The lack of rhodanese RhdA affects the sensitivity of Azobacter vinelandii to oxidative events

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The rhdA gene of Azobacter vinelandii codes for RhdA, a rhodanese-domain protein with an active-site loop structure which has not currently been found in proteins of the rhodanese-homology superfamily. Considering the lack of information on the functional role of the ubiquitous rhodanases, in the present study we examined the in vivo functions of RhdA by using an A. vinelandii mutant strain (MV474), in which the rhdA gene was disrupted by deletion. Preliminary phenotypic characterization of the rhdA mutant suggested that RhdA could exert protection over Fe–S enzymes, which are easy targets for oxidative damage. To highlight the role of RhdA in preserving sensitive Fe–S clusters, in the present study we analysed the defects of the rhdA-null strain by exploiting growth conditions which resulted in enhancing the catalytic deficiency of enzymes with vulnerable Fe–S clusters. We found that a lack of RhdA impaired A. vinelandii growth in the presence of gluconate, a carbon source that activates the Entner–Doudoroff pathway in which the first enzyme, 6-phosphoglucuronate dehydratase, employs a 4Fe–4S cluster as an active-site catalyst. By combining proteomics, enzymatic profiles and model systems to generate oxidative stress, evidence is provided that to rescue the effects of a lack of RhdA, A. vinelandii needed to activate defensive activities against oxidative damage. The possible functionality of RhdA as a redox switch which helps A. vinelandii in maintaining the cellular redox balance was investigated by using an in vitro model system that demonstrated reversible chemical modifications in the highly reactive RhdA Cys230 thiol.

Key words: Azobacter vinelandii, oxidative stress, RhdA, rhodanese-domain protein.

INTRODUCTION

The ubiquitous rhodanese-domain proteins catalyse in vitro the transfer of a sulfur atom from a suitable sulfur donor (thiosulfate for rhodanases and 3-mercaptoppyruvate for 3-mercaptoppyruvate sulfuryltransferases) to cyanide, with concurrent formation of thiocyanate [1]. In the majority of organisms, the proteins of this homology superfamily (Pfam accession number PF00581; http://www.sanger.ac.uk/Software/Pfam/) are present as paralogues and they are found as tandem rhodanase repeats, single-domain proteins or in combination with distinct protein domains [2]. The wide variability in the amino acids of the active-site loop of rhodanese-domain proteins [2] makes it conceivable that substrate recognition and biological interactions are driven by the specific active-site structure. From the findings that bovine rhodanese [3], Escherichia coli GlpE [4] and the 3-mercaptoppyruvate sulfuryltransferase from Leishmania [5] have a higher affinity for reduced thioredoxins than for cyanide, the concept that the abundant rhodanese-like proteins could play roles in managing the processes of stress tolerance is emerging, although in vivo experimental evidence is still lacking. The relevance of specific rhodanese-like proteins in the maintenance of redox homeostasis has been suggested by in vitro studies using rat mercaptoppyruvate sulfuryltransferase [6,7]. As for bacterial rhodanese-domain proteins, possible involvement in physiological processes related to xenobiotic-induced oxidative stress and detoxification was indicated by proteomic analyses [8,9]. Recently, we have found evidence that rhodanese-like enzymes favoured PAH (polycyclic aromatic hydrocarbon) degradation in selected Mycobacterium strains [10]. This experimental evidence suggests that rhodanese domains might function as regulatory devices in specific physiological situations, but the redundancy of these proteins in the same organism makes it difficult to unravel their cellular roles.

Among the redundant rhodanese-homology proteins of Azobacter vinelandii, the tandem domain protein RhdA [11] contains an active-site motif (HCQTHHR) which is not commonly found in rhodanese-domain proteins. Crystallographic investigations [12] showed that the RhdA catalytic centre is surrounded by a strong positive electrostatic field that originates from the peculiar active-site loop structure, and from the vicinity of positively charged groups. Considering the lack of general information on the biological role of rhodanases, we undertook a study of the in vivo function of RhdA by using an A. vinelandii mutant strain (MV474), in which the rhdA gene was disrupted by deletion [11]. Initial characterization of MV474 grown in a sucrose medium revealed that the lack of RhdA enhanced the expression of enzymes of the PHB (polyhydroxybutyrate) biosynthetic operon, giving rise to early accumulation of PHB, and negatively affected the activity of tricarboxylic acid cycle Fe–S enzymes [13]. The effect on aconitate hydratase was dramatic, in spite of the comparable expression of aconitate hydratase polypeptides in both wild-type and rhdA mutant strains. These results [13] led to the proposal that RhdA provides protection against oxidative events that in the aerobe A. vinelandii lead to the inactivation of enzymes containing labile Fe–S [14]. To highlight the role of RhdA in preserving sensitive Fe–S clusters, in the present study, we analysed the defects of the rhdA-null strain MV474 by exploiting growth conditions that enhance the catalytic deficiency of enzymes with vulnerable Fe–S clusters.

Abbreviations used: AhpC, alkyl hydroperoxide reductase; 2-DE, two-dimensional PAGE; DTT, dithiothreitol; ED, Entner–Doudoroff; MALDI-TOF, matrix-assisted laser-desorption ionization–time-of-flight; PHB, polyhydroxybutyrate; PMS, phenazine methosulfate; TST, thiosulfate–cyanide sulfuryltransferase; %V, relative volume.

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[15]. We found that a lack of RhdA impaired A. vinelandii growth in the presence of gluconate, a carbon source that activates the ED (Entner–Doudoroff) pathway [16], in which the first enzyme, 6-phosphogluconate dehydrogenase, employs a vulnerable 4Fe–4S cluster as an active-site catalyst [17]. The use of gluconate as a carbon source proved to be an useful growth condition in which to make an in-depth study into the initially hypothesized [13] role of RhdA in protecting cells from oxidative damage. By combining proteomics, enzymatic profiles and model systems to generate oxidative-stress, in the present report we present evidence to show that to rescue the effects of the lack of RhdA, A. vinelandii needed to activate defensive activities against oxidative damage. The possible functionality of RhdA as a redox switch which helps A. vinelandii in maintaining the redox balance was investigated by using an in vitro model system to demonstrate the occurrence of chemical modifications in the highly reactive RhdA Cys\textsuperscript{236} thiol, the only one present in the molecule.

EXPERIMENTAL

Bacterial strains and growth conditions

The A. vinelandii strains used in this study were UW136 and a derivative of UW136 (MV474), in which disruption of the \textit{rhdA} gene was achieved by the insertion of a KIXX (kanamycin resistance) cassette, following deletion of 584 bp as described previously [11]. Western blot analysis with MV474 [13] clearly showed that no protein corresponding to RhdA is expressed previously [11]. Western blot analysis with MV474 [13] clearly showed that no protein corresponding to RhdA is expressed previously [11]. The possible functionality of RhdA as a redox switch which helps A. vinelandii in maintaining the redox balance was investigated by using an in vitro model system to demonstrate the occurrence of chemical modifications in the highly reactive RhdA Cys\textsuperscript{236} thiol, the only one present in the molecule.

Sample preparation and gel electrophoresis

Cells were harvested by centrifugation at 2000 g for 30 min and suspended in a 10 mM Tris/HCl (pH 8.0) buffer containing 1.5 mM MgCl\textsubscript{2}, 10 mM KCl and 0.1 % SDS. A buffer volume which was approximately equal to the packed cell volume was used. Before analysis, 100 μl of the sample was solubilized in 1 ml of lysis buffer [7 M urea, 2 M thiourea, 4 % (v/v) CHAPS, 65 mM DTT (dithiothreitol) and 2 % (v/v) IPG buffer (pH 4–7) (Amer sham Biosciences)] and incubated for 20 min on ice. Protein concentrations were estimated using a 2-DE (two-dimensional PAGE) Quant Kit (Amersham Biosciences) following the manufacturer’s instructions before adding 0.01 % Bromophenol Blue. Proteins were separated by 2-DE using Immobiline Dry Strips (pH 4–7 linear, 14 cm long; Amersham Biosciences). The sample (180 μg of protein) was loaded on the immobilized pH gradient strip after overnight in-gel re-swelling procedure performed following the manufacturer’s instructions. The isoelectric focusing was carried out at 20°C using a Multiphor II electrophoresis unit (Amersham Biosciences) with the following programme: 75 V for 1 h, 300 V for 1 h, 300–1000 V for 4 h, 1000–8000 V for 6 h, 8000 V for 2 h and 50 V for 1 h. After focusing, Immobiline Dry Strips were equilibrated for 15 min at room temperature (19°C) in equilibration buffer [50 mM Tris/HCl (pH 8.8), 6 M urea, 30 % (v/v) glycerol and 2 % (w/v) SDS] containing 1 % DTT, followed by a second incubation step for 15 min at room temperature in the equilibration buffer in the presence of 2.5 % (w/v) iodoacetamide. Separation in the second dimension (SDS/PAGE) was carried out on homogeneous polyacrylamide gels (12 % gels) at 10 mA per gel for 30 min, and at 25 mA for 4 h in a Hoefer SE600 Electrophoresis Unit. Gels were silver stained as described previously [18]. In order to corroborate the reproducibility of the results, at least three independent experiments for each strain were performed, and three gels were run for each sample. Silver-stained gels were digitized using an Epson Expression 1680 Pro scanner. ImageMaster 2D Platinum software (Amersham Biosciences) was used for gel-image analysis, including quantification of the spot intensities, which is performed on a volume basis (i.e. values were calculated from the integration of spot optical intensity over the spot area, and is referred to as the %V (relative volume)). %V is the ratio of the volume of a spot and the sum of the volume of all the spots detected in the same gel. Gels from independent analyses were matched together, and the %V for each spot in the two gel sets (UW 136 and MV474 A. vinelandii strains) was compared. Significant changes in the abundance of selected proteins (Table 1) were estimated by Student’s t test (P < 0.05). The average expression ratio between the MV474 and UW136 strains was calculated from the averages of %V of five replicates.

MS analysis and identification of protein spots

In-gel enzymatic digestion of the spots and peptide extraction was carried out as described previously [19]. MALDI-TOF (matrix-assisted laser-desorption ionization–time-of-flight) MS analysis was performed in a Voyager DE-Pro spectrometer (PerSeptive BioSystems). External mass calibration was performed with low-mass peptide standards, and the mass-measurement accuracy was ±0.3 Da. The mass spectra were acquired in reflector mode using Delay Extraction technology. Raw data were elaborated using Data Explorer 5.0 software provided by the manufacturer (PerSeptive BioSystems). The software package Protein

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Table 1 Proteins identified by MALDI-TOF MS analysis

<table>
<thead>
<tr>
<th>Spot number</th>
<th>Protein name</th>
<th>Accession number</th>
<th>Sequence coverage (%)</th>
<th>Molecular mass (Da)</th>
<th>pl</th>
<th>Protein ratio (MV474/UW136)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6-Phosphogluconate dehydrogenase</td>
<td>Q4ITN0</td>
<td>32</td>
<td>65348</td>
<td>6.0</td>
<td>n.s.d.</td>
</tr>
<tr>
<td>2</td>
<td>Acylitate dehydrogenase 1</td>
<td>Q4IW32</td>
<td>25</td>
<td>97314</td>
<td>5.3</td>
<td>1.39 ± 0.02</td>
</tr>
<tr>
<td>3</td>
<td>Acylitate dehydrogenase 2</td>
<td>Q4L5V0</td>
<td>22</td>
<td>93347</td>
<td>5.5</td>
<td>−0.69 ± 0.02</td>
</tr>
<tr>
<td>4</td>
<td>Acetoacetyl-CoA reductase</td>
<td>Q93QF0</td>
<td>63</td>
<td>28726</td>
<td>6.2</td>
<td>n.s.d.</td>
</tr>
<tr>
<td>5</td>
<td>β-Oxothiolase</td>
<td>Q9K1H97</td>
<td>50</td>
<td>40875</td>
<td>6.7</td>
<td>1.23 ± 0.07</td>
</tr>
</tbody>
</table>

The relative abundance (%V) of 6-phosphogluconate dehydrogenase (1, Q4ITN0), acylitate dehydrogenase 1 (2, Q4IW32), acylitate dehydrogenase 2 (3, Q4L5V0), acetoacetyl-CoA reductase (4, Q93QF0) and β-oxothiolase (5, Q9K1H97) was determined and used to calculate the protein ratio of MV474 and UW136 [results are means ± S.E.M. (n = 5)]. Accession numbers refer to the Swiss-Prot/TrEMBL database. n.s.d., not significantly different.
Enzymatic activity assays

Enzymatic activities were determined in samples (150–300 μg of protein) of cell-free extracts prepared anaerobically (five 30 s pulses with intermitted 1 min cooling periods) in lysis buffer A [50 mM Tris/HCl (pH 8.0), 100 mM NaCl and 1 mM DTT].

Aconitate hydratase and 6-phosphogluconate dehydratase activities were tested as described by Varghese et al. [20] and Outten et al. [21] respectively. Catalase activity was measured using the method described previously [22], where 1 unit of enzyme is defined as the amount of enzyme that decomposes 1 μmol of H2O2 per min at 37°C.

For re-activation experiments, before the activity tests, cell-free extracts were anaerobically incubated for 10 min at room temperature in the presence of 1 mM FeSO4 and 5 mM DTT.

Rhodanese activity was tested by a discontinuous method that quantifies the product thiocyanate [23] in the presence of thiourea as a sulfur donor [TST (thiosulfate–cyanide sulfurtransferase) activity]. The assays lasted 1 or 2 min, and 1 unit of enzyme is defined as the amount of enzyme that decomposes 1 μmol of thiocyanate per min at 37°C.

For re-activation experiments of PMS-treated purified RhdA (1:1 molar ratio of RhdA/PMS), the mixtures after gel filtration (10 μM RhdA) were incubated at 4°C in the presence of 10 μM E. coli reduced thioredoxin (Sigma), 0.2 μM rat liver thioredoxin reductase (Sigma) and 50 μM NADPH in 20 mM phosphate buffer (pH 7.4) (final volume of 100 μl). In the case of PMS-treated A. vinelandii UW136, the extracts (200 μg of protein) were incubated in the presence of 50 μM thioredoxin, 0.2 μM rat liver thioredoxin reductase and 100 μM NADPH. After 60 min incubation, TST activity was measured as stated above.

In vitro study of overexpressed RhdA

Overexpressed His-tagged RhdA, purified as described previously [24], was used to test the in vitro effects of the oxidative agent PMS. RhdA (100 μM) in 50 mM Tris/HCl (pH 8.0) and 100 mM NaCl was incubated aerobically with PMS (final concentration of 1 mM) at room temperature. At fixed incubation times, sample aliquots were gel-filtered by using Sephadex G-25 columns to remove PMS. The gel-filtered samples were subjected to the rhodanese activity test [23], non-reducing SDS/PAGE [25] and MALDI-TOF MS analyses. Densitometric analyses of the electrophoretic pattern were performed on Coomassie-Blue-stained gels using ImageMaster 1D Elite software (Amersham Biosciences). The MALDI-TOF MS analysis was performed by using a Bruker Daltonics Reflex IV instrument equipped with a nitrogen laser (337 nm) and operated in a positive linear mode with a matrix of α-cyano-4-hydroxycinnamic acid. External standards were used for calibration (Bruker protein II calibration standards) and the mass measurement accuracy was ±1 Da. Each spectrum was accumulated for at least 200 laser shots.

RESULTS

The lack of RhdA affects A. vinelandii growth on gluconate as a carbon source

In the sucrose medium, which we employed for our initial experiments, the rhdA mutant MV474 grew as well as the wild-type UW136 strain, but the metabolic imbalance of the MV474 strain suggested that the absence of RhdA favoured oxidative events in A. vinelandii [13]. To elucidate better the protective cellular roles of RhdA, more critical growth conditions were exploited, and we found that the growth of A. vinelandii MV474 was impaired in the presence of gluconate as a carbon source. Utilization of gluconate via the ED pathway [16] involves the activity of 6-phosphogluconate dehydratase, an enzyme containing a 4Fe–4S cluster which is an easy target for oxidative damage [17]. Under this growth condition (Figure 1), the lack of RhdA caused a significant inhibition of growth at early stages, and, after 24 h of growth, MV474 reached a final culture density of approx. 90% of that of the wild-type strain. At this stage, the accumulation of PHB was 8-fold higher in the rhdA-null mutant than in UW136. PHB content (as determined in three separate growth experiments) was 163 ± 10 μg per mg of protein in UW136, and 1320 ± 25 μg per mg of protein in the mutant MV474 strain, confirming that the metabolic profile of the mutant MV474 strain did favour the synthesis and accumulation of this polymer. Greater accumulation of PHB in the rhdA-null mutant than in UW136 was, indeed, also observed when sucrose was the carbon source provided [13]. PHB accumulation is generally considered a ‘cell-survival mechanism’ [28] that endows bacteria with enhanced stress tolerance [29], thus indicating that PHB accumulation in MV474 represents a response to the physiological imbalance inferred by the lack of RhdA.
Protein expression in <i>A. vinelandii</i> wild-type and <i>rhdA</i>-null mutant strains

Comparative proteomics of gluconate-cultured UW136 and MV474 <i>A. vinelandii</i> strains were performed with the aim of identifying the relative abundance of specific enzymes strictly related to the metabolic picture evidenced in the absence of RhdA. The protein spots listed in Table 1 were identified by peptide mass fingerprinting, and their relative abundance (in terms of %V of each spot) was accurately calculated from independent gels with five experimental repeats for each strain (<i>rhdA</i>-null MV474 mutant and UW136 <i>A. vinelandii</i> strains). Through this comparative analysis, we found that the 6-phosphogluconate dehydratase polypeptide (spot 1 in Figure 2) was present in both strains, and that its expression level in MV474 and in UW136 was not significantly different (Table 1). Further analyses of the 2-DE protein profiles were focused to compare the expression of enzymes differently regulated in MV474 under growth conditions in the presence of sucrose [13]. The enzyme of the PHB biosynthetic operon [30] β-oxothiolase displayed increased expression with statistical significance in MV474 (Figure 2 and Table 1), thus explaining the 8-fold higher production of PHB in the <i>rhdA</i>-null mutant strain than in the wild-type strain. Significantly different expression levels ofaconitate hydratase polypeptides (spots 2 and 3 in Figure 2) between the MV474 and UW136 strains (Table 1) were detected. Aconitate hydratase 2 was present in higher amounts in UW136 than in MV474, whereas aconitate hydratase 1 was overexpressed in MV474. In <i>E. coli</i>, both aconitate hydratase enzymes process the substrate citrate, and it was found thataconitate hydratase 1 (the product of the <i>acnA</i> gene) was induced by oxidative stress [31]. Evidence that a lack of RhdA results in altered protection from oxidative events, thus giving rise to a loss of the functionality of enzymes containing labile Fe–S clusters [15], was provided by enzymatic analyses (Table 2). The activity of 6-phosphogluconate dehydratase in extracts from MV474 was approx. 60% of that found in UW136, although the polypeptide expression in both <i>A. vinelandii</i> strains did not show statistically significant differences, andaconitate hydratase activity in MV474 was approx. 40% of that of UW136. Evident <i>in vitro</i> re-activation (approx. 1.5-fold differences from untreated samples) of both dehydratases was obtained in extracts from MV474 (Table 2) after treatment with ferrous iron and DTT. Re-activation of Fe–S enzymes by treatment with ferrous iron and DTT is considered to be an indication that damage to the clusters stems from an interaction with reactive oxygen species that induces the loss of iron [15]. Moreover, a probable imbalance of oxidative events in the <i>rhdA</i>-null mutant strains of <i>A. vinelandii</i> were separated by 2-DE as described in the Experimental section. The silver-stained gels of UW136 and MV474 are shown, and the numbers represent the spots identified by MALDI-TOF MS listed in Table 1. Molecular-mass markers are shown on the left-hand side (in kDa).

### Table 2 Activity of dehydratase enzymes in <i>A. vinelandii</i> wild-type and <i>rhdA</i> mutant strains

<table>
<thead>
<tr>
<th></th>
<th>Basal activity</th>
<th>Activity after treatment with FeSO₄ and DTT</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>UW136</td>
<td>MV474</td>
</tr>
<tr>
<td>6-Phosphogluconate dehydratase</td>
<td>0.620 ± 0.012</td>
<td>0.39 ± 0.007</td>
</tr>
<tr>
<td>Aconitate hydratase</td>
<td>2.05 ± 0.07</td>
<td>0.84 ± 0.05</td>
</tr>
<tr>
<td>6-Phosphogluconate dehydratase</td>
<td>0.620 ± 0.012</td>
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<tr>
<td>Aconitate hydratase</td>
<td>2.05 ± 0.07</td>
<td>0.84 ± 0.05</td>
</tr>
<tr>
<td>6-Phosphogluconate dehydratase</td>
<td>2.31 ± 0.20</td>
<td>1.27 ± 0.21</td>
</tr>
</tbody>
</table>

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Effects of the lack of RhdA in Azotobacter vinelandii

Figure 3 Effects of the exposure to PMS on A. vinelandii wild-type and rhdA mutant strains

A. vinelandii UW136 and MV474 strains were grown aerobically in BGN (Burk’s gluconate nitrogen) medium (supplemented with 0.2 % gluconate) up to \( D_{600} = 0.8 \), after which the cultures of either UW136 or MV474 strains were divided into two equal samples, one of which was treated with PMS (final concentration of 15 \( \mu \)M). The cultures were grown for 16 h, and 2-DE was performed as described in the legend of Figure 2. Three independent experiments were performed, and the \( D_{600} \) after 16 h exposure to PMS and the value recorded for PMS-untreated cultures are shown (A).

(B) Enlargement of a 2-DE gel of the region showing the AhpC (Q4IS62) protein spot (arrow) in untreated UW136, untreated MV474, PMS-treated UW136, and PMS-treated MV474 (from left- to right-hand side).

was suggested by the finding that the activity of the hydrogen peroxide scavenger catalase [32–34] was higher in MV474 than in UW136 (30 ± 2 units/mg and 50 ± 2 units/mg in UW136 and MV474 respectively).

Defensive action of the rhdA gene product (RhdA) against superoxide stress

To demonstrate the involvement of RhDA in triggering protective processes against oxidative events, we analysed the response of A. vinelandii MV474 and UW136 strains to induced oxidative stress. The resistance of A. vinelandii to exposure to the oxidative agent PMS was severely affected by the absence of RhDA (Figure 3A). With regard to the untreated control cultures, the growth of the rhdA mutant strain was less than 40%, whereas the wild-type strain was not significantly affected by PMS treatment. Upon exposure to PMS, the decrease in aconitate hydratase activity, with regard to that measured in untreated extracts, was more pronounced in the rhdA mutant strain (5-fold) than in the wild-type strain (1.6-fold). As for 6-phosphogluconate dehydratase activity, 60% of the activity of untreated extracts was found in the wild-type strain, and 45% in the rhdA mutant.

To investigate whether treatment of A. vinelandii with the superoxide generator PMS could prime different defensive processes in the wild-type and the rhdA mutant strains, the 2-DE protein profiles of both strains were compared under exposure to PMS. Accurate matching of these protein profiles was performed with the purpose of detecting protein spots which underlie the different responses of MV474 to PMS treatment compared with UW136. In PMS-treated MV474, a protein spot (molecular mass ≈ 20 kDa, pI ≈ 5.2) that was not detected in untreated cells or in the PMS-treated wild-type strain was revealed (Figure 3B). MALDI-TOF MS analysis identified it as Q4IS62_AZOVI, defined as AhpC (alkyl hydroperoxide reductase), a member of the OxyR regulon [32–35]. Regulated adaptive responses of micro-organisms to oxidative stress have been extensively studied using E. coli as a model organism [32,36,37], and it is generally recognized that catalase and AhpC activities are critical to oxidative stress survival.

Even though AhpC was overexpressed in the rhdA-null mutant as a probable defence mechanism, it did not endow A. vinelandii MV474 with resistance to PMS exposure compared with that of the wild-type strain (Figure 3A). This evidence indicated a role of the rhdA gene product in defending A. vinelandii against oxidative stress, and prompted us to explore whether the RhDA protein underwent structural modifications involving sulfur redox chemistry [37–39]. RhDA dimer formation was demonstrated by RhDA immunodetection in UW136 cell extracts exposed to the stressor PMS (Figure 4). Considering that the RhDA Cys\(^{230}\) catalytic thiol, the only one present in the protein [12,40], is mandatory for sulfane/sulfur transfer from thiosulfate to cyanide with concurrent formation of thiocyanate (TST activity) [1], thiocyanate production [23] represents a measure of the availability of the Cys\(^{230}\) thiol. When the wild-type strain UW136 was exposed to PMS, the production of thiocyanate in extracts was 19% of that measured in untreated extracts. Restoration of thiocyanate production to up to 98% of that of untreated cells...
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Figure 4  SDS/PAGE immunoblot

Proteins from cell extracts (40 μg of protein) either from UW136 after 16 h exposure to the stressor PMS (+PMS) or from untreated UW136 (−PMS) were resolved by non-reducing SDS/PAGE and subjected to Western blot analysis with an anti-RhdA antibody [13]. Asterisks indicate the dimeric (*) and monomeric (**) forms of RhdA respectively. The molecular masses are indicated on the left-hand side (in kDa).

was obtained by incubating the extracts from UW136 PMS-treated cells in the presence of the thioredoxin/thioredoxin reductase system. These results indicated that reversible modifications of the RhdA Cys230 thiol occurred in _A. vinelandii_ under oxidative conditions.

In order to investigate how RhdA itself could behave as a redox switch, we undertook an _in vitro_ study to demonstrate RhdA molecular modifications. Incubation of purified RhdA overexpressed in _E. coli_ [24] with PMS resulted in a time-dependent loss of its ability to produce thiocyanate (Figure 5A).

The effects of exposure to PMS on RhdA were concurrently analysed by non-reducing SDS/PAGE and MALDI-TOF MS. Non-reducing SDS/PAGE (Figure 5B), followed by accurate densitometric analysis of the resulting protein pattern (Figures 5C and 5D, grey bars), revealed that exposure to PMS for up to 20 min increased the dimer to monomer ratio of RhdA with regard to the ratio in untreated samples. As shown in Figures 5(B) and 5(C), the presence of the RhdA dimeric form decreased when PMS treatment was prolonged, thus indicating that RhdA inactivation during PMS treatment (Figure 5A) could not only be ascribed to dimer formation. Further details on RhdA modifications that affect Cys230 thiol availability were provided by MALDI-TOF MS. As shown in Figure 5(C) and 5(D) (black bars), MALDI-TOF MS analysis of the dimer to monomer ratios of RhdA during PMS treatment confirmed that formation of RhdA dimer was inhibited by prolonged exposure to PMS. A molecular mass of 31024.5 m/z, corresponding to the protein containing unmodified Cys230 (calculated molecular mass of 31027.9 Da), was measured.

Figure 5 Oxidative events on RhdA

Purified RhdA overexpressed in _E. coli_ (100 μM) in 50 mM Tris/HCl (pH 8.0) and 100 mM NaCl was incubated aerobically at room temperature with PMS (final concentration of 1 mM). At fixed times of PMS exposure, samples were withdrawn and analysed in terms of their ability to produce thiocyanate (TST activity), and by electrophoresis and MS. (A) Time-dependent inactivation of RhdA by PMS, indicated as the percentage activity in relation to untreated RhdA (0 min). (B) Non-reducing SDS/PAGE of the samples at the indicated times of exposure to PMS. Asterisks indicate the dimeric (*) and monomeric (**) forms of RhdA respectively. Molecular masses are indicated on the left-hand side (in kDa). (C and D) Dimer to monomer ratios of RhdA before (0 min) and after PMS treatment for various incubation times determined either by densitometric analyses of electrophoretic patterns (grey bars) or MALDI-TOF MS analyses (black bars). In (D), the values are normalized against those before treatment (0 min).

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by MALDI-TOF MS analyses of untreated RhdA (Figure 6A). When treated with PMS, a second peak with a molecular mass of 31056.7 m/z was detected (Figure 6B), suggesting the formation of a sulfenic acid (Cys$^{390}$–SOH) (+32 Da) [38]. When the experiments were repeated in the presence of an equimolar ratio of RhdA/PMS, MALDI-TOF MS analysis of the sample after 60 min exposure to PMS led to the detection of the peak corresponding to the unmodified protein, and a second peak with an increase in the molecular mass of 16 Da (results not shown), suggesting the presence of sulfenic acid (Cys$^{390}$–SOH). This latter peak disappeared after treatment with 2-mercaptoethanol as a reductant, and the TST activity of PMS-treated RhdA was recovered by up to 60% of that of the untreated sample by incubation in the presence of the thioredoxin/thioredoxin reductase system.

**DISCUSSION**

Among the ten ORFs (open reading frames) of *A. vinelandii* coding for rhodanese-domain proteins displaying different architecture and active-site motifs (Pfam accession number PF00581, Azotobacter vinelandii AvOP), the rhda gene [11] codes for a tandem-domain rhodanese (RhdA) with an active-site loop [12] which provides a strong positive electrostatic field around the catalytic residue Cys$^{390}$. Preliminary phenotypic characterization of the *A. vinelandii* strain disrupted in the *rhdA* gene [13] suggested that RhdA could exert some protection that allowed the cell enzymes containing Fe–S clusters, which are prone to oxidation, to function [15]. Consistent with this hypothesis, in the present study we found that the phenotypic features resulting from the absence of RhdA were elicited using growth conditions that strictly requires enzymes containing labile Fe–S clusters as catalysts. In the presence of gluconate as a carbon source, which implies the functioning of 4Fe–4S 6-phosphogluconate dehydratase [16,17], *A. vinelandii* growth was impaired only in the *rhdA*-null mutant.

Under this growth condition, we found that in the *rhdA*-null mutant aconitate hydratase 1, which in *E. coli* is induced during oxidative stress [31], was overexpressed, and that the activity of the hydrogen peroxide scavenger catalase was enhanced. The presence in *A. vinelandii* of catalase enzymes which are inducible by reactive oxygen intermediates produced during respiration was reported recently [41]. As already found in a sucrose-cultured *rhdA*-null mutant strain [13], PHB accumulation, generally considered to be a cell-survival mechanism [28,29], was increased in the *rhdA* mutant strain. The overall picture confirmed that deletion of the *rhdA* gene makes *A. vinelandii* more prone to stress, and the increased vulnerability of dehydratase Fe–S clusters in the *rhdA* mutant was further evidence proving that in the absence of RhdA, protection from oxidative events was impaired. There is a general consensus that oxidants convert the dehydratase [4Fe–4S]$^{1+}$ form into an unstable [4Fe–4S]$^{3+}$ state, which releases the catalytic iron molecule [15,42], an event that eventually induces the inactivation of the enzymes. Re-activation of the damaged enzymes *in vitro* by treatment with iron and DTT proved that inactivation involves damage to the cluster [42]. The results shown in Table 2, combined with the analyses of polypeptide expression, indicated that the low catalytic efficiency of dehydratases in the *rhdA*-null mutant was the result of oxidative damage of the Fe–S cluster. To overcome oxidative damage resulting from the oxidizing potential of O$_2$, eukaryotic and prokaryotic organisms have evolved strategies to remove reactive oxygen species and repair damage [36,37]. The aerobe *A. vinelandii* was the focus of studies devoted to investigate the mechanisms of protection of the nitrogenase complex from oxygen inactivation [43], but knowledge of the physiological responses to the generation of oxidative species under non-diazotrophic conditions (as examined in the present study) is lacking. When oxidative stress was provoked by the external addition of chemical oxidants (the superoxide generator PMS), we found that the sensitivity of *A. vinelandii* was particularly pronounced only in the *rhdA* mutant strain. Overexpression of AhpC as an adaptive response to oxidative stress [32–37] did not endow the *rhdA* mutant with PMS resistance comparable with that of *A. vinelandii* wild-type. In *E. coli* and in other bacteria, AhpC is a member of the OxyR regulon, which up-regulates peroxide defences [34,36]. In *E. coli*, the model organism of studies on OxyR-mediated adaptive responses, it was found that OxyR can respond to disulfide stress resulting from defects in the systems that maintain an intracellular reducing environment [44], and that the OxyR-regulated *ahpC* transcript was induced by PMS [45]. Although OxyR-regulated responses in *A. vinelandii* are unknown, the presence in *A. vinelandii* chromosome of a gene coding for a protein homologous with OxyR (gi67155513, http://www.ncbi.nlm.nih.gov) could be taken as an indication that similar adaptive mechanisms exist in *A. vinelandii*. The picture that has emerged from the present study, which aimed to define the physiological functions of the rhodanese-like RhdA, indicated that this protein might function as an antioxidant that helps *A. vinelandii* in ‘scavenging’ oxidative species. Elimination of oxidative species does imply that RhdA can function as redox switch via thiol modification, an ability that might be inferred
by the structural peculiarity of the environment of its catalytic cysteine residue [12] that can promote ionization, even at neutral pH, to the thiolate anion (Cys–S⁻). It is generally recognized [37–39] that thiolate anions are easily oxidized to the unstable sulfenic acid form (Cys–SOH), which may react with an accessible thiol to form a disulfide (S–S) bond. The decreased availability of the RhdA Cys³⁰⁰ thiol in extracts from PMS-treated A. vinelandii UW136, proved by RhdA activity tests, and the reversibility of the PMS effects in the presence of the thioredoxin/thioredoxin reductase reducing system, provided evidence that RhdA Cys³⁰⁰ thiol modifications occurred in vivo. Molecular details of RhdA Cys³⁰⁰ thiol modifications (the only one present in the molecule) were explored by using an artificial situation. Formation of the RhdA dimeric form upon exposure for a short period to PMS, and further oxidation producing the stable sulfenic acid species [38,39] was demonstrated by combining activity tests, electrophoresis and MALDI-TOF MS analyses. Also, in the artificial situation of these in vitro studies, in the presence of the thioredoxin/thioredoxin reductase reducing system, RhdA Cys³⁰⁰ thiol modifications were reduced back to the active thiol form, which is mandatory for thiocyanate production. Although to study oxidative modifications of RhdA we used an in vitro approach, the RhdA functionality as a cellular switch is supported by the in vivo evidence that, in the absence of RhdA, A. vinelandii needed to activate defensive activities generally recognized as adaptive responses to a cellular oxidative imbalance [36,37).

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