transfer to RhDA. No fluorescence change was recorded when l-cysteine was added to RhDA-SH alone indicating that l-cysteine alone was not a direct sulfur donor to RhDA (not shown). The addition of purified E. coli IscS to a mixture containing 5-fold molar excess RhDA-SH and l-cysteine decreased the recorded fluorescence (solid line, Fig. 1, panel a) whereas no fluorescence changes were observed when the RhDA Cys230 was chemically blocked by monobromobimane (dashed line, Fig. 1, panel a). This shows that RhDA-SSH was produced and that sulfur was mobilized from l-cysteine in a reaction mediated by IscS. When wild-type IscS was replaced with the mutant IscS C328A no fluorescence change was observed (dashed-dotted line, Fig. 1, panel a) thus demonstrating that the Cys328 residue of IscS is mandatory for the IscS-catalyzed RhDA persulfuration (l-cysteine:RhdA sulfurtransferase activity). According to the proposed mechanism for CDs, the Cys328 residue of IscS is the site where enzyme-bound persulfide is formed (Zheng et al. 1994). Therefore, IscS-mediated production of RhDA-SSH must be the result of a transpersulfuration reaction involving the IscS Cys328 and the RhDA Cys230, being this last the RhDA catalytic residue properly designed to bear a stable persulfide sulfur.

Intrinsic fluorescence of RhDA-SSH was not affected by the addition of 60-fold molar excess of l-cysteine (data not shown) showing that the monothiol l-cysteine does not behave as an acceptor of the sulfane sulfur held on RhDA Cys230. This latter evidence is in line with previous results (Cereda et al. 2003) showing that sulfane sulfur removal from RhDA-SSH, giving rise to the unloaded form (RhdA-SH), occurred in the presence of the dithiol diethiothreitol (DTT), but not in the presence of the monothiol β-mercaptoethanol. The RhDA-SSH formation, monitored
by the fluorescence assay, is not affected by the presence of l-cysteine, and can be used to measure the rate of transpersulfuration between IscS and RhdA. Since this reaction was achieved by multiple cycles according to the stoichiometry of our system, we were prompted to define apparent kinetic parameters of transpersulfuration reaction between the two proteins. Runs monitoring fluorescence changes of RhdA were carried out in the presence of different substrate concentrations (i.e. the sulfane sulfur acceptor RhdA-SH), and the curves were analyzed to calculate initial velocity of the

![Fig. 1](image)

**Fig. 1** Fluorescence kinetic measurements of RhdA persulfuration mediated by *Escherichia coli* IscS. A Representative time-course traces of the fluorescence changes ($\lambda_{\text{exc}} = 280$ nm, $\lambda_{\text{em}} = 340$ nm) following the addition of 0.4 $\mu$M purified *E. coli* IscS (solid line) were monitored in 1 ml of 50 mM Tris-HCl, 100 mM NaCl, pH 8, containing: 2 $\mu$M sulfane sulfur-deprived RhdA, 4 mM L-cysteine and 10 $\mu$M PLP. Trace obtained omitting L-cysteine is reported as a dotted line. As a control: 0.4 $\mu$M IscS$_{CYS}$A (dashed-dotted line) replaced IscS, or 2 $\mu$M RhdA variant having the catalytic cysteine chemically blocked by monobromobimane (RhdA-mBBr; dashed line) replaced RhdA. The arrow denotes the addition of 0.5 mM Na$_2$S$_2$O$_3$. B Example of initial rate determination of IscS-mediated persulfuration of RhdA. Values of time-scale fluorescence changes following the addition of 0.4 $\mu$M IscS to a mixture of 2 $\mu$M RhdA-SH, 4 mM L-cysteine, and 10 $\mu$M PLP in 50 mM Tris-HCl, 100 mM NaCl, pH 8, were converted to RhdA-SSH concentration values (filled circle) using as quantitative standard the value of total fluorescence change obtained after addition of 0.5 mM Na$_2$S$_2$O$_3$, and were fitted to the exponential-rise function (solid line) in order to approximate the initial rate (dashed line). C Double-reciprocal plot of the initial rates of IscS-mediated persulfuration of RhdA determined at different concentrations of RhdA-SH. Values are means ± standard deviation (SD) for three separate experiments.
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Fig. 2 RhDA persulfuration mediated by Azotobacter vinelandii IscS and NiFS. a Intrinsic fluorescence changes ($\lambda_{\text{exc}} = 280$ nm, $\lambda_{\text{em}} = 340$ nm) following the addition of 0.4 $\mu$M purified A. vinelandii IscS (open circles) or NiFS (filled circles) were monitored in 1 ml of 50 mM Tris–HCl, 100 mM NaCl, pH 8, containing 2 $\mu$M sulfane sulfur-deprived RhDA, 4 mM L-cysteine, and 10 $\mu$M PLP. b Double-reciprocal plot of the initial rates of NiFS- (filled circles, solid line) and IscS- (open circles) mediated persulfuration of RhDA determined at different concentrations of RhDA-SH. Values are mean ± standard deviation (SD) for three separate experiments.

A. vinelandii RhDA. Aiming to identify the role of RhDA as a sulfur mediator in sulfur transfer processes of physiological relevance, we studied the transpersulfuration process in the presence of the A. vinelandii CDs, NiFS and IscS.

Transpersulfuration reaction to RhDA occurred with both A. vinelandii CDs, but their efficiencies to produce RhDA-SSH were different (Fig. 2, panel a). After 5 min reaction, 55% of the RhDA-SH present in the mixture was converted into RhDA-SSH when sulfur of L-cysteine was mobilized by NiFS, and 33% in the case of IscS. The observed L-cysteine:RhDA sulfurtransferase activity of NiFS and IscS were, respectively, 0.025 ± 0.006 and 0.014 ± 0.003 $\mu$mol min$^{-1}$ mg$^{-1}$. Lineweaver–Burk plot of sulfur transfer from NiFS to RhDA (Fig. 2, panel b), suggests a productive interaction between the persulfide form of NiFS and RhDA. In the conditions used for fluorescence change measurements, that allowed detection of transpersulfuration activity by a continuous-type enzyme assay, the NiFS apparent (4 mM L-cysteine) $K_m$ for RhDA-SH was 1.88 ± 0.49 $\mu$M, and the NiFS turnover number was 1.05 ± 0.35 min$^{-1}$.

As stated before, A. vinelandii IscS also showed L-cysteine:RhDA sulfurtransferase activity (Fig. 2, panel a), but the data did not yield straight lines in double reciprocal plot analyses in the same range of substrate-RhDA-SH concentrations used for determining kinetic parameters of the NiFS-mediated reaction (Fig. 2, panel b; empty circles). Considering the low L-cysteine:RhDA sulfurtransferase activity of A. vinelandii IscS, probably the sensitivity of the developed time-scale fluorescence assay is decreased by the high background intrinsic fluorescence which is not affected by the quenching effect of the persulide group in RhDA. In A. vinelandii IscS, at least 4 Trp residues contributes to the intrinsic fluorescence of this protein whereas there are only 2 Trp residues in the NiFS sequence. Also in Zheng et al. (1998) was reported that kinetic parameters for L-cysteine desulfurization catalyzed by A. vinelandii IscS could not be obtained and activity inhibition by L-cysteine was claimed.

In A. vinelandii NiFS-catalyzed desulfuration of L-cysteine, the apparent $K_m$ for L-cysteine is about 75 $\mu$M (Zheng et al. 1994), whereas no figure of $K_m$ for L-cysteine in the A. vinelandii IscS-catalyzed desulfuration of cysteine was reported.

Effects of RhDA on the persulfide formation of Azotobacter vinelandii cysteine desulfurases, NiFS and IscS

The formation of persulfide on Cys$_{328}$ (E. coli IscS numbering) residue of CDs is an essential prerequisite for transpersulfuration to RhDA. The relative efficiency of the transpersulfuration reaction paralleled the CD activity figures of 0.124 and 0.168 $\mu$mol min$^{-1}$ mg$^{-1}$ determined for A. vinelandii IscS and NiFS, respectively (Zheng et al. 1998). Since in those determinations persulfide was reductively cleaved to generate sulfide in the presence of DTT, we raised the question of whether the observed sulfur transfer to RhDA reflects the turnover number of generation of the IscS- and NiFS-bound persulfide forms. Persulfide (sulfane sulfur) can be identified by its reactivity with cyanide (Beinert 2000) to produce thiocyanate in the cyanalysis reaction. In this work, cyanalysis assay was used to detect persulfide formation in A. vinelandii IscS and NiFS in the presence of L-cysteine (4 mM) and in the absence of...
Fig. 3 Effect of RhdA on the production of sulfane sulfur of *A. vinelandii* IscS and NifS. a The absorbance at 460 nm due to the production of thiochyanate in mixtures (0.65 ml) of 50 mM Tris–HCl, 100 mM NaCl (pH 8) containing 4 mM l-cysteine, 10 μM pyridoxal phosphate, 0.4 μM cysteine desulfurase, 30 mM KCN was measured after incubation (60 min at 25°C) in the absence of RhdA (black), in the presence of 2 μM RhdA (gray) or 2 μM RhdAc230A (white). Production of sulfane sulfur of NifS in the presence of different concentrations of RhdA is reported relative to that obtained in the absence of RhdA. A.U. arbitrary units. Values are mean ± standard deviation (SD) from three independent experiments.

DTT. Persulfide formation rate on *A. vinelandii* IscS and NifS was similar (Fig. 3, panel a; black bars). When catalytic amounts of RhdA were added in the latter described assay, persulfide formation rates increased 1.6-fold in *A. vinelandii* IscS and 2.0-fold in *A. vinelandii* NifS (Fig. 3, panel b; gray bars). The replacement of RhdA by an RhdA variant (RhdA-mBr), where the C230 thiol was chemically blocked by monobromobimane (data not shown), or by an RhdA mutant (RhdA-c230A), where the Cys230 residue was replaced by alanine, caused a similar increase (Fig. 3, panel a; white bars). Such increase is not observed by the addition of bovine serum albumin (data not shown). This indicates that Cys230, the catalytic residue of RhdA, is not responsible for the RhdA-achieved increase of the persulfide formation rates on IscS and NifS. The RhdA-mediated increase of the persulfide formation in NifS shows a saturation behavior with a maximum reached at about 1:1 RhdA:NifS (molar) ratio (Fig. 3, panel b). These data support the idea that RhdA interacts with NifS leading to an increase in the persulfide formation rate.

Fig. 4 Functional relationship between RhdA and NifS. a Determinations of growth rates of wild type (UW136; triangles) and MV474 (circles) were carried out in the presence (empty symbols) or in the absence (filled symbols) of 15 mM ammonium acetate in the medium. b Thiosulfate-cyanide sulfurtransferase (TST) activity measured in crude extracts prepared from UW136 (black) and MV474 (white) *A. vinelandii* strains grown in the presence (BSN) or in the absence (BS) of 15 mM ammonium acetate. Values are mean ± standard deviation (SD) from three independent experiments.
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Diazotrophic growth of *A. vinelandii* strain lacking a functional RhdA

NiFS was suggested to be required for full activation of *A. vinelandii* nitrogenase and it is partially essential for diazotrophic growth (Jacobson et al. 1989; Zheng et al. 1993). Since data presented here suggested an interaction of RhdA with NiFS, the aerobic growth of *A. vinelandii* strain MV474, lacking a functional RhdA, was carried out in diazotrophic conditions and compared to the growth of the wild-type strain (UW136). The aerobic growth rate in diazotrophic conditions (BS medium) of MV474 was lower respect to that of the wild-type strain, whereas in the presence of ammonia (in the BSN medium) the growth rates of both strains were comparable (Fig. 4, panel a).

This sulfate:cyanide sulfurtransferase activity and NiFS expression in *A. vinelandii*

*Azoctobacter vinelandii* crude extracts prepared from the wild-type strain (UW136) and the mutant strain lacking a functional RhdA (MV474) were assayed for thiosulfate:cyanide sulfurtransferase (TST) activity. Furthermore, the effect of diazotrophic growth (BS medium) on TST activity was evaluated. The strain lacking RhdA retains only a residual TST activity with respect to that revealed in UW136, and most part (94%) of the TST activity of UW136 is assignable to the expressed RhdA (Fig. 4, panel b). Under these conditions RhdA is not immunodetectable in MV474 by anti-RhdA antibodies (Cerden et al. 2007). Thus, the basal TST activity could be due to the redundancy of rhodanese-like genes in the *A. vinelandii* genome and not is modulated by the tested growth conditions (Fig. 4, panel b). The detected TST activity of wild-type strain is 1.5-fold higher in BS than in BSN. Since NiFS is expressed in diazotrophic conditions (Dos Santos et al. 2007) it can be argued that TST activity paralleled NiFS expression. Considering TST activity as a probe for RhdA functionality, it appeared to be dependent on NiFS expression probably as a consequence of a functional/physiological relationship between the cellular processes in which RhdA and NiFS are involved.

Cysteine levels in *A. vinelandii*

l-Cysteine and thiosulfate were identified as the most effective sulfur sources in producing RhdA-SSH during *E. coli* heterologous expression of RhdA (Forlani et al. 2005). Whereas thiosulfate is the sulfane sulfur donor to RhdA in the in vitro catalyzed reaction, l-cysteine can be used as sulfur source for RhdA-SSH formation only in the presence of CDs (this work; Forlani et al. 2005).

To assess if loss of the RhdA function can affect the intracellular levels of cysteine and thiosulfate, monobromobimane-adducts of these metabolites were determined in UW136 and in MV474 *A. vinelandii* strains (Table 1). In the absence of RhdA (MV474), cysteine is 2.1-fold higher than that revealed in wild type (UW136). No important accumulation of thiosulfate can be observed in *A. vinelandii* strain lacking RhdA. When *A. vinelandii* is grown in the absence of ammonia (BS) a much more prominent effect of the RhdA lack was observed on cysteine level, being 7.1-fold more accumulated than wild type. On the other hand thiosulfate levels do not show a clear trend. These analyses evidenced that, in vivo, RhdA affects the cysteine metabolism, whereas thiosulfate does not appear to be metabolically related to RhdA though it is a good in vitro sulfur donor in the RhdA-catalyzed sulfurtransferase reaction.

### Discussion

In this study we showed that the *A. vinelandii* CDs, IscS and NiFS, can mobilize sulfur from l-cysteine for RhdA persulfuration by a transpersulfuration reaction. Peculiar fluorescence features of RhdA allowed us to develop a continuous enzyme assay for defining apparent kinetic parameters of the protein–protein transpersulfuration. Apparent velocity of the transpersulfuration reaction is higher for NiFS than IscS, however persulfuration of RhdA can be achieved by both the *A. vinelandii* CDs. Therefore, there is not a preferential functional relationship of RhdA with NiFS or IscS. The ability of IscS and NiFS to produce cyanide-detectable sulfur (i.e. sulfane sulfur) from

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth medium</th>
<th>Cysteine (pmol/mg cell f. wt.)</th>
<th>Thiosulfate (pmol/mg cell f. wt.)</th>
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</thead>
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<tr>
<td>UW136</td>
<td>BSN</td>
<td>10.3 ± 1.4</td>
<td>42.5 ± 1.1</td>
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<tr>
<td></td>
<td>BS</td>
<td>5.1 ± 1.0</td>
<td>58.9 ± 2.6</td>
</tr>
<tr>
<td>MV474</td>
<td>BSN</td>
<td>22.0 ± 1.8</td>
<td>44.9 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>BS</td>
<td>36.0 ± 2.1</td>
<td>51.5 ± 1.1</td>
</tr>
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Mean ± standard deviation (SD) from three independent experiments

a f. wt. fresh weight
L-cysteine is increased in the presence of RhDA by a mechanism that does not involve the only thiol in RhDA (CyS230). The observation that this increase has a saturation behavior with a maximum when RhDA and NifS are equimolar led us to consider that this effect is in agreement with an interaction between RhDA and NifS. Probably RhDA interacts with NifS (or IscS) inducing some conformational changes which favor the formation of the persulfide on the catalytic cysteine residue of NifS (or IscS). It cannot be excluded that the interaction of RhDA protects the persulfide by the excess of free L-cysteine which was shown to be competitive respect to DTT in the CD reaction when it is assayed probing the labile sulfur (Behshad et al. 2004). Considering that CDs are involved in many pathways for the biogenesis of sulfur-containing compounds (Mihara and Esaki 2002), the RhDA modulation of NifS/IscS-mediated persulfide production could have the regulatory purpose to address the sulfane sulfur flow toward the cellular pathway in which RhDA is involved. Ikeuchi et al. (2006) suggested that the activity of IscS and its paralogs is regulated by specific partner proteins in order to control sulfur flow into various cellular pathways.

Evaluating the ratio of L-cysteine:RhDA sulfurtransferase activity over persulfide production achieved by A. vinelandii CDs, the transpersulfuration efficiency is about 1.6-fold higher in NifS than in IscS. Loss of NifS function drastically impairs diazotrophic growth (Dos Santos et al. 2007; Jacobson et al. 1989) of A. vinelandii, whereas IscS has been shown to be essential under standard culture conditions (Johnson et al. 2006). For these reasons only the NifS/RhDA relationship was further explored, though relationship of RhDA with any of the 8 putative CDs coded by the A. vinelandii genome (Johnson et al. 2005; Sachsal and et al. 2009) cannot be ruled out. In A. vinelandii, TST (rhodanese) activity can be used as a probe of the RhDA expression/functionality. It is higher in conditions in which gene nifS is functional (i.e. diazotrophic growth), which indicates the importance of rhDA for diazotrophic aerobic growth of A. vinelandii and supports a role of RhDA in a cellular process shared with NifS.

Our data show that RhDA contributes to the homeostasis of cysteine concentration avoiding its increase especially under diazotrophic growth conditions where also TST activity, that is RhDA function, is higher with respect to that detected under standard growth conditions. This means that RhDA function is demanded in conditions in which a more stringent control of the cysteine concentration is required. It could be envisaged that RhDA affects the cysteine amount leading to the formation of the persulfurated form of RhDA (RhDA-SSH). According to our in vitro results, this process could be mediated by the CD NifS. The importance of cysteine for the NifS function is witnessed by the presence of a nifS-cotranscribed cysE gene coding for a putative serine transacyltransferase which catalyzes the rate-limiting step in cysteine biosynthesis in bacteria (Johnson et al. 2005). It was shown (Park and Imlay 2003) that, in E. coli, supernormal levels of intracellular cysteine cause sensitivity to oxidative DNA damage. In mammalian, cysteine dioxygenase plays a critical and highly regulated role in the homeostasis of cysteine level (Stipanuk et al. 2009). In bacteria, cysteine catabolic pathways are not fully explored (Sekowska et al. 2000) and systems involved in the regulation of cysteine levels are not described. L-Cysteine is used as a central building block for the synthesis of several sulfur-containing biomolecules (Kessler 2006). Data reported here, about cysteine accumulation, indicate that RhDA, in concert with NifS, is involved in diazotrophic growth, a process that needs cysteine.

A number of proteomic studies evidenced that proteins bearing the same rhodanese-like domain architecture of RhDA, as well as oxidative stress-related proteins, are overexpressed in the mitochondria of liver ethanol-exposed rats (Venkatraman et al. 2004), in phenol-growth Pseudomonas putida KT2440 cells (Santos et al. 2004), in pyrene-growth Mycobacterium sp. cells (Krivobok et al. 2003), in "standard" Mycobacterium bovis BCG cells (Florczyk et al. 2001). Exhibition of Leishmania major promastigotes to the oxidant cumene hydroperoxide caused overproduction of its 3-mercaptoproprionate sulfurtransferase (Williams et al. 2003). Reduced form of thioredoxin was shown to be a good sulfur acceptor substrate in the in vitro sulfurtransferase catalyzed by the bacterial single-domain rhodanases, Aq-477 (Giuliani et al. 2007) and GlpE (Ray et al. 2000), the bovine liver rhodanase (Nandi and Westley 1998), the leishmanial mercaptoproprionate sulfurtransferases LmMST and LmexMST (Williams et al. 2003), and the Trichomonas vaginalis mercaptoproprionate sulfurtransferase TvMST (Westrop et al. 2009). Enzymatic activity of the MST is regulated according to a thioredoxin-dependent redox-sensing molecular switch (Nagahara 2008), and an isoform of bovine liver mitochondrial rhodanase was shown to catalyze the direct oxidation of reduced thioredoxin (Nandi et al. 2000). Thioredoxin is a key intermediate in cellular redox reactions (Winyard et al. 2005). In A. vinelandii, it was found that RhDA triggered protection from oxidants (Cereda et al. 2007) and the possible functionality of RhDA as a redox switch was suggested by using an in vitro model system that demonstrated reversible chemical modifications in the highly reactive RhDA CyS230 thiol (Cereda et al. 2009). Collectively, these reports and the present study suggest that RhDA-dependent modulation of cysteine amount must deal with a redox-related process.
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References

desulfurases Stb0378 from Synecocystis sp. PCC 6803: kinetic
analysis of cleavage of the persulfide intermediate by chemical
reductants. Biochemistry 43:12220–12226


Bordo D, Deriu D, Colnaghi R, Carpen A, Pagani S, Bolognesi M
(2000) The crystal structure of a sulfurtransferase from Azoto-
bacter vinelandii highlights the evolutionary relationship between
the rhodanese and phosphatase enzyme families.
J Mol Biol 296:691–704

Bordo D, Forlani F, Spallarossa A, Colnaghi R, Carpen A, Bolognesi
M, Pagani S (2001) A persulfurated cysteine promotes active site
reactivity in Azobacter vinelandii rhodanese. Biol Chem
382:1245–1252

Bradford MM (1976) A rapid and sensitive method for the
quantitation of microgram quantities of protein utilizing the

Cereda A, Forlani F, Iametti S, Bernhardt R, Ferranti P, Piccardo G,
Pagani S, Bonomi F (2003) Molecular recognition between
Azobacter vinelandii rhodanese and a sulfur acceptor protein.
Biol Chem 384:1473–1481

S (2007) Effects of the deficiency of the rhodanese-like
1630

lack of rhodanese RhDA affects the sensitivity of Azobacter
vinelandii to oxidative events. Biochem J 418:135–143

sequence analysis and overexpression of the rhodanese gene of

Dos Santos PC, Johnson DC, Ragle BE, Unciuleac MC, Dean DR
(2007) Controlled expression of nif and isc iron-sulfur protein
maturation components reveals target specificity and limited
functional replacement between the two systems. J Bacteriol
189:2854–2862

Flint DH (1996) Escherichia coli contains a protein that is homol-
egous in function and N-terminal sequence to the protein
encoded by the nifS gene of Azobacter vinelandii and that can
participate in the synthesis of the Fe-S cluster of dithioxy-acid

Florczyk MA, McCue EA, Stuck RP, Hauer CR, McDonough KA
(2001) Identification and characterization of mycobacterial
proteins differentially expressed under standing and shaking
culture conditions, including Rv2623 from a novel class of

Forlani F, Carpen A, Pagani S (2003) Evidence that elongation of
the catalytic loop of the Azobacter vinelandii rhodanese changed
selectivity from sulfur- to phosphate-containing substrates.

Forlani F, Cereda A, Freuer A, Nimtz M, Leimkuhler S, Pagani S
(2005) The cysteine-desulfurase IscS promotes the production of
the rhodanese RhDA in the persulfurated form. FEBS Lett
579:6786–6790

Giuliani MC, Tron P, Leroy G, Aubert C, Tauc P, Giudici-Orticoni
MT (2007) A new sulfurtransferase from the hyperthermophilic
bacterium Aquifex aeolicus. Being single is not so simple when
temperature gets high. FEBS J 274:4572–4587

Mechanistic insights into sulfur relay by multiple sulfur medi-
ators involved in thiouridine biosynthesis at tRNA wobble
positions. Mol Cell 21:97–108

Jacobson MR, Cash VL, Weiss MC, Laird NF, Newton WE, Dean DR
(1989) Biochemical and genetic analysis of the nifUSVZW
cluster from Azotobacter vinelandii. Mol Gen Genet 219:49–57

Johnson DC, Dean DR, Smith AD, Johnson MK (2005) Structure,
function, and formation of biological iron-sulfur clusters. Anna
Rev Biochem 74:247–281

Johnson DC, Unciuleac MC, Dean DR (2006) Controlled expression
and functional analysis of iron-sulfur cluster biosynthetic
components within Azotobacter vinelandii. J Bacteriol
188:7551–7561

Kambampati R, Lauhon CT (1999) IscS is a sulfurtransferase for the
in vitro biosynthesis of 4-thiouridine in Escherichia coli RNA.
FEMS Microbiol Rev 23:1656–1668

Kambampati R, Lauhon CT (2000) Evidence for the transfer of
sulfur from IscS to Thil during the in vitro biosynthesis of
4-thiouridine in Escherichia coli RNA. J Biol Chem
275:10727–10730

Kato S, Miura H, Kurihara T, Takahashi Y, Tokumoto U, Yoshimura
T, Esaki N (2002) Cys-328 of IscS and Cys-63 of IscU are the
sites of disulfide bridge formation in a covalently bound IscS/
IscU complex: implications for the mechanism of iron–sulfur
cluster assembly. Proc Natl Acad Sci USA 99:5948–5952

into biomolecules in prokaryotes. FEMS Microbiol Rev 30:825–840

Kosower NS, Kosower EM (1987) Thiol labeling with bromobin-
omeanths. Methods Enzymol 143:76–84

Krivobok S, Kuony S, Meyer C, Louwagie M, Willson JC,
Jouanneau Y (2003) Identification of pyrene-induced proteins in
Mycobacterium sp. strain 6P1Y: evidence for two ring-

Substitutions in an active site loop of Escherichia coli IscS result
in specific defects in Fe–S cluster and thioleucine biosyn-

Evidence for the physiological role of a rhodanese-like protein
for the biosynthesis of the molybdenum cofactor in humans. Proc
Natl Acad Sci USA 101:9946–9951

biosynthesis in human: identification of a per sulfide group in
the rhodanese-like domain of MOC53 by mass spectrometry.
Biochemistry 44:7912–7920

function and mechanisms. Appl Microbiol Biotechnol 60:12–23

Mueller EG (2006) Trafficking in persulfides: delivering sulfur in

thioredoxin-dependent redox-sensing molecular switch: a mecha-
anism for the maintenance of cellular redox equilibrium. Mini
Rev Med Chem 8:585–589


Mutagenic analysis of Thr-243 in rhodanese from Azotobacter
vinelandii.
vinelandii highlighted the differences of this prokaryotic enzyme from the known sulfurtransferases. FEBS Lett 472:307–311
411 Urbina JD, Silberg JJ, Hoff KG, Vickery LE (2001) Transfer of sulfur from IscS to IscU during Fe/S cluster assembly. J Biol Chem 276:44521–44526
419