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Evidence that elongation of the catalytic loop of the *Azotobacter vinelandii* rhodanese changed selectivity from sulfur- to phosphate-containing substrates

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Recent investigations have shown that the rhodanese domains, ubiquitous structural modules which might represent an example of conserved structures with possible functional diversity, are structurally related to the catalytic subunit of Cdc25 phosphatase enzymes. The major difference characterizing the active-site of the *Azotobacter vinelandii* rhodanese RhdA, with respect to the closely related Cdc25s (A, B, C), is that in Cdc25 phosphatases the active site loop [His–Cys–$(X)_5$–Arg] is one residue longer than in RhdA [His–Cys–$(X)_4$–Arg]. According to the hypothesis that the length of the RhdA active-site loop should play a key role in substrate recognition and catalytic activity, RhdA scaffold was the starting point for producing mutants with single-residue insertion to generate the catalytic loop HCQTHAHR (in RhdA-Ala) and HCQTHSHR (in RhdA-Ser). Analyses of the catalytic performances of the engineered RhdAs revealed that elongation of the catalytic loop definitely compromised the ability to catalyze sulfur transfer reactions, while it generated 'phosphatase' enzymes able to interact productively with the artificial substrate 3-*O*-methylfluorescein phosphate. Although this study is restricted to an example of rhodanese modules (RhdA), it provided experimental evidence of the hypothesis that a specific mutational event (a single-residue insertion or deletion in the active-site loop) could change the selectivity from sulfur- to phosphate-containing substrates (or vice versa).

Keywords: phosphatase activity/RhdA active-site elongation/rhodanese modules/substrate selectivity/sulfurtransferase activities

Introduction

Genome sequencing has shown that ORFs coding for proteins displaying sequence homology with bovine rhodanese (thiosulfate:cyanide sulfurtransferase) (Ploegman *et al*., 1978) are widely present in all major evolutionary phyla. The observed abundance of potentially functional rhodanese-like proteins also in the same genome, together with the amino acid variability found around the catalytic cysteine residue, suggests that members of this homology superfamily (Accession number: PF00581; http://sanger.ac.uk/cgi-bin/Pfam) may play distinct biological roles. Rhodanese domains were found in a single or tandem arrangement in proteins displaying sulfurtransferase activities *in vitro* (Bordo *et al*., 2000; Papenbrock and Schmidt, 2000; Colnaghi *et al*., 2001; Spallarossa *et al*., 2001; Adams *et al*., 2002; Burow *et al*., 2002) and also as modules in multidomain proteins showing widely different enzymatic activities (Schultz *et al*., 1998; Palenchar *et al*., 2000; Mueller *et al*., 2001). The analysis of the 3D structures of *Azotobacter vinelandii* RhdA (Bordo *et al*., 2000) and *Escherichia coli* GlpE (Spallarossa *et al*., 2001) strongly supports Hofmann's pioneering study (Hofmann *et al*., 1998) that demonstrated the structural relationship among rhodanese domains and the catalytic domain of Cdc25 phosphatases, Cdc25A and Cdc25B (Fauman *et al*., 1998; Reynolds *et al*., 1999). Cdc25s are classified as dual-specificity phosphatase (Nilsson and Hoffmann, 2000; Jackson and Denu, 2001) on the basis of the active-site motif His–Cys–$(X)_5$–Arg. In this motif, His is a highly conserved residue, Cys is the catalytic cysteine and Arg is a highly conserved arginine residue required for binding and transition-state stabilization of the phosphate (Xu and Burke, 1996). Among the rhodaneses of known 3D structure (Ploegman *et al*., 1978; Bordo *et al*., 2000; Spallarossa *et al*., 2001), which share with the catalytic subunit of Cdc25 phosphatases (Fauman *et al*., 1998; Reynolds *et al*., 1999) a common 3D fold and the active-site cysteine at a strictly comparable site, *A.vinelandii* RhdA is unique because it contains either the histidine or the arginine in the active-site loop.

Therefore, RhdA is the member of known rhodaneses that should suit the fundamental requirements for possible phosphatase activity. The major difference characterizing the RhdA active-site with respect to the three closely related Cdc25 phosphatases (Cdc25A, B, C) is that in Cdc25 phosphatases the active–site loop [His–Cys–$(X)_5$–Arg] is one residue longer than in RhdA [His–Cys–$(X)_4$–Arg]. The five X residues in Cdc25s form a loop whose backbone amides hydrogen bond to the phosphate of the substrate (Rudolph, 2002). The absence in RhdA of phosphatase activity supports the relevance of the length of the active-site loop for catalysis. According to this hypothesis, rational mutagenesis was applied to investigate the effect of RhdA active-site loop elongation in substrate recognition. On the structural basis detailed in our previous study (Bordo *et al*., 2001), the position between amino acids His233 and His234, which are structurally equivalent in Cdc25A to Ser433 and Glu435, was considered the most appropriate for a single-residue insertion in *A.vinelandii* RhdA and Ala and Ser residues were chosen for the insertion. Preliminary analysis of the engineered RhdAs (RhdA-Ala and RhdA-Ser mutants) failed to detect phosphatase activity, based on *p*-nitrophenyl phosphate (*p*NPP) hydrolysis (Bordo *et al*., 2001). Since *p*NPP is recognized as a poor substrate for Cdc25 enzymes and the search for good artificial substrates for *in vitro* analysis of phosphatase activity of Cdc25 enzymes is a subject of investigation (Gottlin *et al*., 1996; Chen *et al*., 2000; Kolmodin and Aqvist, 2000; McCain *et al*., 2002), the lack of reactivity towards *p*NPP was not considered sufficient to dismiss the hypothesis that elongation of the RhdA active-site loop could generate enzymes with phosphatase activity.

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Keeping this in mind, we have extended the previous study and analyzed the 'reactivity' of either sulfur- or phosphate-containing compounds with the modified scaffold of RhdA.

The main goal of the present work was to provide evidence that both RhdA-Ala and RhdA-Ser mutants, but not wild-type RhdA, were able to catalyze the hydrolysis of the phosphatase artificial substrate 3-*O*-methylfluorescein phosphate (OMFP). The absence of any sulfurtransferase activities in both mutants indicated that specificity of recognition (S- or P-containing substrate) is essentially determined by the tailored arrangement of the catalytic loop. The results of this investigation should be taken as experimental evidence of the hypothesis (Bordo and Bork, 2002) that the structural difference in the active-site loop length (rhodaneses versus Cdc25 phosphatases) may reflect a specific mutational event (a single-residue insertion or deletion), which changed the selectivity from sulfur- to phosphate-containing substrates (or vice versa).

Materials and methods

Protein expression and purification

The recombinant plasmids for expression of RhdA [pQER1 (Pagani *et al*., 2000)] or of RhdAs with single-residue insertion [pQM22 or pQM5 (Bordo *et al*., 2001)] were transformed into *Escherichia coli* BL21[rep4] and protein overexpression was rapidly induced by addition of 1 mM isopropyl thio-β-D-galactoside to a mid-logarithmic culture (OD_{600} = 0.6). 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 Tris–HCl buffer (pH 8.0) containing 0.3 M NaCl. Cell disruption was carried out by incubation with 0.3 mg/ml lysozyme and sonication. RhdA and the RhdAs with single-residue insertion (RhdA-Ala and RhdA-Ser, respectively) were purified by chromatography on an Ni-NTA agarose column (gel volume, 8 ml). The His-tagged proteins were eluted by addition of 200 mM imidazole.

Activity assays

The discontinuous method that determines the product thiocyanate, based on the absorbance of the ferric–thiocyanate complex at 460 nm, was used to determine either thiosulfate:cyanide sulfurtransferase (rhodanese, TST) or 3-mercaptopyruvate:cyanide sulfurtransferase (MST) activities (Westley, 1981). The assays lasted 1–2 min and 1 U of enzyme is defined as the amount of enzyme that produces 1 μmol of thiocyanate per minute at 37°C.

Sulfurtransferase activity in the presence of dithiothreitol (DTT) as acceptor substrate and thiosulfate as sulfur donor was determined spectrophotometrically by the continuous method described by Pecci *et al.* (Pecci *et al*., 1976). The rate of spontaneous autoxidation of DTT was always subtracted. One unit (U) of enzyme is defined as the amount of enzyme that oxidizes 1 μmol of dithiothreitol per minute at 37°C.

Phosphatase activity was assayed by a continuous fluorimetric method which measures the initial rates of hydrolysis of the artificial substrate OMFP (Gottlin *et al*., 1996). The fluorimetric measurements were carried out with an LS50 luminescence spectrometer (Perkin-Elmer) equipped with a PTP-1 Peltier temperature programmer (Perkin-Elmer) set at 20°C; the excitation and emission wavelengths were 471 and 530 nm, respectively, slit width 5 nm and data pitch 1 s. 3-*O*-Methylfluorescein (OMF) formation was quantitated by fluorescence and fluorescence units were converted to product concentration by using an OMF calibration curve generated by measuring the fluorescence of OMF solutions at various concentrations in the assay buffer. One unit (U) is defined as the amount of enzyme that produces 1 μmol of OMF per minute at 20°C. The assay mixture was 0.5 ml of 50 mM Tris–HCl (pH 8), 0.25 mM OMFP and different enzyme concentrations; when stated, 1 mM dithiothreitol (DTT) was included in the assay mixture. In a set of experiments, the examined proteins were pre-incubated in the assay buffer containing 2 mM DTT for 1 h at 25°C before addition of OMFP; the final DTT concentration in the assay was 1 mM. The rate of spontaneous OMFP hydrolysis was always subtracted and the data presented are the averages of at least three independent determinations.

The protein concentration was determined by dye-binding colorimetric assay (Bradford, 1976).

Table I. Sulfurtransferase activities of wild-type and mutated RhdAs

	Sulfurtransferase activity, U/mg		
Sulfur donor: Sulfur acceptor:	Thiosulfate Cyanide	Thiosulfate DTT	3-Mercaptopyruvate Cyanide
Enzyme			
Wild-type RhdA	899 ± 7	46.0 ± 5.3	50.0 ± 5.4
RhdA-Ala	1.4 ± 0.1	1.5 ± 0.1	6.9 ± 0.1
RhdA-Ser	2.5 ± 0.1	2.2 ± 0.1	8.5 ± 0.3

Results and discussion

With the increasing knowledge of the 3D structures, the structural relationship among rhodanese-related enzymes (Ploegman *et al*., 1978; Bordo *et al*., 2000; Spallarossa *et al*., 2001; Bordo and Bork, 2002) and Cdc25 dual-specificity phosphatases (Fauman *et al*., 1998; Hofmann *et al*., 1998; Reynolds *et al*., 1999) has become evident. An understanding of the structural bases for the catalytic diversity observed in these enzyme families may provide insights for functional analyses of the ~500 gene products containing rhodanese homology domains. As described in the Introduction, the unique features of *A.vinelandii* RhdA, which was defined as a thiosulfate:cyanide sulfurtransferase (EC 2.8.1.1) on the basis of its ability to catalyze *in vitro* the typical rhodanese reactions (Colnaghi *et al*., 1996), made it a suitable model to investigate active-site accessibility of 'novel substrate(s)' by mutagenic analysis.

Sulfurtransferase activities of wild-type and mutated forms of RhdA

The residues surrounding the catalytic Cys230 in RhdA generate a strong positive electrostatic field (Bordo *et al*., 2000), which is in keeping with the RhdA *in vitro* enzymic activity:

$$S_2O_3^{2-} + \text{RhdA} \rightarrow SO_3^{2-} + \text{RhdA-S}$$

$$\text{RhdA-S} + CN^- \rightarrow \text{RhdA} + SCN^-$$

Even in the absence of thiosulfate, RhdA is isolated as a stable Cys230 persulfurated form (Bordo *et al*., 2000; Pagani *et al*., 2000), a distinctive feature not shared by the mutants with elongated catalytic loop RhdA-Ala and RhdA-Ser (Bordo *et al*., 2001). As shown in Table I, single-residue insertion in the catalytic loop of RhdA strongly impaired the ability to transfer sulfur in the presence of thiosulfate as sulfur donor to either

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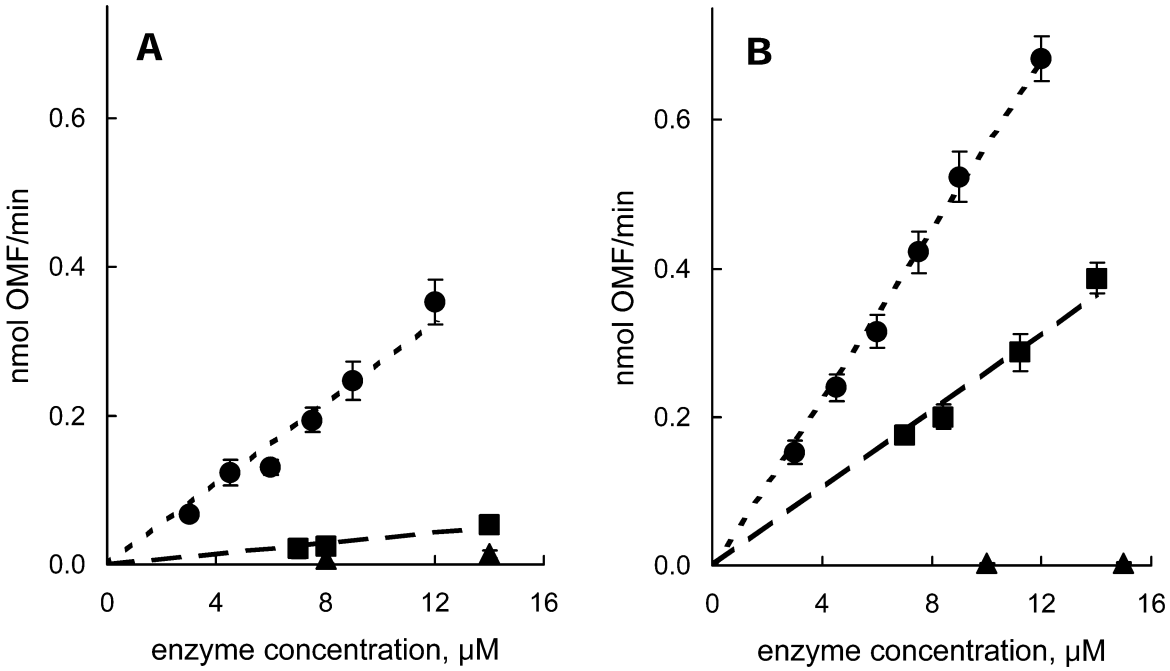

Fig. 1. Phosphatase activity of wild-type and mutated RhdAs. OMFP hydrolysis rates were measured (**A**) in the presence of various enzyme concentrations in the absence of DTT and (**B**) using enzymes pre-incubated with DTT, as described in Materials and methods. Triangles, wild-type RhdA; circles, RhdA-Ala; squares, RhdA-Ser.

cyanide (TST activity) or to the dithiol DTT (DTT-ST activity), the typical rhodanese activities. The MST of wild-type RhdA was low, compared with TST activity. Even single-residue insertion in the catalytic loop of RhdA affected MST activity; the greatest effect of the elongation was found in the sulfur transfer reactions in the presence of thiosulfate as sulfur donor. This result can be taken as evidence that productive binding of thiosulfate requires a specifically arranged catalytic loop, not maintained in the modified scaffold of RhdA-Ala and RhdA-Ser mutants. Our previous study (Pagani *et al*., 2000) showed that RhdA did not follow the consensus rules of substrate donor recognition of eukaryotic sulfurtransferases (Luo and Horowitz, 1994; Nagahara *et al*., 1995; Nagahara and Nishino, 1996). The ability to catalyze sulfur transfer from thiosulfate to cyanide of both Thr232Lys and Thr232Ala RhdAs (Pagani *et al*., 2000), which were expressed in the persulfurated form, was, indeed, not affected by the change of the residue at position +2 with respect to the catalytic cysteine. The above evidence and the data presented here (Table I) might suggest that in RhdA interactions with persulfurated active-sites, cysteine should be the basis for productive binding with sulfur acceptor in the sulfur transfer reactions.

RhdA mutants with elongated active-site loop show phosphatase activity

The rhodanese and Cdc25 phosphatase families display five or six sequential peptide NH groups, respectively, radially arranged around the thiol group of the catalytic cysteine residue, which is located at the bottom of the active-site pocket. Crystallographic analyses (Bordo *et al*., 2001) showed that hypophosphite, but not phosphate, is stably bound to the catalytic pocket of the desulfurated RhdA, thus suggesting that the precise size restrictions in the RhdA catalytic pocket may result in a non-productive accommodation of phosphatase substrate(s). Considering that the selection of proper substrate(s) for 'Cdc25-like phosphatase' could be the reason for the lack of reactivity towards *p*NPP of the mutated RhdAs with an elongated catalytic loop (Bordo *et al*., 2001), we attempted to re-evaluate the effect of single-residue insertion at the position between His233 and His234 of RhdA in generating phosphatase activity. The phosphatase activity of wild-type RhdA and of the mutated RhdAs, tailored to mimick the Cdc25 enzymes active-site loop (RhdA-Ala and RhdA-Ser) was tested by using the artificial substrate OMFP. OMFP has been proved to be an appropriate substrate for Cdc25 phosphatases (Gottlin *et al*., 1996; Chen *et al*., 2000), showing values of k_{cat}/K_m significantly higher than *p*NPP (McCain *et al*., 2002). As is evident in Figure 1A, the hydrolysis product OMF was detectable only in the presence of RhdA-Ala and RhdA-Ser and its production was a linear function of the concentration in the assay of the RhdA mutants with an elongated active-site loop. The calculated values of k_{cat} are listed in Table II, representing the results presented in Figure 1A and Table II (column A) obtained by measuring phosphatase activity in the absence of the reductant DTT, usually present in the assay for Cdc25 enzymes (Dunphy and Kumagai, 1991). Negligible phosphatase activity was found for either wild-type RhdA or sulfur-free RhdA obtained by cyanolysis, indicating that the thiolate of the catalytic cysteine is not the only structural requirement for phosphatase activity in RhdA. The designed loop insertion mutations in RhdA were, however, effective in generating productive interaction with OMFP, although the activity k_{cat} values for these RhdA mutants were low compared with those of Cdc25 enzymes (Gottlin *et al*., 1996; Chen *et al*., 2000; McCain *et al*., 2002).

Given the requirement for reducing agents such as DTT for enzymatic activity of Cdc25s with artificial substrates, we measured the phosphatase activity of wild-type and mutated RhdAs after pre-incubation in the presence of excess

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Table II. Catalytic activities of wild-type and mutated RhdAs with OMFP

Enzyme	k_{cat} (min^{-1})[a]	
	A	B
Wild-type RhdA	n.d.	n.d.
RhdA-Ser	0.003	0.026
RhdA-Ala	0.026	0.055

[a]The k_{cat} figures were calculated from activity assays (A) carried out in the absence of DTT and (B) using enzymes pre-incubated with DTT.
n.d., not detectable.

of DTT (Figure 1B). DTT treatment significantly improved the catalytic behavior of both RhdA-Ser and RhdA-Ala mutants and had no effect on wild-type RhdA (Table II, column B). Since DTT is recognized as a sulfur acceptor of the persulfide sulfur held in wild-type RhdA (data not shown), the ineffectiveness of DTT in enhancing phosphatase activity of RhdA clearly demonstrated that free thiol on Cys230 is not the only requisite for productive interaction of wild-type RhdA with phosphatase OMFP substrate. Precise size restrictions in the RhdA catalytic pocket prevent productive accommodation of the OMFP substrate and single amino acid insertion between RhdA residues His233 and His234 made the enzyme properly tailored for phosphatase activity. Productive interaction with OMFP seems to be favored by introduction of a hydrophobic residue (Ala) rather than the hydrophilic serine, as shown by comparison of the k_{cat} values for RhdA-Ala and RhdA-Ser (Table II).

Effect of reductants on RhdA mutants

The reactive nature of the active-site cysteine and the fact that the sulfenic form of the cysteine is enzymatically inactive support the need for reducing agents in the phosphatase assays of Cdc25s (Claiborne *et al*., 1999). From the molecular point of view, the published 3D structure of Cdc25s (Fauman *et al*., 1998; Reynolds *et al*., 1999) demonstrated that the active-site cysteine can form an intramolecular disulfide bond with another conserved cysteine in the molecule. In a recent report (Savitsky and Finkel, 2002), the effects of oxidative stress on the Cdc25s were examined and it was suggested that in Cdc25C, formation of a disulfide bond between Cys330 and the active-site Cys377 could rescue the protein and prevent the formation of definitely inactivated sulfinic species. In the case of the engineered RhdAs, the presence of only one cysteine residue in the molecule does not allow the formation of any intramolecular disulfide bond. In an attempt to give a rationale for the DTT effect on phosphatase activity of the engineered RhdAs (see Table II), we analyzed whether different experimental conditions of reductant addition could modulate the phosphatase activity of the mutated RhdAs (Figure 2). In the case of RhdA-Ala, comparison of the activity values in the presence of DTT (1 mM final concentration) obtained by using in the assay the enzyme pre-incubated with DTT (k_{cat} = 0.055 min^{-1}) or not (k_{cat} = 0.046 min^{-1}) indicated that under both experimental conditions the phosphatase activity doubled with respect to that measured in the absence of the reductant (k_{cat} = 0.026 min^{-1}). The effectiveness of DTT in enhancing the phosphatase activity of RhdA-Ser was higher and was dependent on the experimental conditions of reductant addition, the k_{cat} values being 0.003 min^{-1} (in the absence of DTT), 0.013 and 0.026 min^{-1} (with DTT only in the assay and after pre-incubation, respectively). The free thiol present in RhdA-Ser and RhdA-Ala could be the target of oxidative events. The significant increase in phosphatase activity of both RhdA mutants 'via reductive pressure' of DTT could be ascribed to the fact that DTT could prevent the formation of a sulfenic form (i.e. Cys-SOH) of the enzyme, cysteine sulfenic acid being readily reversible by thiol reduction (Savitsky and

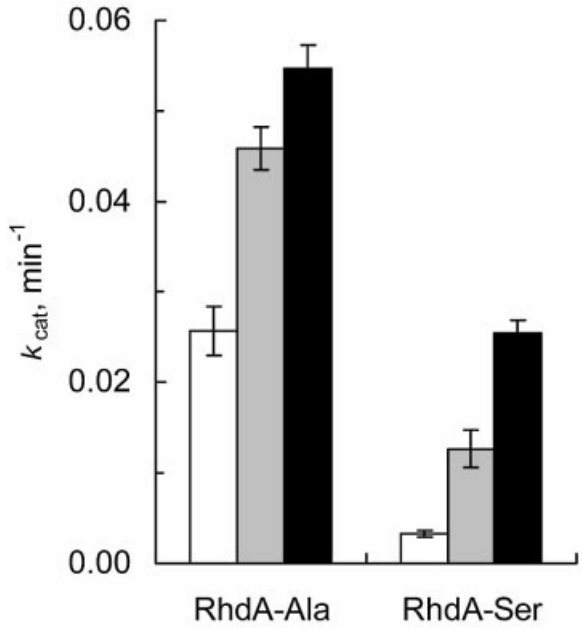

Fig. 2. Effect of DTT on the phosphatase activity of RhdA-Ala and RhdA-Ser. Hydrolysis of OMFP was determined, as described in Materials and methods, in the absence of DTT (white bars), in the presence of 1 mM DTT in the assay mixture (grey bars) and by using enzymes pre-treated with 2 mM DTT (black bars).

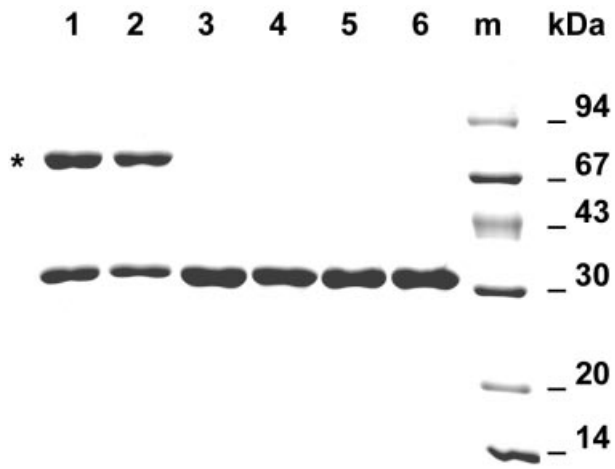

Fig. 3. Electrophoretic patterns of the mutated RhdAs before and after treatment with DTT. SDS–PAGE analysis of RhdA-Ala and RhdA-Ser before treatment with DTT (lanes 1 and 2, respectively) and after 1 h incubation with 2 mM DTT (lanes 3 and 4, respectively). As a control, the electrophoretic patterns obtained by SDS–PAGE analysis in standard reducing conditions (with β-mercaptoethanol) of RhdA-Ala (lane 5) and RhdA-Ser (lane 6) are shown. m, molecular weight standards; *, dimeric form.

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Finkel, 2002). Mass spectrometric measurements (data not shown) were not consistent with cysteine sulfenic acid formation on the molecules of the RhdA mutants with an elongated active-site loop. The formation of an intermolecular disulfide bond, on the other hand, has to be considered since an additional protein band showing a M_r consistent with an RhdA mutant dimeric form was observed by SDS–PAGE analysis (Figure 3, lanes 1–2). In both RhdA-Ala and RhdA-Ser, this form was evaluated to represent ~50% of the enzyme population. According to the formation of an intermolecular disulfide bond, a single protein band was detected in both mutated RhdAs by SDS–PAGE analysis in the presence of β-mercaptoethanol (Figure 3, lanes 5–6). Notably, the electrophoretic patterns of RhdA-Ala and RhdA-Ser after DTT treatment in the same conditions used for phosphatase activity detection showed one only protein band (Figure 3, lanes 3–4), indicating that DTT treatment did prevent the oxidative process involving the catalytic cysteine of the modified RhdAs. Although quantification of the observed molecular forms (i.e. monomeric versus dimeric forms) is rather speculative by SDS–PAGE analysis, in the case of RhdA-Ala the effectiveness of DTT in preserving the form with the cysteine available for catalysis should explain the doubling of phosphatase activity in the presence of the reductant.

Conclusions

Our previous studies on *Azotobacter vinelandii* sulfurtransferase (Bordo *et al*., 2000, 2001; Pagani *et al*., 2000) revealed unique structural features of RhdA which make it a suitable model for the identification of functional diversity determinants among the rhodanese superfamily enzymes. Therefore, from a comparison with the structurally homologous Cdc25 phosphatases (Fauman *et al*., 1998; Hofmann *et al*., 1998; Reynolds *et al*., 1999; Bordo *et al*., 2001), we have engineered the RhdA scaffold to produce an 'artificial' enzyme mimicking the pocket of Cdc25 active-sites. In the mutants RhdA-Ala and RhdA-Ser with single-residue introduction via rational design (Bordo *et al*., 2001), the elongation of the native RhdA catalytic loop definitely resulted in the impairment of productive interaction with substrates involved in sulfur transfer reactions. The modified catalytic loop, on the other hand, made RhdA-Ala and RhdA-Ser able to interact productively with the artificial phosphatase substrate OMFP. Taken together, these results clearly indicate that sulfurtransferase and phosphatase activities are not capable of existing concurrently. Since the identification of artificial substrates suitable for kinetic analyses of Cdc25 enzymes seems to be difficult (Gottlin *et al*., 1996; Chen *et al*., 2000; Kolmodin and Aqvist, 2000; McCain *et al*., 2002), the choice of the artificial substrate OMFP in the present study was dictated by the fact that it has been proven to be more efficient than *p*NPP for Cdc25s. Although there is still much to learn about whether specific structural interactions are responsible for the catalytic and molecular behaviors of RhdA-Ala and RhdA-Ser, their ability to catalyze OMFP hydrolysis highlighted the functional plasticity of the RhdA scaffold. Considering that the recognition of distinctive structural motives in the ubiquitous rhodanese domains should facilitate the identification of possible biological substrates (Bordo and Bork, 2002), here, for the first time, we have identified in a model rhodanese scaffold a structural determinant for selective recognition of substrates (i.e. sulfur- or phosphate-containing compounds).

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