B-subunit of Phosphate-specific Transporter from *Mycobacterium tuberculosis* Is a Thermostable ATPase*

Received for publication, June 12, 2001, and in revised form, August 16, 2001
Published, JBC Papers in Press, September 20, 2001, DOI 10.1074/jbc.M105401200

Jyoti Sarin‡, Sita Aggarwal, Rachna Chaba§, Grish V. Varshney, and Pradip K. Chakraborti§

From the Institute of Microbial Technology, Sector 39A, Chandigarh 160 036, India

The B-subunit of phosphate-specific transporter (PstB) is an ABC protein. *pstB* was polymerase chain reaction-amplified from *Mycobacterium tuberculosis* and overexpressed in *Escherichia coli*. The overexpressed protein was found to be in inclusion bodies. The protein was solubilized using 1.5% N-lauroylsarcosine 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 $K_m$ of ~72 μM and $V_{max}$ of ~0.12 μmol/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 NaN3 had no effect while $	ext{Ca}^{2+}$ was inhibitory to the ATPase activity.

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 critical. Phosphate transporters are involved in the uptake of phosphate and other biomolecules, such as membrane lipids, complex carbohydrates, nucleic acids, etc. Phosphate transporters are important for the survival of microorganisms under adverse conditions. The study of the ATPase activity of the recombinant PstB revealed that it is a thermostable ATPase; thus, our results highlight for the first time the presence of such an enzyme in *Mycobacterium tuberculosis*.

---

* This work was supported in part by a research grant from the Department of Biotechnology, New Delhi, India. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a senior research fellowship from the Council of Scientific and Industrial Research, New Delhi, India.

§ To whom correspondence should be addressed. Tel.: 91-172-695215 (ext. 452); Fax: 91-172-690585; E-mail: pradip@imtech.res.in.

$ The abbreviations used are: Pst, phosphate-specific transporter; ABC, ATP-binding cassette; ATPase, adenosine triphosphatase; F5BA, 5′-p-fluorosulfonylbenzoyl adenosine; IgG, immunoglobulin G; IPTG, isopropyl-β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; $P_o^i$, inorganic phosphate; PstB, B subunit of phosphate-specific transporter.

---

subtilis (3), *Escherichia coli* (4, 5), *Mycobacterium tuberculosis* (6, 7), *Salmonella typhimurium* (5), *Streptococcus pneumoniae* (8), etc. Pst is a tightly regulated highly affinity transport system comprised of ATP-binding cassette (ABC) transporters that includes the 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 multisubunit transporter is known to be operative only during phosphate limitations (see Ref. 5 and references therein). Besides transporting phosphate, the Pst system in bacteria has also shown to be involved in controlling a number of coordinately regulated genes, grouped under the *pho* regulon (5). Interestingly, among the available prokaryotic genome sequences, only in *M. tuberculosis* have three putative *pst* operons 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 (e.g. 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, 12–14).

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). *PstS* is the periplasmic binding protein. The PstA and PstC 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) as well, except for mycobacteria (7, 19). In *M. tuberculosis*, the presence of several copies of all the components of the operon except for PstB 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 *S. 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 hydrolyzing ability (23).

The ATP-binding subunit of the bacterial ABC transporters have also been implicated in diverse biological functions (24–26). We have reported previously 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

---

* This work was supported in part by a research grant from the Department of Biotechnology, New Delhi, India. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a senior research fellowship from the Council of Scientific and Industrial Research, New Delhi, India.

§ To whom correspondence should be addressed. Tel.: 91-172-695215 (ext. 452); Fax: 91-172-690585; E-mail: pradip@imtech.res.in.

$ The abbreviations used are: Pst, phosphate-specific transporter; ABC, ATP-binding cassette; ATPase, adenosine triphosphatase; F5BA, 5′-p-fluorosulfonylbenzoyl adenosine; IgG, immunoglobulin G; IPTG, isopropyl-β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; $P_o^i$, inorganic phosphate; PstB, B subunit of phosphate-specific transporter.
might be important for the microorganisms. We therefore focused our effort to gain an insight on the nature of the mycobacterial PstB protein.

In this article, we report that, unlike other prokaryotic ABC proteins, the ATP hydrolyzing 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 mesophilic bacteria.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction/modifying enzymes and other molecular biological reagents were obtained from either New England Biolabs or Promega Corp. ATP-binding protein detection kit (Roche Molecular Biochemicals), ECL Western blotting detection kit (Amersham Pharmacia Biotech), Expand high fidelity PCR system (Roche Molecular Biochemicals), plasmid preparation kits (Qiagen), protein molecular weight markers (Sigma), x-ray film (Eastman Kodak) were commercially available. All other chemicals including urea, Triton X-100, guanidine hydrochloride, N-lauroylsarcosine (Sarkosyl), etc. were procured from Sigma. All oligonucleotides used in this study were custom synthesized by Integrated DNA Technologies, Coralville, IA.

**PCR Amplification, Construction of Recombinant Plasmids, and Generation of Site-directed Mutant of PstB**—The forward (CS1, 5’-CATATGGCGTGTGAACGGCTC-3’) and reverse (CS2, 5’-CTTTCTTGGACGTCTTAATT-3’) primers for PCR amplification of pstB (Ref9933) 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 at 94 °C for 30 s per cycle, annealing at 50 °C for 30 s per cycle, and elongation at 68 °C for 1 min for first 10 cycles; for the remaining 20 cycles, the elongation step was extended for an additional 5 s in each cycle). Following treatment with Klenow, the blunt-ended pstB was cloned at the EcoRV site of pBluescript (SK+) and the construct was designated as pCSJ1. The nucleic acid sequence of the pstB was confirmed using an automated sequencer (ABI, PerkinElmer Applied Biosystems).

The pstB fragment from pCSJ1 was excised 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 pET23a (28). Following transformation in E. coli strain DH5α, plasmid DNA was extracted and the construct was verified by restriction digestions.

Genomic DNA from E. coli strain K12 (MTC888) was also extracted following standard procedures (29). For PCR amplification of pstB from E. coli, the primers (CS3, 5’-GATTGCaTATAGTATGAT3’; CS4, 5’-GAGACTGTCCATACAAGCA-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 M. tuberculosis PstB. Two forward (CS1 and CS9) and two reverse primers (CS19 and CS21) were used for this purpose. Primers (CS9, 5’-GGTTGCGTCTACAGGACCCACC-3’; CS19, 5’-ACTTACCTCGCGCTGTCG-3’; and CS21, 5’-GGTTGGCTCTGGACGCAACAC-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 wild type pstB (831 base pairs) as template. Primary reactions were carried out with primers CS1/CS21 and CS9/CS19, whereas for secondary PCR reaction CS1 and CS9 were used. Thus, D188K mutation was contained within the amplified fragment of the PstB. Secondary PCR product was restriction-digested with AarII, which yielded a 655-base pair fragment containing the desired mutation, and finally subcloned for the corresponding wild type fragment cloned in expression vector. Mutations were confirmed by sequencing.

**Expression of Recombinant Protein**—The pCS2 was transformed into E. coli strain BL21(DE3) and selected on LB-ampicillin plates (100 µg/ml). Overnight cultures (~15 h at 37 °C) of several colonies were reisolated and grown until A600 reached ~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 Coomasie 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), and sonicated, and different fractions (supernatant and pellet after centrifugation at 22,000 × 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 pCSJ2 or pCRC2) were washed in wash buffer (lysis buffer supplemented with 4 μM 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 ultracentrifugation (107,000 × 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 fast protein liquid chromatography unit (Amersham Pharmacia Biotech) using Superdex 200 column. Protein was eluted with TEN buffer containing 0.1% 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 mM 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.

**Detection of ATP-binding Ability**—ATP-binding ability of the recombinant PstB was monitored by labeling the protein with a nonhydrolyzable ATP analogue, 5′-fluorosulfonpyridylbenzoyl adenosine (FSBA). For the labeling 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. (33). Briefly, PstB protein (0.5 to 1 µg) 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 = 0.6%). 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 increase in inorganic phosphate (P) liberated that forms a phosphomolybdate malaichite complex detected at 650 nm in an enzyme-linked immunosorbent assay plate reader. The values obtained were corrected by subtracting the blank readings obtained for nonenzymatic release of P, because of hydrolysis of ATP and P, 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 nanomoles of P released were calculated. ATPase activity is expressed as nanomoles of P liberated/min/mg of protein, and the data presented in the form of mean ± S.D.

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 cationically linked immunosorbent assay plate reader. The values obtained were corrected by subtracting the blank readings obtained for nonenzymatic release of P, because of hydrolysis of ATP and P, 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 nanomoles of P released were calculated. ATPase activity is expressed as nanomoles of P liberated/min/mg of protein, and the data presented in the form of mean ± S.D.
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 4 h 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 Tris buffer containing 0.06% Sarkosyl) was examined by studying Raleigh’s scattering at 600 nm (λ_{excitation} = λ_{emission}) in a fluorometer (PerkinElmer Life Sciences).

**Southern Hybridization**—To confirm cloning of PCR products in vectors, Southern hybridization was carried out following standard protocols (29) using [α-32P]dCTP-labeled 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 to 3 μg protein/slot) were resolved in SDS-PAGE and transferred at 100 V 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, 0.057% 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 the manufacturer’s recommended protocol (Amersham Pharmacia Biotech).

**RESULTS**

**Overexpression 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 an 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-base pair 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 because codon 235, AAG (coding for phenylalanine), was altered to AAA. PstB was overexpressed following subcloning in pET23a (see “Experimental Procedures”). Several colonies showed the overexpression 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 grown at even lower temperatures did not yield any soluble protein. Such an event is not restricted to PstB from *M. tuberculosis* only because 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 a 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 kDa (mean ± S.D., n = 7), whereas the same samples subjected to SDS-PAGE revealed a molecular mass of 30.5 ± 1 kDa (mean ± S.D., n = 5). Western blot with the anti-PstB antibody recognized the purified protein (Fig. 2). Thus our data argue that the active form of the PstB from *M. tuberculosis* is possibly a dimer. This is not unusual because 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 FSBA at the nucleotide binding sites of such proteins through covalent modification (37). Following labeling of protein (purified in borate buffer) with FSBA (1 or 3 mM) or treating with Me 2SO (solvent control), samples were subjected to SDS-PAGE and immunoblotting using anti-FSBA antibody. As shown in Fig. 2A, anti-FSBA antibody recognized only those samples that 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 hydrolyze ATP at a pH range between 5 and 10 (data not shown), and, unless mentioned otherwise, enzymatic characterization was carried out at pH 7.5. The kinetics of ATP hydrolysis revealed a *K_m* value of ~72 μM for mycobacterial protein as opposed to ~24 μM in *E. coli*.
PstB (Table I). The $K_m$ value is quite comparable with ATPase activity exhibited by S. typhimurium MalK (Tables I and II), whereas velocity of the reaction as reflected in the $V_{max}$ value was lower (see Table I and Ref. 20). PstB was also found to hydrolyze GTP as well as CTP and thus exhibited broad range of substrate specificity. However, compared with ATP, the affinity of the PstB for CTP or GTP as substrate was significantly lower (Table II, compare the $K_m$ values).

Divalent cations are known to influence the ATP hydrolyzing 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 $Mg^{2+}$-specific because 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 (Fig. 3, B 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 3 h of preincubation at room temperature. The results of short term (15 min) incubation of PstB with different inhibitors are depicted in Fig. 4A. Sodium azide, an inhibitor of mitochondrial and bacterial proton ($F_0F_1$) ATPase, up to a concentration of 30 mM did not affect the ATP hydrolyzing ability of PstB. Similarly, sodium orthovanadate, which is known to be a P-type ATPase inhibitor, did not have any effect (up to 10 mM) on the enzyme activity. The enzymatic activity of PstB was also resistant to inhibition up to 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 guanidine hydrochloride (8 M) and taking boiled samples (5 min at 100 °C). As expected, treatment with guanidine hydrochloride caused a significant reduction in the PstB-ATPase activity. Surprisingly, samples following boiling did not show any significant reduction in enzyme activity (Fig. 4A, inset). 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 a 3-h 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 nonhydrolyzable 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 nonspecific reaction of FSBA with free amines in the buffer. Samples were incubated (30 min at 30 °C) with FSBA (0–4 mM), and then ATPase activity was determined following addition of different concentrations (250–750 μM) of substrate solution. FSBA preincubation significantly inhibited the PstB-ATPase activity (Fig. 5A). The $K_i$ 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 $K_m$ value of the enzyme (70 ± 5, 99 ± 11.5, and 155 ± 26 μM in presence of 0, 1.5, and 3 mM FSBA, respectively), but $V_{max}$ remained unaltered (Fig. 5B).

To confirm further that the recombinant PstB is an ATPase, we studied a mutant (D188K) that is known to affect the ATP hydrolyzing ability of the bacterial ABC proteins (22, 39, 40). Unlike wild type 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 wild type and D188K by plotting as a function of amount of protein used in the assay exhibited slopes that are very distinct from each other (Fig. 6B).

**Characterization of Mycobacterial PstB**

**Table I**

<table>
<thead>
<tr>
<th>Organism</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (μmol P/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. tuberculosis</td>
<td>71.5 ± 5.88 (7)</td>
<td>0.122 ± 0.02 (7)</td>
</tr>
<tr>
<td>E. coli</td>
<td>23.9 ± 2.96 (3)</td>
<td>0.322 ± 0.01 (3)</td>
</tr>
</tbody>
</table>

**Table II**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>PstB-ATPase</th>
<th>MalK-ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>Michaelis constant (μM)</td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>71.5 ± 5.88 (7)</td>
<td>70</td>
</tr>
<tr>
<td>CTP</td>
<td>130 ± 8.14 (3)</td>
<td>542</td>
</tr>
<tr>
<td>GTP</td>
<td>211 ± 16.3 (3)</td>
<td>70</td>
</tr>
</tbody>
</table>

The affinities for ATP, CTP, and GTP were determined by monitoring the substrate hydrolyzing 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. $K_m$ values for each substrate were calculated from typical Lineweaver-Burk plots. Results are expressed as mean ± S.D., and number of experiments are indicated in parentheses. Data for S. typhimurium MalK-ATPase are from Ref. 20.

**Characterization of thermo-stable bacterial ATPases**

By guest on September 7, 2017 http://www.jbc.org/ Downloaded from
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 \( \sim 60\% \) of initial value, which was noticed between 30 and 60 min of heat incubation; however, the remaining \( \sim 40\% \) was found to be heat-resistant. Interestingly, mycobacterial PstB even after heat treatment (80 °C) for 1 h retained \( \sim 89\% \) (loss = \( \sim 11\% \)) 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 a congenial environment for their survival, several phosphate importers are present in mycobacteria (2). Pst system is one of such importers belonging to the superfamily of ABC permeases and is known to be operative in...
bacteria in phosphate-limiting conditions (5). Pst is a multisubunit 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. Apart from this, Pst system in *M. tuberculosis* gains additional importance because 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*.

**Fig. 5. Inhibition of mycobacterial PstB-ATPase activity by FSBA.** A, PstB was incubated with indicated concentrations of FSBA at 30 °C for 30 min. ATP hydrolysis was then initiated with addition of indicated concentrations of ATP and monitored as mentioned under “Experimental Procedures.” Points in the graph are interpolated with the data obtained using 250 μM ATP (final concentration). Results are means ± S.D. 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 hydrolyzing ability of the wild type and the mutant was determined in presence of varied concentrations of ATP following standard conditions as mentioned under “Experimental Procedures.” The reproducibility was checked in four independent experiments. B, ATP hydrolyzing ability of the D188K mutant as a function of amount of protein used in the assay. ATPase activity for both wild type and mutant was monitored with increasing concentrations of protein in the presence of 1 mM ATP. Data represent means ± S.D. from three independent experiments.
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 the result of incorrect folding of the expressed protein (43, 44). The renaturation of solubilized protein in urea or guanidine hydrochloride proved to be a difficult task because 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*-lauroylsarcosine (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 been shown previously 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 mass when samples were subjected to SDS-PAGE in nonreducing conditions, suggesting no interaction between intermolecular thiols in forming the putative dimer of the protein (data not shown).

ABC proteins have also been shown to have ATP hydrolyzing 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 nonhydrolyzing ATP analogue with ATP for substrate binding site, which is evidenced by unaltered *V*<sub>max</sub> but increase in apparent *K*<sub>m</sub> 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 hydrolyzing ability of the recombinant PstB (Fig. 6). Thus, these two lines of evidence argue in favor of *M. tuberculosis* PstB being an ATPase.

**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 were expressed in arbitrary units (mean ± S.D.), 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 microcentrifuge tubes (1 μg of protein/reaction). Following addition of 1 mM ATP, samples were incubated for another 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 under “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 microcentrifuge tubes (1 μg of protein/reaction). ATPase activity of each sample was determined as described in A and calculated as percentage of the activity obtained during incubation at 37 °C. Data represent means ± S.D. from three independent experiments.
Although the ATP hydrolyzing ability of mycobacterial PstB exhibited many properties characteristic to typical ABC proteins (Table I), subtle differences made it distinct from others, like MalK or HisP from S. typhimurium. For example, among different nucleotides, ATP has been found to be a preferred substrate for the recombinant PstB (Table II). Similarly, divalent cations (Mg$^{2+}$ and Ca$^{2+}$) that have been reported to be stimulatory for the enzymatic activity (20, 21) had no effect on mycobacterial PstB-ATPase (Fig. 3). Like membrane-bound stimulatory for the enzymatic activity (20, 21) had no effect on activity of these proteins to which had a strong inhibitory effect on their activity, was found to be true for HisP or MalK (20, 21), with excellent laboratory facilities. We are grateful to Dr. T.

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 toward thermophilic nature of PstB will only be unraveled once the crystal structure is solved and complete analysis can 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 a more pivotal role in bacteria than a cursory glance reveals.

Acknowledgments—We are thankful to Dr. A. Ghosh (Director, Institute of Microbial Technology, Chandigarh, India) 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 J. Prasad for providing us with excellent technical assistance during the course of this investigation and acknowledge the secretarial help provided by S. Gupta.

REFERENCES

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

doi: 10.1074/jbc.M105401200 originally published online September 20, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M105401200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 48 references, 18 of which can be accessed free at http://www.jbc.org/content/276/48/44590.full.html#ref-list-1