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Characterization of a rhodanese from the cyanogenic bacterium *Pseudomonas aeruginosa*☆

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Abstract

Pseudomonas aeruginosa, the rRNA group I type species of genus *Pseudomonas*, is a Gram-negative, aerobic bacterium responsible for serious infection in humans. *P. aeruginosa* pathogenicity has been associated with the production of several virulence factors, including cyanide. Here, the biochemical characterization of recombinant *P. aeruginosa* rhodanese (*Pa* RhdA), catalyzing the sulfur transfer from thiosulfate to a thiophilic acceptor, e.g., cyanide, is reported. Sequence homology analysis of *Pa* RhdA predicts the sulfur-transfer reaction to occur through persulfuration of the conserved catalytic Cys230 residue. Accordingly, the titration of active *Pa* RhdA with cyanide indicates the presence of one extra sulfur bound to the Cys230 Sγ atom per active enzyme molecule. Values of K_m for thiosulfate binding to *Pa* RhdA are 1.0 and 7.4 mM at pH 7.3 and 8.6, respectively, and 25 °C. However, the value of K_m for cyanide binding to *Pa* RhdA (=14 mM, at 25 °C) and the value of V_{max} (=750 μmol min^{-1} mg^{-1}, at 25 °C) for the *Pa* RhdA-catalyzed sulfur-transfer reaction are essentially pH- and substrate-independent. Therefore, the thiosulfate-dependent *Pa* RhdA persulfuration is favored at pH 7.3 (i.e., the cytosolic pH of the bacterial cell) rather than pH 8.6 (i.e., the standard pH for rhodanese activity assay). Within this pH range, conformational change(s) occur at the *Pa* RhdA active site during the catalytic cycle. As a whole, rhodanese may participate in multiple detoxification mechanisms protecting *P. aeruginosa* from endogenous and environmental cyanide.

Keywords: Cyanide; *Pseudomonas aeruginosa*; Rhodanese; Sulfurtransferase enzyme

Sulfurtransferases (EC 2.8.1.x) catalyze the transfer of a sulfane sulfur atom to a suitable thiophilic acceptor substrate [1]. These enzymes are classified into two subfamilies depending on the sulfur donor for in vitro reaction: thiosulfate:cyanide sulfurtransferases, referred to as rhodaneses (EC 2.8.1.1), and mercaptopyruvate:cyanide sulfurtransferases (EC 2.8.1.2) (http://www.brenda.uni-koeln.de).

Rhodaneses are characterized by the presence of distinctive structural modules encompassing the so-called rhodanese signatures which can be recognized in single-domain proteins, or in combination with other protein domains, or arranged as tandem repeats with the C-terminal domain harboring the catalytic Cys residue [2]. Bovine liver rhodanese, with its double-domain architecture, represents the reference structure for the rhodanese subfamily [1,3–5]. However, crystal structure analysis of the two-domain rhodanese from

☆ *Abbreviation: Pa* RhdA, His_6-tagged recombinant *Pseudomonas aeruginosa* rhodanese.

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the eubacterium *Azotobacter vinelandii* revealed a striking conservation of the rhodanese fold, arguing for possible common function(s) of rhodanese homologues from evolutionary distant phyla [6].

Recently, the completion of several prokaryotic genome sequences has unraveled that genes encoding rhodanese-related sulfurtransferases are widely distributed among bacteria, and that multiple rhodanese homologs can be encoded by the same genome (COG database, http://www.ncbi.nlm.nih.gov/COG) [2]. In spite of their wide biological distribution, the role of rhodaneses in cell physiology is still uncertain. Proposed functions include formation of iron–sulfur centers [7], involvement in sulfur metabolism [8], and cyanide detoxification [9]. This last function has been proved to be provided by mitochondrial rhodanese in eukaryotic organisms [10,11].

Pseudomonas aeruginosa, the rRNA group I type species of genus *Pseudomonas*, is a Gram-negative, aerobic bacterium responsible for serious infection in patients suffering from cystic fibrosis or severe immune deficiency [12]. *P. aeruginosa* pathogenicity has been associated with the production of several virulence factors, including cyanide [13]. The ability of cyanide to act as a virulence factor for this bacterium was corroborated by the evidence that a non-cyanogenic *P. aeruginosa* mutant is less virulent than the parent strain in an animal model of infection [14]. Thus, *P. aeruginosa* cyanogenesis provides an ideal model to address the involvement of prokaryotic rhodaneses in cyanide detoxification. Noticeably, constitutive rhodanese activity has been documented in *P. aeruginosa* [15], but the underlying enzymatic basis has not yet been investigated.

Here, the biochemical characterization of recombinant *P. aeruginosa* rhodanese (*Pa* RhdA) is reported. The sulfur-transfer reaction catalyzed by rhodanese may participate in multiple detoxification mechanisms protecting *P. aeruginosa* from endogenous and environmental cyanide.

Materials and methods

Gene cloning and protein expression and purification. To clone the *P. aeruginosa rhdA* gene under the control of the T_7 promoter, ORF PA4956 (*rhdA* at www.pseudomonas.com) was amplified by PCR with primers 5′-GGAATTCCATATGTCCGTTTTCTCCGACCTGC-3′ (RhdAfw) and 5′-GGGCAAGCTTCCTCAAACCTCTACAGGGG-3′ (RhdArv), using *P. aeruginosa* PAO1 genomic DNA as the template. Primers were designed to introduce *Nde*I and *Hin*dIII restriction sites at the 5′- and 3′-ends, respectively. The digested PCR product was directionally cloned in pET-28a (Novagen), downstream of the His_6-coding sequence, and checked by DNA sequencing. The resulting plasmid, named pET*rhdA*, was introduced in *Escherichia coli* BL21(DE3) by transformation. Cells of *E. coli* BL21(DE3)(pET*rhdA*) were grown to mid-log phase ($OD_{600} \sim 0.6$) at 37 °C in Luria–Bertani (LB) medium supplemented with kanamycin (25 μg/ml) and chloramphenicol (30 μg/ml). Protein expression was obtained by 4-h induction with 0.1 mM isopropyl thio-β-D-galactopyranoside (IPTG). Then, cells were harvested, incubated for 15 min at 30 °C in the lysis buffer (50 mM Tris–HCl, pH 7.5) containing 100 μg/ml lysozyme, and disrupted by sonication (8 cycles × 15 s in a W-225R sonicator, Ultrasonics). The lysate was centrifuged at 14,000*g* for 20 min, and the supernatant was loaded onto a Ni^{2+}–NTA affinity chromatography column (Qiagen). The His_6-tagged protein was eluted under native conditions with a stepwise (5–500 mM) imidazole gradient, according to the manufacturer's protocol. The eluate was analyzed by 15% (w/v) SDS–PAGE with Coomassie blue staining [16]. Pure fractions of *Pa* RhdA were pooled, extensively dialyzed against 50 mM Tris–HCl, pH 7.5, and then stored at 4 °C until used. Immunoblot analysis was performed with murine anti-His_6 monoclonal antibodies (Qiagen) as described elsewhere [17].

The concentration of *Pa* RhdA was estimated using the extinction coefficient ($\varepsilon_{280\ nm} = 58{,}320\ M^{-1}\ cm^{-1}$), calculated according to the deduced amino acid composition [18], and by the Bradford colorimetric assay [19].

Enzyme activity assay. The *Pa* RhdA activity was determined colorimetrically according to the Sörbo method [9] with thiosulfate or 3-mercaptopyruvate or sodium sulfide as sulfur donors, and cyanide as the sulfur acceptor substrate. The enzyme activity was determined at pH 7.3 and 8.6 (50 mM Tris–HCl), between 5 and 60 °C. The enzyme concentration ranged between 0.2 and 100 nM, the sulfur donor and cyanide concentration ranged between 0.6 and 68 mM. The product (i.e., thiocyanate) formation was linear on the time assay (5 min). The spontaneous reaction between different sulfur donors and cyanide was taken into account in the determination of catalytic parameters. The actual cyanide concentration was determined by stoichiometric cyanide binding to human hemoglobin (Sigma) [20]. Steady-state kinetics has been analyzed according to the classical Michaelis–Menten equation.

Spectroscopic measurements. Static fluorescence emission of *Pa* RhdA was measured between 300 and 400 nm in the absence and presence of sulfur donors or cyanide, at pH 7.3 and 8.6 (50 mM Tris–HCl), and 25 °C. The excitation wavelength was 280 nm, with excitation and emission slits of 5 and 3 nm, respectively. The cuvette pathlength was 1 cm. The enzyme concentration was 1.0 μM, the sulfur donor and cyanide concentration ranged between 0.2 and 250 μM. The incubation time was 10 min [21,22].

Far-UV circular dichroism spectra were recorded at pH 7.3 and 8.6 (50 mM Tris–HCl), and 25 °C. The cuvette pathlength was 0.05 cm. The enzyme concentration was 10 μM, the sulfur donor and cyanide concentration ranged between 0.2 and 250 μM. The incubation time was 10 min.

Spectroscopic data were analyzed using the GraFit 5.0 software (Erithacus Software).

Results and discussion

Eight putative gene products harboring at least one rhodanese module (COG database, http://www.ncbi.nlm.nih.gov/COG) were retrieved from the complete genome sequence of *P. aeruginosa* PAO1 (www.pseudomonas.com) [23]. One of these, encoded by ORF PA4956 (annotated as *rhdA*), shows 79% and 22% sequence identity with *A. vinelandii* and *Bos taurus* rhodanese, respectively (Fig. 1) [6,24,25]. All amino acid residues determining the architecture of the active site pocket of *A. vinelandii* rhodanese are fully conserved in the predicted *P. aeruginosa rhdA* product, with 100% identity in the region surrounding the catalytic

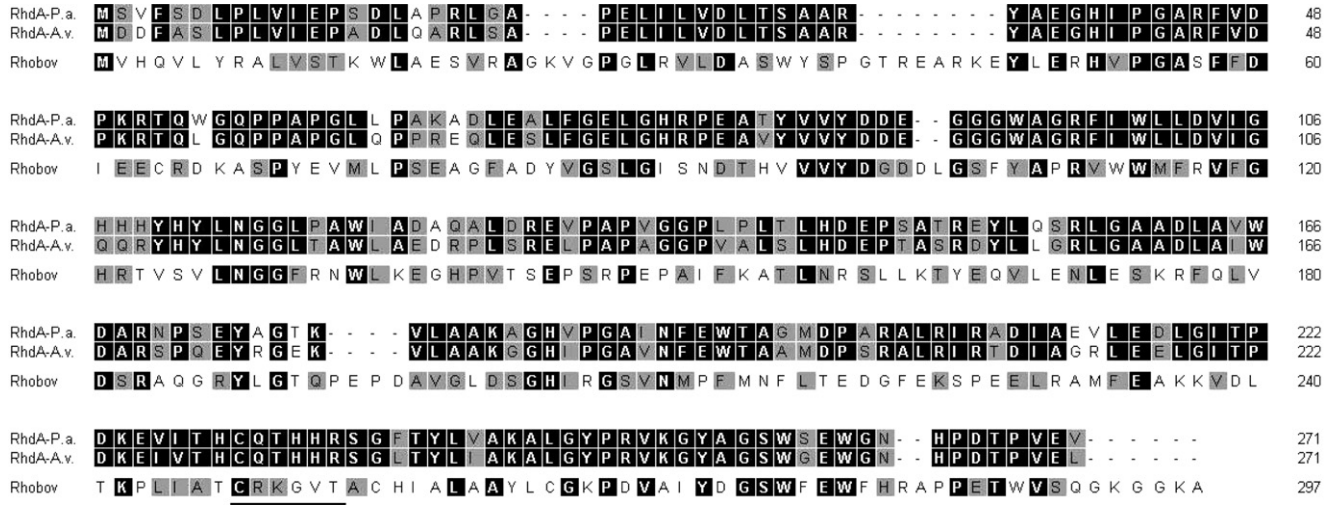

Fig. 1. Sequence comparison between rhodaneses from *P. aeruginosa* PAO1 (RhdA-P.a.), *A. vinelandii* (RhdA-A.v.), and *B. taurus* (Rhobov). Identical residues and conservative substitutions are indicated by black and grey boxes, respectively. Residues forming the six-amino acid active-site loop [2] are underlined. Amino acid sequences (Accession Nos. Q9HUK9, Q44557, and P52197, respectively) were retrieved from Swiss-Prot/TrEMBL database and aligned with the program CLUSTALW [23]. For details, see text.

Cys230 residue (Fig. 1) [6]. Accordingly, substantial rhodanese activity (0.12 μmol min^{-1} mg^{-1} of soluble cellular proteins) was detected in crude extracts of *P. aeruginosa* PAO1 (data not shown).

The *P. aeruginosa* PAO1 *rhdA* gene was cloned in pET-28a under the control of the T_7 promoter for heterologous expression in *E. coli* BL21(DE3). IPTG-induction of *E. coli* BL21(DE3)(pET*rhdA*) resulted in overexpression of a 32-kDa protein, consistent with the predicted molecular mass of *Pa* RhdA (Fig. 2A, lanes 2 and 3). Coherently, the rhodanese activity was 450-fold increased in lysates of IPTG-induced *E. coli* BL21(DE3)(pET*rhdA*) cells, relative to the uninduced control (135 μmol min^{-1} mg^{-1} vs. 0.3 μmol min^{-1} mg^{-1} of soluble cellular proteins). The overexpressed protein was localized in the soluble cell fraction (Fig. 2A, compare lanes 4 and 5), and could be purified to homogeneity by single-step affinity chromatography on a Ni^{2+}–NTA column (Fig. 2A, lane 6). *Pa* RhdA eluted between 20 and 50 mM imidazole, yielding ca. 50 mg of pure protein per liter of culture. Immunoblot analysis with commercial monoclonal antibodies against the His_6 epitope recognized the 32-kDa band, thereby confirming the identity of *Pa* RhdA (Fig. 2B).

Fluorescence emission spectra were measured to define the extent of *Pa* RhdA persulfuration. As expected from the high sequence identity with *A. vinelandii* RhdA (Fig. 1), the quantum yield of *Pa* RhdA intrinsic fluorescence is lower in the persulfurated active enzyme than in the sulfur-free inactive form, depending on energy transfer from the excited Trp residues to the catalytic Cys230 persulfide group and on local conformational changes in the active site pocket upon persulfide formation [21,22,26]. The spectral transition of purified *Pa* RhdA following cyanide-dependent sulfur removal is shown in Fig. 3A.

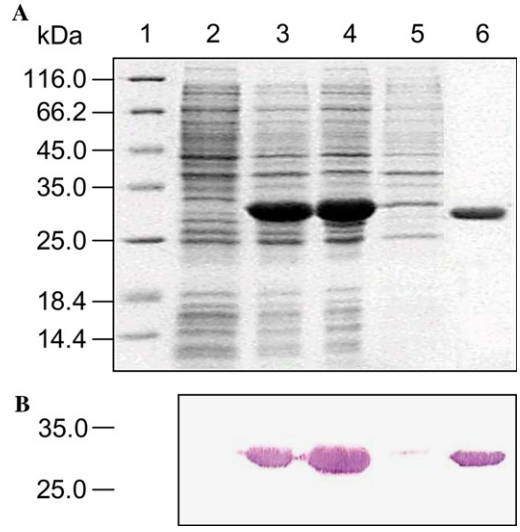

Fig. 2. Overexpression and purification of *Pa* RhdA. (A) SDS–PAGE and (B) Western blot analysis of enzyme preparations. The gel was stained with Coomassie brilliant blue, then blotted onto a nitrocellulose filter, and hybridized with anti-His_6 monoclonal antibodies. Lane 1, molecular mass markers; lanes 2 and 3, total cellular proteins (20 μg each) from uninduced and IPTG-induced *E. coli* BL21(DE3) (pET*rhdA*), respectively; lanes 4 and 5, soluble and insoluble fractions from the IPTG-induced cell lysate (20 and 10 μg), respectively; lane 6, purified *Pa* RhdA eluting with 20 mM imidazole (2 μg). For details, see text.

Fluorescence spectra of sulfur-free and persulfurated *Pa* RhdA are pH-dependent (Fig. 3A). The quantum yield of both the sulfur-free and the persulfurated form was decreased by ca. 20% on lowering the pH from 8.6 (i.e., the conventional pH of rhodanese activity assays)

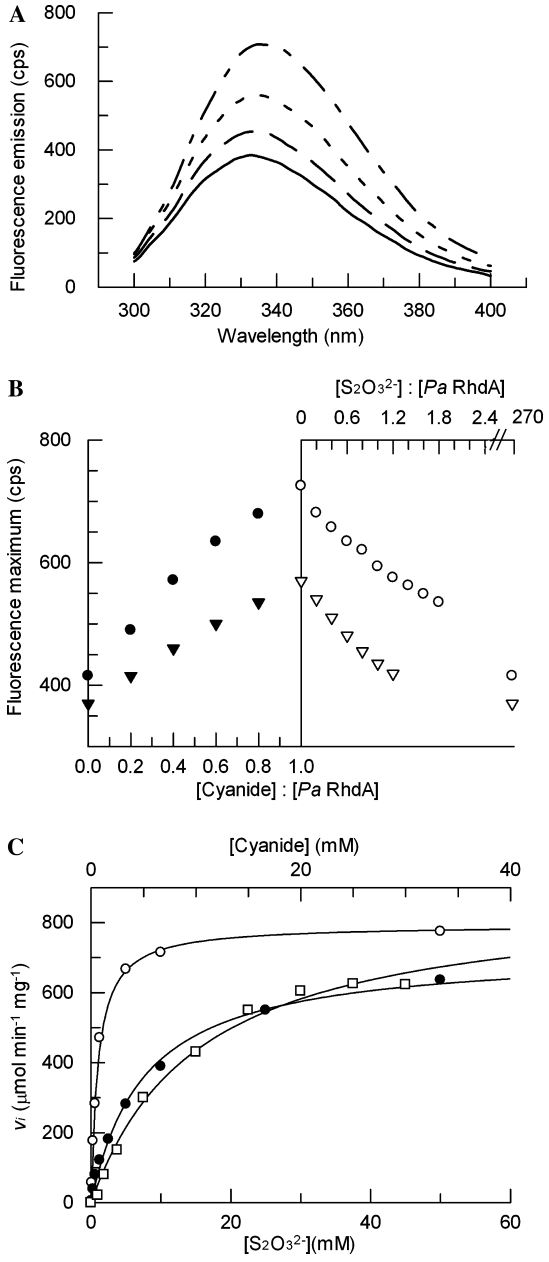

Fig. 3. Spectroscopic and kinetic properties of *Pa* RhdA. (A) Intrinsic fluorescence emission ($\lambda_{exc} = 280$ nm) of 1.0 μM *Pa* RhdA at pH 7.3 and pH 8.6 (50 mM Tris–HCl). Spectra were determined before (—, pH 7.3; ——, pH 8.6) and after (– – –, pH 7.3; – – – –, pH 8.6) addition of an equimolar amount of cyanide, at 25 °C. (B) Intrinsic fluorescence emission at 334 nm ($\lambda_{exc} = 280$ nm) of 1.0 μM *Pa* RhdA as a function of cyanide concentration (filled symbols), and of the depersulfurated enzyme as a function of thiosulfate concentration (open symbols). Experiments have been performed at pH 7.3 (triangles) and pH 8.6 (circles) (50 mM Tris–HCl), and 25 °C. No fluorescence changes were observed at [cyanide]:[*Pa* RhdA] molar ratio higher than 1. (C) Effect of thiosulfate (circles) and cyanide (squares) concentration on the initial velocity (v_i) for the *Pa* RhdA-catalyzed sulfur-transfer reaction at pH 7.3 (open symbols) and pH 8.6 (filled symbols), and 25 °C. Because data on the effect of cyanide concentration on v_i are essentially superimposable at the two pH values (see Table 1), only data at pH 7.3 are shown. The continuous lines were calculated according to the Michaelis–Menten equation with values of catalytic parameters given in Table 1. For details, see text.

to pH 7.3 (i.e., the *P. aeruginosa* cytosolic pH). Noteworthy, the 4-nm red-shift of the fluorescence maximum of the sulfur-free form, resulting from higher exposure to the solvent of Trp residues [22], occurs only at pH 8.6. These findings suggest that energy transfer between the excited Trp residues and the persulfurated Cys230 is facilitated at pH 7.3, reflecting higher proximity of fluorescence donor and acceptor. By contrast, the pH- and substrate-independent profiles of far-UV CD spectra suggest that no significant secondary and tertiary conformational change(s) occur in *Pa* RhdA during the catalytic cycle (data not shown).

Pa RhdA appears to be produced by *E. coli* cells in the persulfurated form, as inferred by the evidence that the maximum increase in fluorescence is attained upon addition of an equimolar amount of cyanide to the protein (Fig. 3B). Then, the inactive sulfur-free *Pa* RhdA can be fully persulfurated upon addition of 250 molar excess thiosulfate (Fig. 3B). Thus, while the sulfur-transfer reaction to cyanide is apparently irreversible, thiosulfate-dependent persulfide formation seems to be governed by a reversible equilibrium, similar to what was reported for *A. vinelandii* and *B. taurus* rhodaneses [21,27].

The sulfur-transfer reaction catalyzed by *Pa* RhdA follows simple Michaelis–Menten kinetics at pH 7.3 and 8.6 (Fig. 3C). The product (i.e., thiocyanate) formation at fixed substrate concentration is linear with *Pa* RhdA concentration (data not shown). Values of K_m for thiosulfate binding to *Pa* RhdA are 1.0 and 7.4 mM at pH 7.3 and 8.6, respectively, and 25 °C. However, the value of K_m for cyanide binding to *Pa* RhdA (=14 mM, at 25 °C) and the value of V_{max} (=750 μmol min^{-1} mg^{-1}, at 25 °C) for the *Pa* RhdA-catalyzed sulfur-transfer reaction are essentially pH- and/or substrate-independent. The increase in the catalytic efficiency of *Pa* RhdA (expressed by V_{max}/K_m) at pH 7.3 is consistent with the enzyme cytoplasmic location.

A comparative evaluation of the catalytic parameters of rhodaneses from *B. taurus*, *A. vinelandii*, and *P. aeruginosa* is given in Table 1. Values of K_m for thiosulfate binding to prokaryotic and eukaryotic rhodaneses range between 1.0 and 7.4 mM (at pH 8.6 and 25 °C). The affinity of cyanide for *B. taurus* rhodanese ($K_m = 0.063$ mM, at pH 8.6 and 25 °C) is higher than those reported for *P. aeruginosa* ($K_m = 16$ mM, at pH 8.6 and 25 °C) and *A. vinelandii* ($K_m = 8.7$ mM, at pH 8.6 and 25 °C) enzymes. Moreover, V_{max} values for the sulfur-transfer reaction are pH- and substrate-independent, and similar for the three enzymes considered (the average V_{max} value is 830 μmol min^{-1} mg^{-1}, at pH 8.6 and 25 °C).

Finally, *Pa* RhdA shows temperature-dependent activity, with maximum between 20 and 32 °C, corresponding to the growth-temperature range of *P. aeruginosa* (data not shown). Moreover, the putative sulfur

Table 1
Enzyme activity and kinetic parameters of the rhodanese catalyzed sulfur-transfer reaction, at 25 °C

Source organism	pH	Substrate			
		Thiosulfate		Cyanide	
		V_{max} (μmol min^{-1} mg^{-1})	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})	K_m (mM)
B. taurus[a]	8.6	600	7.0	n.d.	0.063
A. vinelandii[b]	8.6	1250	1.0	n.d.	8.7
P. aeruginosa[c]	7.3	720	1.0	800	12
	8.6	790	7.4	840	16

n.d., not determined.
[a] From [32,33].
[b] From [34,35].
[c] Present study.

donors sodium sulfide and 3-mercaptopyruvate [1,28] do not affect the catalytic activity and the spectroscopic properties of *Pa* RhdA (data not shown).

Concluding remarks

Cyanogenesis has been documented among organisms belonging to all three major evolutionary phyla, which have developed sophisticate strategies to face cyanide toxicity (reviewed in [29]). Many species of genus *Pseudomonas*, including *P. aeruginosa*, synthesize up to 300 μM cyanide from glycine through the activity of the HCN–synthase complex, and are believed to circumvent cyanide poisoning by the inducible expression of a cyanide-insensitive terminal oxidase, called CIO [30]. Cyanogenesis by group I *Pseudomonas* species has important biological implications, influencing the dynamics of microbial communities in the soil, the biocontrol of plant pathogens, and pathogenicity [31].

Here, we have just reported the existence in *P. aeruginosa* of multiple rhodanese paralogs whose contribution to in vivo cyanide detoxification awaits further assessment. Concerning *Pa* RhdA, our in vitro results are coherent with those previously reported for *A. vinelandii* rhodanese, whose primary involvement in cyanide detoxification has been excluded [27]. Although *P. aeruginosa* and *A. vinelandii* belong to the same eubacterial family, only the former has been documented to produce cyanide [31]. This poses the question of whether *P. aeruginosa* has developed multiple detoxification mechanisms for a safe response to endogenous cyanide. Speculatively, RhdA-dependent cyanide scavenging could prevent intoxication prior to the expression of the cyanide-inducible cyanide-insensitive terminal oxidase.

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