B subunit of Phosphate Specific Transporter from *Mycobacterium tuberculosis* is a thermostable ATPase

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Running title: Characterization of mycobacterial PstB
SUMMARY

The B-subunit of phosphate specific transporter (PstB) is an ABC protein. *pstB* was PCR amplified from *Mycobacterium tuberculosis* and overexpressed in *E. coli*. The overexpressed protein was found to be in inclusion bodies. The protein was solubilized using 1.5% N-lauroyl sarcosine and was purified by gel permeation chromatography. The molecular mass of the protein was ~31 kDa. The eluted protein showed ATP-binding ability and exhibited ATPase activity. Among different nucleotide triphosphates, ATP was found to be the preferred substrate for *M. tuberculosis* PstB-ATPase. The study of the kinetics of ATP-hydrolysis yielded Km of ~72 µM and Vmax of ~0.12 µmoles/min/mg of protein. Divalent cation like manganese was inhibitory to the ATPase activity. Magnesium or calcium, on the other hand, had no influence on the functionality of the enzyme. The classical ATPase inhibitors like sodium azide, sodium vanadate and N-ethyl maleimide were without any effect but an ATP analogue, 5’-p-fluorosulfonylbenzoyl adenosine, inhibited the ATPase function of the recombinant protein with a Ki of ~0.40 mM. Furthermore, there was hardly any ATP hydrolyzing ability of the PstB due to mutation of the conserved aspartic acid residue to lysine in the Walker motif B, confirming the recombinant protein is an ATPase. Interestingly, analysis of the recombinant PstB revealed that it is a thermostable ATPase and thus our results highlight for the first time the presence of such an enzyme in any mesophilaic bacteria.
INTRODUCTION

Importance of phosphate as an essential component of several biomolecules, such as membrane lipids, complex carbohydrates, nucleic acids etc., is well known. Therefore, assimilation of phosphate from the environment and its metabolism are essential events for microorganisms for their survival. As phosphate is often a limiting nutrient, its import in bacteria is accomplished through several parallel transport systems (1, 2). Phosphate specific transporter (Pst) is one of them which has been reported to be present in several bacteria like *Bacillus subtilis* (3), *Escherichia coli* (4, 5), *Mycobacterium tuberculosis* (6, 7), *Salmonella typhimurium* (5), *Streptococcus pneumoniae* (8) etc. Pst is a tightly regulated high affinity system grouped under ATP-binding cassette (ABC) \(^1\) transporters which includes largest family of paralogous proteins that are present in wide variety of cells including those of mammals (9 - 11). Expression of Pst is operon controlled and the import function of this multi-subunit transporter is known to be operative only during phosphate limitations (5 and references therein). Besides transporting phosphate, the Pst system in bacteria has also shown to be involved in controlling a number of co-ordinately regulated genes, grouped under the *pho* regulon (5). Interestingly, among the available prokaryotic genome sequences only in *M. tuberculosis* three putative *pst* operons have been identified (6,7). Therefore, it has been thought that many copies of the same phosphate transporter in mycobacteria might be involved in subtle biochemical adaptations of this microorganism for its growth and survival under highly varying (eg. phosphate limiting) conditions during infectious cycle (2). Besides phosphate transport, the role of this transporter in coping up with adverse situations in mycobacteria has also been postulated (6, 12, 13, 14).

In most of the prokaryotes Pst is found as a membrane associated complex. In *E. coli* it is composed of four distinct subunits encoded by *pstS*, *pstA*, *pstC and pstB* genes (4) and arranged in an operon as *pstSCAB* (5, 15). Pst S is the periplasmic binding protein. The PstA and Pst C are integral membrane channel proteins and are hydrophobic in nature. PstB subunit, which is often referred as ABC protein (16), provides energy for transport through ATP hydrolysis (5). Available reports indicated the similar organization of the genes of the *pst* operon in other prokaryotes (17, 18) also except for mycobacteria (7, 19). In *M. tuberculosis* presence of several copies of all the components of the operon except for PstB

\(^1\) Available for download at [http://www.jbc.org/](http://www.jbc.org/)
has been reported (6, 7).

Bacterial ABC proteins have been shown to be responsible for ATP binding as well as ATP hydrolysis which is evident from the studies with histidine and maltose transporters from *Salmonella typhimurium* (16, 20, 21). In fact, structural data with the ATP binding component of the *S. typhimurium* histidine permease corresponded well with the biochemical studies (22). Even the homologous component of the Pst in *E. coli* has already been shown to possess ATP hydrolysing ability (23).

The ATP-binding subunit of the bacterial ABC transporters have also been implicated in diverse biological functions (24 - 26). We have reported earlier that *pstB* is overexpressed as well as amplified in a fluoroquinolone resistant colony of *Mycobacterium smegmatis* suggesting a novel role of this subunit in addition to its involvement in the process of phosphate import (13, 14, 27). Furthermore, among all the prokaryotic genome sequences available so far, *pstB* has been found to be present throughout, giving strong indications that this gene might be important for the microorganisms. We therefore focussed our effort to gain an insight on the nature of the mycobacterial PstB protein.

In this communication, we report that unlike other prokaryotic ABC proteins, the ATP hydrolysing ability of PstB from *M. tuberculosis* is rather magnesium independent and resistant to known ATPase inhibitors. Furthermore, our results convincingly established that the mycobacterial PstB is a thermostable ATPase and thus highlighted the presence of such an enzyme in any mesophillic bacteria.
EXPERIMENTAL PROCEDURES

Materials – Restriction/modifying enzymes and other molecular biological reagents were obtained either from New England Biolabs or Promega Corporation, USA. ATP-binding protein detection kit (Roche Molecular Biochemicals, Germany), ECL Western blotting detection kit (Amersham Pharmacia, England), Expand high fidelity PCR system (Roche Molecular Biochemicals, Germany), plasmid preparation kits (Qiagen, Germany), protein molecular weight markers (Sigma Chemical Company, USA), X-ray film (Eastman Kodak, USA) were commercially available. All other chemicals including urea, triton X 100, guanidine hydrochloride, N-lauroyl sarcosine (sarkosyl), etc. were procured from Sigma Chemical Company, USA. All oligonucleotides used in this study were custom synthesized from PMK International, USA. \(\alpha^{32P}\)-dCTP was supplied by Jonaki Labs, BRIT, Hyderabad, India.

PCR amplification, construction of recombinant plasmids and generation of site directed mutant of PstB – The forward (CS1: 5’-CATATGGCGTGTGAACGGCTC-3’) and reverse (CS2: 5’-CTTTCTGAGCTCTTCAATT-3’) primers for PCR amplification of \(pstB\) (Rv0933) were designed on the basis of the published \(M.\) tuberculosis genome sequence (7). ‘CAT’ in primer CS1 does not correspond to the genome sequence. It was introduced in CS1 to incorporate a \(NdeI\) site at the 5’ end of the amplified PCR fragment. Genomic DNA from \(M.\) tuberculosis H37Rv (obtained as a gift from Dr. Jaya Tyagi, Department of Biotechnology, All India Institute of Medical Sciences, New Delhi, India) was used as the template in Expand high fidelity PCR system (Roche Molecular Biochemicals). PCR was carried out for 30 cycles (denaturation: 94\(^{\circ}\)C for 30 sec per cycle; annealing: 50\(^{\circ}\)C for 30 sec per cycle; elongation: 68\(^{\circ}\)C for 1 min for first 10 cycles and then for the remaining 20 cycles the elongation step was extended for an additional 5 sec in each cycle). Following treatment with klenow the blunt ended \(pstB\) was cloned at the \(Eco\)RV site of pBluescript (SK+) and the construct was designated as pCJS1. The nucleic acid sequence of the \(pstB\) was confirmed using an automated sequencer (ABI, PE Applied Biosystems).

The \(pstB\) fragment from pCJS1 was excised out following restriction digestions with \(NdeI\) and \(BamHI\) (the enzyme site is absent in \(pstB\) but present in the vector and located at 3’
end after the stop codon) and subcloned at the corresponding sites in pET 23a (28). Following transformation in E. coli strain DH5α, plasmid DNA was extracted and the construct (pCJS2) was verified by restriction digestions.

Genomic DNA from E. coli strain K12 (MTCC 1302) was also extracted following standard procedures (29). For PCR amplification of pstB from E. coli, the primers (CS3: 5′GATTGCATATGAGTATG 3′; CS4: 5′ GAGACTGTCCATAACGCA 3′) were designed based on the published sequence (4) and the same strategy was adopted for cloning (pCRC2) into expression vector.

PCR was employed to generate D188K (aspartic acid is replaced by lysine at amino acid residue 188) mutant in the Walker B motif (30) of the M. tuberculosis PstB. Two forward (CS1 and CS9) and two reverse primers (CS19 and CS21) were used for this purpose. Primers (CS9: 5′ GTTGCTGCTCAAGGAGCCCACC 3′; CS19: 5′ACTTCAATTTCCGCCTTGGC 3′ and CS21: 5′ GGTGGGCTCCTTGAGCAGCAAC 3′) were designed based on M. tuberculosis pstB sequences and base mismatches (underlined) were incorporated to obtain desired mutations. To generate the mutant two sets of primary and one set of secondary PCR reactions were carried out (31) using the gel purified wildtype pstB (831 bp) as template. Primary reactions were carried out with primers CS1/CS21 and CS9/CS19, while for secondary PCR reaction CS1 and CS19 were used. Thus, D188K mutation was contained within the amplified fragment of the PstB. Secondary PCR product was restriction digested with Aat II, which yielded a 655 bp fragment containing the desired mutation, and finally substituted for the corresponding wildtype fragment cloned in expression vector. Mutations were confirmed by sequencing.

Expression of recombinant protein – The pCJS2 was transformed into E. coli strain BL21(DE3) and selected on LB- ampicillin plates (100µg/ml). Overnight cultures (~15 h at 37°C) of several colonies were reinoculated and grown till O.D_{600} reached to ~0.45. Cultures were then induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested after 2 h, lysates were prepared and expression was checked by running 12 % SDS-PAGE followed by coomassie brilliant blue staining. To know the solubility of the expressed protein, cells after induction were suspended in lysis buffer (100 mM Tris, pH 7.5 containing 5 mM EDTA, 5 mM dithiothreitol and 5 mM phenylmethylsulfonyl fluoride),
treated (20 min at 24°C) with lysozyme (200 µg/ml), sonicated and different fractions (supernatant and pellet after centrifugation at 22,000 x g for 30 min at 4°C) were subjected to SDS-PAGE followed by coomassie brilliant blue staining.

**Purification of recombinant proteins** – Pellet fractions obtained following centrifugation of IPTG induced sonicated cells (transformed either with pCJS2 or pCRC2) were washed in wash buffer (lysis buffer supplemented with 4 M urea and 5% Triton X 100) following the procedure described elsewhere (32). The washed pellets (inclusion bodies containing expressed protein) were suspended in TEN buffer (10 mM Tris, 1 mM EDTA and 150 mM NaCl, pH 7.5) containing 1.5% sarkosyl, and were subjected to ultra centrifugation (1,07,000 x g for 1 h at 4°C in an ultracentrifuge, Beckman). The supernatant fractions collected in this way contained solubilized protein and were subjected to gel permeation chromatography in a FPLC unit (Pharmacia) using Superdex 200 column. Protein was eluted with TEN buffer containing 0.15% sarkosyl.

**Antibodies** – The inclusion body containing PstB protein from *M. tuberculosis* was solubilized using buffer (50 mM Tris, 5 mM EDTA and 5 mM dithiothreitol, pH 7.5) containing guanidine hydrochloride (8 M) following standard procedures (32). The denatured protein obtained in this way (purity ~ 85% as evidenced by SDS-PAGE) was precipitated by removing guanidine hydrochloride through dialysis (buffer: 50 mM Tris, 5 mM EDTA, 5 mM dithiothreitol and 1 M NaCl, pH 7.5) and was used to raise polyclonal antibodies in rabbit. Briefly, purified protein (~800 µg) emulsified in complete Freund’s adjuvant was injected subcutaneously at multiple sites. Boosters were emulsified in incomplete Freund’s adjuvant and were given at the same dose at intervals of 21 days. After the third booster dose blood was collected and sera prepared was decomplimented at 56°C for 30 min. The antibody titer was determined by indirect ELISA using horseradish peroxidase conjugated anti-rabbit IgG as secondary antibody and 2, 2’-azinobis (3-ethylbenzthiazoline-sulfonic acid) as substrate. The antisera showed cross reactivity to PstB from *E. coli*. Similarly antibody raised against *E. coli* PstB (obtained as a gift from Dr. A. Torriani, MIT, USA) recognized mycobacterial PstB.

**Detection of ATP-binding ability** – ATP-binding ability of the recombinant PstB was monitored by labelling the protein with a nonhydrolyzable ATP analogue, 5’-p-
fluorosulfonylbenzoyl adenosine (FSBA). For the labelling reaction the recombinant PstB protein (dissolved in borate buffer, pH 7.4 supplemented with 0.15% sarkosyl) was incubated (30 min at 30°C) with 1-3 mM FSBA. The samples were run on SDS-PAGE followed by detection through Western blotting using anti-FSBA antibody.

ATPase assay – The ATPase activity of the protein was quantitated by a colorimetric assay performed in microtiter plates following the method described by Henkel et al., 1988 (33). Briefly, PstB protein (0.5 - 1µg) was diluted with TEN buffer to a final volume of 25 µl. The reaction was initiated by adding equal volume of substrate solution (ATP final concentration = 1 mM; unless mentioned otherwise) followed by incubation at 37°C for 5 min (final sarkosyl concentration = 0.06%). The enzymatic reaction was terminated by addition of an acidic solution (200 µl) of malachite green, ammonium molybdate and polyvinyl alcohol (33). The activity was measured as the amount of inorganic phosphate (Pi) liberated which forms a phosphomolybdate malachite complex detected at 650nm in an ELISA plate reader. The values obtained were corrected by subtracting the blank readings obtained for nonenzymatic release of Pi due to hydrolysis of ATP and Pi contamination in the absence of enzyme as well as substrate. A standard curve with sodium phosphate monobasic was run concurrently with each experiment and thus nmole of Pi released was calculated. ATPase activity is expressed as nmole Pi liberated/ min / mg of protein and the data presented in the form of Mean ± SD.

EDTA was omitted from the sarkosyl supplemented TEN buffer in carrying out studies with divalent cations. The effect of different divalent cations (Ca²⁺, Mg²⁺ and Mn²⁺) on the ATPase activity was monitored by adding them in the presence or absence of 5 mM EDTA prior to incubation with substrate solution. Different inhibitors used in this study were sodium azide, N-ethylmaleimide and sodium orthovanadate. All inhibitors were dissolved in assay buffer, except for N-ethylmaleimide, which was in ethanol. To elucidate the effect of N-ethylmaleimide, final concentration of ethanol during the assay was adjusted to 0.6% and accordingly proper blank was maintained. The influence of inhibitors was determined by incubating (15 min or 3h at room temperature) them with the protein prior to the addition of substrate solution.

Raleigh’s scattering – Effect of temperature (80°C) for different time periods (0 - 60 min) on
aggregation pattern of PstB proteins from *M. tuberculosis* and *E. coli* in solution (protein concentration of ~100 µg/ml in TEN buffer containing 0.06% sarkosyl) was examined by studying Raleigh’s scattering at 600 nm ($\lambda_{\text{excitation}} = \lambda_{\text{emission}}$) in a fluorometer (Perkin Elmer).

**Southern hybridization** – To confirm cloning of PCR products in vectors, Southern hybridization was carried out following standard protocols (29) using [$\alpha^{32}$P]d-CTP labelled probes.

**Western blotting** – Western blotting was employed to examine the expression of PstB protein or to detect FSBA-bound protein. Protein was estimated following Bradford's method (34). Purified proteins or cell extracts (800 ng - 3 µg protein/slot) were resolved in SDS-PAGE and transferred at 100 volts for 45 min to nitrocellulose membrane (0.45 µm) in a mini-transblot apparatus (Bio-Rad) using Tris-Glycine buffer (48 mM Tris, 39 mM glycine, 0.037% SDS and 20% methanol, pH ~8.3). Blots were probed with primary (anti-PstB or anti-FSBA) and secondary (horseradish peroxidase conjugated anti-rabbit IgG) antibodies and processed with ECL detection system as described elsewhere (31). Stripping of the blots, if necessary, was done following manufacturers recommended protocol (Amersham Pharmacia, England).
RESULTS

Over production of the protein – The pstB gene from M. tuberculosis strain H37Rv was amplified by PCR. pstB specific primers (CS1 and CS2) were designed based on the published M. tuberculosis genome sequence (7). PCR was carried out at annealing temperature of 50°C with primers and genomic DNA utilizing a mixture of Taq and Pwo DNA polymerase which resulted in the amplification of expected ~831 bp fragment. Only those reactions, which contained template DNA, primers and enzymes, showed the amplification (data not shown). The PCR amplified fragment was cloned in pBluescript (SK+) and was sequenced. A base pair change was observed at codon 235. However, it did not result in alteration of any amino acid since codon 235 AAG (coding for phenylalanine) was altered to AAA.

PstB was over-expressed following subcloning in pET23a (see Experimental procedures). Several colonies showed the over expression of the protein as evidenced by an expected band of ~31 kDa in SDS-PAGE following staining with coomassie brilliant blue in cultures transformed with pCJS2 and induced with IPTG. One of these colonies was selected for further processing (Fig. 1). However, the expressed protein was observed in the pellet fraction (inclusion bodies) in SDS-PAGE analysis (Fig. 1, lane 4). Cultures even grown at lower temperatures did not yield any soluble protein. Such an event is not restricted to PstB from M. tuberculosis only since homologous protein from E. coli when expressed following transformation of pCRC2 was also found to be in inclusion bodies (data not shown). PstB aggregates were partially solubilized with 1.5% sarkosyl (Fig. 1, compare lanes 6 and 7) and were purified by gel permeation chromatography. Column elutes formed single peak within the separation range of Superdex 200, SDS-PAGE analysis of which is also shown in Fig. 1 (lanes 8 and 9). The molecular weight of the eluted protein obtained through gel permeation chromatography was found to be 69.3 ± 3.25 (Mean ± SD, n = 7) while the same samples subjected to SDS-PAGE revealed a molecular mass of 30.5 ± 1 kDa (Mean ± SD, n = 5). Western blot with the anti-PstB antibody recognized the purified protein (Fig.2). Thus our data argues that the active form of the PstB from M. tuberculosis is possibly a dimer. This is not unusual since the nucleotide binding subunits of bacterial ABC transporters are known to
be active as dimer (21, 23, 35).

PstB is an ATP-binding protein — Nucleotide-binding subunits of different bacterial ABC transporters including PstB from *E. coli* have been shown to bind ATP (23, 36). To gain insight on this aspect, we utilized the binding ability of 5'-p-fluorosulfonylbenzoyl adenosine (FSBA) at the nucleotide binding sites of such proteins through covalent modification (37). Following labelling of protein (purified in borate buffer) with FSBA (1 or 3 mM) or treating with DMSO (solvent control), samples were subjected to SDS-PAGE and immuno-blotting using anti-FSBA antibody. As shown in Fig. 2A, anti-FSBA antibody recognized only those samples, which were incubated with FSBA (lanes 2 and 3). On the other hand, the same blot, following stripping, when probed with anti-PstB antibody, recognized all the samples (Fig. 2B). This result reflected the binding ability of the purified protein specifically to FSBA and thus argues that the PstB of *M. tuberculosis* is also an ATP-binding protein.

PstB of *M. tuberculosis* is an ATPase — The ATP-binding subunit of bacterial ABC transporters has been reported to exhibit the ATPase activity (20, 21, 36). We therefore investigated the ATPase activity of the recombinant PstB of *M. tuberculosis*. The protein was able to hydrolyse ATP at a pH range between 5 to 10 (data not shown) and unless mentioned otherwise, enzymatic characterization was carried out at pH 7.5. The kinetics of ATP hydrolysis revealed a Km value of ~72 µM for mycobacterial protein as opposed to ~24 µM in *E. coli* PstB (Table 1). The Km value is quite comparable to ATPase activity exhibited by *S. typhimurium* MalK (Tables 1 and 2) while velocity of the reaction as reflected in the Vmax value was lower (see Table 1 and reference 20). PstB was also found to hydrolyze GTP as well as CTP and thus exhibited broad range of substrate specificity. However, compared to ATP, the affinity of the PstB for CTP or GTP as substrate was significantly lower (Table 2, compare the Km values).

Divalent cations are known to influence the ATP hydrolysing ability of the nucleotide binding subunit of bacterial ABC transporters (20, 21). As shown in Fig. 3A, ATPase activity of PstB was inhibited by Mn⁺². Such an inhibition of the enzyme activity was Mn⁺² specific since the effect was abolished when incubation was carried out in the presence of metal ion chelator, EDTA. On the other hand, ATP hydrolysis of PstB was unaffected when incubated either with Mg⁺² or Ca⁺² in the presence or absence of EDTA (Figs. 3B and C).
In contrast, MalK-ATPase activity in *S. typhimurium* was found to be strongly Mg\(^{2+}\) dependent (20).

ATPases are often grouped into F-, P- and V-types based on the effect of their specific inhibitors (38). The influence of these inhibitors on the ATPase activity associated with *M. tuberculosis* PstB was examined following 15 min or 3h of preincubation at room temperature. The results of short-term (15min) incubation of PstB with different inhibitors are depicted in Fig. 4A. Sodium azide, an inhibitor of mitochondrial and bacterial proton (F\(_0\)F\(_1\)) ATPase, upto a concentration of 30 mM did not affect the ATP hydrolysing ability of PstB. Similarly sodium orthovanadate which is known to be a P-type ATPase inhibitor, did not have any effect (upto 10 mM) on the enzyme activity. The enzymatic activity of PstB was also resistant to inhibition upto a concentration of 30 mM by N-ethylmaleimide, a V-type ATPase inhibitor. To know whether these results are experimental artifacts, ATPase activity of PstB was determined following treatment with a denaturant like guanidium hydrochloride (8 M) and taking boiled samples (5 min at 100\(^0\)C). As expected, treatment with guanidium hydrochloride caused a significant reduction in the PstB-ATPase activity. Surprisingly, samples following boiling did not show any significant reduction in enzyme activity (inset of Fig. 4A). Furthermore, to rule out the possibility that short term incubation (15 min) with these inhibitors was not a factor in affecting the enzyme activity, PstB-ATPase activity was monitored following treatment with them at 10 mM concentrations for 3h period. As shown in Fig. 4B, none of these inhibitors could affect the ATPase activity.

To confirm ATPase activity of PstB, enzymatic hydrolysis of ATP was monitored following incubation with non-hydrolysable substrate analogue, FSBA. This study was carried out with protein extracted and purified in borate buffer, pH 7.4 containing 0.15% sarkosyl to prevent non-specific reaction of FSBA with free amines in the buffer. Samples were incubated (30 min at 30\(^0\)C) with FSBA (0 - 4 mM) and then ATPase activity was determined following addition of different concentrations (250 - 750 \(\mu\)M) of substrate solution. FSBA preincubation significantly inhibited the PstB-ATPase activity (Fig. 5A). The Ki value for FSBA was 0.4 ± 0.05 mM (n = 3). FSBA inhibited the enzyme activity by competing with ATP for the substrate binding site which is evident from the shift in apparent Km value of the enzyme (70 ± 5, 99 ± 11.5 and 155 ± 26 \(\mu\)M in presence of 0, 1.5 and 3 mM
Further to confirm that the recombinant PstB is an ATPase, we studied a mutant (D188K), that is known to affect the ATP hydrolysing ability of the bacterial ABC proteins (22, 39, 40). Unlike wildtype PstB, incubation with increasing concentrations of ATP (62.5 - 2000 µM), the mutant protein hardly displayed any ATPase activity (Fig. 6A). Similarly, use of increasing concentrations of mutant protein (0.5 - 2 µg) showed negligible enzyme activity. In fact, ATPase activity when compared between wildtype and D188K by plotting as a function of amount of protein used in the assay exhibited slopes which are very distinct from each other (Fig. 6B).

PstB-ATPase is thermostable – Characterization of thermostable bacterial ATPases have already been reported (41, 42), however, none of them was identified from any mesophillic bacteria. Since boiling of protein did not affect the enzymatic activity of the PstB-ATPase of *M. tuberculosis* (Fig. 4A), we therefore concentrated on determining its thermostability profile. Aggregation pattern of the recombinant PstB protein in response to temperature was monitored through Raleigh’s scattering. In addition to the buffer (TEN buffer containing 0.06% sarkosyl, the condition used for assaying ATPase activity) as a control for this study, the well characterized *E. coli* PstB expressed and purified in a similar manner (see Experimental procedures) was also examined. In a preliminary experiment, aggregation patterns of these proteins were examined after incubating for 15 min at different temperatures (25°C to 80°C). Interestingly, the *E. coli* PstB precipitated following incubation at 80°C while mycobacterial protein hardly showed any aggregation (data not shown). We further monitored the aggregation profile of these two proteins by incubating at 80°C for different time periods (0 - 60 min). As depicted in Fig. 7A, the aggregation of *E. coli* protein increased with the time of incubation. Incubation of these proteins for an hour indicated low level of aggregation with PstB from *M. tuberculosis* (30.2 ± 7.23 arbitrary units; only buffer: 7.3 ± 1.89 arbitrary units) compared to that of the *E. coli* (188 ± 7.4 arbitrary units). Furthermore, when the heated samples (after centrifugation to remove precipitated proteins) were subjected to SDS-PAGE followed by coomassie brilliant blue staining, unlike *M. tuberculosis* protein a decrease in signal intensity of the bands could be visualized clearly with the *E. coli* samples (Fig. 7B). The identity of these proteins was confirmed by Western
blotting using anti-PstB antibody (data not shown). As reflected in the ATPase assays of these samples, thermal denaturation of E. coli protein was evident within 5 min of its incubation at 80°C (Fig. 8A). The maximum loss of enzyme activity for E. coli PstB was ~60% of initial value which was noticed between 30 to 60 min. of heat incubation, however, remaining ~40% was found to be heat resistant. Interestingly, mycobacterial PstB even after heat treatment (80°C) for an hour retained ~89% (loss = ~11%) of its initial ATPase activity (Fig. 8A). We further compared the enzymatic activities of both the proteins by incubating with the substrate (1 mM ATP for 5 min) at different temperatures (24 - 80°C). The PstB from E. coli showed substantial loss in the ATPase activity at temperatures above 37°C. On the other hand, M. tuberculosis enzyme was found to be heat resistant (Fig. 8B). Thus our results argue that the ATP binding subunit of the phosphate specific transporter from M. tuberculosis is a thermostable ATPase.
DISCUSSION

Phosphate is an essential but often limiting nutrient especially for pathogenic bacteria, like *M. tuberculosis* which faces rapidly changing environment within the host during the infectious cycle. To create congenial environment for their survival several phosphate importers are present in mycobacteria (2). Pst system is one of such importers belonging to the super family of ABC permeases and is known to be operative in bacteria in phosphate limiting conditions (5). Pst is a multi-subunit transporter and its expression is operon controlled. PstB of *M. tuberculosis* is the nucleotide binding subunit of the phosphate specific transporter (6). Like other ABC proteins, being the energy transducing unit, PstB in *M. tuberculosis* has a vital role in the ATP-consuming import of phosphate. Besides this, Pst system in *M. tuberculosis* gains additional importance since several copies of all the components of the operon is present in the genome but there is single copy of *pstB* gene (6, 7). Furthermore, our earlier reports indicated that pstB is overexpressed as well as amplified in a fluoroquinolone resistant colony of *M. smegmatis* where drug efflux plays a pivotal role in conferring resistance (13, 14, 27). These consequences together with the fact that ABC proteins are involved in diverse biological processes (24 - 26), led us to characterize the PstB subunit from *M. tuberculosis*.

Like other ABC proteins (21, 23, 36), overexpression of PstB in *E. coli* resulted in its accumulation as aggregates and it was found to be in pellet fractions (inclusion bodies) following centrifugation of sonicated cultures (Fig. 1). The formation of such aggregates was not unusual and could be due to incorrect folding of the expressed protein (43, 44). The renaturation of solubilized protein in urea or guanidine hydrochloride proved to be a difficult task since it yielded completely denatured protein which could not be refolded following conventional methods (32). Finally the protein was obtained mostly in soluble form using a mild ionic detergent N-lauroyl sarcosine (Fig. 1). Furthermore, the binding of the ATP analogue, FSBA, ensured the correct folding of this recombinant protein (Fig. 2). Analyses of the size of the native (obtained through gel filtration chromatography) and denatured (as in SDS PAGE gels as well as in Western blots) proteins revealed that the PstB presumably existed as a dimer. ABC proteins in bacteria have already been shown to be active as dimer
(21, 23, 36, 45). Therefore, this did not seem to be a unique feature for mycobacterial protein. Additionally, the monomeric form (~31 kDa) did not exhibit any shift in the molecular weight when samples were subjected to SDS-PAGE in non-reducing conditions suggesting no interaction between inter-molecular thiols in forming the putative dimer of the protein (data not shown).

ABC proteins have also been shown to have ATP hydrolysing ability, which is very important for the functionality of the transporter (40). Our results indicated that ATPase activity could be inhibited if incubated with FSBA (Fig. 5A). This suggests a possible competition of the non-hydrolysing ATP-analogue with ATP for substrate binding site which is evidenced by unaltered Vmax but increase in apparent Km during the activity assay in presence of inhibitor (Fig. 5B). Moreover, mutation in the conserved aspartic acid residue in the Walker motif B which is known to affect ATPase activity in other ABC proteins (22, 39, 40) exhibited hardly any ATP hydrolysing ability of the recombinant PstB (Fig. 6). Thus these two lines of evidence argue in favor of *M. tuberculosis* PstB being an ATPase.

Although ATP hydrolysing ability of mycobacterial PstB exhibited many properties characteristic to typical ABC proteins (Table 1), subtle differences made it distinct from others, like MalK or HisP from *S. typhimurium*. As for example, among different nucleotides ATP has been found to be a preferred substrate for the recombinant PstB (Table 2). Similarly, divalent cations (Mg$^{+2}$ and Ca$^{+2}$) which have been reported to be stimulatory for the enzymatic activity (20, 21) had no effect on mycobacterial PstB-ATPase (Fig. 3). Like membrane bound thermostable ATPase from *Sulfolobus acidocaldarus* (46), the enzymatic activity of mycobacterial PstB was found to be resistant to known inhibitors such as sodium orthovanadate and sodium azide. Although such an observation has also been found to be true for HisP or MalK (20, 21), N-ethylmaleimide which had a strong inhibitory effect on their activity, was unable to affect the mycobacterial enzyme (Fig. 4). The sensitivity of these proteins to N-ethylmaleimide have often been correlated to the presence of a cysteine residue at or near the highly conserved Walker motif A (20). Interestingly, no such cysteine residue is present in *M. tuberculosis* PstB.

Several thermostable ATPases have been characterized (41, 42, 46), however, they all are present in thermophillic bacteria. Therefore, what makes our finding significant is not
only the fact that the recombinant PstB being an ATPase is thermophilic but the presence of such a thermostable enzyme in a mesophillic bacteria like *M. tuberculosis*. Studies regarding thermal aggregation profile of the recombinant protein through Raleigh’s scattering (Fig. 7A) and subsequently monitoring of enzymatic activities indeed showed that PstB-ATPase from *M. tuberculosis* is thermostable compared to its *E. coli* counterpart (Fig. 8A). Furthermore, activity assays with both the ATPases confirmed that *M. tuberculosis* PstB was more efficient in exhibiting its functionality at temperatures above 37°C (Fig. 8B). However, 38 - 49% of *E. coli* PstB protein was found to be heat resistant (Fig. 8). Given the fact that during the process of thermal inactivation of an enzyme, a significant amount of unfolding intermediates could be reactivated by addition of the substrate (47, 48) might explain why such an event is not be unusual.

Like other thermostable proteins, comparison of nucleotide derived amino acid sequences of *M. tuberculosis* PstB revealed that there is an increase in β-branched (valine, ~8%) as well as charged (aspartic acid, arginine, glutamic acid and lysine, total ~23%) residues which presumably confers rigidity and stability of the protein (49, 50). Furthermore, PstB has decreased content of polar uncharged amino acids, such as asparagine (3.2%) or glutamine (3.2%), which is known to minimize deamidation and backbone cleavages of a protein (50). However, many other factors, such as improved hydrogen bonding, better hydrophobic packing, enhanced secondary structure propensity, helix dipole stabilization, improved electrostatic interactions etc. might have significant contribution in the process. Insight into these properties as well as their specific contribution towards thermophillic nature of PstB would only be unravelled once the crystal structure is solved and complete analysis could be carried out.

Finally, the detailed study on the thermostability of other ABC proteins is not available as yet, therefore, such a property brings them in sharp focus and indicates that they might be playing pivotal role in bacteria than a cursory glance reveals.
Acknowledgements – We are thankful to Dr. A. Ghosh, Director, Institute of Microbial Technology for providing us with excellent laboratory facilities. We are grateful to Dr. T. Chakrabarti for critical reading of the manuscript. We acknowledge the helpful suggestions of Drs. A. Mondal and S. Mande during this investigation. We thank Mr. J. Prasad for providing us with excellent technical assistance during the course of this investigation and acknowledge the secretarial help provided by Ms. S. Gupta. Two of the authors (JS and RC) are the recipients of Senior Research Fellowships from the CSIR, New Delhi, India. This project has partly been supported by a research grant from the DBT, New Delhi, India.
REFERENCES

Foot note:
1 Abbreviations used: ABC, ATP binding cassette; ATP, adenosine triphosphate; ATPase, adenosine triphosphatase; bp, base pair; CTP, cytosine triphosphate; DMSO, dimethyl sulphoxide; EDTA, ethylenediamine-tetra-acetic acid; ELISA, enzyme linked immunosorbent assay; FSBA, 5′-p-fluorosulfonylbenzoyl adenosine; GTP, guanosine triphosphate; IgG, immunoglobulin G; IPTG, isopropyl-β-D-thiogalactopyranoside; kDa, kilo Dalton; PAGE, poly acrylamide gel electrophoresis; PCR, polymerase chain reaction; Pi, inorganic phosphate; Pst, phosphate specific transporter; PstB, B subunit of phosphate specific transporter; SDS, sodium dodecyl sulphate.
Fig 1. **Summary of purification of *M. tuberculosis* PstB expressed in *E. coli***. Overnight cultures BL21(DE3) cells transformed with pCJS2 were reinoculated and grown till O.D$_{600}$ was ~0.45. Cultures were then induced with 0.4 mM IPTG. Cells were harvested after 2 h, lysates were prepared and processed for purification as mentioned in the ‘Experimental procedures’. Protein samples at various stages of purification were subjected to 12.5% SDS-PAGE followed by coomassie brilliant blue staining. *Lane 1*, crude extract of cells harboring plasmid pCJS-2 without IPTG induction; *Lane 2*, crude extract of cells harboring plasmid pCJS-2 induced with IPTG; *Lane 3*, low speed supernatant fraction; *Lane 4*, pellet fraction obtained after low speed centrifugation; *Lane 5*, insoluble fraction of protein (inclusion body); *Lane 6*, insoluble fraction of protein in the pellet following treatment with sarkosyl; *Lane 7*, soluble protein obtained after treatment with sarkosyl; *Lanes 8 and 9*, purified PstB obtained after gel permeation chromatography. Position of PstB is indicated by an arrow. Numbers denote the position of molecular weight standards.

Fig 2. **ATP-binding ability of mycobacterial PstB**. PstB following incubation with different concentrations of FSBA were subjected to SDS-PAGE and immunoblotting using anti-FSBA (aFSBA) or anti-PstB (aPstB) antibodies. *Lane 1*, PstB with no FSBA; *Lane 2*, PstB with 1mM FSBA; *Lane 3*, PstB with 3 mM FSBA.

Fig 3. **Effect of divalent cations on PstB-ATPase activity**. The ATPase activity of the recombinant PstB was determined using 1mM ATP with indicated concentrations of manganese (A), magnesium (B) and calcium (C) in presence or absence of EDTA. The data was expressed as percentage control in each case and reproducibility was checked in 4 - 7 independent experiments.
Fig 4. **Effect of inhibitors on ATPase activity exhibited by PstB.** PstB was preincubated with different ATPase inhibitors for 15 min (A) and 3h (B) at room temperature. 10 mM of each inhibitor was used for the experiment where preincubation was carried out for 3h. ATP hydrolysis was measured under standard conditions as described in the ‘Experimental procedures’. The reproducibility was checked in four independent experiments. Inset: Effect of denaturing agents or boiling of the enzyme on the PstB-ATPase activity. Different notations used: NONE, preincubation without any inhibitor; AZ, sodium azide; NEM, N-ethyl maleimide; VAN, sodium ortho vanadate.; BOIL, heated at 100\(^\circ\)C for 5 min; GUA, 8 M guanidine hydrochloride.

Fig 5. **Inhibition of mycobacterial PstB-ATPase activity by FSBA.** (A) PstB was incubated with indicated concentrations of FSBA at 30\(^\circ\)C for 30 min. ATP hydrolysis was then initiated with addition of indicated concentrations of ATP and monitored as mentioned in the ‘Experimental procedures’. Points in the graph are interpolated with the data obtained using 250 µM ATP (final concentration). Results are in Mean ± SD from three independent experiments. (B) Lineweaver-Burk plot of ATP affinity in the presence of indicated concentrations of FSBA. The figure shows a representative experiment. The reproducibility was checked in three independent experiments.

Fig. 6. **D188K mutant hardly shows any ATPase activity.** (A) ATP hydrolysing ability of the wildtype and the mutant were determined in presence of varied concentrations of ATP following standard conditions as mentioned in the ‘Experimental procedures’. The reproducibility was checked in four independent experiments. (B) ATP hydrolysing ability of the D188K mutant as a function of amount of protein used in the assay. ATPase activity for both wildtype and mutant was monitored with increasing concentrations of protein in the presence of 1 mM ATP. Data represents Mean ± SD from three independent experiments.
Fig. 7. **Aggregation profile of PstB proteins in response to temperature.** (A) Purified PstB proteins from *E. coli* and *M. tuberculosis* (in TEN buffer containing 0.06% sarkosyl at a concentration of ~100 µg/ml) were incubated at 80°C for indicated time periods and subsequently Raleigh’s scattering was monitored at 600 nm in a fluorometer. Results expressed in arbitrary units (Mean ± SD) and reproducibility was checked in three independent experiments. (B) Samples following incubation at 80°C for 0 (lane 1), 5 (lane 2), 10 (lane 3), 30 (lane 4) and 60 (lane 5) min were centrifuged. Supernatant fractions were subjected to 12% SDS-PAGE and visualized after coomassie brilliant blue staining.

Fig. 8. **Effect of high temperature incubation on ATPase activity of PstB.** Purified PstB proteins from *E. coli* and *M. tuberculosis* were incubated at 80°C for indicated time periods in microfuge tubes (1µg protein/reaction). Following addition of 1 mM ATP, samples were further incubated for 5 min at 37°C. The reaction was stopped by addition of an acidic solution of malachite green, ammonium molybdate and polyvinyl alcohol. Precipitates (obtained during incubation at 80°C) were pelleted by centrifugation, supernatant fractions of each samples were transferred to microtiter plates and amount of inorganic phosphate (Pi) liberated was monitored following standard conditions as mentioned in the ‘Experimental procedures’. Reproducibility was checked in three independent experiments. (B). Purified PstB proteins from *E. coli* and *M. tuberculosis* following addition of 1 mM ATP were incubated for 5 min at indicated temperatures in microfuge tubes (1µg protein/reaction). ATPase activity of each sample was determined as described in ‘A’ and calculated as percent of the activity obtained during incubation at 37°C. Data represents Mean ± SD from three independent experiments.
### TABLE I

**Kinetics of ATPase activity exhibited by PstB**

ATPase assays were carried out as described under ‘Experimental procedures’ following incubation of 1 µg of protein with different concentrations (0.06 - 2 mM) of ATP at 37°C for 5 min. Km and Vmax values were calculated from typical Lineweaver-Burk plots and results are expressed as Mean ± SD. Number of experiments is indicated in parenthesis.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Km (µM)</th>
<th>Vmax (µmole Pi/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mycobacterium. tuberculosis</em></td>
<td>71.5 ± 5.88 (7)</td>
<td>0.122 ± 0.02 (7)</td>
</tr>
<tr>
<td><em>Escherichia. coli</em></td>
<td>23.9 ± 2.96 (3)</td>
<td>0.322 ± 0.01 (3)</td>
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</tbody>
</table>
TABLE II  

_Affinity of mycobacterial PstB-ATPase for different substrates_

The affinity for ATP, CTP and GTP were determined by monitoring the substrate hydrolysing ability of the recombinant mycobacterial PstB. Assays were carried out as described under ‘Experimental procedures’ with 1 µg of recombinant protein following incubation with different concentrations (0.06 - 2 mM) of substrates for 5 min at 37°C. Km values for each substrate were calculated from typical Lineweaver-Burk plots. Results are expressed as Mean ± SD and number of experiments are indicated in parenthesis. Data for _S. typhimurium_ MalK-ATPase are from Morbach _et al._, 1993 (20).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Michaelis constant (µM)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>PstB-ATPase</td>
</tr>
<tr>
<td>ATP</td>
<td>71.5 ± 5.88 (7)</td>
</tr>
<tr>
<td>CTP</td>
<td>130 ± 8.14 (3)</td>
</tr>
<tr>
<td>GTP</td>
<td>211 ± 16.3 (3)</td>
</tr>
</tbody>
</table>
Fig. 3
Fig. 4
Fig. 5

A

B

![Graph A](image1)

![Graph B](image2)

Fig. 5
Fig. 6
Fig. 8
B subunit of phosphate specific transporter from mycobacterium tuberculosis is a thermostable ATPase
Jyoti Sarin, Sita Aggarwal, Rachna Chaba, Grish C. Varshney and Pradip K. Chakraborti

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