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Mycobacterium tuberculosis *S*-adenosyl-L-homocysteine hydrolase is negatively regulated by Ser/Thr phosphorylation

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ABSTRACT

S-Adenosylhomocysteine hydrolase (SahH) is known as an ubiquitous player in methylation-based process that maintains the intracellular *S*-adenosylhomocysteine (SAH) and *S*-adenosylmethionine (SAM) equilibrium. Given its crucial role in central metabolism in both eukaryotes and prokaryotes, it is assumed that SahH must be regulated, albeit little is known regarding molecular mechanisms governing its activity. We report here that SahH from *Mycobacterium tuberculosis* can be phosphorylated by mycobacterial Ser/Thr protein kinases and that phosphorylation negatively affects its enzymatic activity. Mass spectrometric analyses and site-directed mutagenesis identified Thr2 and Thr221 as the two phosphoacceptors. SahH_T2D, SahH_T221D and SahH_T2D/T221D, designed to mimic constitutive phosphorylation, exhibited markedly decreased activity compared to the wild-type enzyme. Both residues are fully conserved in other mycobacterial SahH orthologues, suggesting that SahH phosphorylation on Thr2 and Thr221 may represent a novel and presumably more general mechanism of regulation of the SAH/SAM balance in mycobacteria.

1. Introduction

S-adenosylhomocysteine hydrolase (SahH), or adenosylhomocysteinase, catalyzes the hydrolysis of *S*-L-adenosylhomocysteine (SAH) into free adenosine and L-homocysteine (Fig. 1A). SAH is generated from *S*-adenosylmethionine (SAM) as a by-product of SAM-dependent methyltransferase reactions. Methylation plays a role in a vast array of biological processes, such as DNA replication and repair [1], quorum sensing [2], methionine metabolism or phospholipid biosynthesis [3]. In mycobacteria, a family of highly related SAM-dependent methyltransferases is also involved in functionalization of mycolic acids [4,5], which are very long α-branched β-hydroxylated fatty acids, responsible for the highly impermeable cell envelope and playing a crucial role in *Mycobacterium tuberculosis* virulence [6].

Because SAH acts as a feedback inhibitor of many SAM-dependent methyltransferases, SahH plays a critical role in clearing the cofactor product and maintaining the proper SAM/SAH equilibrium. Perturbation of this equilibrium has been shown to inhibit growth of different cell lines [7]. As a central element in metabolism, SahH has been regarded as a druggable target in many diseases, such as cancer [8], hypercholesterolemia [9], parasitic [10,11] and viral [12] infections. Pharmacological research on SahH has been stimulated by the large number of inhibitors such as aristeromycin [13] or nucleoside analogues available [14], although these are not considered clinically relevant due to cytotoxicity issues [12]. SahH has also been proposed as an attractive target to combat tuberculosis (TB), one of the most threatening diseases worldwide. Despite the progress that has been made to reduce global incidence of TB, emergence of multidrug (MDR) resistant TB threatens to undermine these advances. Therefore, novel therapeutic intervention strategies effective against drug-sensitive and -resistant strains of *M. tuberculosis* are urgently needed as adducts in the present treatment regimen. SahH represents a potential target in *M. tuberculosis* because it is essential for growth [15]. In order to delineate structural differences with the human SahH structure and possible implications for the design of selective inhibitors, the crystal determination of *M. tuberculosis* SahH

Abbreviations: MS, mass spectrometry; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; STPK, Ser/Thr protein kinase; TB, tuberculosis.

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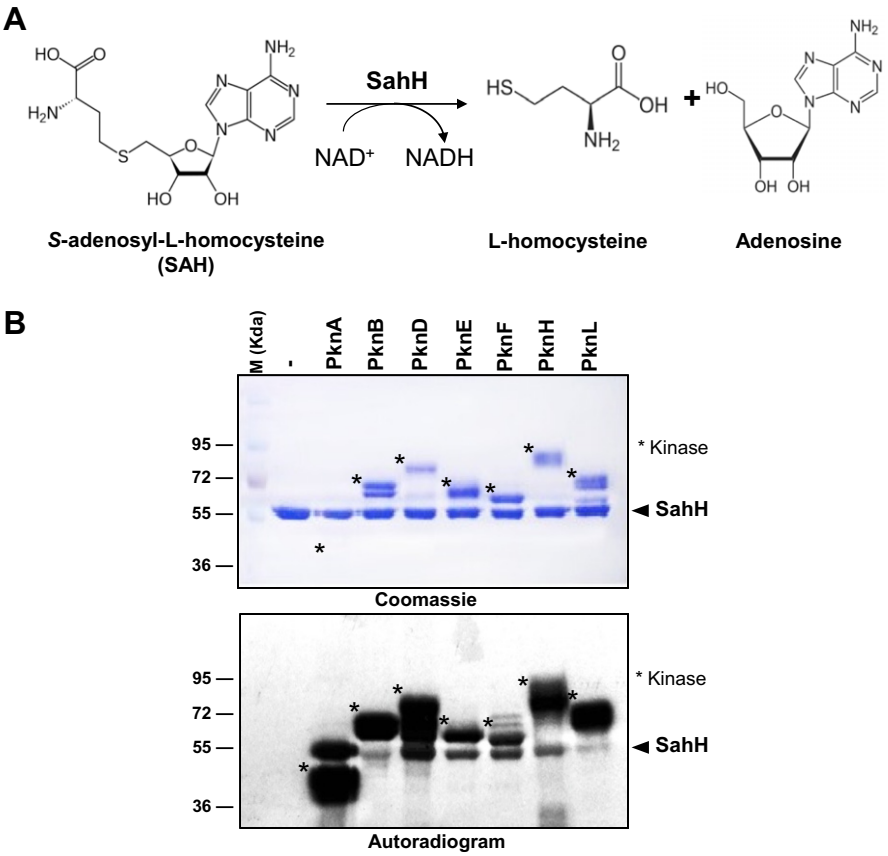

Fig. 1. (A) Enzymatic reaction catalyzed by SahH. (B) *In vitro* phosphorylation of SahH by multiple STPKs. Purified His-tagged SahH was individually incubated with several *M. tuberculosis* STPK purified as GST-tag fusion proteins in the presence of [γ-^{33}P]ATP. Depending on the STPK, 0.6–4.2 μg were necessary for obtaining optimal autophosphorylation activity for each specific kinase. Samples were separated by SDS–PAGE and stained with Coomassie Blue (upper panel) and visualized by autoradiography after overnight exposure to a film (lower panel). Bands labeled with ∗ indicate the position of each kinase in the upper panel and illustrate the autokinase activity of each STPK in the lower panel. Molecular weights are indicated in the left margin.

(Rv3248c) in ternary complex with substrate and inhibitors has been solved [16], representing the first SahH crystal structure in a prokaryote. SahH has been shown to be up-regulated in infected mouse lungs as determined using a promoter-trap system [17], and despite the essential role of this enzyme in the physiology of the bacteria, molecular regulatory mechanisms of SahH activity remain unknown. SahH has been recently identified as one of the proteins upregulated/expressed during growth in macrophages by comparative proteome analysis of broth-cultured and intraphagosomally-grown mycobacteria [18]. The occurrence of several equidistant spots with the same molecular weight on two-dimensional polyacrylamide gels suggests the presence of different SahH isoforms [18]. We thus reasoned that these isoforms may correspond to various phosphorylation states of SahH, since each phosphate group changes the charge of the protein and makes it to migrate toward the acidic end of the two-dimensional strip. Because signalling through Ser/Thr phosphorylation by mycobacterial Ser/Thr protein kinases (STPKs) has recently emerged as a key regulatory mechanism in mycobacteria [19,20], this prompted us to investigate the possible regulation of *M. tuberculosis* SahH activity by STPKs.

2. Materials and methods

2.1. Bacterial strains, media, and growth conditions

Strains used for cloning and expression of recombinant proteins were *Escherichia coli* DH5α (Invitrogen) and BL21(DE3)Star (Novagen) grown in LB medium at 37 °C. Media were supplemented with ampicillin (100 μg ml^{-1}) or spectinomycin (100 μg ml^{-1}), as required.

2.2. Cloning, expression and purification of recombinant SahH and mutant proteins

The *sahH* gene (Rv3248c) was amplified by PCR using *M. tuberculosis* H37Rv chromosomal DNA as a template and primers containing a NdeI and BamHI site listed in Table 1. Amplified products were cloned into the pETPhos vector [21], a variant of pET15b (Novagen) that includes the replacement of the thrombin

Table 1
Primers used in this study.

Primers	5′ to 3′ Sequence[a,b]
SahH WT dir	CTACCTACATATGACCGGAAATTTGGTGACCAAA (NdeI)
SahH WT rev	ATGCCTAT GGATCCTCAGTAGCGGTAGTGGTCCG (BamHI)
SahH T2A dir	CTACCTACATATG**G**CCGGAAATTTGGTGACCAAA (NdeI)
SahH T2A rev	ATGCCTAT GGATCCTCAGTAGCGGTAGTGGTCCG (BamHI)
SahH T221A dir	ACCGAGGAGACCACC**G**CCGGCGTGCTGCGGCTC
SahH T221A rev	GAGCCGCAGCACGCCGG**C**GGTGGTCTCCTCGGT
SahH T2D dir	CTACCTACATATG**GA**CGGAAATTTGGTGACCAAA (NdeI)
SahH T2D rev	ATGCCTAT GGATCCTCAGTAGCGGTAGTGGTCCG (BamHI)
SahH T221D dir	ACCGAGGAGACCACC**GA**CGGCGTGCTGCGGCTC
SahH T221D rev	GAGCCGCAGCACGCCG**TC**GGTGGTCTCCTCGGT

[a] Restriction sites are underlined and specified into brackets.
[b] Mutagenized bases are shown in bold.

site coding sequence with a tobacco etch virus (TEV) protease site and with a N-terminal His-tag free of Ser/Thr/Tyr residues, generating pETPhos_*sahH*. Site-directed mutagenesis was directly performed on pETPhos_*sahH* using inverse PCR amplification with self-complementary primers (Table 1). The duet strategy [22] was used to generate phosphorylated SahH in the pCDFDuet-1 vector that allows to co-express the PknA kinase domain along with SahH. All constructs were verified by DNA sequencing. The different His-tagged recombinant proteins were over-expressed in *E. coli* BL21(DE3)Star and purified using TALON Metal affinity resin (Clontech) as described previously [23] with slight modifications. Briefly, purification buffer was modified (20 mM Tris–HCl, pH 8, 500 mM NaCl, 10% glycerol, 0.1% Tween 20, 20 mM imidazole) and sonication of bacterial pellet was performed for 2 min at 4 °C in the form of 15-s pulses with 10-s cooling intervals between pulses.

2.3. In vitro kinase assay

In vitro phosphorylation was performed as described with 4 μg of SahH in 20 ml of buffer P (25 mM Tris–HCl, pH 7.0; 1 mM DTT; 5 mM $MgCl_2$; 1 mM EDTA; 50 mM ATP) with 200 μCi ml^{-1} [γ-^{33}P]-ATP corresponding to 65 nM (PerkinElmerPerkinElmer, 3000 Ci/mmol), and 0.6–4.2 μg of kinase in order to obtain for each specific kinase its optimal autophosphorylation activity for 30 min at 37 °C. Cloning, expression and purification of the eight recombinant GST-tagged STPKs from *M. tuberculosis* were described previously [24].

2.4. Enzymatic assay

The assay of SahH activity in the hydrolytic direction used the spectrophotometric method described earlier [25]. Briefly, Ellman's reagent (5,5′-dithiobis (2-nitrobenzoic acid), or DTNB), was used to convert HCY into an HCY–TNB complex with an absorption maximum at 412 nm. Assays were performed in the presence of a 50 mM (pH 8.0) phosphate buffer, 1.5 μM SahH, and 400 μM DTNB for a total assay volume of 600 μl. Concentrations of the SAH substrate were varied from 5 μM to 80 μM. Readings were performed on a NanoDrop 2000c Spectrophotometer (Thermo Scientific).

2.5. Mass spectrometry analysis

Phosphorylated form of SahH (pETduet_*sahH*) were purified from *E. coli* as described above and directly analyzed by mass spectrometry without additional kinase treatment, as previously described [26].

2.6. Circular dichroism analysis

UV-CDspectra were recorded using a Chirascan spectrophotometer (Applied Photophysics) at a protein concentration of 2 mg/ml in buffer (20 mM Tris–HCl, pH 8, 500 mM NaCl, 10% glycerol, 0.1% Tween 20). The data (average of three spectra) were analyzed with the program CDNN to estimate the secondary structure composition [27].

2.7. Immunoblotting analysis

The purified recombinant proteins from pETPhos_*sahH*, pETDuet_*sahH*, and pETDuet_*sahH_T2A/T221A* (SahH_Ala) were used for immunoblotting using anti-phosphothreonine antibodies according to the manufacturer's instructions (Invitrogen) and revealed with secondary antibodies labeled with IRDye infrared dyes (Odyssey Classic) to increase the detection sensitivity of phosphorylated proteins.

3. Results and discussion

3.1. STPKs-mediated phosphorylation of SahH

Several components of the central metabolism have recently been shown to be regulated by Ser/Thr phosphorylation [28–30], thus prompting us to investigate whether SahH might be regulated *via* post-translational modification and particularly by phosphorylation, a reaction that changes the physicochemical properties of defined Ser or Thr residues by introducing negative charges, which can ultimately affect the overall activity of the protein. Therefore, the *M. tuberculosis* STPKs PknA, B, D, E, F, H and L were expressed as GST-tagged fusions and purified from *E. coli* as described previously [24]. Recombinant SahH was expressed and purified as a soluble protein from *E. coli* BL21(DE3)Star harbouring the pETPhos_*sahH*. When STPKs were individually incubated with SahH and [γ-^{33}P]ATP, phosphorylation was observed with most of the kinases tested (Fig. 1B). As expected, no radioactive signal was detected when kinases were omitted from the reaction mixture. These results clearly indicate that SahH is a specific substrate and interacts with various STPKs *in vitro*, suggesting that this central metabolic protein might be regulated in mycobacteria by multiple extracellular signals. The "overlapping" substrate specificity of the different kinases is in agreement with earlier studies demonstrating that multiple mycobacterial STPKs are able to phosphorylate the same substrates [23,26,31,32].

3.2. Identification of the SahH phosphorylation sites

In order to identify the nature and position of the phosphorylation sites, a purified hyperphosphorylated isoform of SahH was prepared using the duet system, allowing to co-express both PknA and SahH, and directly analyzed by mass spectrometry after tryptic digestion following a method previously reported [22]. The ProteinPilot® database searching software, using the Paragon method with phosphorylation emphasis, was used to detect and identify the phosphorylated peptides. The MS/MS spectra not only confirmed the occurrence of phosphate groups on SahH, but also allowed to identify two phosphorylated peptides: peptide [Histag-7] carrying a single phosphorylation site at Thr2 (Fig. S1A) and peptide [214–225] carrying a single phosphorylation site at Thr221 (Fig. S1B).

Definitive identification and localization of the phosphorylation sites was next achieved by site-directed mutagenesis to introduce either single or double mutations (Thr to Ala replacements) in SahH, to prevent specific phosphorylation. The different mutants were expressed using the pETPhos constructs, purified as His-tagged proteins and individually analyzed following incubation with [γ-^{33}P]ATP and PknF. The mixtures were then separated by SDS–PAGE and analyzed by autoradiography. As illustrated in Fig. 2A, phosphorylation was partially inhibited in the SahH_T221A mutant and this inhibition was even more pronounced in the SahH_T2A mutant. Complete abrogation of the phosphorylation reaction was evidenced in the double SahH_T2A/T221A mutant, thus confirming the proper identification of the sites (Fig. 2A). This phosphoablative mutant protein also failed to generate a radioactive signal in the presence of other kinases, namely PknA, PknB, PknD, PknE, PknH or PknL (Fig. 2B), thus indicating that all kinases phosphorylate SahH solely on Thr2 and Thr221.

To further address the relevance of *in vitro* phosphorylation, the phosphoablative *sahH* allele was cloned into the pCDFDuet-1 vector [22], which allows its co-expression together with PknA in *E. coli*. The purified SahH_T2A/T221A was next assessed for phosphorylation in an *in vivo* context by Western blotting using anti-phosphothreonine antibodies. The reactivity of the antibodies for

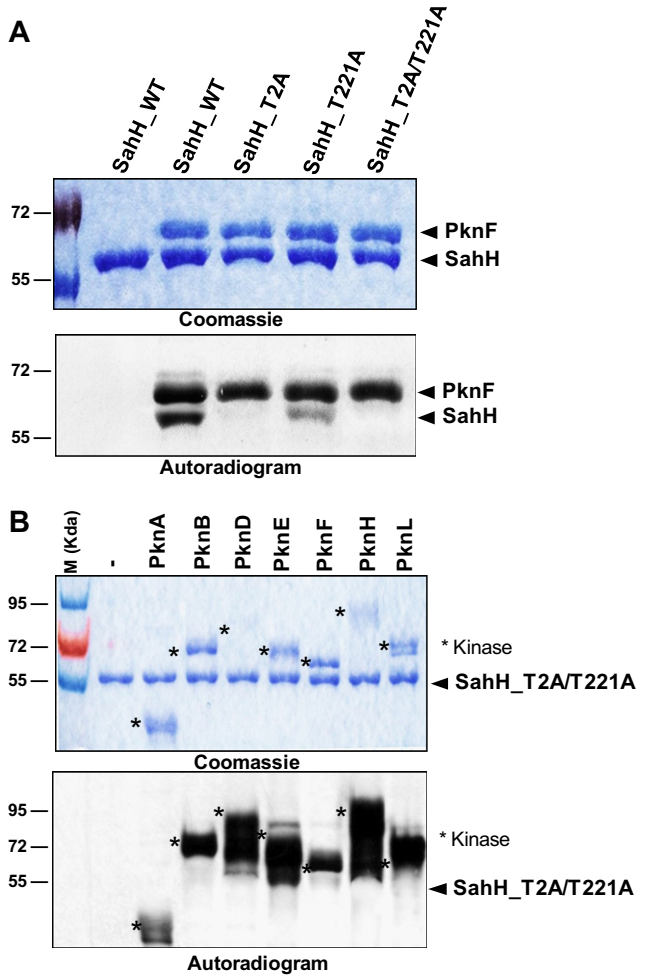

Fig. 2. Phosphorylation of SahH variants with Ala-substituted phosphosites. (A) Purified SahH_WT, SahH_T2A, SahH_T221A, and SahH_T2A/T221A mutants were incubated with PknF and [^{33}P]ATP, separated by SDS–PAGE, stained with Coomassie Blue (upper panel) and visualized by autoradiography (lower panel). (B) *In vitro* phosphorylation of SahH_T2A/T221A by multiple kinases. Recombinant STPKs encoded by the *M. tuberculosis* genome were expressed and purified as GST fusions and incubated with purified His-tagged SahH_T2A/T221A and radiolabelled [^{33}P]ATP. Depending on the STPK, 0.6–4.2 μg were necessary for obtaining optimal autophosphorylation activity for each specific kinase. Samples were separated by SDS–PAGE, stained with Coomassie Blue (upper panel) and visualized by autoradiography after overnight exposure to a film (lower panel) and (C) phosphorylation status of SahH_WT or SahH_T2A/T221A in a PknA co-expressing *E. coli* strain. The *sahH_WT* and *sahH_T2A/T221A* (SahH_Ala) alleles were cloned into pETDuet_*pknA*. Four μg of the purified His-tagged SahH variants were probed with anti-phosphothreonine antibodies according to the manufacturer's instructions (Invitrogen) and revealed with secondary antibodies labeled with IRDye infrared dyes (Odyssey Classic) to increase the detection sensitivity of phosphorylated proteins.

the phosphorylated SahH isoform was demonstrated using the protein purified from *E. coli* co-expressing PknA together with SahH_WT (Fig. 2C), corresponding to pETDuet_*sahH*. As anticipated pETDuet_*sahH_Ala*, corresponding to the SahH_T2A/T221A mutant co-expressed with PknA failed to react with the antiphosphothreonine antibodies, indicating that Thr2 and Thr221 are the unique phosphoacceptors in SahH. These results are in agreement with a recent study reporting the whole phosphoproteome of *M. tuberculosis*, in which SahH was identified and found to be phosphorylated at position Thr221 *in vivo*, although this report failed to identify the Thr2-containing phosphopeptide [32].

3.3. Phosphorylation negatively regulates SahH hydrolase activity

We next investigated the effect of phosphorylation on SahH activity using a colorimetric assay as reported earlier [25]. The enzymatic parameters of both the non-phosphorylated and the phosphorylated SahH isoforms, produced in pETPhos_*sahH* and pETduet_*sahH* respectively, were determined and compared (Fig. 3). Although the apparent K_m was found to be similar (18.5 μM for non-phosphorylated SahH and 17.7 μM for phosphorylated SahH), phosphorylation of SahH was associated with a reduced V_{max} value (1859 pmol/min compared to 3783 pmol/min for the non-phosphorylated isoform). These data suggest that phosphorylation of SahH modifies the catalytic rate of the enzyme but does not affect substrate binding.

Previous studies have shown that aspartate is an acidic residue that qualitatively mimics the effect of phosphorylation with regard to functional activity and thus commonly used to elucidate the role of phosphorylation on various mycobacterial proteins [23,26,33]. Thus, this approach was applied to SahH by generating the single and double phosphomimetic mutants in which Thr2 or/and Thr221 were substituted by Asp. The corresponding SahH_T2D, SahH_T221D and SahH_T2D/T221D variants were expressed *via* the pETPhos_derivatives, purified and assessed for hydrolase activity. The graph depicted in Fig. 3 unambiguously shows that SahH_T2D exhibits a significant reduced activity, comparable to that of the phosphorylated isoform produced in the pETDuet. Careful examination indicates that, indeed, SahH_T2D, exhibits apparent K_m and V_{max} values that are similar to those of the pETD-

	pETPhos-SahH	pETDuet-SahH	pETPhos-SahH_T2D
V_{Max} pmol/min	3783	1859	1730
K_m μM	18.5	17.7	19.9

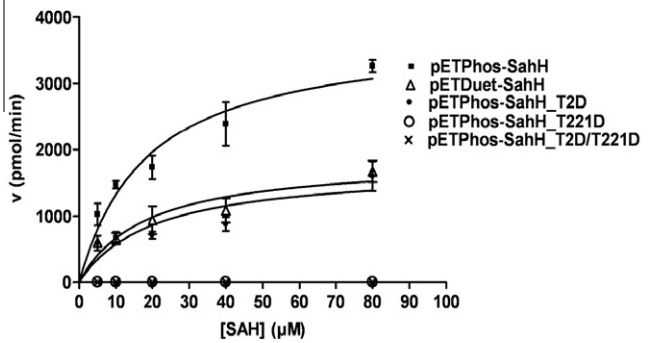

Fig. 3. Hydrolase activity of SahH_WT and phosphorylated derivatives. Unphosphorylated and phosphorylated SahH isoforms were prepared from pETPhos_*sahH* and pETDuet_*sahH* in *E. coli*, purified and assayed for hydrolase activity in the presence of SAH to determine the kinetic parameters. Enzymatic activities of SahH phosphomimetic mutants carrying either T2D, T221D or T2D/T221D replacements were also assayed in the presence of increasing SAH concentrations. Values are means ± SEM of triplicates representative of three sets of experiments with independent protein preparations.

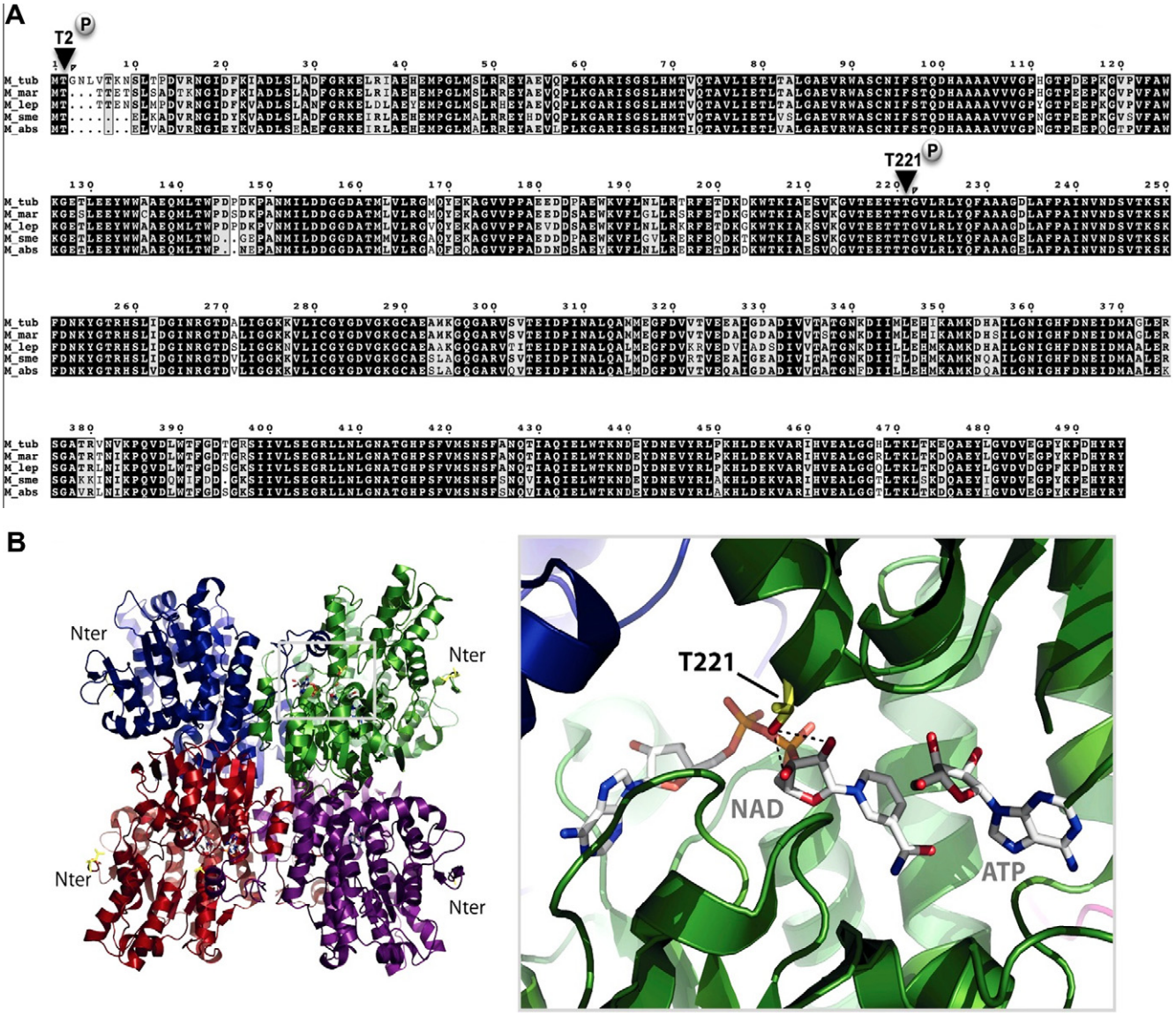

Fig. 4. Conservation and localization of the phosphoacceptors on SahH proteins. (A) Conservation of the phosphoacceptors in SahH orthologues. The multiple sequence alignment of SahH orthologues was performed using ClustalW and Espript (M_tub, *Mycobacterium tuberculosis*; M_mar, *Mycobacterium marinum*; M_lep, *Mycobacterium leprae*; M_sme, *Mycobacterium smegmatis*; M_abs, *Mycobacterium abscessus*). Numbering of the amino acids corresponds to the SahH protein from *M. tuberculosis*. Residues conserved in all species are presented in black boxes. The phosphorylated sites of SahH and their positions are indicated. (B) Localization of the Thr221 phosphosite in the three-dimensional structure of SahH. The quaternary structure of the *M. tuberculosis* SahH is represented in the left panel (PDB: 3CE6). The first nine N-terminal residues are not visible in the crystal structure, thus resulting in the lack of structural information regarding Thr2. The Thr221 residue is present in the catalytic site and is directly involved in NAD binding. Its phosphorylation is very likely to result in the loss of affinity for the NAD cofactor.

uet_derivative proteins (Fig. 3). Moreover, the enzymatic activity was found to be completely abrogated with SahH_T221D or SahH_T2D/T221D, suggesting that phosphorylation of SahH negatively regulates SahH activity.

To exclude the possibility that the T221D or T2D/T221D mutations affect the proper folding of the protein, which might explain the reduced activity of these proteins compared to the SahH_WT, circular dichroism was performed to check the protein folding and to estimate secondary structures (Fig. S2). The CD spectra of SahH_WT, SahH_T221D and SahH_T2D/T221D suggest that the Asp replacements did not significantly affect the folding of the protein. Therefore, it can be inferred that Asp replacements are very likely to affect catalysis of SahH. Taken collectively, these results indicate that STPK-phosphorylation at Thr2 and Thr221 negatively affects the catalytic activity of SahH rather than altering the overall folding of the protein.

3.4. Localization of the phosphoacceptors on the SahH structure

Multiple sequence alignments showed that SahH is an ubiquitous and highly conserved protein in all mycobacterial species, as would be expected for an essential protein. Both phosphorylation sites, Thr2 and Thr221, were found to be conserved in all the species presented in Fig. 4A, including pathogenic and non-pathogenic mycobacteria. For more distantly related organisms, only the Thr221 appears very well conserved, including human, rat or *Plasmodium falciparum* [34]. This, not only emphasizes an important role of Thr221 in catalysis, but also suggests that regulation of SahH activity by phosphorylation may not be restricted to *M. tuberculosis*, but may represent a more general and conserved regulatory mechanism.

To gain insight into the possible effect(s) of Thr2 and Thr221 phosphorylation on the enzymatic behaviour of SahH, the positions

of both residues were mapped with respect to the substrate-binding site and the catalytic residues, taking advantage of the available three-dimensional structure of the protein (PDB3CE6) [16] and the structure of close homologues (i.e. PDB3OND and PDB3N58). In the crystal structure of *M. tuberculosis* SahH, the first 9 residues are not defined, so are supposedly very mobile. Therefore, due to the lack of structural information, the Thr2 phosphoacceptor could not be defined on the three-dimensional structure and hence its contribution in catalysis of SahH could not be predicted. Nevertheless, several recent studies reported that *Corynebacterium glutamicum* OdhI or *M. tuberculosis* GarA proteins possess disordered N-terminal extensions which, following phosphorylation by STPK, undergo major conformational changes characterized by the binding of the phosphorylated and ordered N-terminal part of the proteins to their respective C-terminal FHA domain, and consequently inhibiting them [29,35]. Although a direct effect of Thr2 phosphorylation on the catalytic site cannot be proposed due to the distance between the peripheral locations of the N-terminal domain in the tetramer compared to the more central localization of the active site, it may possibly affect entry of the ligand or induce allosteric changes. In contrast to Thr2, Thr221 is located within or close to the catalytic site of the protein. The hydroxyl group of Thr221 is in close contact with the ribose 3′OH of the NAD nicotinamide moiety (Fig. 4B). Therefore, phosphorylation of this critical residue is very likely to profoundly impact and even to abolish binding of the cofactor, consistent with the total loss of activity observed with SahH_T221D and SahH_T2D/T221D mutants.

In summary, we provide here the first evidence of a post-translational modification involved in the regulation of SahH activity in *M. tuberculosis*. Our results strongly suggest that phosphorylation of SahH may play a critical role in regulating the levels of three important biological molecules (adenosylhomocysteine, adenosine and homocysteine) in mycobacteria. Thus, it appears that *M. tuberculosis*, and presumably also other mycobacterial species, may control in a very subtle manner the SAM/SAH equilibrium. Whether phosphorylation occurs under certain circumstances such as stationary phase or during dormancy and participates in regulating the SAM metabolism during persistence or intracellular survival requires further investigation.

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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.2012.11.038.

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