Regulation of glucose metabolism in the actinomycetes amycolatopsis methanolica and streptomyces coelicolor A3(2)
Alves, Alexandra Maria da Costa Rodrigues

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Chapter 3

Characterization and phylogeny of the *pfp* gene of *Amycolatopsis methanolica* encoding PPi-dependent phosphofructokinase


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Chapter 3

The actinomycete Amycolatopsis methanolica employs a PP_i-dependent phosphofructokinase (PP_i-PFK; E.C. 2.7.1.90) during growth on glucose with biochemical characteristics similar to both ATP and PP_i-dependent enzymes. A 2.3-kb Pvu II fragment hybridizing to two oligonucleotides based on the amino-terminal amino acid sequence of PP_i-PFK was isolated from a genomic library of A. methanolica. Nucleotide sequence analysis of this fragment revealed the presence of an open reading frame encoding a protein of 340 amino acids with high similarity to phosphofructokinase proteins. Heterologous expression of this open reading frame in Escherichia coli gave rise to a unique 45-kDa protein displaying high PP_i-PFK activity. The open reading frame was therefore designated pfp encoding the PP_i-PFK of A. methanolica. Upstream and transcribed divergently from pfp a partial open reading frame (aroA) similar to 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase encoding genes was identified. The partial open reading frame (chiA) downstream from pfp was similar to chitinase genes from Streptomyces species. A phylogenetic analysis of the ATP and PP_i-dependent proteins showed that PP_i-PFK enzymes are monophyletic, suggesting that both types of phosphofructokinase evolved from a common ancestor.

INTRODUCTION

The actinomycete Amycolatopsis methanolica is one of the few known Gram-positive bacteria which can use methanol as growth substrate (de Boer et al., 1990a). Until recently, research on A. methanolica focussed on methanol metabolism (Alves et al., 1994) and on regulation of aromatic amino acid biosynthesis (Euverink et al., 1992, 1995). It was shown previously that A. methanolica metabolizes glucose via the Embden-Meyerhof-Parnas pathway (Alves et al., 1994)

In most organisms the glycolytic flux is controlled at the level of phosphofructokinase (PFK) (Uyeda, 1979), which catalyses the irreversible ATP-dependent phosphorylation of fructose-6-phosphate (E.C. 2.7.1.11). The ATP-dependent PFKs are allosterically regulated, have a high $K_m$ for their substrate fructose-6-phosphate and a neutral pH optimum. Three classes can be recognized. The bacterial enzyme has a tetrameric structure of identical subunits with a molecular mass of 35-kDa and is allosterically regulated by phosphoenolpyruvate and ADP (Uyeda, 1979). Like the bacterial enzyme, the mammalian enzyme is composed of four identical subunits, although the subunits are substantially larger (85-kDa). The yeast enzyme is an octamer composed of two non-identical subunits with molecular mass of 112 and 118-kDa. Sequence comparisons between the three classes of ATP-dependent PFK proteins indicate that the yeast and mammalian enzymes have arisen as a result of gene duplication and fusion events. The activity of the yeast and mammalian enzymes is generally regulated by citrate, ATP and fructose-2,6-bisphosphate (Uyeda, 1979; Fothergill-Gilmore and Michels, 1993).
In some bacteria, plants and protozoa a pyrophosphate (PP\textsubscript{i}) dependent PFK (E.C. 2.7.1.90) is encountered (Mertens, 1991). In contrast to the ATP-dependent PFK, the PP\textsubscript{i}-dependent enzyme catalyzes the reversible phosphorylation of fructose-6-phosphate (Reeves et al., 1974). A correlation between anaerobic metabolism and the presence of PP\textsubscript{i}-dependent PFK has led to the suggestion that this enzyme is better adapted to anaerobiosis than its ATP-dependent counterpart. In Propionibacterium freudenreichii and a number of protists a homodimeric PP\textsubscript{i}-PFK is present that is not allosterically regulated (O’Brien et al., 1975; Mertens, 1991). This class of PP\textsubscript{i}-PFK is characterized by a low Km for fructose-6-phosphate and an acidic pH optimum for the phosphorylation of fructose-6-phosphate. In plants PP\textsubscript{i}-PFK is present as a heterotetrameric enzyme composed of two non-identical subunits with a molecular mass of 60 and 65-kDa (Balogh et al., 1984). Like the yeast and mammalian enzymes, the activity of the plant PP\textsubscript{i}-PFK is regulated by fructose-2,6-bisphosphate, the Km for fructose-6-phosphate is high and it has a neutral pH optimum for the glycolytic reaction.

We have previously purified and characterized the PP\textsubscript{i}-dependent PFK of \textit{A. methanolica} (Alves et al., 1994). As is typical for PP\textsubscript{i}-PFK enzymes, the enzyme activity was not allosterically regulated and catalyzed the reversible phosphorylation of fructose-6-phosphate. However, the \textit{A. methanolica} PFK has a number of biochemical characteristics in common with the bacterial ATP-dependent enzymes: it is a tetramer of identical subunits, the Km for fructose-6-phosphate is high and it has a neutral pH optimum. Furthermore, the amino terminal amino acid sequence of this enzyme is more similar to ATP-dependent than to PP\textsubscript{i}-dependent enzymes.

These characteristics of the PP\textsubscript{i}-PFK of \textit{A. methanolica} which are intermediary between ATP and PP\textsubscript{i}-dependent PFKs prompted us to characterize this enzyme in greater detail. This paper describes the cloning, primary structure and phylogeny of this unusual PP\textsubscript{i}-PFK from \textit{A. methanolica}.

MATERIALS AND METHODS

**Bacterial strains and plasmids.** Bacterial strains and plasmids used in this study are listed in Table 1.

**Media and growth conditions.** \textit{E. coli} strains were grown on Luria-Bertani (LB) medium at 37°C (Sambrook et al., 1989). \textit{A. methanolica} was grown on complete medium (Vrijbloed et al., 1994) at 37°C. When appropriate the following supplements were added (in micrograms per milliliter): ampicillin (Ap), 100; 5-bromo-4-chloro-3-indolyl-ß-D-galactoside, 20; isopropyl-ß-D-thiogalactoside (IPTG), 0.1 mM; kanamycin (Km), 25. Agar was added for solid media (1.5% [wt/vol]).

**DNA manipulations.** Plasmid DNA was isolated using the alkaline lysis method of Birnboim and Doly (Birnboim and Doly, 1979) followed by equilibrium centrifugation in a cesium chloride gradient for preparative DNA isolations (Sambrook et al., 1989). Chromosomal DNA was isolated.
following cell lysis with sodium dodecyl sulphate as described by Hintermann et al. (Hintermann et al., 1981). DNA modifying enzymes were obtained from Boehringer (Mannheim, Germany) and were used according to the manufacturer’s instructions. E. coli strain DH5α was electrotransformed with the electrocell manipulator 600, (Biotechnology & Experimental Research Inc, San Diego, California) according to the manufacturer’s instructions. Other DNA manipulations were done according to standard protocols (Sambrook et al., 1989).

**Southern hybridizations.** Chromosomal DNA from *A. methanolica* was digested with the appropriate restriction enzymes, separated on a 0.8% (w/v) agarose gel and blotted onto a high-bond nylon membrane supplied by Qiagen (Basel, Switzerland), via the alkaline transfer method (Sambrook et al., 1989). Southern hybridizations were done at 68°C with oligonucleotides (100 pmol) labelled with the DIG oligonucleotide tailing kit from Boehringer (Mannheim, Germany). The membrane was subsequently washed twice with 2XSSC (1 X SSC is 0.15 M NaCl plus 0.015M sodium citrate), 0.1% [w/v] sodium dodecyl sulphate (SDS) for 5 minutes and twice with 0.1XSSC, 0.1% [w/v] SDS for 5 minutes.

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**Table 1. Bacterial strains and plasmids used in this study.**

<table>
<thead>
<tr>
<th>Strains and plasmids</th>
<th>Relevant genotype or characteristicsa</th>
<th>Source or reference</th>
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</thead>
<tbody>
<tr>
<td>Strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>supE44 ΔlacU169 (Δ80lacZΔ M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 F thr-1 ara-13 leu-6 azi-8 tonA2 lacY1 minA1 glnU44 gal-6 minB2 rpsL135 malA1 xyl-7 m1t-2 thi-1</td>
<td>Bethesda Research Laboratories</td>
</tr>
<tr>
<td>P678.54</td>
<td></td>
<td>Adler et al. 1967</td>
</tr>
<tr>
<td>MC1061</td>
<td>hsdR mcrB araD139Δ (araABC-leu) 7679 ΔlacX74 galU galK rpsL thi</td>
<td>Meisner et al. 1987</td>
</tr>
<tr>
<td>A. <em>methanolica</em> WVI</td>
<td>pMEA300-free strain</td>
<td>Vrijbloed et al. 1995a</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pWV138</td>
<td>Km', E. coli-A. <em>methanolica</em> shuttle vector</td>
<td>Vrijbloed et al. 1995b</td>
</tr>
<tr>
<td>pAA601</td>
<td>Ap', 6-kb ApaI fragment in pBluescriptKS*</td>
<td>This study</td>
</tr>
<tr>
<td>pAA101</td>
<td>Ap', 2.3-kb PvuII fragment in pBluescriptKS*, lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>pAA108</td>
<td>Ap', 2.3-kb PvuII fragment in pBluescriptKS*</td>
<td>This study</td>
</tr>
<tr>
<td>pAA123</td>
<td>Km', 12.3-kb fragment in pWV138</td>
<td>This study</td>
</tr>
<tr>
<td>pBluescriptKS*</td>
<td>Ap', phagemid derived from pUC18, lacZ</td>
<td>Stratagene</td>
</tr>
</tbody>
</table>

*aKm', kanamycine resistant; Ap', ampicillin resistant*
Construction of a genomic library. Plasmid pWV138 was derived from pMEA300 which is indigenous to A. methanolica (Vrijbloed et al., 1994, 1995b); it was digested with BamHI and subsequently dephosphorylated with calf intestine phosphatase according to manufacturer’s instructions (Boehringer, Mannheim Germany). Chromosomal DNA of A. methanolica was partially digested with SauIII. DNA fragments ranging in size from 5 to 10-kb were purified from agarose gels and subsequently ligated into the BamHI site of pWV138. E. coli MC1061 was transformed with 0.02 µg ligation mixture. Transformants were scraped off the LB plates and combined into six pools each containing approximately 2,500 colonies.

Isolation and labeling of E. coli minicells. E. coli P678.54 was transformed with the appropriate plasmids and minicells were isolated and labeled with [35S] methionine according to a modified protocol (Clark-Curtis and Curtis, 1983) as described previously (Eggink et al., 1988). The labeled proteins were separated on a 8%(w/v) denaturing acrylamide gel and subsequently analyzed by fluorography (Wensink and Witholt, 1981).

Nucleotide sequencing. A nested set of unidirectional deletions of pAA101 was constructed using exonuclease III and mung bean nuclease (Henikoff, 1984). Dideoxy sequencing reactions were done using T7 DNA polymerase, with unlabeled primers and fluorescein-labeled ATP (Voss et al., 1992). Nucleotide sequencing was done with the Automated Laser Fluorescent DNA sequencer (Pharmacia, Uppsala, Sweden). The nucleotide sequence data were compiled and analyzed using the programs supplied in the PC/GENE software package (Intelligenetics, Mountain View, California).

Tree construction. The amino acid sequences were aligned using ClustalW (Thompson et al., 1994). The programs supplied in the PHYLIP 3.5c package were used to analyze the phylogenetic relationships of the PFK proteins (Felsenstein, 1985). Distance matrices were calculated with PROTDIS using Dayhoff’s PAM 001 matrix (Dayhoff et al., 1978). A phylogenetic tree was subsequently constructed via the neighbor joining method (Saitou and Nei, 1987) as implemented in the NEIGHBOR program. A maximum parsimony tree was constructed with the program PROTPARS (jumble option selected, value =10). The reliability of the branches of the phylogenetic tree was tested via bootstrapping (Felsenstein, 1985) using SEQBOOT and CONSENSE (500 replicates).

Preparation of cell extracts and enzyme assays. E. coli cells containing the appropriate plasmids were grown on LB medium at 37°C. Growth was followed by measuring the optical density of the culture at 660 nm in a 1 cm cuvette on a Hitachi U-1100 spectrophotometer. At A660 = 0.5, IPTG (1 mM) was added to induce transcription from the lac promotor, and growth was allowed to continue for 4 hours. Cells were harvested via centrifugation at 7,000 x g for 15 min at 4°C and resuspended in 50 mM Tris-HCl (pH 7.5). Preparation of cell extracts and determination of PFK activity was done as described previously (Alves et al., 1994). Protein concentrations were determined (Bradford, 1976) using the Bio-Rad (Hercules, California) protein determination kit with bovine serum albumin as standard.

Accession number. The nucleotide sequence presented in this paper was entered into GenBank under accession number U32177.
RESULTS

Design of pfp specific probes

We previously purified the PPi-dependent PFK from *A. methanolica* and determined the amino-terminal amino acid sequence (Alves et al., 1994). Based on the obtained amino-terminal amino acid sequence two degenerated oligonucleotides were designed which corresponded to the segments: DCPGLNAVIRAVV (P38) and KGIEAHGWEIVGF (P37) of the PFK protein of *A. methanolica* (Fig. 3). Since both probes are based on the amino-terminus of PPi-PFK it is very likely that they will hybridize to the same restriction fragments of *A. methanolica* chromosomal DNA. Southern hybridization experiments showed that this was indeed the case (data not shown). We therefore concluded that the probes specifically hybridized to the *pfp* gene.

Cloning the pfp gene from *A. methanolica*

Total plasmid DNA was isolated from two pools of the *A. methanolica* genomic library and digested with restriction enzymes. The *pfp* probes hybridized to restriction fragments from both pools, indicating that the *pfp* gene is present in the *A. methanolica* genomic library. One of the genomic library pools was diluted, plated on LB plates containing kanamycin and replica plated. The colonies were scraped off, total plasmid DNA was isolated and tested for hybridization with the *pfp* probes. This procedure was repeated until a single colony was identified, which contained a plasmid with a 12.3-kb insert. Further subcloning reduced the size of the DNA fragment containing the sequences hybridizing to the *pfp* probe to a 2.3-kb *PvuII* fragment.

The 2.3-kb *PvuII* fragment encodes a 45-kDa protein

In order to determine the number and molecular weights of the proteins encoded on the cloned DNA fragments, a minicell producing *E. coli* strain was transformed with pAA601, containing a 6-kb *ApaI* fragment, and pAA101 which harbors a 2.3-kb *PvuII* fragment (Fig. 1). Newly synthesized polypeptides were labeled with $^{35}$S methionine and were analyzed on denaturing acrylamide gels followed by fluorography (Fig. 2). Two unique proteins with molecular masses of 40-kDa and 45-kDa were present in minicells harboring pAA601, whereas only the latter protein was present in minicells containing pAA101. The subunits of the previously purified PPi-PFK of *A. methanolica* have a molecular mass of 45-kDa (Alves et al., 1994), which strongly suggests that the 2.3-kb *PvuII* fragment contains the complete *pfp* gene.
Expression of the pfp gene in E. coli

Further confirmation of this was obtained by determining the PP\textsubscript{i}-PFK activity in the crude extracts of E. coli DH5\textalpha{} transformed with pAA101 and pAA108, before and after induction with IPTG (Table 2). E. coli cells harboring pAA101, which contains the pfp gene in the same orientation as the lac promoter, had a 100-fold higher PP\textsubscript{i}-PFK activity than E. coli cells transformed with pAA108, which has the pfp gene in opposite orientation. This shows that expression of pfp gene in E. coli is dependent on the lac promoter. PP\textsubscript{i}-PFK activity was absent in E. coli containing pBluescript. We therefore conclude that the pfp gene is located on the 2.3-kb Pvu\textit{II} fragment.

DNA sequence of the A. methanolica pfp gene

The nucleotide sequence of the 2.3-kb Pvu\textit{II} fragment containing the pfp gene was determined in both directions by the dideoxy method (Zimmermann et al., 1990) using

Table 2. Heterologous expression of the gene encoding PP\textsubscript{i}-PFK of A. methanolica in E. coli DH5\textalpha{}.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>PFK activity\textsuperscript{a}</th>
<th>Before induction</th>
<th>After induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAA101</td>
<td>&lt;1</td>
<td>210</td>
<td></td>
</tr>
<tr>
<td>pAA108</td>
<td>&lt;1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>pBluescriptKS\textsuperscript{*}</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Enzyme activity is measured in nanomoles per minute per milligram of protein.
unlabeled primers and fluorescein-labeled ATP (Voss et al., 1992). The nucleotide sequence contains an open reading frame (ORFA) of 340 codons starting with a ATG codon at position 806 which is preceded by a plausible ribosome binding site (Fig. 3). The deduced amino terminal sequence of the protein encoded by ORFA is identical to the amino terminal amino acid sequence of PPi-PFK of *A. methanolica* (Alves et al., 1994). Furthermore, a search of the non-redundant nucleotide database at the National Center for Biotechnology Information with the deduced amino acid sequence of ORFA as query sequence using BlastX revealed extensive similarities to PFK proteins (Fig. 4). We therefore identified ORFA as the PPi-PFK encoding gene (*pfp*) from *A. methanolica*.

The predicted molecular mass of the 340 amino acid from the Pfp protein is 36,229 Da, which is lower than that of the purified PFK subunit. This could be due to the hydrophobic nature of the PFK subunit, which may cause it to migrate anomalously on a denaturing acrylamide gel (Llanos et al., 1993).

Two partial open reading frames were detected upstream (ORFB) and downstream (ORFC) from the *pfp* gene (Fig. 3). The deduced amino acid sequence of ORFB is very similar to 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHP synthase) of *E. coli* (AroG, 52%) (Davies and Davidson, 1982) and *Corynebacterium glutamicum* (AroF, 52%) (Chen et al., 1993) which catalyze the first step in the biosynthesis of aromatic amino acids via the Shikimate pathway. ORFC is transcribed in the same direction as the *pfp* gene. The protein which can be encoded by ORFC is 70% identical with chitinase proteins from various *Streptomyces* species (Fujii and Miyashita, 1993;
PPi-dependent phosphofructokinase of A. methanolica
Tsujibo et al., 1993). We therefore tentatively identified ORFB as \( \text{aroA} \), encoding DAHP synthase, and ORFC as \( \text{chiA} \), encoding chitinase, respectively.

Phylogenetic relationships of PFKs

A previously observed correlation between the presence of \( \text{PPi} \)-dependent PFK and anaerobic metabolism has led to the suggestion that \( \text{PPi} \)-dependent PFK proteins arose on various occasions from ATP-dependent enzymes in response to anaerobic growth conditions (Mertens, 1991). To test this hypothesis a phylogeny of PFK proteins was inferred. A striking feature of the primary structures of PFK is the huge difference in length, which makes an alignment of the full-length protein sequence difficult. Only the sequences containing the amino acids which participate in substrate binding were therefore used in constructing a PFK sequence alignment.

Initially a phylogenetic tree of ATP-dependent PFK proteins was constructed with the neighbor joining method in order to examine their relationships. The PFK proteins from mammals, yeasts, insects and bacteria each formed tight clusters (data not shown). Subsequently two representatives of each cluster were taken and aligned with the sequences of \( \text{PPi} \) dependent proteins, after which a phylogenetic tree was constructed using the neighbor joining method (Fig. 4). The \( \text{PPi} \)-dependent PFK enzymes form a monophyletic group. The branch leading to the \( \text{PPi} \)-dependent enzymes is supported by a high bootstrap value (100%). A parsimony analysis resulted in one most parsimonious
Figure 4. Phylogenetic relationships of ATP and PPi-dependent PFKs. The tree is based on a distance analysis of the PFK segments involved in ATP and fructose-6-phosphate binding from A. methanolica (residues 1 to 223), Bacillus stearothermophilus (residues 2 to 224) (Sakai and Ohta, 1993), Drosophila melanogaster (residues 18 to 269) (Currie and Sullivan, 1994), E. histolytica (residues 36 to 264) (Huang et al., 1995), G. lamblia (residues 69 to 311) (Rozario et al., 1995), Haemonchus contortus (residues 32 to 262) (Kleig et al., 1991), human muscle (residues 16 to 266) (Nakajima et al., 1987), Lactococcus lactis (residues 2 to 224) (Llanos et al., 1993), Naegleria fowleri (residues 18 to 263) (Green et al., 1992), Potato α subunit (residues 87 to 328) (Carlisle et al., 1990), Potato β subunit (residues 16 to 266) (Lee et al., 1987), P. freudenreichii (residues 3 to 268) (Ladror et al., 1991), rabbit muscle (residues 16 to 266) (Lee et al., 1987), S. cerevisiae α subunit (residues 206 to 457) (Heinisch, 1986); S. cerevisiae β subunit (residues 197 to 449) (Heinisch, 1986). Bootstrap values (in percentages) are based on 500 replicates and are given at each internal node. The structural and biochemical characteristics of the PFK proteins, subunit composition, allosteric behavior, pH optimum of the glycolytic reaction and the Km for fructose-6-phosphate (Km-F-6-P), are given next to the brackets. The arrows indicate a trend from non-allosteric, dimeric enzymes with an acidic pH optimum and a low Km-F-6-P to allosteric multimeric enzymes with a neutral pH optimum and a high Km-F-6-P. (a), The enzyme of G. lamblia represents the only exception; this protein has the potato enzyme as its closest relative, whereas it shares the biochemical and structural properties of the enzymes from E. histolytica and P. freudenreichii.
tree with the same topology as the tree constructed with the neighbor joining method (data not shown).

**DISCUSSION**

We have previously purified and characterized the PP\(_i\)-PFK of *A. methanolica* (Alves *et al.*, 1994). The properties of this enzyme are intermediate between ATP and PP\(_i\) dependent enzymes: the use of PP\(_i\) and the lack of allosteric regulation is typical for PP\(_i\)-dependent enzymes, but the amino terminal amino acid sequence, pH optimum, the Michaelis constant for fructose-6-phosphate and the quaternary structure (tetramer) are similar to the ATP-dependent enzymes (Fothergill-Gilmore and Michels, 1993). This paper describes the cloning of the *pfp* gene, the primary structure and phylogenetic position of this unusual PFK enzyme.

The *pfp* gene of *A. methanolica* encoding the PP\(_i\)-dependent PFK was identified on a 2.3-kb *Pvu*II fragment. The bacterial *pfk* genes encoding the ATP-dependent PFK are usually organized in an operon together with the pyruvate kinase encoding gene (Chevalier *et al.*, 1990; Branny *et al.*, 1993; Llanos *et al.*, 1993; Sakai and Ohta, 1993). The *pfp* gene is flanked by two open reading frames which were tentatively identified as *aroA* and *chiA* based on the high similarity with DAHP synthase and chitinase encoding genes, respectively. The *aroA* gene is located upstream and transcribed divergently from the *pfp* gene, indicating that the transcription of the *pfp* gene is initiated in the *aroA*-*pfp* intergenic region. Since the intergenic region between *aroA* and *pfp* is small (195-bp), the *aroA* and *pfp* promoters may overlap.

PFK is one of the key enzymes in glycolysis, and its expression level may have a dramatic effect on the intracellular concentrations of glycolytic intermediates such as phosphoenolpyruvate, glyceraldehydephosphatase and fructose-6-phosphate. DAHP synthase (*AroA*) catalyzes the first step of the shikimate pathway for which phosphoenolpyruvate and erythrose-4-phosphate, which is produced from the glycolytic intermediates fructose-6-phosphate and glyceraldehydephosphate by transketolase, are the substrates. The activity of PP\(_i\)-PFK may therefore influence the flux through the shikimate pathway by controlling the substrate levels of DAHP synthase. Overlapping *aroA* and *pfp* promoters may provide the cell with a means to balance the carbon-flux through glycolytic and shikimate pathways via the inter-dependent regulation of the expression levels of DAHP synthase and PP\(_i\)-PFK.

The eleven residues which have been implicated in binding of fructose-6-phosphate by the ATP-dependent enzyme of *E. coli* (Shirakihara and Evans, 1988; Lador *et al.*, 1991), are all conserved in the *A. methanolica* enzyme (Fig. 4). Two out of ten residues
(Gly-12, Arg-73) of the *E. coli* which are involved in binding of ATP are conserved in the PP\textsubscript{i}-dependent enzyme of *A. methanolica*. The Tyr-42, Ser-106 and Arg-78 residues of the *E. coli* enzyme are replaced by Trp, Thr and Lys in the *A. methanolica* enzyme, which represent conservative substitutions. The four other residues of the *E. coli* enzyme, Phe-74, Gly-105, Met-108 and Gly-109 are replaced by Thr-73, Asp-104, Gly-107 and Val-108 in the *A. methanolica* enzyme. The conservation of residues involved in ATP-binding in the PP\textsubscript{i}-dependent enzyme of *A. methanolica*, would indicate that these residues are also involved in PP\textsubscript{i}-binding. Current research focuses on the biochemical characterization of mutant *A. methanolica* PFK proteins in which these residues have been altered.

It has been suggested that the PP\textsubscript{i} dependent PFK is more suited for a role in anaerobic metabolism than its ATP-dependent counterpart, and may have evolved from ATP-PFK on several independent occasions in response to an anaerobiosis (Mertens, 1991). In this scenario the *A. methanolica* enzyme would have arisen from a bacterial ATP-dependent enzyme. The phylogenetic analysis of PFK proteins clearly shows that the PP\textsubscript{i}-dependent enzymes form a monophyletic group. This contradicts the hypothesis that the PP\textsubscript{i}-dependent enzymes evolved many times from ATP-dependent enzymes (Mertens, 1991) and supports the view that both types of PFK evolved from a common ancestor.

Since anaerobic metabolism existed before oxygen evolved, the early PFK proteins must have been adapted to anaerobiosis. Interestingly, the simplest PFK proteins, PP\textsubscript{i}-dependent homodimeric enzymes lacking allosteric control, are encountered in *P. freudenreichii* (Ladror *et al.*, 1991), *Giardia lamblia* (Mertens, 1990) and *Entamoeba histolytica* (Huang *et al.*, 1995), which have an obligate anaerobic metabolism. The latter two species are considered to have branched off the eukaryotic lineage before Eukarya acquired mitochondria (Mertens, 1991). The phylogenetic analysis places the enzymes from *P. freudenreichii* and *E. histolytica* in the middle of the tree. Interestingly, there is a strong correlation between the structural and biochemical complexity of the PP\textsubscript{i} and ATP dependent PFK proteins and the phylogenetic distance between these proteins and those from *P. freudenreichii* and *E. histolytica* (Fig. 4). Based on this correlation we propose that the ancestral PFK resembled the enzymes encountered in *P. freudenreichii* and *E. histolytica* from which the allosteric hetero-tetrameric PP\textsubscript{i}-dependent enzymes and the tetrameric and hetero-octameric ATP dependent enzymes encountered in plants, bacteria, insects, yeasts and mammals were derived (Fig. 4). The discovery of an enzyme from *A. methanolica* with molecular and biochemical characteristics which are intermediary between those of bacterial ATP-dependent enzymes and the PP\textsubscript{i}-dependent enzymes of the type found in *P. freudenreichii* and *E. histolytica* strengthens this hypothesis.
Chapter 3

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REFERENCES
References are listed on pages 129-140.