Biochemical and Biophysical Research Communications 444 (2014) 651–655

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Serine 83 in DosR, a response regulator from *Mycobacterium tuberculosis*, promotes its transition from an activated, phosphorylated state to an inactive, unphosphorylated state

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ARTICLE INFO

Article history:
Received 22 January 2014
Available online 1 February 2014

Keywords:
Mycobacterium tuberculosis
DosS
Sensor kinase
DosR
Response regulator
Autodephosphorylation

ABSTRACT

A sensor kinase, DosS, and its corresponding response regulator, DosR, constitute a two component system for regulating gene expression under hypoxic conditions in *Mycobacterium tuberculosis*. Among response regulators in *M. tuberculosis*, NarL has high sequence similarity to DosR, and autophosphorylated DosS transfers its phosphate group not only to DosR but also to NarL. Phosphorylated DosR is more rapidly dephosphorylated than phosphorylated NarL. DosR and NarL differ with respect to the amino acids at positions T + 1 and T + 2 around the phosphorylation sites in the N-terminal phosphoacceptor domain; NarL has S83 and Y84, whereas DosR has A90 and H91. A DosR S83A mutant shows prolonged phosphorylation. Structural comparison with a histidinol phosphate phosphatase suggests that the hydroxyl group of DosR S83 could play a role in activating the water molecule involved in the triggering of autodephosphorylation.

1. Introduction

A two component system (TCS) consisting of a sensor histidine kinase (HK) and its cognate response regulator (RR) is a signal transduction pathway that plays a major role in the adaptive response to environmental changes [1]. A typical bacterial TCS involves sensing internal or external changes by a HK, resulting in autophosphorylation of a conserved histidine residue in the kinase domain. The phosphoryl group is then transferred to an Asp residue in the N-terminal phosphoacceptor (receiver; REC) domain of the cognate RR, which affects the properties of its C-terminal DNA binding domain [2,3], resulting in changes in downstream gene expression. Genes activated in this manner tend to remain active until the RR is deactivated by autodephosphorylation.

The REC domain contains a conserved Asp residue that receives the phosphoryl group from an autophosphorylated HK. The structure of the typical RR regulatory domain has the canonical $(\beta\alpha)_5$-fold [4,5]. Six residues (three Asp/Glu, one Thr/Ser, one Tyr/Phe, and one Lys) are conserved in the REC domain and are functionally important for phosphorylation of the RR and the activation of the effector domain through phosphorylation-induced conformational changes [6–8]. The two consecutive acidic residues that coordinate a Mg^{2+} ion and the phosphoaccepting Asp are located in the β1-α1 and β3-α3 loops, respectively [8,9]. The conserved Lys residue is located in the β5-α5 loop and forms an ionic interaction with the phosphoryl oxygen of phosphoaspartate in the phosphorylated RR. The phosphorylation of the REC domain involves nucleophilic attack on the phosphorus atom by Asp carboxylate oxygen [10]. Its autodephosphorylation is generally presumed to proceed via a mechanism similar to that of autophosphorylation, but in reverse. A water molecule has been suggested to perform nucleophilic in-line attack on the phosphorus, causing a planar PO_3 transition state coordinated by the conserved Thr/Ser, conserved Lys, and Mg^{2+} ion.

Mycobacterium tuberculosis undergoes a metabolic transformation to its non-replicating state under the influence of environmental stimuli such as hypoxia [11]. *M. tuberculosis* has 12 TCSs, involving 13 contributing HKs [12]. Among these HKs, two, DosS and DosT, function with a single RR, DosR, which functions to up-regulate genes essential for the survival of *M. tuberculosis* under hypoxic conditions [13,14]. Although phosphotransfer from a HK to its cognate RR is very specific, some cross-talk (cross-phosphorylation) between HKs and non-cognate RRs has been observed [15–17]. This cross-phosphorylation has been suggested to

Abbreviations: TCS, two component system; HK, histidine kinase; RR, response regulator; REC domain, receiving domain; HAD, haloacid dehydrogenase.

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http://dx.doi.org/10.1016/j.bbrc.2014.01.128
0006-291X/

contribute to the integration of multiple signals into a single response or to the expansion of a single signal to multiple responses [18]. Recently, DosT and NarL were found to interact using a yeast two-hybrid assay [19].

Here, we show cross-phosphorylation of NarL by DosS using purified proteins. A comparison of the phosphorylation and dephosphorylation reactions of DosR, NarL and mutants thereof showed that S83 next to the conserved Thr/Ser (T82) in DosR was a key residue for autodephosphorylation.

2. Materials and methods

2.1. Gene cloning

The DosS (Rv3132c) gene was amplified by PCR from the chromosomal DNA of the *M. tuberculosis H37Rv* strain. To obtain N-terminal truncated DosS, which possesses autophosphoryaltion activity, genes spanning amino acid residues 63–578 (DosSΔ62) and 231–578 (DosSΔ230) of DosS were amplified by PCR using primers for the *Nde*I and *Hin*dIII restriction sites. The PCR products were cloned into the expression vector pET30a (Invitrogen) containing a C-terminal hexa-His tag. The genes for the response regulators, NarL (Rv0844c), PdtaR (Rv1626), DosR (Rv3133c), and MtrA (Rv3246c) were amplified by PCR from the genomic DNA of *M. tuberculosis H37Rv* and cloned into the pET30a vector using *Nde*I and *Xho*I restriction sites. The regions encoding the REC domains of DosR (residues 1–122) and NarL (residues 1–129) were also cloned into the pET30a vector.

2.2. Protein expression and purification

To obtain DosS, PdtaR, MtrA, DosR, NarL, and REC domains of DosR and NarL, the recombinant plasmids were transformed into *Escherichia coli* BL21 (DE3) and the recombinant proteins were overexpressed in *E. coli* cultivated in LB medium at 18 °C. After sonication of the harvested cells, the proteins were purified from the soluble fraction using Ni–NTA affinity chromatography. The purification steps were performed as previously described for the purification of the hexa-His tagged DosS ATP binding domain [20].

2.3. Site-directed mutagenesis

To introduce mutations S83A and Y84H into DosR and A90S and H91Y into NarL, mutagenesis was carried out using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Mutations in all constructs were confirmed by DNA sequencing

2.4. Autophosphorylation and phosphotransfer assays

Protein autophosphorylation reactions were carried out with 15 μM of protein for 15 min at 30 °C in kinase buffer (50 mM Tris–HCl, pH 8.0, 50 mM KCl, and 25 mM $MgCl_2$). The reactions were initiated by the addition of [γ-^{32}P]ATP (10 mCi/ml, 3000 Ci/mmol) and unlabeled ATP to a final concentration of 10 μM, and were terminated by the addition of 2X gel-loading buffer containing 4% SDS and 0.2 M dithiothreitol. For the phosphotransfer assay, the autophosphorylation reaction of DosS was followed by the addition of 15 μM of response regulator proteins and the reaction mixture was further incubated for the times indicated at 30 °C. The reaction was terminated by adding 2X gel-loading buffer containing 4% SDS and 0.2 M dithiothreitol, and subjected to SDS–PAGE.

3. Results

3.1. Two REC domain subgroups for twelve response regulators from *M. tuberculosis*

The classification of RRs, which comprise a REC domain and effector modules, has been based primarily on the domain architecture of the effector module because the REC domain exhibits relatively high sequence conservation [21]. A DNA binding helix-turn-helix (HTH) domain is found in NarL-type RRs, while the OmpR-type family has winged HTHs. Although the REC domains have high sequence conservation, we aligned the amino acid sequences of 12 REC domains from *M. tuberculosis* RR into two subgroups (Supplementary Fig. S1). REC domains had the conserved residues, D/E-D, D, S/T, K at the ends of β1, β3, β4, and β5 strands in canonical $(\beta\alpha)_5$ structures, respectively. They were divided into two subgroups depending on other conserved sequences, especially in β2 and β5 strands. The first group included RegX3, TcrA, PhoA, PrrA, MprA, KdpE, TrcR, MtrA, and TcrX, and the second group included NarL, PdtaR, and DosR. Among these, the REC domain of DosS had the highest sequence identity to that of NarL (33%).

3.2. Phosphorylation of NarL by DosS

Autophosphorylated DosS can transfer its phosphoryl group to its cognate RR, DosR. To check for cross-talk between DosS and other RRs, NarL and PdtaR, a phosphorylation dependent transcriptional antitermination regulator, from the same subgroup and MtrA, the first *M. tuberculosis* RR A, from another subgroup were purified (Fig. 1A). Both PdtaR and MtrA are likely to be involved in *M. tuberculosis* adaptation within macrophages [12]. The phosphotransfer assay was performed using autophosphorylated DosS and purified RRs. In addition to phosphorylation of DosR at 0.5 min, NarL was phosphorylated at 15 min, but the other RRs were not (Fig. 1B). No radioactivity was detected at the positions of PdtaR and MtrA even after 4 h (data not shown).

3.3. Fast phosphorylation and dephosphorylation of DosR

Although DosR was phosphorylated at 0.5 min, the phosphorylated DosR band disappeared after 15 min (see Fig. 1B). To determine whether this was due to autodephosphorylation, the phosphorylated DosR REC domain band was monitored for 10 min (Fig. 1C). The intensity of the phosphorylated DosR band became gradually weaker as the incubation time progressed and most of it had disappeared by 10 min. In addition, no autophosphorylated DosS remained at this time. The phosphorylation of NarL was also monitored, but for 60 min. The phosphorylation of NarL only became detectable after 5 min. However, the phosphorylated protein continued to accumulate up to 60 min, whereas the level of autophosphorylated DosS gradually decreased over the same interval (Fig. 1D). When we compared the kinetics of DosR and NarL phosphorylation/autodephosphorylation, we found that both phosphorylation and autodephosphorylation occurred faster for DosR than for NarL.

3.4. REC domains of DosR and NarL

To determine whether the differences in the kinetics of phosphorylation and autodephosphoryation between DosR and NarL were due to differences in their REC domains, the phosphotransfer assay was performed with the purified REC domains of DosR and NarL (Fig. 2A). When the DosR REC domain was incubated with autophosphorylated DosS, DosR was phosphorylated at 0.5 min

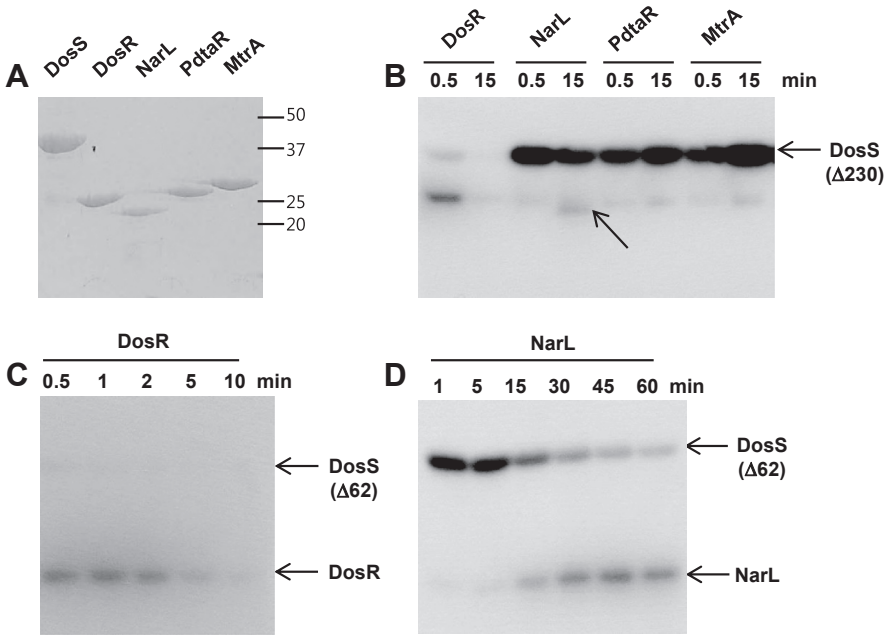

Fig. 1. Phosphosphorylation and autodephosphorylation of RRs. (A) Purified DosS and RRs (DosR, NarL, Pdta, and MtrA). (B) Autoradiogram of RR phosphorylation by autophosphorylated DosSΔ230 in the presence of [γ-^{32}P]ATP. Arrows indicate phosphorylated RRs. (C) Phosphorylation and autodephosphorylation of DosR after 10 min incubation (D) Phosphorylation of NarL after 60 min incubation. DosSΔ62 autophosphorylated with [γ-^{32}P]ATP was used for (C) and (D).

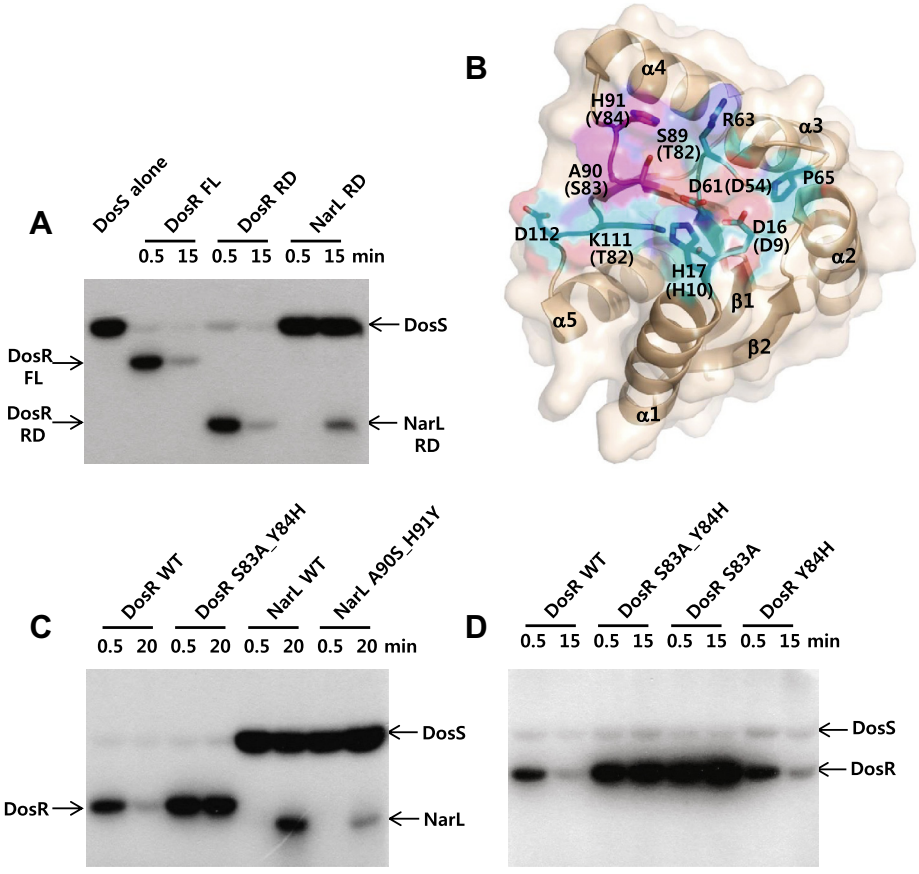

Fig. 2. Effect of the S87 residue on the dephosphorylation of DosR. (A) Full-length (FL) DosR and REC domains (RD) of DosR and NarL were phosphorylated for 0.5 and 15 min by DosSΔ230 autophosphorylated with [γ-^{32}P]ATP. (B) Active site of the NarL REC domain. Catalytically important residues at the loops connected to the C-terminal ends of b-strands are shown with sticks. The positions of the conserved Thr/Ser and two residues (T, T + 1, and T + 2, respectively) are shown in magenta. The corresponding residues for DosR are in parentheses. (C) Phosphorylation and autodephosphorylation of DosS S83A_Y84H and NarL A90S_H91Y mutants for 0.5 and 20 min. (D) Phosphorylation and autodephosphorylation of DosS S83A and Y84H mutants for 0.5 and 15 min. DosSΔ230 autophosphorylated with [γ-^{32}P]ATP was used for (C) and (D).

whereas phosphorylated DosS had almost disappeared by this time. At 15 min, the phosphorylated DosR REC domain had almost disappeared to the same extent as the phosphorylated DosS. In the case of NarL, phosphorylated NarL RD was detected after 15 min, but most of autophosphorylated DosS still remained at this time. This suggests that the NarL REC domain is also autodephosphorylated more slowly than the DosR REC domain.

The crystal structure of the NarL REC domain [22] shows that the residues surrounding the phospho-accepting Asp (D61) include the catalytically important residues D16, S89, and K111, and the residues next to the conserved ones (Fig. 2B). These residues are conserved in DosR except for A90 and H91 (T + 1 and T + 2 positions, respectively), which are located next to conserved Thr/Ser in the loop4 connecting to the α4 helix. The residues at T + 1 and T + 2 in DosR are S83 and Y84, respectively (see Supplementary Fig. S1).

3.5. Ser83 is a key residue for fast dephosphorylation of DosR

Since the two residues (T + 1 and T + 2) after the conserved Thr/Ser are exposed as part of the active site, differences in the amino acids at these positions could have caused the differences in the phosphorylation and autodephosphorylation kinetics between DosR and NarL. We generated a DosR S83A_Y83H mutant and a NarL A90S_H91Y mutant by introducing reciprocal mutations at their T + 1 and T + 2 sites and performed the phosphotransfer assay with these mutant proteins in the presence of autophosphorylated DosS (Fig. 2C). Unlike wild-type DosR, which lost its phosphate group at 20 min, the DosR S83A_Y84H mutant remained phosphorylated up to 20 min, indicating reduced autodephosphorylation. The NarL A90S_H91Y mutant exhibited a lower level of phosphorylation at 20 min than phosphorylated wild-type NarL, implying that these mutations accelerated its autodephosphorylation.

To identity the residue conferring accelerated DosR autodephosphorylation, two mutants, DosR S83A and DosR Y84H, were generated and subjected to the phosphotransfer reaction (Fig. 2D). The DosR S83A mutant was still phosphorylated after 15 min as observed for the DosR S83A_Y84H mutant, whereas DosR Y84H showed rapid dephosphorylation like wild-type DosR.

4. Discussion

Phosphatases of the haloacid dehydrogenase (HAD) superfamily of hydrolases are a large class of enzymes that have evolved to dephosphorylate a wild range of substrates. According to the general catalytic mechanism of HAD phosphatases, the release of a phosphate from a phosphor-Asp intermediate occurs via nucleophilic attack of a water molecule activated by another Asp residue [23]. The crystal structure of one HAD phosphatase, histidinol phosphate phosphatase (HisB) from *E. coli*, revealed a phosphorylated intermediate, suggesting a HisB reaction pathway [24]. Comparison of the phosphorylation site in NarL from *M. tuberculosis* with the active site of HisB (PDB Id. 2FPW) showed that conserved Asp, Thr/Ser, and Lys residues in NarL are occupied at corresponding positions in the HisB active site [22]. The crystal structure of DosR (PDB Id. 3W3C) adopts a $(\beta\alpha)_4$-fold with α4 on the same side of the β-sheet as α1, and a helical rearrangement mechanism for phosphorylation activation of DosR was suggested [25]. As superimposition of the REC domain of DosR on that of NarL [26] showed, when DosR α5 occupies the place of α4 and α4 moves next to α3, DosR is structurally and functionally similar to NarL with K104 located at the corresponding position to K111 of NarL. A model of the reconstituted REC domain of DosR (based on that of NarL) superimposed on the HisB structure is shown in Fig. 3A. The conserved residues T82, K104, D7 and D8 are located at positions corresponding

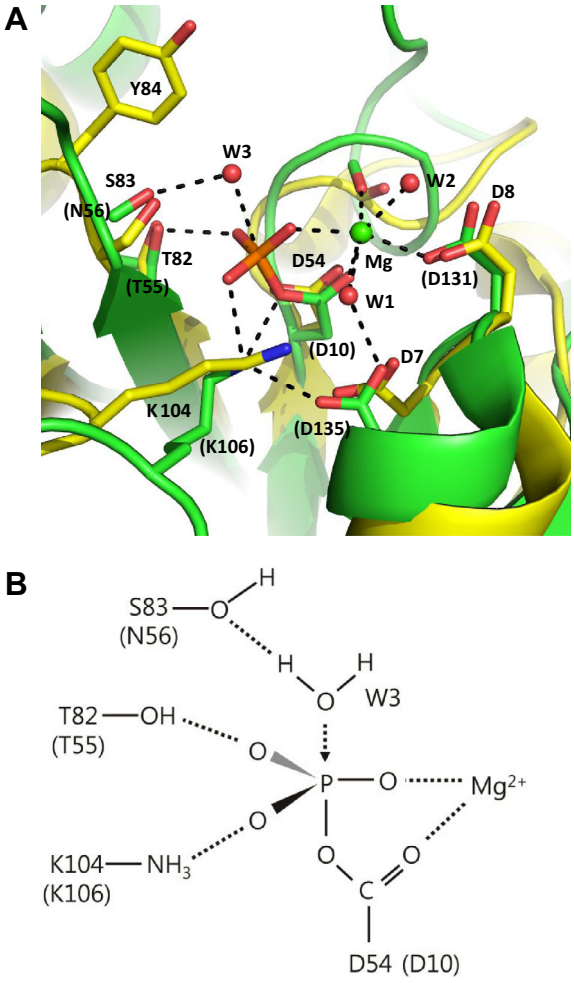

Fig. 3. Proposed mechanism for DosR autodephosphorylation. (A) Superimposition of a reconstituted DosR REC domain l, based on that of NarL (PDB Id. 3EUL), on the structure of histidinol-phosphate phosphatase (HisB) containing a phospho-aspartate intermediate (PDB Id. 2FPW). The conserved residues in DosR are located at positions that have residues interacting with the phospho-Asp group and Mg^{2+} ion in HisB. The hydroxyl group of DosR S83 is positioned at the carbonyl oxygen of the HisB N56 interacting phosphate group via a W3 water molecule (W3). The residues for HisB are in parentheses. The residues are shown as sticks, and water molecules and the Mg^{2+} ion are shown as spheres. Hydrogen bonds are shown as dashes. (B) A proposed model for nucleophilic substitution. During the autodephosphorylation process, a water molecule positioned and activated by the hydroxyl group of S83 would perform a nucleophilic in-line attack on the phosphorus, causing a planar PO_3 transition state coordinated by conserved T82, K106, and the Mg^{2+} ion.

to those of residues T55, K106, D135, and D131 in HisB that interact with phospho-Asp and Mg^{2+}. The position of the oxygen atom of the S83 hydroxyl group corresponds to that of the carbonyl oxygen of N56 of HisB, which has a hydrogen bond to a water molecule that interacts with the phosphate group of the phospho-Asp intermediate (see W3 in Fig. 3A). This provides a hint as to the possible mechanism of DosR autodephosphorylation. While the conserved Thr/Ser (T82) holds the phosphate group with K104, S83 (T + 1 residue) could help position a water molecule via a hydrogen bond to the water molecule attacking the phosphorus as a nucleophile (Fig. 3B). The hydroxyl group of S83 might participate in activating the water molecule by proton abstraction. This could be the reason why the DosR S87A mutant showed slower autodephosphorylation than the wild type.

Removal of the phosphoryl group from the REC domain to terminate a response is a critical aspect of signal transduction. A wide range of RR autodephosphorylation rates has been reported despite the extensive structural and chemical similarities in REC domains

[10]. Variations in amino acid residues at active sites in different REC domains may influence the autophosphorylation rate. In particular, variation in two amino acids at positions on the C-terminal side of the Asp phosphorylation site and the conserved Thr/Ser (residues D + 2 and T + 2) could cause a change in the autodephosphorylation rate [27]. Previously, the degree of accessibility of the water molecule attacking the phosphoryl group was shown to be responsible for some differences in autodephosphorylation rates [28]. It has been suggested that residue T + 1 affects access to the phosphorylation site [29] and hence, it might have an effect on autodephosphrylation kinetics, but this has not been tested [10]. Here, we propose that Ser83 at the T + 1 position in DosR is important for providing both space and positioning for the water molecule for autodephosphorylation.

Acknowledgments

This research was supported by Kyungpook National University Research Fund, 2012, and a National Research Foundation of Korea (NRF) Grant funded by the Korean government (MEST)(2009-0077493 and 2011-0015987).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.01.128.

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