ORIGINAL ARTICLE

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 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 et al. 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 S^0 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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63 sulfide (Beinert 2000; Mueller 2006). Specifically, sulfur
64 incorporation systems that rely on persulfide chemistry
65 often involve rhodanese homology domain proteins. It was,
66 indeed, shown that the essential catalytic Cys_{456} in the
67 rhodanese homology domain of *Escherichia coli* ThiI was
68 a recipient of the terminal sulfur of the persulfide form
69 of IscS in the biosynthesis of 4-thiouridine in tRNA
70 (Palenchar et al. 2000), and that the rhodanese homology
71 domain of the human enzyme MOCS3 transiently bears a
72 persulfide group on the pathway of sulfur incorporation
73 into molybdopterin (Matthies et al. 2004, 2005).
74   *Azotobacter vinelandii* expresses a unique rhodanese
75 called RhdA (Colnaghi et al. 1996). The RhdA active site
76 cysteine (Cys_{230}) is located at a structural loop (Bordo et al.
77 2000, 2001; Pagani et al. 2000) that appears to be properly
78 designed to stabilize persulfide bond on its catalytic resi-
79 due. Moreover, in *E. coli*, recombinant RhdA is expressed
80 either in the persulfurated form (RhdA-SSH) or in the
81 sulfane sulfur-deprived form (RhdA-SH), depending on the
82 sulfur source present during growth. We reported that
83 L-cysteine was the effective sulfur source of RhdA per-
84 sulfuration, and that the CD IscS present in *E. coli* pro-
85 moted the production of RhdA in the persulfurated form
86 (Forlani et al. 2005).
87   Considering that RhdA's active site favors the stabil-
88 ization of its persulfurated form, we sought to determine if
89 RhdA-SSH could function as "escort" protein during sul-
90 fur mobilization processes. In order to frame RhdA-SSH
91 functions in a cellular context, we first investigated direct
92 transfer of S^0 from the *A. vinelandii* CDs NifS, and IscS to
93 RhdA. In the present study, we report the effect of RhdA
94 inactivation on the levels of cysteine and on diazotrophic
95 growth of *A. vinelandii*.

Materials and methods

Bacterial strains and growth conditions

98 The *A. vinelandii* strains used in this study were UW136, a
99 derivative of UW136 (MV474) in which disruption of the
100 *rhdA* gene was achieved by the insertion of a KIXX cas-
101 sette, following deletion of 584 bp as described in Coln-
102 aghi et al. (1996). Cells were grown aerobically in Burk's
103 medium for 24 h at 30°C, supplemented with 15 mM
104 ammonium acetate and 1% sucrose. For diazotrophic
105 growth, ammonium acetate was omitted and culture time
106 was 48 h. Cells were spun down at 3,800*g* for 10 min, and
107 stored at −80°C. For protein expression, the *E. coli* strains,
108 BL21[pRep4] (for RhdA and $RhdA_{C230A}$) and BL21(DE3)
109 (for *E. coli* IscS, *A. vinelandii* IscS and NifS) were grown
110 in Luria–Bertani medium (containing 100 μg/ml ampicillin
111 and, only for BL21[pRep4], 25 μg/ml kanamycin) at 37°C

112 and, when absorbance at 600 nm was 0.4, 1 mM IPTG was
113 added. After 4 h of growth cells were collected and used
114 for protein purification.

Protein preparations

116 In the RhdA mutant ($RhdA_{C230A}$) the catalytic Cys_{230}
117 residue was replaced by an alanine residue. Site-directed
118 mutagenesis of *rhdA* generated the plasmid pQER1MP and
119 was performed using pQER1 plasmid as template (Pagani
120 et al. 2000), and the mutagenic primers 5′-CCG GAC AAG
121 GAA ATC GTC AC*g CAt gcg* CAG ACC CAT CAC CGC
122 TCC-3′ (FMP1) and 5′-GGA GCG GTG ATG GGT CTG
123 *cgc aTG c*GT GAC GAT TTC CTT GTC CGG-3′ (RMP1)
124 where lower case letters indicate the mutated bases and
125 italic letters indicate the *Sph*I restriction site introduced for
126 quick verification of mutagenesis. The accuracy of muta-
127 genesis was checked by DNA sequencing. His-tagged
128 RhdA and $RhdA_{C230A}$ were expressed in *E. coli* strains
129 (BL21[pRep4]) harboring pQER1 and pQER1MP, respec-
130 tively. Expressed proteins were purified by Ni–NTA
131 affinity chromatography (Forlani et al. 2003), and gel-fil-
132 tered using a G25 column equilibrated in 50 mM Tris–HCl,
133 100 mM NaCl (pH 8). Sulfane sulfur-deprived RhdA and
134 recombinant *E. coli* IscS were prepared as described in
135 Forlani et al. (2005). Cys_{230}-blocked RhdA (RhdA-mBBr)
136 was obtained by incubation of 156 μM sulfane sulfur-
137 deprived RhdA in 50 mM Tris–HCl, 100 mM NaCl buffer
138 (pH 8) with 5 mM monobromobimane (mBBr, thiolyte,
139 Calbiochem, Darmstadt, Germany; diluted from a 100 mM
140 stock solution in acetonitrile). After 2 h in the dark at room
141 temperature with continuous stirring, the reaction mixture
142 was gel-filtered on a G-25 column to remove reagent
143 excess. RhdA-bound mBBr was determined by measuring
144 the fluorescence ($\lambda_{exc} = 345$ nm; $\lambda_{em} = 465$ nm)(Kosow-
145 er and Kosower 1987). *E. coli* $IscS_{C328A}$ was obtained
146 courtesy of Prof. L. E. Vickery (UC Irvine, CA, USA).
147   The plasmids pDB943 (Zheng et al. 1998) and pDB551
148 (Zheng et al. 1993) were used for *E. coli* heterologous
149 expression of *A. vinelandii* IscS and NifS, respectively.
150 Purification of recombinant *A. vinelandii* IscS and NifS
151 were performed as described previously (Zheng et al.
152 1993), except that after the ammonium-sulfate-fractiona-
153 tion the material was solubilized in 25 mM Tris–HCl (pH
154 7.5) and desalted by gel-filtration chromatography on G25-
155 Sephadex. Gel-filtrated protein fraction was submitted to
156 anion exchange chromatography that was carried out in the
157 same buffer on Mono Q Sepharose (5/50 GL; Tricorn™),
158 in the case of IscS, or on DEAE 15 h (16/100 Protein-
159 Pak™, Water™), in the case of NifS, in a HPLC system
160 (Waters™ 600S). Protein elution was achieved with a
161 0–1 M NaCl gradient (*A. vinelandii* IscS and NifS eluted at
162 ~0.3 M NaCl). To complete NifS purification, the eluted

163 protein was concentrated using ultrafiltration devices
164 (Amicon® Ultra 5 K) and submitted to Superose 12 (10/
165 300 GL, Tricorn™) HPLC chromatography in 25 mM
166 Tris–HCl 0.1 M NaCl (pH 8). Purification of *A. vinelandii*
167 IscS and NifS was continuously monitored by determining
168 the ratio OD_{425}/OD_{280} and assessed by SDS–PAGE anal-
169 yses. Purified proteins were fractionated and stored at
170 −30°C until used.

171 Analytical procedures

172 Cell-free extracts of *A. vinelandii* were prepared by soni-
173 cation (five 30 s pulses with intermitted 1 min cooling
174 periods in Soniprep 150; UK) in 10 mM Tris–HCl,
175 100 mM NaCl (pH 8), and cell debris was removed by
176 centrifugation (30 min at 10,000*g*). Protein concentration
177 was determined by the Bradford assay (Bradford 1976)
178 using bovine serum albumin as standard. Thiosulfate:cya-
179 nide sulfurtransferase (TST) activity was tested by the
180 discontinuous method described in Sörbo (1953) that
181 quantifies the product thiocyanate. One unit (U) of TST
182 activity is defined as the amount of enzyme that produces
183 1 μmol thiocyanate/min at 37°C. Persulfide formation in
184 CDs was tested as cyanolizable sulfur that was revealed as
185 thiocyanate (Sörbo 1953). In particular, for thiocyanate
186 quantification, to 0.65 ml of assay mixtures, 0.1 ml 37%
187 formaldehyde and 0.25 ml Sörbo's reagent (10% ferric
188 nitrate in 13% nitric acid) were added sequentially before
189 the spectrophotometric determination of the absorbance of
190 the ferric-thiocyanate complex at 460 nm ($\varepsilon = 2{,}890$ M^{-1}
191 cm^{-1}). Detection of cysteine and thiosulfate was carried
192 out by a monobromobimane HPLC method (Riemen-
193 schneider et al. 2005) that was applied to 50 mg (f. wt.) cell
194 samples.

195 Spectroscopic determinations

196 Fluorescence measurements were carried out in a Perkin–
197 Elmer LS-50 instrument, and data were analyzed as
198 previously described (Pagani et al. 2000). Time-course
199 fluorescence experiments were carried out at 25°C in
200 50 mM Tris–HCl, 100 mM NaCl (pH 8) in the presence
201 of 4 mM ʟ-cysteine, 10 μM pyridoxal phosphate and
202 0.5–3 μM sulfane-sulfur deprived RhdA (RhdA-SH). For
203 the evaluation of the formation rates of RhdA-SSH,
204 the intrinsic fluorescence ($\lambda_{exc} = 280$ nm, $\lambda_{em} = 340$ nm)
205 change, obtained after the addition of CD (0.4 μM), was
206 converted in RhdA-SSH concentration. For conversion, the
207 value of total fluorescence change obtained after the final
208 addition of 0.5 mM $Na_2S_2O_3$ was used as quantitative
209 standard. Time-course data of RhdA-SSH concentration
210 were fitted to an exponential-rise function in order to
211 approximate the initial rate.

Results

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ʟ-Cysteine:RhdA sulfurtransferase activity of *E. coli* IscS

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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 ʟ-cys-
teine (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 ʟ-cysteine was added to RhdA-
SH alone indicating that ʟ-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 ʟ-cysteine decreased the recorded fluores-
cence (solid line, Fig. 1, panel a) whereas no fluorescence
changes were observed when the RhdA Cys_{230} was
chemically blocked by monobromobimane (dashed line,
Fig. 1, panel a). This shows that RhdA-SSH was produced
and that sulfur was mobilized from ʟ-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 Cys_{328} residue of IscS is mandatory for the IscS-
catalyzed RhdA persulfuration (ʟ-cysteine:RhdA sulfur-
transferase activity). According to the proposed mechanism
for CDs, the Cys_{328} 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 Cys_{328} and the RhdA Cys_{230}, being this last the RhdA
catalytic residue properly designed to bear a stable per-
sulfide sulfur.

Intrinsic fluorescence of RhdA-SSH was not affected by
the addition of 60-fold molar excess of ʟ-cysteine (data not
shown) showing that the monothiol ʟ-cysteine does not
behave as an acceptor of the sulfane sulfur held on RhdA
Cys_{230}. 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

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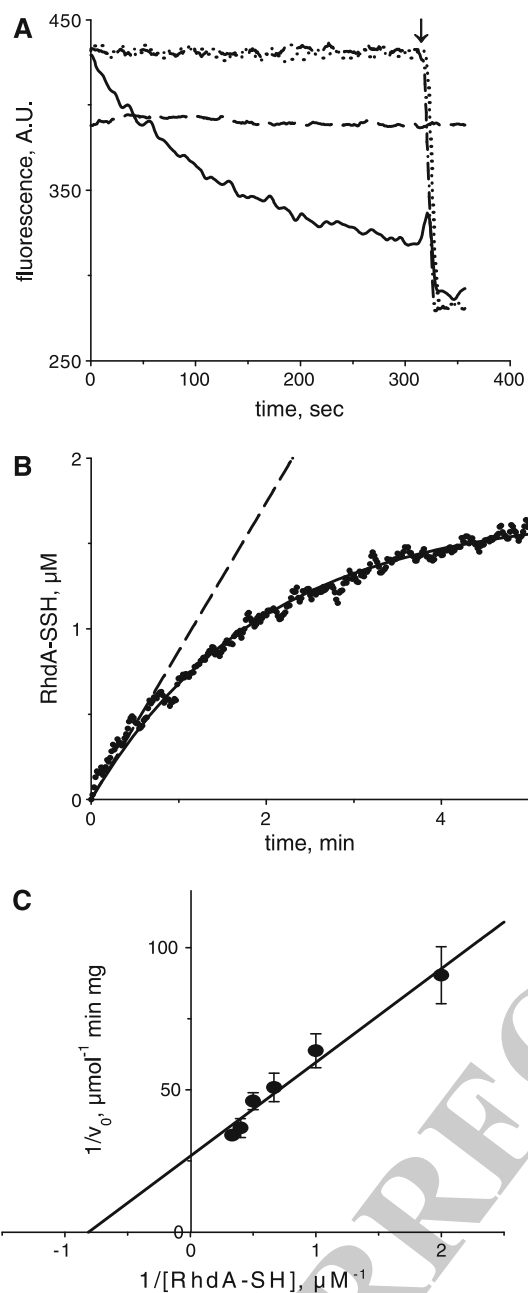252
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◄ **Fig. 1** Fluorescence kinetic measurements of RhdA persulfuration mediated by *Escherichia coli* IscS. **a** Representative time-course traces of the fluorescence changes ($\lambda_{exc} = 280$ nm, $\lambda_{em} = 340$ nm) following the addition 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 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 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 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

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

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
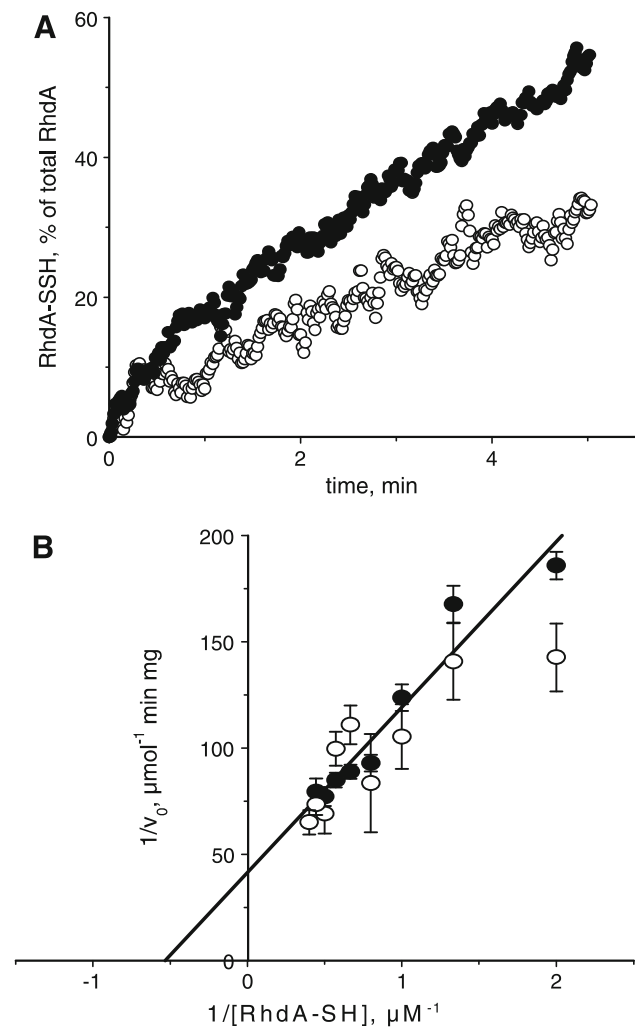
Fig. 2 RhdA persulfuration mediated by *Azotobacter vinelandii* IscS and NifS. **a** Intrinsic fluorescence changes ($\lambda_{exc} = 280$ nm, $\lambda_{em} = 340$ nm) following the addition of 0.4 μM purified *A. vinelandii* IscS (*open circles*) or NifS (*filled circles*) were monitored in 1 ml of 50 mM Tris–HCl, 100 mM NaCl, pH 8, containing: 2 μM sulfane sulfur-deprived RhdA, 4 mM L-cysteine, and 10 μM 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 311
sulfur mediator in sulfur transfer processes of physiological 312
relevance, we studied the transpersulfuration process in the 313
presence of the *A. vinelandii* CDs, NifS and IscS. 314

Transpersulfuration reaction to RhdA occurred with 315
both *A. vinelandii* CDs, but their efficiencies to produce 316
RhdA-SSH were different (Fig. 2, panel a). After 5 min 317
reaction, 55% of the RhdA-SH present in the mixture was 318
converted into RhdA-SSH when sulfur of L-cysteine was 319
mobilized by NifS, and 33% in the case of IscS. The 320
observed L-cysteine:RhdA sulfurtransferase activity of 321

NifS and IscS were, respectively, 0.025 ± 0.006 and 322
0.014 ± 0.003 μmol min^{-1} mg^{-1}. Lineweaver–burk plot 323
of sulfur transfer from NifS to RhdA (Fig. 2, panel b), 324
suggests a productive interaction between the persulfide 325
form of NifS and RhdA. In the conditions used for fluo- 326
rescence change measurements, that allowed detection of 327
transpersulfuration activity by a continuous-type enzyme 328
assay, the NifS apparent (4 mM L-cysteine) K_m for RhdA- 329
SH was 1.88 ± 0.49 μM, and the NifS turnover number 330
was 1.05 ± 0.35 min^{-1}. 331

As stated before, *A. vinelandii* IscS also showed L-cys- 332
teine:RhdA sulfurtransferase activity (Fig. 2, panel a), but 333
the data did not yield straight lines in double reciprocal plot 334
analyses in the same range of substrate-RhdA-SH concen- 335
trations used for determining kinetic parameters of the 336
NifS-mediated reaction (Fig. 2, panel b; empty circles). 337
Considering the low L-cysteine:RhdA sulfurtransferase 338
activity of *A. vinelandii* IscS, probably the sensitivity of the 339
developed time-scale fluorescence assay is decreased by the 340
high background intrinsic fluorescence which is not affected 341
by the quenching effect of the persulfide group in RhdA. In 342
A. vinelandii IscS, at least 4 Trp residues contributes to the 343
intrinsic fluorescence of this protein whereas there are only 344
2 Trp residues in the NifS sequence. Also in Zheng et al. 345
(1998) was reported that kinetic parameters for L-cysteine 346
desulfuration catalyzed by *A. vinelandii* IscS could not be 347
obtained and activity inhibition by L-cysteine was claimed. 348

In *A. vinelandii* NifS-catalyzed desulfuration of L-cys- 349
teine, the apparent K_m for L-cysteine is about 75 μM 350
(Zheng et al. 1994), whereas no figure of K_m for L-cysteine 351
in the *A. vinelandii* IscS-catalyzed desulfuration of cysteine 352
was reported. 353

Effects of RhdA on the persulfide formation 354
of *Azotobacter vinelandii* cysteine desulfurases, 355
NifS and IscS 356

The formation of persulfide on Cys$_{328}$ (*E. coli* IscS num- 357
bering) residue of CDs is an essential prerequisite for 358
transpersulfuration to RhdA. The relative efficiency of the 359
transpersulfuration reaction paralleled the CD activity fig- 360
ures of 0.124 and 0.168 μmol min^{-1} mg^{-1} determined for 361
A. vinelandii IscS and NifS, respectively (Zheng et al. 362
1998). Since in those determinations persulfide was reduc- 363
tively cleaved to generate sulfide in the presence of DTT, 364
we raised the question of whether the observed sulfur 365
transfer to RhdA reflects the turnover number of generation 366
of the IscS- and NifS-bound persulfide forms. Persulfide 367
(sulfane sulfur) can be identified by its reactivity with 368
cyanide (Beinert 2000) to produce thiocyanate in the 369
cyanolysis reaction. In this work, cyanolysis assay was used 370
to detect persulfide formation in *A. vinelandii* IscS and NifS 371
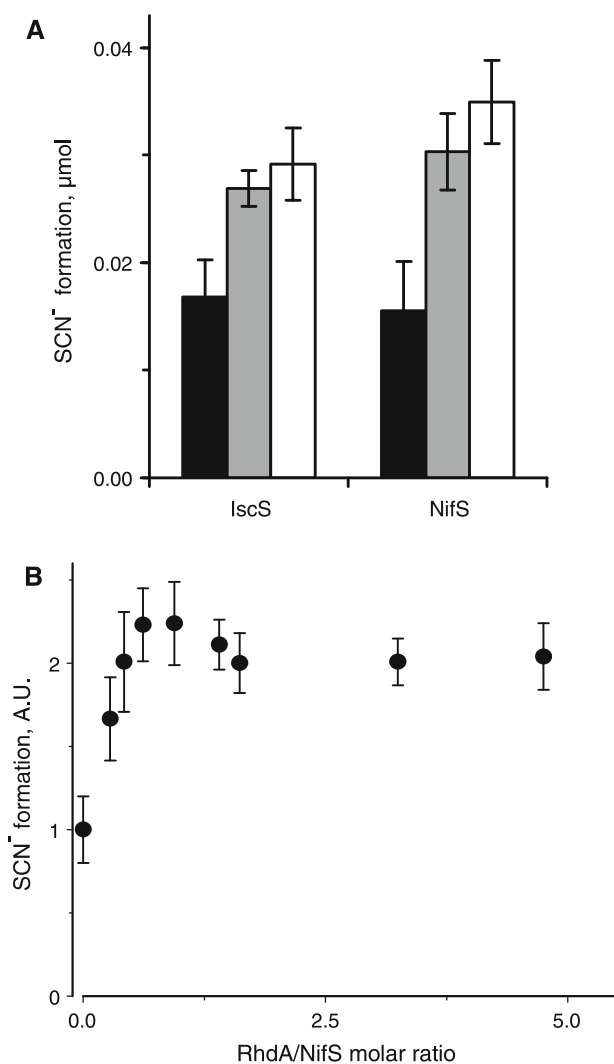in the presence of L-cysteine (4 mM) and in the absence of 372

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 $\pm$ standard deviation (SD) from three independent experiments
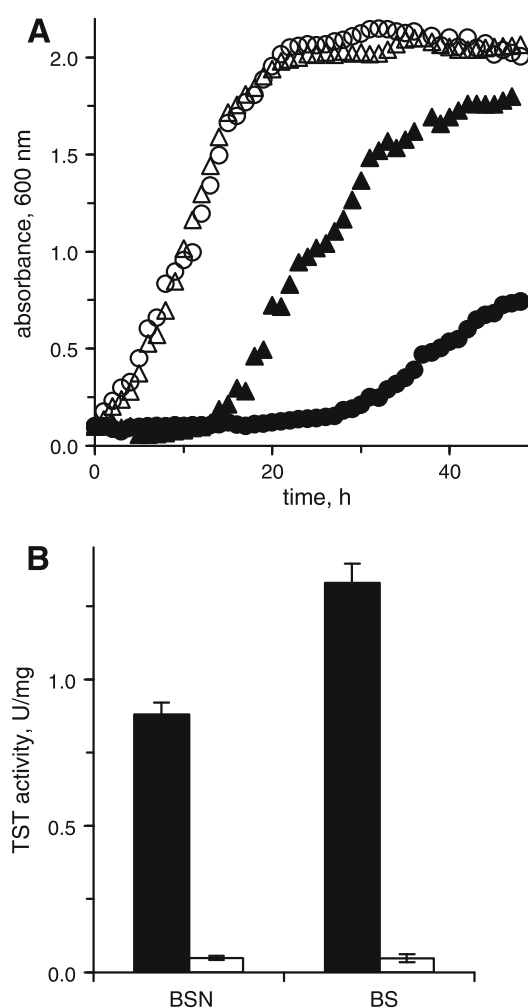
Fig. 4 Functional relationship between RhdA and NifS. **a** Determinations of growth rates of wild type (UW136; *triangles*) and MV474 (*circles*) were carried out in the presence (empty symbols) or in the absence (*filled symbols*) of 15 mM ammonium acetate in the medium. **b** Thiosulfate:cyanide sulfurtransferase (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 $\pm$ 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 C$_{230}$ thiol was chemically blocked by monobromobimane (data not shown), or by an RhdA mutant (RhdA$_{C230A}$), where the Cys$_{230}$ 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 Cys$_{230}$, 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 (Cys$_{230}$). 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 mammalians, 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). 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 rhodanes, 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 Cys$_{230}$ 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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