Expression, Purification, and Characterization of Pyruvate Kinase from *Mycobacterium tuberculosis*: A Key Allosteric Regulatory Enzyme

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Abstract

**Background:** The current research aims to isolate pyruvate kinase (Pyk) gene from *Mycobacterium tuberculosis* and expression of the gene (Rv1617) to obtain a purified enzyme. The enzyme activity and secondary structural features were assessed through biochemical assays and circular dichroism (CD) spectroscopy, respectively. **Methods:** The Pyk-encoding gene from the complete genome of *M. tuberculosis* was cloned, sequenced, and expressed in *Escherichia coli* BL21 (DE3). The enzyme was purified by nickel-nitrilotriacetic acid affinity chromatography and enzyme activity was determined by a lactose dehydrogenase-coupled assay system. Further, far ultraviolet CD spectra of the enzyme and the substrate bound enzyme were analyzed using a Jasco J712 spectrophotometer. **Results:** A single protein with an approximate molecular mass of 54 kDa was purified and a specific activity of 5.31 units/mg was determined from purified *M. tuberculosis* Pyk. The activity of the enzyme indicating a protein is defined by separate domain for each catalytic function. The secondary structure analysis of CD spectra of the recombinant Pyk has revealed a content of 17% α-helix, 34% β-sheet, and 49% turns in the enzyme. **Conclusion:** The growing evidence has impacted *M. tuberculosis* central carbon metabolism as a key determinant of the survival and pathogenicity in the host. The purified Pyk was observed to have increased enzyme activity in all steps of purification. Retention of Pyk activity indicates a possible catalytic role for the lower part of the glycolytic pathway. The overall results of the spectra obtained from the CD suggest that the substrate phosphoenolpyruvate and adenosine diphosphate binding to the enzyme can cause conformational changes resulting in the exposure or shielding of residues susceptible to modification.

**Keywords:** Allosteric regulation, bacterial metabolism, circular dichroism, *Mycobacterium tuberculosis*, pathogenesis, pyruvate kinase

INTRODUCTION

Over the past several decades, our knowledge concerning the metabolism and physiology of *Mycobacterium tuberculosis* during the host infection is very limited.[1-4] During infection, the bacteria use nutrients such as carbon and nitrogen sources for growth, survival, and replication.[5] It is observed that a metabolic shift from an aerobic to anaerobic modes and expression of genes encoding enzymes of the glycolytic pathway are found to be upregulated in adapting the hypoxic host environment inside the macrophage. Bai et al. (2009) have demonstrated that in the persistent state of *M. tuberculosis*, nearly 70% of the energy required by the bacteria is provided by the glycolysis and found to be central to the mycobacterial survival in the hostile environment.[6] Several in vivo studies have suggested that *M. tuberculosis* maintains a functional glycolytic pathway which plays a crucial role during the growth of the organism.[7] The final step in glycolysis involves the production of pyruvate and adenosine triphosphate (ATP) from phosphoenolpyruvate (PEP) and adenosine diphosphate (ADP) catalyzed by pyruvate kinase (Pyk). The tricarboxylic acid cycle is then primed by the...
pyruvate with other carbon metabolites.[8] The flux through and out of glycolysis is monitored by Pyk, which acts as the rate-limiting step.[7] The switch between glycolysis and gluconeogenesis is regulated by the Pyk that averts a futile metabolic cycle.[9,10] In most studied organisms, Pyk activity is regulated by the upper glycolytic metabolite fructose-1, 6-bisphosphate.[11-13]

*Mycobacterium bovis* which causes bovine tuberculosis lacks the ability to metabolize fermentable carbon source due to the presence of inactive Pyk.[14] It was shown that gluconeogenesis through the combined reaction of pyruvate carboxylase and PEP carboxykinase was indispensable for the survival of *M. tuberculosis* in mice, which indicate that glycolytic substrate cannot be scavenged from this host.[1] In *M. tuberculosis* H37Rv, it was observed that the Pyruvate kinase A upregulates the expression of a gene which is involved in fatty acid β-oxidation in the absence of Pyk.[14] Since from several decades, the research toward uncovering the role of Pyk in the physiology of *M. tuberculosis* is very limited. Recently, Tahel *et al.* using biochemical and genetic approaches defined the essential role of Pyk and glycolysis and as a sole carbon source for odd-chain fatty acid metabolism in *M. tuberculosis*.[15] The primary route of the oxidation of glucose by *M. tuberculosis* was found to be the glycolysis pathway.[16] Several studies based on the enzyme assay and radiorepiometric techniques reveal that about 70%–90% of glucose oxidized in *Mycobacterium smegmatis* as well as in *M. tuberculosis* H37Rv was routed through Embden–Meyerhof–Parnas pathway and the rest through the pentose-phosphate pathway, thus appear to be absent in these species.[16]

From various sources such as *E. coli,*[17] *Bacillus subtilis,*[18] and *Bacillus licheniformis,*[19] the Pyk has been purified to homogeneity but failed to study their structures due to their instability. Pyk was purified from *M. smegmatis* by severe step procedure resulting in a 410-fold increase in specific activity with 16% recovery. The enzyme having four identified subunits of molecular weight 57,400 appeared to be a regulatory enzyme and exhibited a unique property of its activation by glucose-6-phosphate.[20]

Genome sequencing of *M. tuberculosis* has identified probable genes involved in glucose metabolism through homology search. Computational biology approaches aid the process of characterization of the pathway and further facilitates in deciphering species-specific enzyme differences. Specific differences in the biochemical properties of metabolic enzymes between the host and the pathogen have been exploited to develop drugs targeting the pathogen. In contrast to the above review, our earlier research has been reported that the interaction of the proteins in the host is essential for the pathogen to establish infection, growth, and survival in the diverse environment. We used computational methods to predict and overlaid the predicted interactions onto construct the network. Analysis of the biological processes of the protein suggests that the pathway regulating the key nutrient source for the growth of the pathogen in the host was investigated. The proteins involved in the network were analyzed to identify as a hub protein. The Pyk was found to be one of the essential proteins for the bacteria for infection and survival in the host. In this relevance, we suggest that Pyk can be considered as the potential target in the *M. tuberculosis* H37Rv to develop a new molecule that can knockout the pathway.[19] To explore the relation between the structure of the enzyme and its activity at the molecular level, we proceeded to isolate, purify, and characterize Pyk from *M. tuberculosis.*

### Methods

**Bacterial strains, plasmids, and enzymes**

The plasmids pUC29 and pET21a were propagated in *E. coli* strain (Chromos Biotech Pvt Ltd, India). *M. tuberculosis* H37Rv was used as a source of genomic DNA for the amplification and cloning of the mycobacterial Pyk gene in *E. coli* BL21 (DE3) strain. All DNA manipulations, plasmid isolation, restriction endonuclease digestion, and transformation were carried out according to the standard protocol.[21]

**Construction of expression plasmid-containing pyruvate kinase gene**

Each forward and reverse primers contained additional sequences at the 5’ end including Ndel and Xhol restriction sites (yellow colored nucleotides), respectively, are used for efficient cloning of polymerase chain reaction (PCR)-amplified DNA in the cloning and expression vectors. The DNA sequences of forward and reverse primers for the gene shown below:

**PK-Ndel-FP:** 5’–GCTGTCCATATGACGACGCGGGAA AATCGTCTGC– 3’.

**PK-Xhol-RP:** 5’–GCTGTCCCTCGAGGACGTAC TCTTCCCCGGATCGT– 3’.

The PCR reaction mix containing template (gDNA), forward and reverse primer (100 mg/μl), deoxynucleotide triphosphates (10 mM), ×10 ChromTag assay buffer, ChromTag enzyme (3U/μl), GC solution (×2.5), and nuclear-free water was set up as given in Table 1.

**Preparation of pyruvate kinase gene for cloning**

Pyk gene (1 μg) in deionized water was mixed with 10 μl of ×10 assay buffer, 30 μl of Xhol, and 10 u/μl of Ndel. The total reaction mix of volume 100 μl was incubated at room temperature

| Steps                  | Temperature (ºC) | Time     | Number of cycles |
|------------------------|------------------|----------|------------------|
| Initial                | 94               | 5 min    | 1                |
| denaturation           | 94               | 30 s     | 35               |
| Denaturation           | 55               | 30 s     |                  |
| Annealing              | 72               | 1.5 min  |                  |
| Extension              | 72               | 15 min   | 1                |
| Final extension        | 10               | Infinite |                  |
| Hold                   |                  |          |                  |

Table 1: Cyclic conditions for the polymerase chain reaction of pyruvate kinase gene
for 2 h. About 4 μl of the reaction mixture was subjected to electrophoresis in 1% of agarose gel to conform the digestion, remaining 96 μl of the reaction mixture was subjected for salt precipitation to remove the XhoI and Ndel traces by adding one-third volume of 10M ammonium acetate and incubated at 4°C for 15 min. The suspension was centrifuged at 10,000 rpm for 15 min, followed by the addition of 1 μl of glycogen and an equal volume of isopropanol and was centrifuged at 10,000 rpm for 10 min. The pellet was washed with 1 ml of 70% of ethanol and air-dried and stored at −20°C until further use.

**Transformation into competent cells**

The amplified DNA fragment corresponding to Pyk gene was gel purified, restriction digested, and ligated into the cloning vector pUC29 at Ndel and XhoI restriction sites giving construct pUC29/Pyk. The probable clones were screened and confirmed again by restriction digestion of vector and PCR product, where the reaction mixture was incubated at 37°C for 2 h. Further, 5 μl of the ligation reaction mix was transformed into 50 μl of DH5α competent cells. Incubation was carried out at 4°C on ice for 30 min and subjected to heat shock at 42°C for 45 min. Thus, the volume was made up to 1 ml with Lysogeny broth (LB) by quick chilled on ice for 2 min and incubated at 37°C for 1 h in shaking incubator. The cells were then pelleted at 3,000 rpm for 3 min. The supernatant was discarded and cells were plated on Ampicillin – Xgal – IPTG culture plate (to 100 μl of LB media add 100 μl ampicillin [initial concentration: 100 mg/ml], 120 μl X-gal [initial concentration: 100 mg/ml], and 24 μl isopropylβ-D-thiogalactopyranoside (IPTG) [initial concentration: 1 M]) and incubated overnight at 37°C for the growth of the colonies.

**DNA sequencing**

The Pyk gene cloned into the pUC29 vector was sequenced using Thermal Cycler ABI2720 with model number: ABI 3500 XL Genetic Analyzer. The amplification was performed with the above-mentioned sequencing reaction mixture and the PCR cycle conditions such as initial denaturation at 96°C for 1 min followed by 25 cycles of denaturation at 96°C for 10 s, hybridization at 50°C for 5 min, and elongation at 60°C for 4 min. The obtained DNA sequence data were further analyzed by aligning to the reference sequence using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.gov/Blast.cgi). The DNA sequence was submitted for publication to the GenBank database for no change in base pairs.

**Subcloning of pyruvate kinase in pET21a vector**

As illustrated earlier, the cloned DNA was taken and digested will EcoRI. The released insert was gel eluted and ligated into pET21a vector at Ndel and XhoI restriction sites. A 10 μg of the ligation reaction mixture was transformed into 100 μg of E. coli BL21 (DE3) cells. Incubation at 4°C (ice) for 30 min and heat shock was given at 42°C for 90 s, which was followed by quick chilled on ice for 2 min and increased the volume up to 1 ml with LB media. The above reaction mixture was incubated at 37°C for 1 h in a shaking incubator. Finally, the cloned cells were then pelleted at 3,000 rpm for 3 min by centrifugation and the supernatant was discarded to collect the cells, which were plated on ampicillin by incubating overnight at 37°C. The probable clones were screened and confirmed for further expression.

**Protein expression and purification**

**Small-scale expression**

A single clone of E. coli culture consisting of recombinant Pyk gene has been grown by incubated into 1 mM IPTG in a flask and induced at 30°C in shaking incubator until culture reaches an optical density (OD) 600 = 0.6 to ensure the growth of the cells at log phase. Before incubation, 4 h after induction and 16 h (overnight) induction samples were processed for protein extraction and loaded on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**Large-scale induction and protein purification**

Purification of protein was performed from 4 h induction culture. A single colony was incubated for big batch expression and purification of the protein of interest. The culture was grown till OD 600 = 0.6 and incubated with 1 mM IPTG at 37°C for 18 h. Followed by sonication of the cell pellet in ×1 lysis buffer, 2M and 4M urea lysis buffer centrifuged at 10,000 rpm for 15 min at 4°C. After centrifugation, the supernatant was processed for protein purification using nickel-nitritolriacetic acid (Ni-NTA)-based affinity column purification with imidazole gradient elution. Thus, the purified fraction of the Ni-NTA column was loaded at 12% of SDS-PAGE, and the resolved protein was stained with Coomassie brilliant blue. Finally, the purified fraction was dialyzed against ×1 phosphate-buffered saline.

**Enzyme activity by pyruvate kinase assay**

The activity of M. tuberculosis Pyk was determined by a lactose dehydrogenase-coupled assay system by measuring the decrease in absorbance at 340 nm, resulting from the oxidation of nicotinamide adenine dinucleotide (NADH). The reaction is as given below:

\[ \text{NADH} + \text{pyruvate} \rightarrow \text{lactate} + \text{NAD} \]

The standard reaction mixture contained 0.05M imidazole-HCL buffer, pH 7.6, 0.12M potassium chloride, 0.062M magnesium sulfate, 5 mM ADP, 6.6 mM NADP, 45 mM PEP, and diluted lactate dehydrogenase to a concentration of 1300–1400 units/ml in the above imidazole buffer to make the final volume. The Bradford assay method was also used to determine the concentration of total Pyk in the sample. The protein concentration was determined from the absorbance at 595 nm on a spectrophotometer.

**Circular dichroism of Mycobacterium tuberculosis pyruvate kinase**

Far-ultraviolet (UV) circular dichroism (CD) measurement was conducted using a Jasco J712 spectrophotometer. Wavelength scans in the far-UV regions of 190–260 nm were performed in 0.1-cm path length Quartz SUPRASIL (HELLMA) precision cell at room temperature. The scan rate was 100 nm/min and the bandwidth was 2 nm. The protein concentration of 0.8 mg/ml was used for obtaining the CD spectrum.
RESULTS

Cloning and sequencing of pyruvate kinase from Mycobacterium tuberculosis

PCR using gene-specific primers and genomic DNA from M. tuberculosis resulted in the amplification of DNA fragment corresponding to the Pyk gene. The amplified DNA fragment was ligated to pUC19 vector DNA, yielding recombinant plasmid pUC19/Pyk. The DNA fragment corresponding to Pyk gene from recombinant pUC19 was subcloned in the expression vector pET21a, and their identity was confirmed by DNA sequencing results demonstrating that Pyk gene been cloned in this study was identical to the published sequence, and there was no any changes of base pairs (GenBank Accession No. KU043311).

Expression and purification of recombinant pyruvate kinase

E. coli BL21 (DE3) was transformed with the pET21a/Pyk construct and after 5 h of incubation by IPTG; high levels of recombinant Pyk were observed. Small-scale culture was used to test the solubility of the recombinant Pyk, which expressed in the cytoplasm in the insoluble form and hence it was treated with 2M and 4M urea lysis buffer. The molecular mass of the expressed protein in the bacterial lysis extract was approximately 54 kDa. Twenty milliliters of the soluble lysate extract were incubated with 1 ml of Ni-NTA affinity resin, which efficiently retained the recombinant protein as demonstrated by the SDS-PAGE profile of the flow-through sample. After washing of the column, the recombinant Pyk was eluted with 250 mM of imidazole. The eluted sample presented a single band in the SDS-PAGE, corresponding to purified recombinant Pyk [Figure 1].

Activity of pyruvate kinase

The specific activity determined from purified M. tuberculosis Pyk is good agreement with values determined for other bacterial Pyk enzymes. In the ADP-dependent conversion of PEP, the purified Pyk enzyme showed a good catalytic activity at specific conditions [Figure 2a]. The concentration of the protein Pyk was found to be 0.16 mg/ml. The quantity of protein within the unknown protein sample was 160 ppm (μg/ml) that having 5.31 units/mg of specific activity [Figure 2b].

Secondary structural studies of pyruvate kinase

The secondary structure of the recombinant Pyk was evaluated by CD. The far-UV CD spectrum of the recombinant Pyk from M. tuberculosis was similar to the far UV-CD spectra reported for other Pyks. The spectrum shows peaks at 194 nm and troughs at 216 and 223 nm, which indicates a presence of mixed α-helix and β-sheet secondary structures [Figure 3a]. The secondary structure from the CD spectra of the recombinant Pyk was yielding a content of 17% α-helix, 34% β-sheet, and 49% turns. The possibility of changes in the conformation of the enzyme was accomplished by the binding of the substrates such as PEP and ADP as determined by the CD under far-UV. The native enzyme shows well-determined negative cotton effect at approximately 220 nm indicating the presence of β-sheet structure in the enzyme. The Pyk (enzyme) with PEP (substrate) in contrast exhibits the same negative cotton effect at 224 nm [Figure 3b], whereas enzyme PEP and ADP complex has a spectrum with negative cotton effect at 224 nm [Figure 3c] with an early increase in the band curve [Figure 3d].

DISCUSSION

This study was carried out to clone, express, and purify the glycolytic enzyme Pyk, encoded by the Pyk gene from M. tuberculosis. The purified protein was further characterized by the enzyme activity and secondary structure analysis. The sequence of this ubiquitous enzyme is highly conserved across prokaryotic and eukaryotic species and a number of amino acids are essential for catalytic activity. These results support that pET21a is useful for high-level expression and efficient purification of recombinant mycobacterial proteins. The enzyme is also stable at a low temperature, at which many bacterial Pyks is unstable. For example, the Pyk from Bacillus licheniformis is unstable in a dilute buffer but stable only in a buffer containing 50% of glycerol or in the presence of the PEP substrate. The enzyme is also stable at a low temperature, at which many bacterial Pyk is unstable. However, the enzyme from M. tuberculosis was found to be stable for at least 2 weeks or more at 4°C and for more periods at −20°C in 50% of glycerol. Even during carbohydrate catabolism in M. tuberculosis, few enzymes are a key for glycolysis because of the irreversibility of the reactions they catalyze such as glucokinase, phosphofructokinase, and Pyk. The first step of glycolysis is catalyzed by glucokinase when glucose is phosphorylated to form glucose 6-phosphate which is encoded by ppgk gene. By the approach of saturation mutagenesis, ppk gene was shown to be required for survival of M. tuberculosis during infection of mice. These studies have suggested the requirement of glucose as a significant energy source. The phosphofructokinases encoded by pfkA and pfkB genes were observed to be essential for growth of M. tuberculosis in vivo by saturation mutagenesis. [11]
Till the date, no report confirmed that Pyk is essential to \textit{in vivo} growth of \textit{M. tuberculosis}, although Rubin et al. reported that optimum growth of \textit{M. tuberculosis} in glucose-containing media requires Pyk gene.\textsuperscript{[13]} Some results report that Pyk is not essential for \textit{M. tuberculosis} pathogenesis in mice, it is important to note that mouse models do not exactly mimic the human disease, and that may enzymatic reaction, though nonessential for mouse infection was shown to be essential to infect higher organisms.\textsuperscript{[25]} Studies of Tahel et al. describe the central role of Pyk in actively growing \textit{M. tuberculosis} and its pathogenesis.\textsuperscript{[15]} Their data suggest that Pyk is crucial to facilitate catabolism of glycolytic and gluconeogenesis substrates and is essential for the detoxification of sugar phosphates during the glucose metabolism. Pyk is needed to keep \textit{M. tuberculosis} primed at all times to rapidly react to potential changes in carbon availability in its environment.

We focus on analyzing the key secondary structural elements of Pyk during the catalyzing the final and irreversible step in glycolysis, the transphosphorylation of PEP and ADP to pyruvate and ATP. The allosteric regulation of Pyk on binding to the fructose 1, 6-bisphosphate was also characterized by CD analysis. The apparent changes in the conformations of the protein on binding of the substrate are whether related to the changes in catalytic activity of Pyk remains to be estimated in further studies. It was reported that the true substrate of the Pyk is an ADP-Mg\textsuperscript{2+} complex, whereas free ADP is a potent inhibitor.\textsuperscript{[26]}

\textbf{Figure 2:} Plot illustrating enzyme activity of pyruvate kinase and its concentration in unknown sample. (a) Plot illustrating the enzyme activity of pyruvate kinase isolated from \textit{Mycobacterium tuberculosis} and (b) Plot showing the concentration of protein in the unknown sample.

\textbf{Figure 3:} Circular dichroism spectrum of pyruvate kinase in unbound and bound state with phosphoenolpyruvate and adenosine diphosphate. Circular dichroism spectrum of the (a) Purified native pyruvate kinase from \textit{Mycobacterium tuberculosis}; (b) Protein with substrate phosphoenolpyruvate; (c) Protein with phosphoenolpyruvate and adenosine diphosphate; (d) Spectra of native pyruvate kinase enzyme (blue), enzyme (pyruvate kinase) bound to phosphoenolpyruvate substrate (red), and pyruvate kinase bound with phosphoenolpyruvate and adenosine diphosphate (black). A Jasco J712 circular dichroism spectrophotometer machine was used to record the circular dichroism values.
It was also suggested that PEP is bound by the enzyme before pyruvate is released and that both events precede the ordered binding of ADP and the release of magnesium ATP.[27] The nucleotide and the substrate bind independently and their individual binding spectra can be visualized during the formation of the abortive complex ADP + enzyme + acceptor substrate which is shown in the Figure 3d. This spectrum illustrates the changes in the conformation of the Pyk upon binding with the substrate and with ADP in the presence of the PEP.

The CD spectra analysis revealed that the *M. tuberculosis* Pyk retained the characteristic α-helical and β-sheet structure. It has been revealed that all the known Pyk have the absolute requirement for two divalent metal ions per monomer, usually Mg²⁺ or Mn²⁺ and one monovalent metal ion such as K⁺ or NH₄⁺ per monomer for its activity.[28,29] In these two metal ions, one divalent metal ion binds to free enzyme and facilitates the binding of the substrate PEP, while the second metal ion binds to the enzyme through a complex with the substrate ADP.[30,31] Earlier work has suggested that the conformational changes and their effect on rate may be more dramatic with an alternate substrate. Such conformational changes in protein structure and substrate orientation have been revealed to have dramatic effects on the rate constants of enzyme-catalyzed reaction.[32]

Since the CD spectrum of protein is dependent on conformations,[33] the evidence for a conformational change in Pyk on binding with substrate was studied by employing this technique. The actual mechanism in the secondary structural changes for alteration of the protein conformation can be clearly understood by both far- and near-UV CD spectra.[31] But at present, we recorded only the far-UV CD spectrum, which can show only the secondary structural changes just by the binding of substrates PEP and ADP at room temperature. The spectra show the band as having a negative cotton effect at the wavelength of 220–230 nm. The conformational changes in protein relative to these secondary structural changes can be more clearly understood by scanning the protein at near-UV CD spectrum.

**Conclusion**

The information available regarding Pyk was limited and the structures of the bacterial Pyk owing to their instability were very minimum. This instability encounters more difficulty in isolating and purifying them in a large amount. It is well known that Pyk is one of the three regulatory enzymes of glycolysis from which all the prokaryotic organisms derive their energy at maximum and which control the activity of the pathway that catalyzes the formation of pyruvate from PEP by producing a molecule of ATP. Since the enzyme is the key intersection in the network of metabolic pathways, in the present work, the purification protocol was adopted. The purified Pyk was observed to have increased enzyme activity in all steps of purification. Retention of Pyk activity indicates a possible catalytic role for the lower part of the glycolytic pathway. The overall results of the spectra obtained from the CD suggest that the substrate PEP and ADP binding to the enzyme can cause conformational changes, resulting in the exposure or shielding of residues susceptible to modification. The amount of secondary structure of the enzyme as determined by spectral analysis is virtually identical, indicating that there is a small structural change in enzyme by the addition of PEP and ADP. This small structural change in the secondary structure could bring about changes in the whole protein conformation.

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**Conflicts of interest**

There are no conflicts of interest.

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