The N-terminal rhodanese domain from *Azotobacter vinelandii* has a stable and folded structure independently of the C-terminal domain

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Abstract Sulfurtransferase are enzymes involved in the formation, conversion and transport of compounds containing sulfane-sulfur atoms. Although the three-dimensional structure of the rhodanese from the nitrogen-fixing bacterium *Azotobacter vinelandii* is known, the role of its two domains in the protein conformational stability is still obscure. We have evaluated the susceptibility to proteolytic degradation of the two domains of the enzyme. The two domains show different resistance to the endoproteinases and, in particular, the N-terminal domain shows to be more stable to digestion during time than the C-terminal one. Cloning and overexpression of the N-terminal domain of the protein was performed to better understand its functional and structural role. The recombinant N-terminal domain of rhodanese *A. vinelandii* is soluble in water solution and the spectroscopic studies by circular dichroism and heteronuclear NMR spectroscopy indicate a stable fold of the protein with the expected α/β topology. The results indicate that this N-terminal domain has already got all the elements necessary for an C-terminal domain independent folding. Its solution structure by NMR, actually under course, will be a valid contribution to understand the role of this domain in the folding process of the sulfurtransferase.

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Keywords: *Azotobacter vinelandii* rhodanese; Sulfurtransferase; Limited proteolysis; ^{15}N NMR; Domain protein expression

1. Introduction

Sulfurtransferase enzymes are widely distributed among plants, animals and bacteria [1]. The role of these enzymes is still widely debated. In vitro these enzymes catalyze the transfer of the outer sulfur atom from thiosulfate to cyanide and, during catalysis, the enzyme cycles between two catalytic intermediates: the sulfur loaded form (ES) and sulfur unloaded enzyme (E). Given the abundance in all the major evolutionary phyla of genes coding for rhodanese-like proteins, it appears unlikely that their physiological function is limited to cyanide scavengers. Bovine rhodanese (Rhobov, thiosulfate-cyanide sulfurtransferase, E.C.2.8.1.1) is the best characterized enzyme [2–4] and it represents the reference structure of the rhodanese superfamily (Accession number: PF00581; http://sanger.ac.uk/cgi-bin/Pfam). For this enzyme, a general role in detoxification mechanisms of the reactive oxygen species in aerobic tissues was suggested by the finding that it shows a 1000-fold higher affinity for the reduced form of thioredoxin than for cyanide [5]. In vitro interaction with thioredoxin was also demonstrated for the rhodanese-like protein GlpE from *Escherichia coli* [6], and recently for a *Leishmania* 3-mercaptopyruvate sulfurtransferase [7]. In addition, it has been demonstrated that rhodanese could act as a sulfur insertase in the biosynthesis and/or repair of Fe-S centers of proteins [8–10]. Sulfurtransferases have also been implicated in the synthesis of biotin- [11] and molybdopterin [12]. A role in the assimilatory sulfate reduction by transferring a sulfide molecule of sulfide to O-acetyl-L-serine in the synthesis of cysteine has also been postulated [13]. The three-dimensional structures of the Rhobov [2] and *Azotobacter vinelandii* (RhdA) [14] rhodaneses have been solved and, although these proteins exhibit only 22% sequence identity, they display very similar three-dimensional structures. In both cases, a single polypeptide chain is folded in two domains, each about 120 amino acid long, which are connected by a peptide bridge. The essential Cys residue, located in the C-terminal domain (i.e., the catalytic domain), is the first residue of a 5 amino acid loop (the active-site loop) that folds in a cradle-like structure defining the catalytic pocket. Each domain displays α/β topology, with a central parallel five-stranded β-sheet surrounded by α-helices on both sides. Keim et al. [15] proposed that this structure arose from the gene duplication of an ancestral single-domain enzyme and subsequent divergent evolution of the two proteins and a fusion event. Most sulfurtransferases have an N-terminal "structural" domain and a C-terminal domain containing the active site. In fact, the catalytic function of the rhodanese is located close to a cleft in between the two domains, and all side chains essential for the catalysis are provided by the C-terminal domain only. The presence of interactions between some structural elements of the N-terminal domain and the catalytic one close to active site indicates that the first domain can be involved in the stability of the active site and in the enzyme's substrate selectivity.

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Abbreviations: ES, sulfur-loaded rhodanese; E, sulfur-free form of the rhodanese; RhdA, rhodanese *A. vinelandii*; HSQC, heteronuclear single quantum coherence; RhoBov, bovine rhodanese; IPTG, isopropyl-β-D-thiogalactoside; N-RhdA, N-terminal domain of RhdA

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Recent structural and functional studies of a sulfurtransferase from *E. coli* composed of a single rhodanese domain (GlpE) [6,16] indicate that the presence of the N-terminal domain is not essential for the catalysis. Furthermore, single rhodanese domain proteins have been associated with specific stress conditions such as PspE (phage-shock protein E of *E. coli*) [17] and the *Vibrio cholerae* shock protein Q9KN65 [18]. Single rhodanese domain proteins have also been associated with the process of leaf senescence in *Arabidopsis thaliana* (Sen1) [19], *Nicotiana tabacum* (Ntdin) and *Raphanus sativus* (Din1) [20]. More recently, a 121 amino acidic hypothetical rhodanese domain (At4g01050) from *A. thaliana* has been expressed and its NMR assignment performed [21].

The role of the rhodanese's N-terminal domain is yet to be elucidated, but the vertebrate rhodaneses have been extensively studied in attempts to understand the role played by this domain in the correct folding and stability of the enzyme [22,23]. It has been demonstrated that the deletion in the N-terminal signal sequence (1–23) of the bovine rhodanese (RhoBov) causes inactivation as well as instability of the rhodanese molecule [24]. Moreover, this region of the enzyme has been found important in the initial steps of the folding process. In particular, Horowitz and coworkers [25] have found that the first 40 amino acids of the bovine enzyme interact with molecular chaperones (as Gro-EL) involved in the protein folding process. Numerous attempts at preparing individual domain of the Rhobov's have been unsuccessful, presumably due to the extensive hydrophobic surfaces that would be exposed on the individual domains [26].

Our previous structural and functional correlations studies on *A. vinelandii* sulfurtransferase [27–29] revealed a characteristic stable folding of this enzyme during the catalytic cycle. To better characterize the structural organization of RhdA protein, we have investigated the proteolytic susceptibility of recombinant RhdA to trypsin and endoprotease Asp-N by limited proteolysis, which is a useful method for studying the folded state of the proteins. The results presented here suggest that the two domains of the RhdA have different proteolytic susceptibility: in particular, the N-terminal domain is more resistant than the C-terminal to proteolysis. Moreover, we report the cloning, expression and structural characterization of the RhdA N-terminal domain and provide results on its possible role in the structural stabilization and folding of this prokaryotic sulfurtransferase.

2. Materials and methods

2.1. Sample preparation

The *A. vinelandii* RhdA was overexpressed in *E. coli* cells and purified by the procedure previously described [14,30]. Rhodanese was desalted by overnight dialysis against 20 mM Tris–HCl buffer, pH 7.4, before the analyses. The protein concentration was determined using a $A^{0.1\%}_{280\,nm} = 1.3$ [8]. The rhodanese activity was monitored spectrophotometrically by absorbance at 460 nm of the complex formed between ferric ion and the reaction product, thiocyanate, according to Sörbo [31].

2.2. Proteolysis of RhdA

Rhodanese (0.5 mg/ ml) in 0.1 M ammonium bicarbonate buffer, pH 8, was proteolyzed with 1% trypsin (Sigma) (w/w) at 23 °C. The tryptic digestion of persulfurate rhodanese (ES form) was performed in the absence of thiosulfate and in the presence of 0, 2, 3 and 4.5 M urea, for the times indicated in the figure legends.

RhdA (1 mg/ml) in 10 mM Tris–HCl buffer, pH 8, was treated with endoproteinase Asp-N (Sigma) (1% w/w) at 23 °C and the proteolysis was monitored during the time by SDS–PAGE.

The reactions were stopped by addition of 4 × SDS–PAGE sample buffer and the samples were boiled for 5 min. The rhodanese digests were analyzed by SDS–PAGE. Gels were run by the method of Laemmli [32] using 12% or 15% in the separating gels and 4% in the stacking gels. The gels were stained with Coomassie brillant blue (Bio-Rad).

2.3. Western blotting and protein sequencing

After SDS–PAGE, the separated protein fragments were electrotransferred to PVDF membrane (ProBlot; Applied Biosystems) by the method of Matsudaira [33]. Sequence analysis of the first ten N-terminal amino acids of the proteolytic products was performed using Applied Biosystems 473A-model pulsed liquid sequencer, with an online phenyl-thiohydantoin amino acid analyzer. The sequences obtained were compared with the *A. vinelandii* rhodanese sequence.

2.4. Cloning, expression and purification of N-terminal domain of RhdA

The plasmid coding the N-terminal domain was made by performed strategic restriction digestion of pQRE-1 [30], using *Hin*dIII and *Sma*1 enzymes, for cutting the nucleotide fragment of the rhda gene coding the C-terminal domain. The nucleotide fragment about 4.3 kbp, corresponding to the pQRE plasmid with the gene-fragment coding the His-tagged N-terminal domain of the RhdA, was purified by gel extraction after electrophoresis and enzymatic reactions with Klenow fragment and DNA ligase was performed. The plasmid pQRE-N-rhda coding the N-terminal domain of the rhodanese was used in the transformation of the *E. coli* XL1-blue strain. Sequences were verified by dye terminator cycle sequencing. The N-termnal protein domain was overexpressed and purified by affinity chromatography using a Ni–NTA column (Qiagen). The composition of the sample was analyzed by SDS–PAGE using 15% polyacrylamide slab gels under reducing condition. The gels were stained with Coomassie brillant blue G-250.

The molecular mass of protein was calculated using standard techniques for mass estimation by SDS–PAGE and the concentration of the recombinant N-term RhdA was measured using the extinction coefficient of $A^{0.1\%}_{280\,nm} = 1.5$. The extinction coefficient was estimated with ExPASy's ProtParam (hpp://us.expasy.org/tools/protparam.html).

2.5. Circular dichroism

CD spectra were performed with a Jasco 600 CD spectropolarimeter calibrated with camphor-sulfonic acid. Spectra were recorded between 200 and 250 nm using a path length of 0.1 cm, a time constant of 1.0 s, a 2 nm bandwidth and a scan rate of 2 nm/min. A total of 4 scans were used for each experiment. The average was corrected by 4 scans of the solvent alone. A 0.1 cm sealed and thermostatically controlled quartz cell was used for all CD spectra.

2.6. Expression of the uniformly labeled N-terminal domain

Cell mass is predominately grown on unlabeled rich media allowing growth to high cell densities. Following growth in Luria–Bertani medium (LB), cells are exchanged into an isotopically defined minimal media at higher cell densities optimized for maximal protein expression. The protein was expressed in the *E. coli* XL1-blue using the pQRE-N-rhda plasmid and the antibiotic for the selection of *E. coli* transformants was used at the following concentration: 100 μg/ml ampicillin. The general protocol for isotope labeling was as described by Marley et al. [34]. Cells were grown in 600 ml of LB at 37 °C shaken at 180 rpm. Upon reaching optical cell densities at 600 nm ~ 0.7, the cells were pelleted by a 25 min centrifugation at 5000 rpm. The gels were then washed and pelleted using an M9 salt solution (1 L of autoclaved 5 × M9 salt: 15 g KH_2PO_4, 64 g $NaHPO_4 \cdot 7H_2O$, 2.5 g NaCl, and 5 g NH_4Cl, pH 7.2). The cell pellet was resuspended in 400 ml of minimal media isotopically labeled with $^{15}NH_4Cl$ (99%; Spectra 2000) (1 L of minimal media was composed of 200 ml of 5 × M9 salts, 20 ml of D-glucose Stock 20 g/100 ml, 10 ml basal vitamins Eagle Media, Life Sciences Technology, 2 ml of 1 M $MgSO_4$, and 0.1 ml of 1 M $CaCl_2$) and the protein expression was induced after 1h by addition of isopropyl-β-D-thiogalactoside (IPTG) to a concentration of 1 mM. After a 4 h incubation period, the cells were harvested. The incorporation rate for ^{15}N was estimated by NMR to be ~90%. Cell disruption was carried out by incubation with 0.6 mg/ml lysozyme and sonication.

RhdA was purified by chromatography on Ni–NTA agarose column. The His-tagged protein was eluted by addition of 200 mM imidazole.

2.7. NMR spectroscopy

Samples of ^{15}N labeled RhdA and N-terminal domain of RhdA were prepared and concentrated to 0.1 mM in 50 mM NH_4HCO_3, pH 6.9, and 0.3 M sodium chloride. Two-dimensional ^{15}N–^{1}H HSQC were measured at 20 °C on a Bruker Avance 700 MHz NMR spectrometer equipped with a z-gradient triple resonance probe. Data were processed and analyzed on IRIS O2 work-station (Silicon Graphics) using NMRPipe [35] and NMRView [36].

3. Results and discussion

The protein dissection strategy has been used in the past in a number of studies [37] and it has been demonstrated that some protein fragments can fold autonomously into a partially or fully native structure. The limited proteolysis appears to be the best experimental technique for splicing out fragments that can fold autonomously. In fact, the limited proteolysis of a globular protein occurs at sites which are the most flexible regions of the polypeptide chain within domains or at the flexible hinges between domains. Therefore, a limited proteolysis of the RhdA was used to investigate the stability of the domains. Rhodanese was equilibrated in buffers with 0, 2, 3 and 4.5 M urea for 2 h at 23 °C (trypsin is susceptible to denaturation by urea beyond 4.5M concentrations) and was then digested with trypsin, in the absence of thiosulfate. As shown in Fig. 1, the RhdA was susceptible to partial tryptic digestion also with 0 and 2 M urea; one daughter band (“b” band), approximately 21 kDa, appears after 30 min of digestion and traces of small

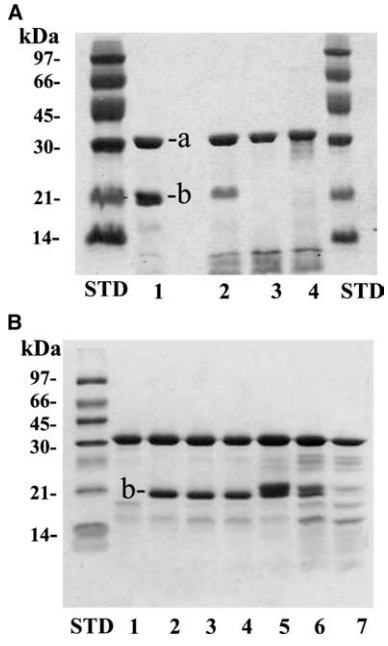

Fig. 1. SDS – PAGE 12% acrylamide gel of proteolysis of RhdA in the absence of thiosulfate. (A) RhdA (0.5 mg/ml) was equilibrated with 0, 2, 3 and 4.5 M urea for 2 h, then was proteolyzed with 1% trypsin for 30 min at 23 °C. Lane 1, 0 M urea; lane 2, 2 M urea; lane 3, 3 M urea and lane 4, 4.5 M urea. (B) Time course of the trypsin digestion of RhdA, without urea, at same ratio and temperature used in A. Lanes 1–7 are proteolysis products at 0, 1, 5, 15, 30, 60 min and 24 h, respectively. Band “a” is the parent band (31 kDa) and band “b” is the daughter band. Molecular markers are indicated at the left.

fragments migrating near the dye front were also observed. The N-terminal amino acid sequence of the daughter band at 21 kDa (Tr b) was determined as discussed under Section 2 and the sequence corresponding to the N-terminal of the rhodanese (Fig. 2). The linker between the two globular domains has two aspartic residues and a glutamic one. Thus, a proteolysis of the RhdA with endoproteinase Asp-N has been performed for obtaining the separation of the domains. Fig. 3 shows the SDS–PAGE of the time course of the endoproteinase Asp-N limited proteolysis. Three major fragments appear simultaneously after 1 min incubation with endoproteinase Asp-N, while an increase of about 17 kDa band and the disappearance of the other fragments are observable after 1 and 24 h. In Fig. 2 are shown the N-terminal sequences of the protein bands (“b” and “c” bands), which are approximately 17 and 12 kDa. The sequence results as well as the sizes of the bands obtained indicate that the resulting fragments represent the N-terminal and C-terminal regions of RhdA, respectively.

In our experiments, RhdA appears quite resistant to further proteolysis. In fact, as shown in Fig. 1, a limited fraction of the enzyme is rapidly proteolyzed by trypsin, and a large amount of the protein remains intact even in the presence of 4.5 M urea. Moreover, the structural stability of the RhdA protein was confirmed by an activity essay which gave a 55% residual activity after 2 h incubation with 4.5 M urea and by the experimental data of the proteolysis and of the CD spectra performed in the presence of urea (data not shown, supplementary materials available on request). One intensive band at 21 kDa and other bands of lower intensity are observable in SDS–PAGE in the absence of urea. Despite the fact that the amino acid sequence of the enzyme reveals the presence of the several lysine and arginine residues, the time course of the digestion, shows that the 21 kDa band was susceptible to further degradation only after some hours. The daughter band “b” appears after 30 min as a double band (Fig. 1B). We attributed the presence of these two bands to the presence in the protein solution of the two forms of the enzyme, ES and E, and this point is actually under investigation. Similarly to trypsin digestion the limited Asp-N digestion shows that the fragment corresponding to the N-terminal domain was accumulated over time, whereas the C-terminal domain is present at first and begins to disappear after a few minutes of digestion. It is clear that the stable daughter band (“b” band) that remains on the gel (Fig. 3) results from the cleavages within the C-terminal domain. These results suggest that the N-terminal domain of RhdA possesses sufficient stability and rigidity to prevent fast proteolysis. This domain is more stable than the C-terminal one. Furthermore, these results suggesting a very stable folding of the recombinant RhdA protein are in agreement with the structural characterizations of the bovine enzyme [26]. The Rhobov is the first prototype of tandem domain rhodanese and it has been the subject of many investigations aimed at elucidating the role of the non-catalytic N-terminal domain in the correct folding and stability of the enzyme [26,38]. In fact, although the sequence of Rhobov would indicate the interdomain tether, consisting of a long loop (residues 143–158), as readily susceptible to proteolytic attack, the separation of the domains could not be performed by trypsin digestion, presumably because of the strict conformational constraints and limited accessibility imposed by the high stability of the interdomain interactions [22,38]. Digestion of Rhobov with trypsin and subtilisin showed no fragmentation

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1          10          20          30          40          50          60
MRGSHHHHHHGIMDDFASLPLVIEPADLQARLSAPELILVDLTSAARYAEGHIPGARFVDPKR
|______ Tr b...
|____ Asp-N b.. |______ Asp-N b...

TQLGQPPAPGLQPPREQLESLFGELGHRPEAVYVVYDDEGGGWAGRFIWLLDVIGQQRYHYLN


GGLTAWLAEDRPLSRELPAPAGGPVALSLHDEPTASRDYLLGRLGAADLAIWDARSPQEYRGE
                                                        |__Asp-N c__.

KVLAAKGGHIPGAVNFEWTAAMDPSRALRIRTDIAGRLEELGITPDKEIVTHCQTHHRSGLTY


LIAKALGYPRVKGYAGSWGEWGNHPDTPVEL
```

Fig. 2. Amino acid sequence of the *A. vinelandii* rhodanese. Underlined sequences indicate the N-terminal amino acid sequence of the 21 kDa fragment obtained after trypsin digestion (Tr b), and the N-terminal sequences of the fragments at about 17 and 12 kDa, Asp-N b and Asp-N c, respectively, obtained after proteolysis with endoprotease Asp-N.

after 1h incubation [37]. Interdomain separation by trypsin digestion was successful in the presence of 4.25 M urea, suggesting that only in this condition RhoBov loses the interactions between the N- and C-terminal domains [38]. Nevertheless, the RhdA appears more sensitive to the proteolysis than Rhobov in the same experimental condition. However, recent studies on the Rhobov showed that the mutation of all non-essential cysteine residues (C3S) turns the enzyme into an active but less stable form of the enzyme [23]. A 21 kDa band, corresponding to the N-terminal domain, appeared when the C3S-Rhobov was treated with trypsin. This fragment remains as an apparently stable product, suggesting that the C-terminal domain of the C3S-Rhobov, in the absence of thiosulfate, is less stable than the corresponding region of wt Rhobov. The data suggested that C3S-Rhobov has less compact structure in its C-terminal domain [23].

On the other hand, our previous fluorescence studies on RhdA show that the interdomain region of the prokaryotic enzyme possesses a better accessibility to ions and to the hydrophobic quencher than that of the Rhobov enzyme [27]. Moreover, the acrylamide is slightly more effective in quenching the fluorescence of the E form than the ES form, indicating that tryptophan residues are less solvent-exposed in the ES form than in the E form. The sulfur-free form of RhdA shows a non-linear Stern–Volmer plot in the fluorescence quenching by acrylamide, like the E form of the double mutant C254/263S [27,39] does. This non-linearity, that can be described by a kinetic model that assumes two parallel processes of the quenching, is probably due to an increased solvent accessibility to some of tryptophan residues changing the hydrophobic exposure after the removal of the transferable sulfur atom. This is an effect of local conformational change leaving the global shape of the protein unaffected [27]. Thus, although RhdA shows the same peculiar tandem domain architecture of Rhobov [2,14], in the vicinity of the catalytic center, the two enzymes display different protein surface, shape and amino acid composition. An inspection of it in the structure of the bacterial enzyme reveals that, in addition to the entirely different amino acid composition of the active-site loops, two loops (amino acids 33–42 and 189–201), located next to the catalytic loop of Rhobov, are eight and four residues shorter, respectively, in RhdA. Thus, the existing local structural differences, between RhdA and Rhobov, could explain, also, the different behavior observed in the limited proteolysis experiments.

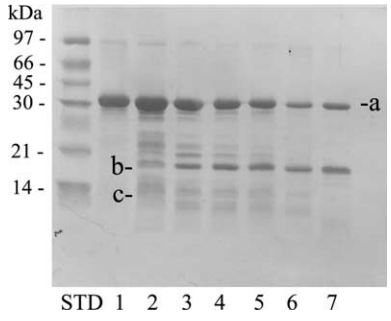

Fig. 3. Time course of endoprotease Asp-N digestion of RhdA. 100 μg of a solution of 1 mg/ml of RhdA was subjected to limited digestion with 1% endoprotease Asp-N (w/w), in 10 mM Tris–HCl buffer, pH 8, at 23 °C. After the reaction, the samples were subjected to SDS–PAGE. Lanes 1–7 are proteolysis products at 0, 1, 5, 15, 30, 60 min and 24 h, respectively. Band "a" is the parent band, bands "b" and "c" are the daughter bands, at about 17 and 12 kDa, respectively, analyzed by N-term sequence analysis (as reported in Fig. 2). Molecular markers are at the left.

The plasmid coding and the expression of the recombinant His-tagged N-terminal domain of RhdA (N-RhdA) was performed, as reported in Section 2. The recombinant N-RhdA protein was obtained as a soluble protein. Fig. 4 shows the SDS–PAGE of the crude extract before and after the induction. The overexpressed His-tagged N-terminal RhdA was present in the soluble fraction and this procedure gives yields of 7–10 mg of protein from 1 L of bacterial culture. The molecular weight of the recombinant protein calculated by SDS–PAGE (about 16 kDa) is in agreement with that calculated from primary sequence (Fig. 4).

Fig. 5 shows the far-UV CD spectra of the purified N-term-RhdA in 50 mM Tris–HCl and 0.3 M NaCl, pH 7.25. The dichroic profile indicates that the protein assumes a typical α/β conformation in solution. An estimate of the secondary structure was determined using the program K2d [40] giving, as a result of a content of 37% α-helix, 26% β-structure and 38% other structures.

These values are in agreement with the evaluation of the extent of secondary structure elements from crystallographic, structure which gave 35% of α-helix, 19% of β–structures and 46% coil. Therefore, the three-dimensional structure of the N-

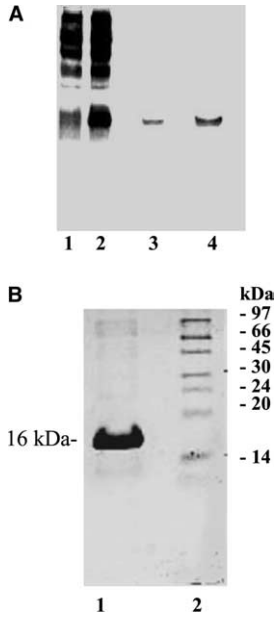

Fig. 4. SDS–PAGE gels showing overproduction and purification of N-terminal domain of RhdA. (A) 1, total cellular proteins of *E. coli* XL1-Blue, harboring pQRE–His-tag-N-term rhda, before IPTG induction; lane 2, at 4 h after IPTG; lanes 3 and 4, after purification by Ni–NTA chromatography; (B) Lane 1, N-terminal domain of RhdA after Ni–NTA chromatography; lane 2, molecular weight markers.

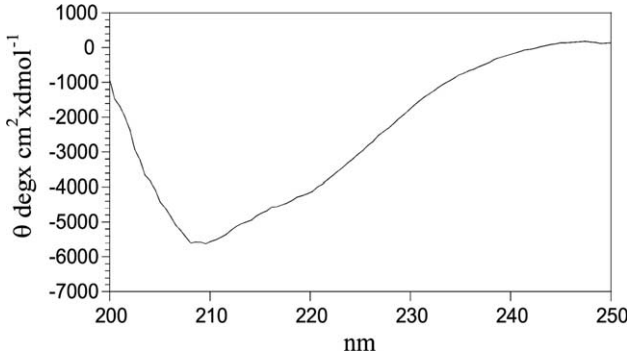

Fig. 5. Far-UV CD spectrum of N-term-RhdA. 8 μM of N-terminal domain of RhdA in 50 mM Tris–HCl, 0.3 M NaCl, pH 7.2, buffer.

termRhdA has a high probability to have a conformation like the N-terminal domain of the crystallographic structure of the RhdA. Fig. 6 shows the putative molecular model of the three-dimensional structure of the N-RhdA obtained using Geno3D program [41].

To better characterize the fold of the protein, an isotopic ^{15}N-labeling of the protein was performed as reported in Section 2. The two-dimensional ^{1}H–^{15}N NMR spectra (HSQC) of the uniformly ^{15}N labeled N-RhdA were performed (Fig. 7), they showed a very good dispersion of the ^{15}N–^{1}H cross peaks, which is typical of a protein with a definite and stable fold and structured compatibly with the expected α/β topology. The resolution of the HSQC spectrum is good, as are also all the three indolic ^{15}N–^{1}H cross peaks observed in the spectral region from 10 to 11 ppm.

The CD spectrum shows a well-defined secondary structure and the ^{15}N–^{1}H HSQC NMR spectrum shows a remarkable

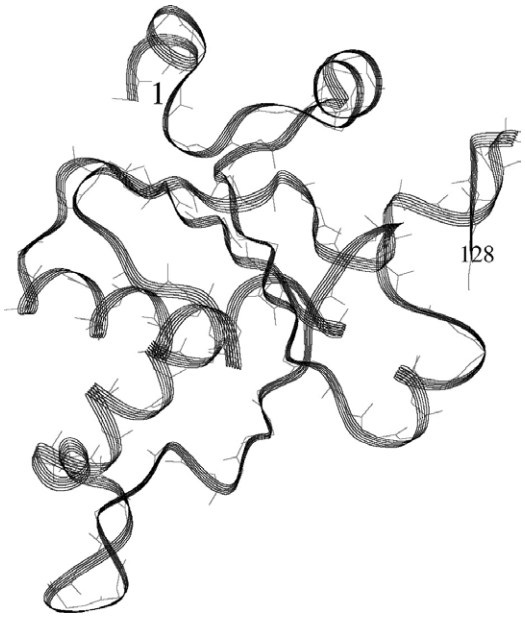

Fig. 6. Model of three-dimensional structure of N-terminal domain of RhdA obtained by Geno3D (http://geno3d-pbil.ibcp.fr) [41].

dispersion of chemical shift. The quality of line-shape of resonances indicates the absence of aggregation and a stable fold of the protein.

These results show that the N-terminal domain of the rhodanese is stably folded and contains elements that could be important in the folding process of the native RhdA. Moreover, the N-terminal domain has an independent folding on the presence of the C-terminal domain and could be an example of a compact, autonomous folding unit. Rhodanese has been one of the most studied substrates for assisted folding by the chaperonin GroEL and much is known about the GroEL–GroES dependent folding of the unfolded Rhobov rhodanese

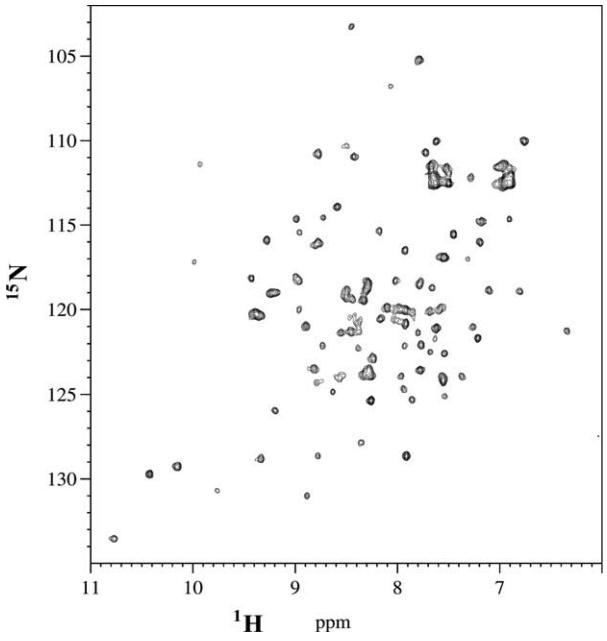

Fig. 7. HSQC spectrum of the uniformly ^{15}N isotopically labeled N-terminal domain of RhdA. The sample was 0.2 mM, in 50 mM Tris–HCl buffer, pH 7.2, and 0.3 M NaCl.

[42–44]. Moreover, recent studies performed on the mutant form C3S-Rhobov showed that the addition of the GroEL/GroES/ATP to C3S-E after complete inactivation generates 100% active protein and furthermore that it is the C-terminal domain of C3S-Rhobov that preferentially binds to GroEL [23]. No experimental data exist about the GroEL–GroES dependent folding of the RhdA, but the fluorescent and proteolysis data show a similar behavior to the mutant form C3S-Rhobov. Thus, it would be of interest to know whether the N-terminal domain of *A. vinelandii* rhodanese has a refolding process that is dependent on the GroEL–GroES chaperonin system and how it is expressed in an *E. coli* strain that carries a mutation in either GroEL or GroES [45].

In addition to the presence of the rhodanese catalytic domain in enzymes displaying sulfurtransferase activity, catalytic inactive rhodanese domains (i.e., domain lacking the active-site Cys) are found as N-terminal domains in a number of dual-specific phosphatases (DSP) and in several ubiquitinating enzymes [46,47]. The role of the inactive rhodanese domain has not been established, but recently data show that the presence of the N-terminal domain of the sulfurtransferase from *A. thaliana* is important for the activity of the C-terminal domain alone [48]. In this work, the N- and C- domain of the *Arabidopsis* ST1 protein were overexpressed in the *E. coli* and the addition of the inactive N-terminal domain to the isolated C-terminal domain, which contains the active site, led to a linear increase in the specific activity of the C-terminal domain. The determination of the solution structure of the N-RhdA by NMR spectroscopy, which is under study in our laboratory, may be of significant help in better understanding the role of this domain in folding process of the rhodanese.

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