

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, it is suggested that RhdA-dependent modulation 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 molybdopterin, 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 and co-workers defined the mechanism for L-cysteine desulfuration 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 eukaryotes 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 Lauhon 1999; Kato 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 NifU (Smith et al. 2001), and IscS to IscU and ThiI proteins (Kambampati and Lauhon 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

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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 Cys₄₅₆ in the rhodanese homology domain of *Escherichia coli* ThiI was a recipient of the terminal sulfur 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 MOCS3 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 (Cys₂₃₀) 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 residue. Moreover, in *E. coli*, recombinant RhdA is expressed either in the persulfurated form (RhdA-SSH) 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 persulfuration, and that the CD IscS present in *E. coli* promoted the production of RhdA in the persulfurated form (Forlani et al. 2005).

Considering that RhdA's active site favors the stabilization of its persulfurated form, we sought to determine if RhdA-SSH could function as "escort" protein during sulfur mobilization processes. In order to frame RhdA-SSH functions in a cellular context, we first investigated direct transfer of S⁰ from the *A. vinelandii* CDs NifS, 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 cassette, following deletion of 584 bp as described in Colnaghi et al. (1996). Cells were grown aerobically in Burk's medium for 24 h at 30°C, supplemented with 15 mM ammonium acetate and 1% sucrose. For diazotrophic growth, ammonium acetate was omitted and culture time was 48 h. Cells were spun down at 3,800×g 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 NifS) 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 Cys₂₃₀ 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 GTC ACg *CAr* gcg CAG ACC CAT CAC CGC TCC-3' (FMP1) and 5'-GGA GCG GTG ATG GGT CTG *cgc aTG cGT* 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 mutagenesis was checked by DNA sequencing. His-tagged RhdA and RhdA_{C230A} were expressed in *E. coli* strains (BL21[pRep4]) harboring pQER1 and pQER1MP, respectively. 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). Cys₂₃₀-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, thiolyle, 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 excess. RhdA-bound mBBR was determined by measuring the fluorescence ($\lambda_{exc} = 345$ nm; $\lambda_{em} = 465$ nm) (Kosower and Kosower 1987). *E. coli* IscS_{C328A} 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 NifS, respectively. Purification of recombinant *A. vinelandii* IscS and NifS were performed as described previously (Zheng et al. 1993), except that after the ammonium-sulfate-fractionation the material was solubilized in 25 mM Tris-HCl (pH 7.5) and desalted by gel-filtration chromatography on G25-Sephadex. Gel-filtrated protein fraction was submitted to anion exchange chromatography that was carried out in the same buffer on Mono Q Sepharose (5/50 GL; TricornTM), in the case of IscS, or on DEAE 15 HR (16/100 Protein-PakTM, WaterTM), in the case of NifS, in a HPLC system (WatersTM 600S). Protein elution was achieved with a 0–1 M NaCl gradient (*A. vinelandii* IscS and NifS eluted at ~0.3 M NaCl). To complete NifS purification, the eluted

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 sonication (five 30 s pulses with intermitted 1 min cooling periods in Soniprep 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. Thiosulfate:cyanide sulfurtransferase (TST) activity was tested by the discontinuous method described in Sörbo (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 ($\epsilon = 2,890 \text{ M}^{-1} \text{ cm}^{-1}$). Detection of cysteine and thiosulfate was carried out by a monobromobimane HPLC method (Riemenschneider et al. 2005) that was applied to 50 mg (f. wt.) cell samples.

Spectroscopic determinations

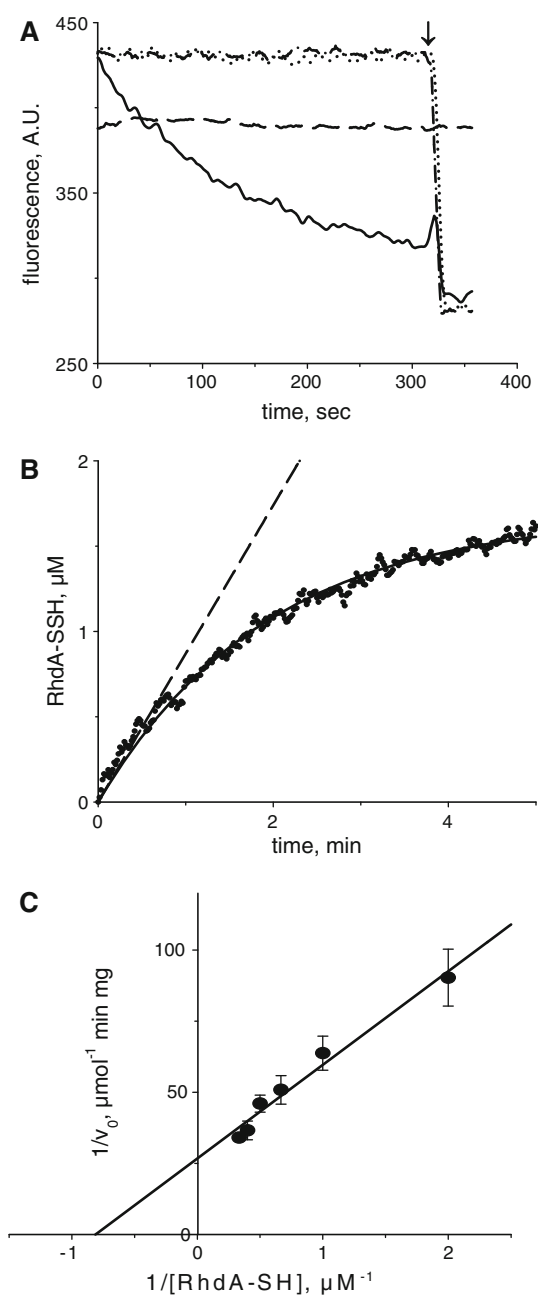
Fluorescence measurements were carried out in a Perkin–Elmer LS-50 instrument, and data were analyzed as previously described (Pagani et al. 2000). Time-course fluorescence experiments were carried out at 25°C in 50 mM Tris–HCl, 100 mM NaCl (pH 8) in the presence of 4 mM L-cysteine, 10 μM pyridoxal phosphate and 0.5–3 μM sulfane-sulfur deprived RhdA (RhdA-SH). For the evaluation of the formation rates of RhdA-SSH, the intrinsic fluorescence ($\lambda_{\text{exc}} = 280 \text{ nm}$, $\lambda_{\text{em}} = 340 \text{ nm}$) 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 are investigated. 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 dithiothreitol (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 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 μM purified *E. coli* IscS (solid line) were monitored in 1 ml of 50 mM Tris-HCl, 100 mM NaCl, pH 8, containing: 2 μM sulfane sulfur-deprived RhdA, 4 mM L-cysteine and 10 μM PLP. Trace obtained omitting L-cysteine is reported as a dotted line. As a control: 0.4 μM IscS_{C328A} (dashed-dotted line) replaced IscS, or 2 μM 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 Na₂S₂O₃. **b** Example of initial rate determination of IscS-mediated persulfuration of RhdA. Values of time-scale fluorescence changes following the addition of 0.4 μM IscS to a mixture of 2 μM RhdA-SH, 4 mM L-cysteine, and 10 μM 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 Na₂S₂O₃, 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 $\pm$ standard deviation (SD) for three separate experiments

transpersulfuration reaction as exemplified (Fig. 1, panel b). The transpersulfuration reaction catalyzed by *E. coli* IscS (Fig. 1, panel c) exhibited Michaelis-Menten behavior, suggesting interaction between the persulfide form of IscS and RhdA. Under the used conditions (4 mM L-cysteine), the K_m for RhdA-SH is 1.21 ± 0.43 μM , and turnover number is 1.71 ± 0.09 min^{-1} (IscS monomer).

To our knowledge, kinetic parameters of an enzyme-catalyzed reaction, where sulfane sulfur is transferred from an enzyme-bound persulfide to a cysteine residue of another protein (i.e. protein-protein transpersulfuration) were never calculated. The K_m figure for RhdA-SH in the IscS-catalyzed transpersulfuration is in the same magnitude to that estimated (2.7 μM ; Urbina et al. 2001) for L-cysteine in the *E. coli*-IscS-catalyzed desulfuration reaction achieved in the presence of 5 mM DTT as reductant for the decomposition of the enzyme-bound persulfide. It is worth noting that DTT is always present in a molar excess with respect to the substrate L-cysteine when cysteine desulfuration catalyzed by CDs was monitored (Flint 1996; Urbina et al. 2001; Lauhon et al. 2004).

The evidence that the *A. vinelandii* RhdA is a stable acceptor of sulfane sulfur held on the catalytic cysteine residue of *E. coli* IscS, prompted us to investigate whether it could promote sulfur transfer from *A. vinelandii* CDs.

RhdA is an acceptor of sulfur mobilized by *A. vinelandii* cysteine desulfurases NifS and IscS

Most of the ability of *A. vinelandii* RhdA to be an acceptor of the sulfur generated by the *E. coli* IscS might be inferred by its peculiar active-site structure and environment (Bordo et al. 2000). None of the rhodanese-like proteins present in *E. coli*, indeed, contains the same active-site structure of

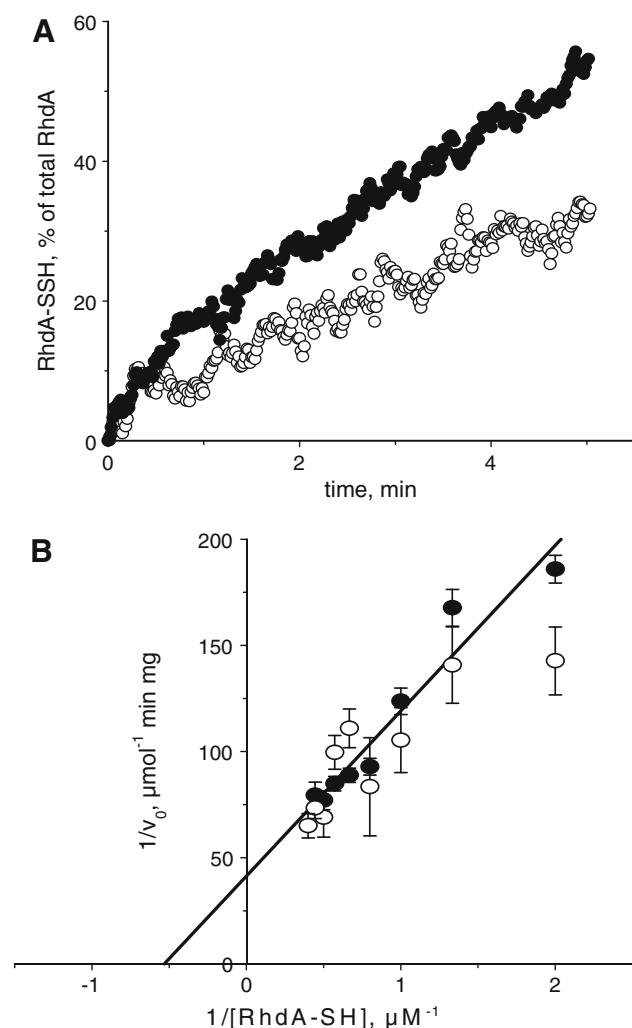


Fig. 2 Rhda persulfuration mediated by *Azotobacter vinelandii* IscS and NifS. **a** Intrinsic fluorescence changes ($\lambda_{\text{exc}} = 280 \text{ nm}$, $\lambda_{\text{em}} = 340 \text{ nm}$) following the addition of $0.4 \text{ } \mu\text{M}$ purified *A. vinelandii* IscS (open circles) or NifS (filled circles) were monitored in 1 ml of 50 mM Tris-HCl, 100 mM NaCl, pH 8, containing: $2 \text{ } \mu\text{M}$ sulfane sulfur-deprived Rhda, 4 mM L-cysteine, and $10 \text{ } \mu\text{M}$ 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 $\pm$ standard deviation (SD) for three separate experiments

A. vinelandii Rhda. Aiming to identify the role of Rhda as 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 $0.014 \pm 0.003 \text{ } \mu\text{mol min}^{-1} \text{ mg}^{-1}$. 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 $1.88 \pm 0.49 \text{ } \mu\text{M}$, and the NifS turnover number was $1.05 \pm 0.35 \text{ min}^{-1}$.

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 persulfide 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 desulfuration 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 $75 \text{ } \mu\text{M}$ (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 Cys₃₂₈ (*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 $0.168 \text{ } \mu\text{mol min}^{-1} \text{ mg}^{-1}$ 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 cyanolysis reaction. In this work, cyanolysis 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

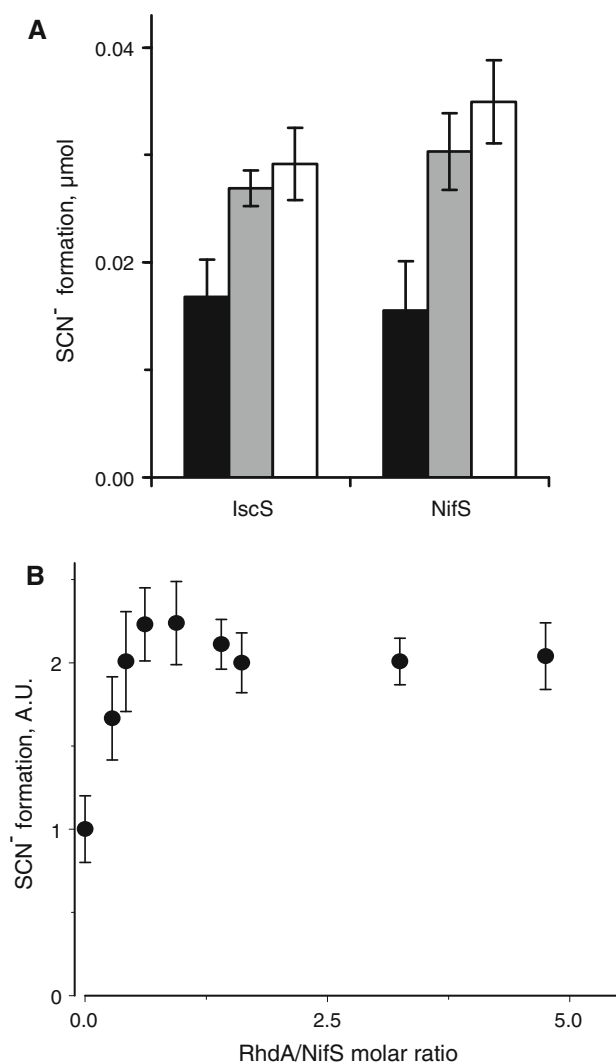


Fig. 3 Effect of Rhda on the production of sulfane sulfur of *A. vinelandii* IscS and NifS. **a** The absorbance at 460 nm due to the production of thiocyanate in mixtures (0.65 ml) of 50 mM Tris-HCl, 100 mM NaCl (pH 8) containing 4 mM L-cysteine, 10 μM pyridoxal phosphate, 0.4 μM cysteine desulfurase, 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 Rhda_{C230A} (white). **b** 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 a; gray bars). The replacement of Rhda by an Rhda variant (Rhda-mBBR), where the C₂₃₀ thiol was chemically blocked by monobromobimane (data not shown), or by an

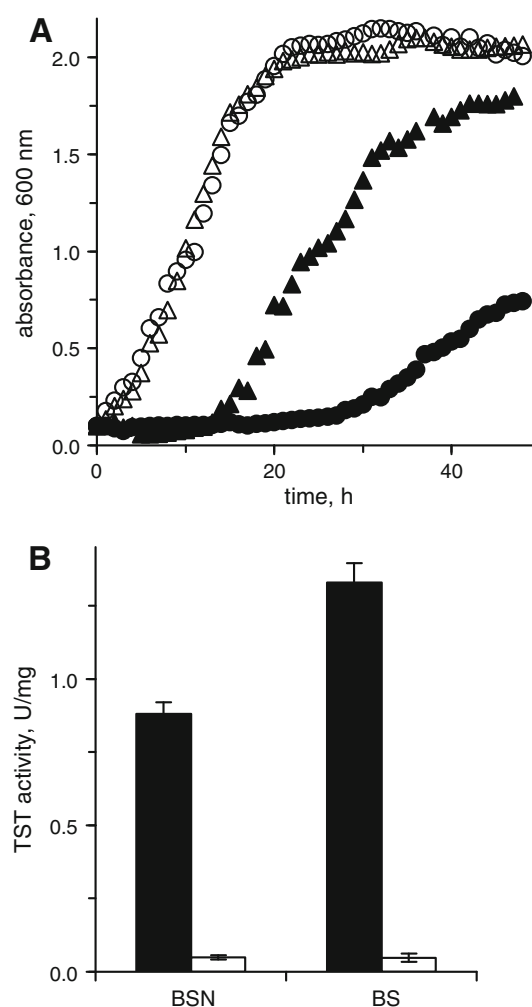


Fig. 4 Functional relationship between Rhda and NifS. **a** Determinations of growth rates of wild type (UW136; triangles) and MV474 (circles) were carried out in the presence (empty symbols) or in the absence (filled symbols) of 15 mM ammonium acetate in the medium. **b** Thiosulfate:cyanoide sulfurtransferase (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

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 (monomer) ratio (Fig. 3, panel b). These data support the idea that Rhda interacts with NifS leading to an increase in the persulfide formation rate.

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).

Thiosulfate:cyanide sulfurtransferase activity and NifS expression in *A. vinelandii*

Azotobacter 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 (Cereda et al. 2007). Thus, the basal TST activity could be due to the redundancy of rhodanese-like genes in the *A. vinelandii* genome and is not 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 1 Cysteine and thiosulfate amount in *A. vinelandii* strains grown under standard or diazotrophic conditions

Strain	Growth medium	Cysteine (pmol/mg cell f. wt.) ^a	Thiosulfate (pmol/mg cell f. wt.) ^a
UW136	BSN	10.3 ± 1.4	42.5 ± 1.1
	BS	5.1 ± 1.0	58.9 ± 2.6
MV474	BSN	22.0 ± 1.8	44.9 ± 1.4
	BS	36.0 ± 2.1	51.5 ± 1.1

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 favors 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; Setubal 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 transacetylase 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 mammals, 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, mainly under diazotrophic growth, in 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 standing growth *Mycobacterium bovis* BCG cells (Florczyk et al. 2001). Exposition of *Leishmania major* promastigotes to the oxidant cumene hydroperoxide caused overproduction of its 3-mercaptopyruvate 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 rhodanese (Nandi and Westley 1998), the leishmanial mercaptopyruvate sulfurtransferases LmajMST and LmexMST (Williams et al. 2003), and the *Trichomonas vaginalis* mercaptopyruvate sulfurtransferase TvMST (Westrop et al. 2009). Enzymatic activity of the rat MST is regulated according to a thioredoxin-dependent redox-sensing molecular switch (Nagahara 2008), and an isoform of bovine liver mitochondrial rhodanese 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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