Mutagenic analysis of Thr-232 in rhodanese from *Azotobacter vinelandii* highlighted the differences of this prokaryotic enzyme from the known sulfurtransferases

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Abstract ***Azotobacter vinelandii*** **RhdA uses thiosulfate as the only sulfur donor in vitro, and this apparent selectivity seems to be a unique property among the characterized sulfurtransferases. To investigate the basis of substrate recognition in RhdA, we replaced Thr-232 with either Ala or Lys. Thr-232 was the target of this study since the corresponding Lys-249 in bovine rhodanese has been identified as necessary for catalytic sulfur transfer, and replacement of Lys-249 with Ala fully inactivates bovine rhodanese. Both T232K and T232A mutants of RhdA showed significant increase in thiosulfate-cyanide sulfurtransferase activity, and no detectable activity in the presence of 3-mercaptopyruvate as the sulfur donor substrate. Fluorescence measurements showed that wild-type and mutant RhdAs were overexpressed in the persulfurated form, thus conferring to this enzyme the potential of a persulfide sulfur donor compound. RhdA contains a unique sequence stretch around the catalytic cysteine, and the data here presented suggest a possible divergent physiological function of *A. vinelandii* sulfurtransferase.**

Key words: Sulfurtransferase; Site-directed mutagenesis; Sulfur donor substrate; *Azotobacter vinelandii* rhodanese

1. Introduction

Azotobacter vinelandii rhodanese (RhdA) is the only prokaryotic sulfurtransferase structurally and functionally characterized [1–3]. RhdA, the product of *A. vinelandii rhdA* gene, which was cloned and overexpressed in *Escherichia coli* [3], catalyzes in vitro the sulfur transfer either to cyanide or to the dithiol dihydrolipoate in the presence of thiosulfate as donor substrate. To date, the best studied rhodanese is that from bovine liver which represents the reference enzyme among sulfurtransferases [4–7]. The active site of bovine rhodanese is characterized by the presence of a cysteine residue (Cys-247), which promotes formation of a persulfide intermediate during the catalytic cycle [5,6,8,9]. The catalytic cysteine is considered a structural feature common to all sulfurtransferases, including 3-mercaptopyruvate sulfurtransferase (3-MST), claimed to be evolutionarily related to mitochondrial rhodanese [10,11]. Crystallographic investigations have shown that in bovine rhodanese the catalytic cysteine is surrounded by polar and apolar residues which are deemed important for substrate specificity [5,6]. The residues in the active site pocket of the bovine enzyme [6] are fully conserved in all vertebrate rhodaneses [12–15], all showing a high degree of similarity to the bovine enzyme. In RhdA, on the other hand, the only conserved residue is the catalytic cysteine (the only cysteine in this protein), which is surrounded by residues that are entirely different from those found in the vertebrate enzymes [3,16]. In bovine rhodanese, the cationic residues Arg-186 and Lys-249 have been identified as catalytic requirements for the sulfur transfer function [17]. The critical role of Lys-249 in determining sulfur donor selectivity (thiosulfate, for the rhodanese reaction) has been assessed by site-directed mutagenesis experiments on bovine rhodanese, rat liver rhodanese and 3-MST [17,10,11]. In *A. vinelandii* RhdA, the corresponding residues are glutamic acid (Glu-173) and threonine (Thr-232), and thiosulfate is the only sulfane sulfur donor used for catalysis in vitro [3]. This apparent selectivity seems to be a unique property among characterized sulfurtransferases, since rat 3-MST and rhodanese show both sulfurtransferase activities [10,11].

To investigate the basis of substrate recognition in *A. vinelandii* RhdA, and to determine the role of Thr-232 in catalysis and substrate(s) binding, a study was carried out on selectively engineered RhdAs. The amino acid substitutions were designed taking into account that: (i) cationic side chains are crucial for thiosulfate binding and not essential for 3-mercaptopyruvate binding [10,11,17]; (ii) the replacement of Lys-249 with a hydrophobic residue (Ala) knocks out bovine rhodanese ability to transfer sulfane sulfur from thiosulfate to cyanide [17]; (iii) the replacement of Ser-249 with Lys in rat liver 3-MST does not alter the binding of 3-mercaptopyruvate [11]. Thr-232 was replaced with Lys and Ala. The biochemical characterization of the mutant RhdAs highlighted differences between *A. vinelandii* sulfurtransferase and vertebrate rhodaneses, thus suggesting possible divergent functions.

2. Materials and methods

2.1. DNA manipulation and sequencing

E. coli 71-18 [18] and M15 (Qiagen) strains and their transformed derivatives were grown at 37°C in Luria–Bertani medium [19]. Antibiotics for the selection of *E. coli* transformants were used at the following concentration: 100 μg/ml (ampicillin); 30 μg/ml (kanamycin). All enzymes used for DNA manipulation were from Boehringer Mannheim, New England Biolabs and Pharmacia. Oligonucleotide primers were synthesized by Boehringer Mannheim. The 'Silver

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Sequence DNA Sequencing System' from Promega was used for non-radioactive DNA sequence analysis, which was performed according to the supplier's instructions.

2.2. Site-directed mutagenesis and overexpression of His-tagged proteins

The *Pfl*MI-*Kpn*I fragment containing *rhdA* from plasmid pRC9189 [3], was cloned into overexpression vector pQE32 (Qiagen) after trimming the *Pfl*MI site with T4 DNA polymerase and filling in the *Bam*HI site on the vector. In the resulting plasmid (named pQER1), there are eight additional codons upstream of the ATG starting codon of *rhdA*: the ATC codon for isoleucine, the GGG codon for glycine and six histidine codons. Site-directed mutagenesis of *rhdA* was performed by subcloning the 1 kb *Eco*RI/*Hin*dIII fragment from pQER1 into pTZ18 (resulting in the plasmid pMC1). Single stranded pMC1 DNA was prepared by using the bacteriophage M13K07 (Pharmacia) as superinfecting helper phage and used as template for elongation of mutagenic primers. Replacement of Thr-232 with Lys (T232K) and Ala (T232A) was achieved by using two mutagenic primers: CGTCACCCACTGCCAGaaACATCACCGCTCCGG and CGTCACCCACTGCCAGgcACATCACCGCTCCGG, respectively, where lower case letters indicate the mutated bases.

The 'Gene Editor in vitro Site-Directed Mutagenesis System' (Promega) was used for rapid screening of the mutated recombinant plasmids. The accuracy of mutagenesis and cloning were checked by sequencing the mutated recombinant plasmids (pMC1A and pMC1B). For overexpression, the 1 kb *Eco*RI/*Hin*dIII fragments from pMC1A and pMC1B were subcloned into pQE32, giving rise to plasmids pQER3 and pQER4, respectively. The recombinant plasmids containing the wild-type or mutant RhdAs were transformed into *E. coli* M15, and protein overexpression was rapidly induced by addition of 1 mM isopropyl-thio-β-D-galactoside to a mid-log culture ($OD_{600} = 0.600$).

2.3. Purification of overexpressed His-tagged proteins

Cell-free extracts were prepared from 500 ml of culture. After 4 h of induction cells were harvested by centrifugation, and resuspended in 5 ml of 50 mM sodium phosphate buffer (pH 8.0), containing 0.3 M NaCl. Cell disruption was carried out by incubation with 0.3 mg/ml lysozyme and sonication. RhdAs were purified by chromatography on Ni–NTA agarose column (gel volume, 8 ml). The His-tagged proteins were eluted by addition of 200 mM imidazole. The samples from Ni–NTA agarose column were dialyzed against 50 mM Tris–HCl buffer (pH 7.6) for activity assays and fluorescence experiments.

2.4. Enzyme assays

Rhodanese activity was assayed by a discontinuous method that quantitates the product thiocyanate, based on the absorbance of the ferric–thiocyanate complex at 460 nm [20]. One unit of rhodanese activity is defined as the amount of enzyme that produces 1 μmol thiocyanate/min at 37°C. 3-MST activity was measured as lactate formation (from the product pyruvate) in a coupled reaction in the presence of lactate dehydrogenase and NADH at 340 nm [21]. One unit of 3-MST activity is defined as the amount of enzyme that produces 1 μmol NAD^+/min at 37°C. Protein concentration was determined by a dye-binding colorimetric assay [22].

2.5. Fluorescence spectroscopy

Fluorescence measurements were performed using an LS50 luminescence spectrometer (Perkin Elmer, UK) equipped with a thermostatted (20°C) stirred cell holder. The excitation wavelength was 280 nm in all the fluorescence experiments, with excitation and emission slit of 5 and 3 nm, respectively. Emission spectra were recorded from 300 to 400 nm 1 min after reagent addition, and the samples were continuously stirred. In the titration experiments changes in fluorescence intensity at 336 nm (F_{obs}) are given as ΔF (%).

$$\Delta F\ (\%) = \frac{F_{obs} - F_o}{F_o} \times 100$$

where F_o is the original fluorescence intensity of the studied RhdAs.

3. Results and discussion

Overexpression of *A. vinelandii rhdA* gene led to a significant increase in thiosulfate-cyanide sulfurtransferase activity in cell-free extracts, and a further increase in rhodanese activity was found when the gene was mutated (Table 1). No significant changes in 3-MST activity were observed following overexpression of either wild-type or mutant RhdAs (Table 1). The residual sulfurtransferase activities in cells not overexpressing RhdA can be ascribed to the presence of other sulfurtransferases in the *E. coli* host strain [23,24]. The overexpressed RhdA proteins were purified in only one fast chromatography step, taking advantage of the inserted histidine tag. None of the purified RhdAs showed detectable activity in the presence of 3-mercaptopyruvate as the sulfur donor (Table 2). The thiosulfate-cyanide sulfurtransferase activity of His-tagged wild-type RhdA was indistinguishable from that of the wild-type enzyme not carrying the histidine tag, and purified by conventional gel-exclusion chromatography. The ability to transfer sulfane sulfur from thiosulfate to cyanide increased about three-fold in both mutant RhdAs, compared to that of the wild-type enzyme. The activity increase observed following substitution of Thr-232 with Lys is consistent with the site-directed mutagenesis results obtained for other rhodaneses [10,17]. Surprisingly, replacement of Thr-232 with Ala not only did not inactivate RhdA, as the replacement of Lys-249 with Ala did in the bovine enzyme [17], but significantly increased the ability of RhdA in transferring sulfur from thiosulfate to cyanide. These results might be interpreted as evidence that the catalytic properties of RhdA could be different from those of vertebrate rhodaneses.

Obligatory intermediates in rhodanese catalysis, in the presence of the donor thiosulfate [8], are E (sulfur-free enzyme) and ES (sulfur-substituted enzyme). The intrinsic fluorescence of rhodanese is quenched when a stable persulfide is formed in ES, this quenching being due to energy transfer between the persulfide and the initially excited tryptophan residues [25,26]. The fluorescence spectra of purified wild-type RhdA and its mutant forms are shown in Fig. 1 (solid lines). The addition of cyanide, to ensure the removal of sulfur from the ES form of the enzymes [25], determined a significant increase in the intrinsic fluorescence intensity for all RhdAs, without affecting the shape of the spectra (Fig. 1, dashed lines). All RhdAs

Table 1
Enzyme activities of *E. coli* cells overexpressing RhdAs

E. coli cells	Cloned gene	Sulfurtransferase activity (U/mg protein)	
		A	B
M15 (pQE32)	–	0.60	0.52
M15 (pQER1)	wild-type *rhdA*	225	0.25
M15 (pQER3)	mutant *rhdA* T232A	440	0.52
M15 (pQER4)	mutant *rhdA* T232K	550	0.35

Cell-free extracts were tested for rhodanese (A) and 3-MST (B) activities.

Table 2
Sulfurtransferase activities of the purified RhdAs in the presence of either thiosulfate (A) or 3-mercaptopyruvate (B) as sulfur donor

Sample	Sulfurtransferase activity (U/mg protein)	
	A	B
Wild-type RhdA[a]	330	not detectable
Wild-type RhdA	350	not detectable
T232K RhdA	1015	not detectable
T232A RhdA	950	not detectable

[a]Wild-type enzyme not containing the histidine tag.

showed the same emission maximum (336 nm), irrespective of whether the excitation wavelength was 280 or 295 nm. The persulfide sulfur at the catalytic cysteine was restored by the addition of the sulfane donor thiosulfate, as demonstrated by quenching of the intrinsic fluorescence (Fig. 1, dotted lines). Analysis of the percent variation in fluorescence as a function of the added cyanide gave the plots in the insets to Fig. 1. The $\Delta F\%$ figures obtained at the end of cyanide titration are higher in both mutants than in wild-type RhdA. This might be ascribed to a greater efficiency of energy transfer between the excited tryptophans and the persulfide at Cys-230 in either mutant. This suggests that the relative position of the persulfide and of the excited tryptophans are slightly different in the mutants with respect to wild-type RhdA, and this might be correlated to their increased rhodanese activities. In bovine rhodanese, Trp-107 and Phe-212 are located in the walls of the active site pocket [6]. In *A. vinelandii* RhdA the corresponding residues are Trp-94 and Trp-195 [3,16], which might be important in the catalytic reaction. The fluorescence experiments indicated that overexpressed RhdAs were in the ES form, even if they were purified in the absence of the sulfur donor, thiosulfate. The presence of thiosulfate during the purification steps [27] was required for bovine liver rhodanese, since the E form of this enzyme was very sensitive to oxidative reactions [28]. In addition, the functional stability of bovine rhodanese in the sulfur-substituted form (ES) could be increased in the presence of thiosulfate, this latter being an effective scavenger of free radicals in solution [29]. In contrast, the functional stability of *A. vinelandii* RhdA was not affected by the presence of thiosulfate, and the enzyme appeared more stable compared to the bovine enzyme (data not shown). These results are consistent with a model in which in the 'native' RhdAs, irrespective of the amino acid substitutions at position 232, the transferable sulfur is held on the active site sulfhydryl group (Cys-230) as a persulfide linkage (E–S–SH). Formation of an enzyme-bound cysteinyl persulfide was identified as an intermediate in the NifS-catalyzed reaction [30], and the cysteine persulfide found in Y13C *A. vinelandii* ferredoxin I was probably generated in vivo via the action of NifS [31]. A NifS-like protein has recently been isolated from *E. coli* [32], and it might account for the persulfuration of overexpressed RhdAs. The cysteinyl residue of RhdA seems to function as a primary sulfur acceptor, thus conferring to this enzyme the potential of a persulfide sulfur donor compound, and suggesting a possible divergent function of the *A. vinelandii* RhdA with respect to the known sulfurtransferases. Defence against cyanide toxication has been claimed as an in vivo function for mammalian rhodanese and 3-MST [33–35]. The *A. vinelandii rhdA* null mutant, with internal deletion/insertion mutation, showed a similar level of growth in media containing cyanide, in comparison to wild-type [36]. Therefore, involvement of RhdA in the cyanide detoxification process should be ruled out.

The phylogenetic tree generated by multiple sequence alignment [37] shown in Fig. 2 might give a rationale for divergent 'functions' of a number of prokaryotic proteins referred to as putative rhodaneses. The vertebrate sulfurtransferases for which the catalytic behaviors in the presence of thiosulfate or 3-mercaptopyruvate has been demonstrated [11,17,38,39], cluster in the same group. The conserved stretch around the catalytic cysteine is CRKGVTA for vertebrate rhodaneses and CGSGVTA for 3-MST. Other unknown eukaryotic proteins which share the sequence stretch CGTGVTA are clustered close to the vertebrate group. *E. coli* SseA (THTR_ECOLI) [24], which contains the motif CGSGVTA, is the only prokaryotic protein closely related to the vertebrate sulfurtransferases, and it has recently been shown to have 3-MST activity (Colnaghi, R. and Drummond, M., unpublished). The above sequence stretches are not present in the other prokaryotic

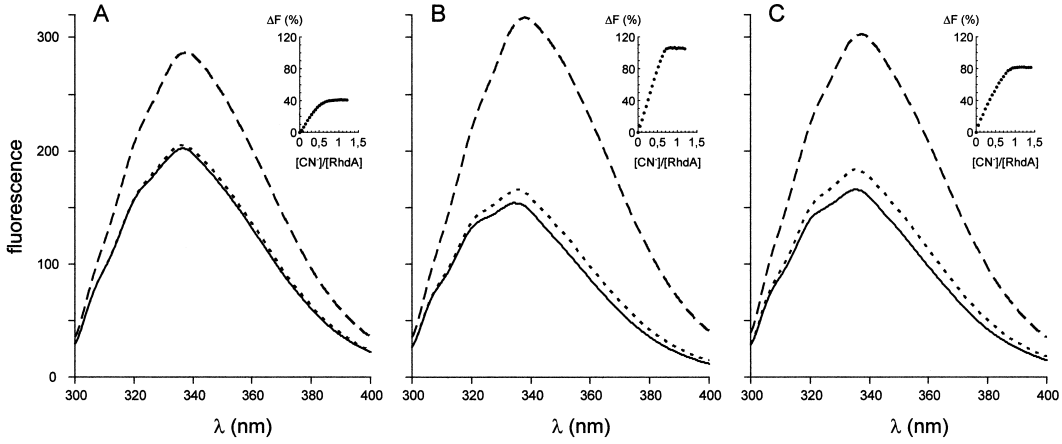

Fig. 1. Intrinsic fluorescence of wild-type and mutant RhdAs. Emission fluorescence spectra ($\lambda_{exc} = 280$ nm) were recorded for wild-type (A), T232K (B) and T232A (C) RhdAs. Proteins were 6 μM in 50 mM Tris–HCl buffer (pH 7.6). Spectra were taken on the protein as purified (solid lines) and after addition of a 1.2-fold molar excess of cyanide (dashed lines), followed by a 130-fold molar excess of thiosulfate (dotted lines). Insets: percent changes in fluorescence intensity upon titration with cyanide. The fluorescence intensities observed were corrected for dilutions due to KCN additions.

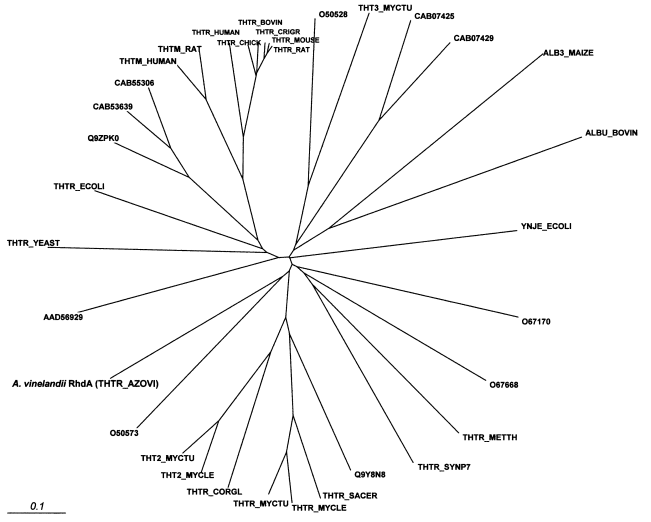

Fig. 2. Phylogenetic tree of known and putative sulfurtransferases. The tree is represented in the unrooted form, and was generated by multiple sequence alignment (Clustal X ver. 1.8) [37] of the sequences chosen for their similarity to RhdA complete sequence (Fasta ver. 3.28 [40]; SWall, rel. 8th Dec 1999). Branch length (divergence distance) is expressed as number of amino acid substitutions per site. Database sequence entries are from: *Arabidopsis thaliana* (CAB55306, CAB53639), *Bos taurus* (THTR_BOVIN), *Caenorhabditis elegans* (CAB07429, CAB07425), *Cricetulus griseus* (THTR_CRIGR), *Datisca glomerata* (Q9ZPK0), *Gallus gallus* (THTR_CHICK), *Homo sapiens* (THTM_HUMAN, THTR_HUMAN), *Mus musculus* (THTR_MOUSE), *Rattus norvegicus* (THTM_RAT, THTR_RAT), *Saccharomyces cerevisiae* (THTR_YEAST), *Aeropyrum pernix* (Q9Y8N8), *Aquifex aeolicus* (O67668, O67170), *A. vinelandii* (THTR_AZOVI), *B. firmus* (O50573), *Corynebacterium glutamicum* (THTR_CORGL), *E. coli* (THTR_ECOLI, YNJE_ECOLI), *Methanobacterium thermoautotrophicum* (THTR_METTH), *Mycobacterium tubercolosis* (THT2_MYCTU, THTR_MYCTU, THT3_MYCTU), *Mycobacterium leprae* (THT2_MYCLE, THTR_MYCLE), *Saccharopolyspora erythraea* (THTR_SACER), *Streptomyces coelicolor* (O50528), *Synechococcus* SP. (THTR_SYNP7), *Zymomonas mobilis* (AAD56929). ALBU_BOVIN (bovine serum albumin) and ALB3_MAIZE (maize ribosome-inactivating protein) were used as outgroup sequences.

proteins, including RhdA, which all are separately clustered from the known sulfurtransferases. The motif CRIGER is present in the majority of the putative prokaryotic rhodaneses, while other prokaryotic proteins (i.e. O67170, O67668, THTR_METH, THTR_SYNP7, YNJE_ECOLI, THT3_MYCTU, O50528) do not share any of the sequence stretches mentioned above. Interestingly, the sequence stretch of RhdA (CQTHHR) appears to be unique, and it is not even present in protein O50573 from *Bacillus firmus*, which shows the highest degree of similarity to RhdA. The residues surrounding the persulfide linkage of Cys-230 in RhdA generate a strong positive electrostatic field of the active-site loop [16] which is not significantly altered by substitution of Thr-232 with either the positively charged Lys residue or the uncharged Ala residue. Electrostatic interactions are likely to be the driving force for the binding of the anions (cyanide, sulfite, thiosulfate) used in the in vitro activity assay, which may not represent the only catalytic reaction of *A. vinelandii* RhdA.

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