1987

Molecular Cloning and Sequencing of the Bacillus Stearothermophilus 6-Phosphofructo-1-Kinase Gene and of a Partial Rabbit Muscle 6-Phosphofructo-1-Kinase Complementary-Dna.

Brent Arthur French
Louisiana State University and Agricultural & Mechanical College

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_disstheses

Recommended Citation

This Dissertation is brought to you for free and open access by the Graduate School at LSU Digital Commons. It has been accepted for inclusion in LSU Historical Dissertations and Theses by an authorized administrator of LSU Digital Commons. For more information, please contact gradetd@lsu.edu.
INFORMATION TO USERS

While the most advanced technology has been used to photograph and reproduce this manuscript, the quality of the reproduction is heavily dependent upon the quality of the material submitted. For example:

- Manuscript pages may have indistinct print. In such cases, the best available copy has been filmed.

- Manuscripts may not always be complete. In such cases, a note will indicate that it is not possible to obtain missing pages.

- Copyrighted material may have been removed from the manuscript. In such cases, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, and charts) are photographed by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each oversize page is also filmed as one exposure and is available, for an additional charge, as a standard 35mm slide or as a 17"x 23" black and white photographic print.

Most photographs reproduce acceptably on positive microfilm or microfiche but lack the clarity on xerographic copies made from the microfilm. For an additional charge, 35mm slides of 6"x 9" black and white photographic prints are available for any photographs or illustrations that cannot be reproduced satisfactorily by xerography.
Molecular cloning and sequencing of the *Bacillus stearothermophilus* 6-Phosphofructo-1-kinase gene and of a partial rabbit muscle 6-Phosphofructo-1-kinase cDNA

French, Brent Arthur, Ph.D.
The Louisiana State University and Agricultural and Mechanical Col., 1987
PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark √.

1. Glossy photographs or pages ____
2. Colored illustrations, paper or print ______
3. Photographs with dark background ✓
4. Illustrations are poor copy ______
5. Pages with black marks, not original copy ✓
6. Print shows through as there is text on both sides of page ______
7. Indistinct, broken or small print on several pages ✓
8. Print exceeds margin requirements ______
9. Tightly bound copy with print lost in spine ______
10. Computer printout pages with indistinct print ______
11. Page(s) _______ lacking when material received, and not available from school or author.
12. Page(s) _______ seem to be missing in numbering only as text follows.
13. Two pages numbered ______. Text follows.
14. Curling and wrinkled pages ______
15. Dissertation contains pages with print at a slant, filmed as received ______
16. Other ______________________________________________________________

University
Microfilms
International
Molecular Cloning and Sequencing of the *Bacillus stearothermophilus* 6-Phosphofructo-1-kinase Gene and of a Partial Rabbit Muscle 6-Phosphofructo-1-kinase cDNA

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in

The Department of Biochemistry

by

Brent Arthur French
B.S., Louisiana State University, 1982
May 1987
ACKNOWLEDGEMENTS

I would like to acknowledge the academic and financial support of my major professor, Dr. Simon H. Chang. I would also like to thank the members of my Committee for their kind help and advice.

Last but by no means least, I would like to thank my wife Eileen for her patience and support as well as my daughters Whitney and Lindsey for being such beautiful little sources of motivation.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>LIST OF TABLES AND FIGURES</td>
<td>iv</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>vi</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>SECTI ON I: Nucleotide sequence of the 6-phosphofructo-1-kinase gene from <em>Bacillus stearothermophilus</em> and comparison with the homologous <em>Escherichia coli</em> gene</td>
<td>23</td>
</tr>
<tr>
<td>SECTI ON II: High-level expression of <em>Bacillus stearothermophilus</em> 6-phosphofructo-1-kinase in <em>Escherichia coli</em></td>
<td>41</td>
</tr>
<tr>
<td>SECTI ON III: Molecular cloning and sequencing of a partial cDNA for rabbit muscle 6-phosphofructo-1-kinase</td>
<td>59</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>78</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>79</td>
</tr>
<tr>
<td>APPENDIX</td>
<td>91</td>
</tr>
<tr>
<td>CURRICULUM VITAE</td>
<td>105</td>
</tr>
</tbody>
</table>
# LIST OF TABLES AND FIGURES

## Introduction:

<table>
<thead>
<tr>
<th>Figure 1</th>
<th>Schematic views of two subunits in the Bs-PFK tetramer</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>Table 1</td>
<td>A comparison of residues in the ligand-binding sites of three related PFK's</td>
<td>10</td>
</tr>
</tbody>
</table>

## Section I:

<table>
<thead>
<tr>
<th>Figure 1</th>
<th>Southern blot analysis of genomic and cloned Bs-pfk</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>30</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Sequencing strategy for the Bs-pfk gene</td>
<td>32</td>
</tr>
<tr>
<td>Figure 3</td>
<td>The deduced amino acid sequence of Bs-PFK, and the aligned nucleotide sequences of Bs-pfk and Ec-pfkA</td>
<td>33</td>
</tr>
<tr>
<td>Table 1</td>
<td>G+C content of codons &amp; percentage of codons with G or C in the third position for pfk and tyrTS genes</td>
<td>34</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Alignment of three related PFK sequences</td>
<td>36</td>
</tr>
</tbody>
</table>

## Section II:

<table>
<thead>
<tr>
<th>Figure 1</th>
<th>Complementation of PFK deficiency in E. coli strain DF1020</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>48</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Coomassie-stained SDS-polyacrylamide gel of cell lysates and purified Bs-PFK</td>
<td>50</td>
</tr>
</tbody>
</table>
Table 1. PFK expression in various strains and recombinants ................ 52

Figure 3. Homologies between the Bs-pfk promoter region and E. coli consensus sequences ............... 54

Section III:

Figure 1. Southern blots of pOB220/RM-pfk .. 68

Figure 2. Sequencing strategy for RM-PFK cDNA ............... 70

Figure 3. Nucleotide and deduced amino acid sequences of RM-PFK cDNA .... 72
ABSTRACT

A system is presented for determining the relationships between structure and function in the allosteric enzyme 6-phosphofructo-1-kinase (PFK). The ATP-dependent phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate catalyzed by PFK is the first reaction unique to the glycolytic pathway.

The structure-function relationships in PFK can now be addressed by site-specific mutagenesis. This is a method for directly testing hypotheses concerning the functions of individual amino acids in a protein molecule.

The main body of this dissertation is composed of three sections. The first two sections describe the development of a system by which the structure-function relationships in Bacillus stearothermophilus PFK (Bs-PFK) can be investigated using the technique of site-specific mutagenesis. The third section describes preliminary efforts made toward establishing a similar system for the PFK from rabbit muscle.

I. Nucleotide sequence of the 6-phosphofructo-1-kinase gene from Bacillus stearothermophilus and comparison with the homologous Escherichia coli gene: This section describes the cloning and sequencing of the gene encoding
Bs-PFK. A significant degree of homology exists when the deduced amino acid sequence of *Bs-PFK* is compared with the sequences of rabbit muscle PFK or the major PFK from *E. coli*.

II. High-level expression of *Bacillus stearothermo­philus* 6-phosphofructo-1-kinase in *Escherichia coli*: This section describes the subcloning of the *Bs-PFK* gene into a plasmid vector and the high level of *Bs-PFK* expression which results when this construction is introduced into a PFK null strain of *E. coli*. This high level of *Bs-PFK* expression completes the system required for determining the relationships between structure and function in *Bs-PFK* by site-specific mutagenesis.

III. Molecular cloning and sequencing of a partial cDNA for rabbit muscle 6-phosphofructo-1-kinase: The nucleotide sequence of the cDNA described in this section confirms corresponding portions of the genomic sequence for rabbit muscle PFK. The cloning of the rabbit muscle PFK cDNA fragment represents significant progress toward the long-term goal of using site-specific mutagenesis to determine the structure-function relationships in this allosteric enzyme.
INTRODUCTION

I. Overview

This dissertation summarizes the development of systems for determining the relationships between structure and function in the enzyme 6-phosphofructo-1-kinase [EC 2.7.1.11, phosphofructokinase (PFK)]. PFK catalyzes the ATP-dependent phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate. This reaction represents the first step unique to the glycolytic pathway, and the activity of PFK is therefore regulated by an array of physiological modulators.

The mechanisms by which PFK responds to intracellular metabolites in order to regulate glycolysis have been the subject of intensive biochemical research. This research has focused upon the attempt to relate the three-dimensional structure of the enzyme to its allosteric and catalytic functions. The main thrust of this dissertation is on the development of systems in which the function of key amino acid residues can be directly tested. In order to achieve this goal, we have used the techniques of molecular biology to build upon foundations which were established by more classical methods of biochemistry.

The advent of recombinant DNA technology has enabled the biological sciences to make great strides forward in
only a few short years. One of the most basic applications of this technology involves the cloning and sequencing of genes. This is of fundamental importance to biochemists since the amino acid sequence of an enzyme can easily be deduced from the nucleotide sequence of its gene. Cloning and sequencing is therefore a valuable alternative to the more classical method of peptide sequencing. In practice, both methods are often used for determining a single amino acid sequence since each method tends to compensate for shortcomings in the other.

A second application of recombinant DNA technology involves the amplified expression of cloned genes in the form of proteins. For example, it is now possible to express a cloned gene in *Escherichia coli* at levels representing up to 50% of the total soluble protein. This is again of fundamental importance to biochemists since proteins that were difficult if not impossible to isolate by classical separations can now be produced in abundance using recombinant DNA technology. High-level expression is particularly important to structure-function studies which require relatively large amounts of pure protein.

A third application of recombinant DNA technology is site-specific mutagenesis. This is a powerful technique by which defined changes can be made in the nucleotide sequence of a cloned gene. Site-specific mutagenesis is an important tool for enzymologists because it allows one to make predetermined changes in the amino acid sequence of a
protein. Hypotheses concerning the function of a particular amino acid residue can now be tested by changing that residue and analyzing the effect it has upon the enzymology of the "mutant" protein.

It is obvious that these applications of recombinant DNA technology are interdependent. In order to establish a system for performing site-specific mutagenesis on a particular enzyme; one must clone the gene for the protein, determine its nucleotide sequence, and express the protein product at reasonably high levels. The first two sections in this dissertation summarize the work involved in establishing a system for the site-specific mutagenesis of the PFK from *Bacillus stearothermophilus*. The third section describes preliminary efforts made toward establishing a similar system for rabbit muscle PFK.

II. Background

The following review of PFK will be restricted to the ATP: D-fructose-6-phosphate-1-phosphotransferase which catalyzes the transfer of Y-phosphate from ATP to the C-1 hydroxyl of D-fructose 6-phosphate (F6P2−) to form ADP and D-fructose 1,6-bisphosphate (F1,6P2−).

\[ \text{F6P}^2- + \text{MgATP}^2- \rightleftharpoons \text{F1,6P}^4- + \text{MgADP}^- + \text{H}^+ \]

This reaction is the first step which is unique to the glycolytic pathway. Glycolysis was the first major enzymatic pathway to be elucidated so it is not surprising that PFK has been the subject of intensive research.
PFK's can generally be classified into three groups based upon their molecular weights. The tetrameric bacterial PFK's have subunit molecular weights of 32 to 38 kDa, the tetrameric mammalian PFK's have subunit molecular weights of 75 to 95 kDa, and the octomeric yeast PFK's have subunit molecular weights of 112 and 118 kDa (Evans et al., 1981). The PFK's can be divided into two groups based upon their allosteric nature. The prokaryotic PFK's respond to a rather limited range of physiological modulators while the eukaryotic PFK's are much more complex in their allosteric behavior.

PFK's have been isolated in pure form from a wide variety of sources so a comprehensive account of all of them is beyond the scope of this review. General reviews on the subject have been made by Hofmann (1976) and Uyeda (1979). The following review summarizes information pertinent to the study of structure and function in the PFK's from B. stearothermophilus and rabbit muscle. These two enzymes are among the best characterized of all the known PFK's.

A. Prokaryotic PFK's

1. Structures of Bs-PFK and Ec-PFK-1

The PFK isolated from B. stearothermophilus (Bs-PFK) is a stable tetramer composed of four 34 kDa subunits. The primary structure of the enzyme was first determined by peptide sequencing (Kolb et al., 1980). The same laboratory has also determined the crystal structures for
the R-state (Evans and Hudson, 1979) and the T-state of the enzyme (Evans et al., 1986). The 2.4 Å resolution which was obtained for the R-state was adequate to identify not only the tertiary structure but also the binding sites for the various ligands (Hellinga and Evans, 1985).

The major PFK from *E. coli* is a tetramer composed of four 35 kDa subunits and accounts for 90% of the total PFK activity in that organism. The amino acid sequences of *Bs*-PFK and the major PFK from *E. coli* (*Ec*-PFK-1) share a 55% homology (Hellinga and Evans, 1985; French and Chang, 1987). Furthermore, nearly all of the amino acids believed to play a critical role in the function of *Bs*-PFK are conserved in *Ec*-PFK-1 (Hellinga and Evans, 1985).

The minor PFK (*Ec*-PFK-2) accounts for the remaining 10% of the PFK activity in *E. coli*. This enzyme is a tetramer of four 36 kDa subunits and it is quite distinct from *Ec*-PFK-1 in both its immunogenic (Kotlarz and Buc, 1977) and kinetic properties. *Ec*-PFK-2 does not display cooperative behavior and it does not respond to phosphoenolpyruvate. The enzyme has been referred to as the non-allosteric PFK from *E. coli*; however, it will display some inhibition by ATP (Kotlarz and Buc, 1977) and fructose 1,6-bisphosphate (Babul, 1978).

2. Kinetics of *Bs*-PFK and *Ec*-PFK-1

The PFK from *B. stearothermophilus* displays cooperative kinetics with respect to fructose 6-phosphate but not with respect to ATP, and is subject to allosteric
activation by ADP and inhibition by phosphoenolpyruvate (unpublished work of H. Hengartner cited in Evans and Hudson, 1979). The allosteric nature of Bs-PFK is therefore quite similar to that of the major PFK from E. coli (Ec-PFK-1).

The allostery of Ec-PFK-1 has been studied extensively (Blangy et al., 1968) and explained in terms of the model advanced by Monod, Wyman, and Changeux (1965). In this model, the enzyme can exist in two states: an active R-state and an inactive T-state. The difference between the two states of the enzyme can be characterized by either a differential in Vmax (V-system) or a differential affinity for the cooperative ligands (K-system). Bs-PFK has been characterized as a pure K-system since the two states differ in affinity for the homotropic and heterotropic ligands but not in Vmax (Evans and Hudson, 1979). For example, the binding of the cooperative substrate fructose 6-phosphate by a single subunit can induce the entire tetramer to make the conformational transition from the low affinity T-state to the high affinity R-state. The sigmoidal dependence of Bs-PFK reaction rate on the concentration of fructose 6-phosphate has therefore been explained in terms of positive cooperativity.

3. Genes Encoding Bs-PFK and Ec-PFK-1

The gene encoding Bs-PFK has been cloned and sequenced by French and Chang (1987). The deduced amino acid sequence predicts a subunit molecular weight of 34,122 and
is nearly identical to the sequence which was determined by peptide sequencing (Kolb et al., 1980). The deduced sequence published by French and Chang (1987) corrects errors which were present in previously published amino acid sequences for Bs-PFK (Kolb et al., 1980; Poorman et al., 1984; Hellinga and Evans, 1985).

The gene encoding Ec-PFK-1 has also been cloned (Thomson et al., 1979) and sequenced (Hellinga and Evans, 1985). However, the nucleotide and deduced amino acid sequences for Ec-PFK-1 published by Hellinga and Evans (1985) contain a number of errors and the reader is referred to French and Chang (1987) for sequences which were revised according to a personal communication from Philip R. Evans (MRC, Cambridge, England). The gene encoding Ec-PFK-2 has been cloned (Daldal, 1983) and completely sequenced (Daldal, 1984). A mutant of the gene has also been selected which causes a high level of Ec-PFK-2 expression (Daldal, 1983).

4. Structure-function of Bs-PFK and EcPFK-1

The PFK from B. stearothermophilus is the only PFK for which crystal structures have been published (Evans and Hudson, 1979; Evans et al., 1986). Figure 1 shows schematic views of two Bs-PFK subunits in the Bs-PFK tetramer. By detailed analysis of the crystal structures for Bs-PFK, Hellinga and Evans (1985) have identified 31 amino acid residues which appear to bind the various ligands of the enzyme.
Fig. 1. Schematic views of two subunits in the Bs-PFK tetramer: a, viewed along the z-axis; b, viewed along the x-axis. In each case, the other two subunits lie behind those shown. The alpha-helices are indicated by cylinders (1-13) and the beta-strands by arrows (A-K). The substrates ATP and fructose 6-phosphate are shown in the active site and the activator ADP in the effector site. (Taken from Evans et al., 1981.)
Table 1 lists these 31 amino acids and describes the critical roles which they appear to play in the function of the enzyme. Table 1 also lists amino acids in Ec-PFK-1 and rabbit muscle PFK which may serve similar functions. The determination of the 3-D structure of Bs-PFK and the identification of 31 critical amino acids make Bs-PFK the best understood PFK in terms of structure-function relationships.

The four identical subunits of the Bs-PFK tetramer are related by three orthogonal dyad axes x, y, and z with 222 symmetry (Evans and Hudson, 1979). The y-axis runs along the solvent filled hole through the center of the tetramer. The x- and z-axes define the two interface planes. The binding site for the cooperative substrate fructose 6-phosphate is located at the interface of subunits related by the z-axis. The activator ADP and the inhibitor phosphoenolpyruvate are bound at the same effector site located at the interface of subunits related by the x-axis. However, all of the amino acid residues involved in binding the substrate ATP are confined within each subunit.

The three-dimensional structure of Bs-PFK is therefore consistent with the kinetics of the enzyme. The hyperbolic kinetics of ATP binding follow a simple Michaelis-Menten pattern because this substrate is bound independently by each subunit. The cooperative ligands display sigmoidal kinetics because they are bound between adjacent subunits.
<table>
<thead>
<tr>
<th>Binding site</th>
<th>Residue in B. starothermophilus</th>
<th>Residue type</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A, fructose 6-phosphate binding</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asp-127</td>
<td>Asp</td>
<td>Asp</td>
<td>Ser</td>
</tr>
<tr>
<td>Arg-162* (r)</td>
<td>Arg</td>
<td>Arg</td>
<td>Arg</td>
</tr>
<tr>
<td>Met-169</td>
<td>Met</td>
<td>Met</td>
<td>Met</td>
</tr>
<tr>
<td>Glu-222</td>
<td>Glu</td>
<td>Glu</td>
<td>Glu</td>
</tr>
<tr>
<td>Arg-245* (r)</td>
<td>Arg</td>
<td>Arg</td>
<td>Arg</td>
</tr>
<tr>
<td>His-249</td>
<td>His</td>
<td>His</td>
<td>His</td>
</tr>
<tr>
<td>Arg-252</td>
<td>Arg</td>
<td>Arg</td>
<td>Glu</td>
</tr>
<tr>
<td>Asp-12</td>
<td>Asp</td>
<td>Asp</td>
<td>Pro</td>
</tr>
<tr>
<td>Thr-155* (r)</td>
<td>Thr</td>
<td>Thr</td>
<td>Thr</td>
</tr>
<tr>
<td>His-150* (r)</td>
<td>His</td>
<td>His</td>
<td>His</td>
</tr>
<tr>
<td>Ser-9</td>
<td>Ser</td>
<td>Ser</td>
<td>Ser</td>
</tr>
<tr>
<td>Gly-11</td>
<td>Gly</td>
<td>Gly</td>
<td>Gly</td>
</tr>
<tr>
<td>Tyr-41</td>
<td>Tyr</td>
<td>Tyr</td>
<td>Tyr</td>
</tr>
<tr>
<td>Cys-73</td>
<td>Cys</td>
<td>Cys</td>
<td>Cys</td>
</tr>
<tr>
<td>Lys-77</td>
<td>Arg</td>
<td>Lys</td>
<td>Arg</td>
</tr>
<tr>
<td>Asp-103</td>
<td>Asp</td>
<td>Asp</td>
<td>Asp</td>
</tr>
<tr>
<td>Gly-104</td>
<td>Gly</td>
<td>Gly</td>
<td>Gly</td>
</tr>
<tr>
<td>Gin-107</td>
<td>Met</td>
<td>Gin</td>
<td>Thr</td>
</tr>
<tr>
<td>C. effector site (ADP)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg-63* (q)</td>
<td>Arg</td>
<td>Arg</td>
<td>Leu</td>
</tr>
<tr>
<td>Arg-154</td>
<td>Arg</td>
<td>Arg</td>
<td>Thr</td>
</tr>
<tr>
<td>Gly-185</td>
<td>Gly</td>
<td>Gly</td>
<td>Gly</td>
</tr>
<tr>
<td>Glu-187</td>
<td>Glu</td>
<td>Glu</td>
<td>Asp</td>
</tr>
<tr>
<td>Arg-211</td>
<td>Lys</td>
<td>Arg</td>
<td>Thr</td>
</tr>
<tr>
<td>Lys-214</td>
<td>Lys</td>
<td>Lys</td>
<td>Arg</td>
</tr>
<tr>
<td>C-terminus</td>
<td>Tyr</td>
<td>Tyr</td>
<td>(Val)</td>
</tr>
</tbody>
</table>

Table 1. A comparison of residues in the ligand-binding sites of three related PFK's: Ec-PFK-1 (EC), Bs-PFK (BS), and the N-terminal and C-terminal halves of RM-PFK [RM(N) and RM(C)]. Residues are listed under their number in the Bs-PFK sequence. Residues contributing to sites A and C are divided between neighboring subunits in the tetramer. Residues marked * are not in the subunit containing the main part of the site. The dyad symmetry axis relating the subunit containing these residues to that containing the main site are given as (p), (q) or (r). (Adapted from Hellinga and Evans, 1985.)
Comparison of crystal structures for the R- and T-
states of Bs-PFK suggests a physical basis for the
cooperativity displayed by the homotropic effector fructose
6-phosphate (Evans et al., 1986). The structural change
between the two conformations can be described as a twist
along the y-axis of symmetry. The twist from the T- to the
R-state rotates one pair of subunits relative to the other
in such a way that the fructose 6-phosphate binding sites
are made more accessible.

The physical bases for the heterotropic interactions
are more complex and less well understood. This is a
challenging problem since the activator ADP and the
inhibitor phosphoenolpyruvate bind to the same effector
site with opposite effects. Comparison of the crystal
structures for the T- and the R-states shows no detectable
change in the effector site.

The crystal structures of Bs-PFK have been of great
value in elucidating its structure-function relationships.
However, it is important to remember that the information
derived from X-ray crystallography is static rather than
dynamic by its very nature. There is also the real
possibility that the 3-D conformation of a protein in
solution is significantly different from its conformation
in the crystalline state. It is therefore important that
the structure-function relationships deduced from the
crystal structures of Bs-PFK be verified by independent
means. Site-specific mutagenesis of critical amino acids
in *Bs-PFK* is perhaps the best way to achieve this goal.

The laboratory of Dr. Philip R. Evans at MRC (Cambridge, England) is currently using site-specific mutagenesis to investigate the structure-function relationships in the major PFK from *E. coli*. The gene encoding *Ec-PFK-1* has been cloned (Thomson et al., 1979), sequenced, and expressed at high levels in *E. coli* (Hellinga and Evans, 1985). Evans' laboratory at MRC is also working on crystal structures for both the T- and R-states of *Ec-PFK-1* (personal communication). The MRC laboratory has recently used site-specific mutagenesis to probe the function of two amino acids at the catalytic site of *Ec-PFK-1* (personal communication). They found that Asp-127 plays a key role in the rate enhancement of the enzyme by serving as a general base in the reaction mechanism. They also found that Arg-171 plays only a minor role in the catalytic mechanism. These two residues are conserved between *Ec-PFK-1* and *Bs-PFK* so it is likely that their functions in *Bs-PFK* are similar.

The gene encoding *Bs-PFK* has been subcloned into the plasmid vector pBR322 and expressed in *E. coli* at levels representing approximately 20% of the total soluble protein (Section II of this work, pp. 41-58). The cloning, sequencing, and high level expression of the gene encoding *Bs-PFK* have thus created a system in which structure-function relationships deduced from crystal structures of the enzyme can be tested by site-specific mutagenesis.
B. Mammalian PFK Isozymes

1. Structure of Rabbit PFK Isozymes

The widely diverse requirements for glycolytic regulation in the tissues of higher animals are met by tetrameric combinations of three different isozymic subunits of PFK. These are the muscle (A in rabbit or M in human), liver (B or L) and brain (C or P) isozymic subunits of PFK (Foe and Kemp, 1985). The composition of the homotetramers and random heterotetramers is determined by the relative abundance of each subunit in a given tissue. The PFK isolated from skeletal and heart muscle is a tetramer of A subunits, while the PFK isolated from liver and erythrocytes is a tetramer of B subunits. The PFK's isolated from most other tissues are made up of various combinations of the A and B subunits; however, the PFK's isolated from brain, thymus, and several other tissues contain the C subunit as well (Tsai and Kemp, 1973). For example, the molar ratio of the C, A, and B subunits isolated from rabbit brain is approximately 5:4:1.5 (Foe and Kemp, 1984).

The molecular weights of the three subunits are quite similar: 85 kDa for A, 80 kDa for B, and 86 kDa for C subunits (Lee et al., 1987; Foe and Kemp, 1984). All three subunits are phosphorylated to some extent in vivo. This phosphorylation can be completed in vitro by the catalytic subunit of cAMP-dependent protein kinase (Foe and Kemp, 1984). Phosphorylation of the A isozyme (Foe and Kemp,
1982) or the B isozyme (Sakakibara and Uyeda, 1983) causes a minor increase in their sensitivity to allosteric inhibition. However, phosphorylation does not affect the specific activity or allosteric response of the C isozyme to ATP or fructose 2,6-bisphosphate (Foe and Kemp, 1984). The physiological significance of these observations has not yet been resolved.

The immunological relationships between the various rabbit subunits has been studied by Foe and Kemp (1985) using polyclonal antibodies. They found no cross-reactivity between anti-A serum and either the purified B or C subunits. They did find a small amount of cross-reactivity between the anti-B serum and the purified C subunits, but contamination of the original B antigen by C subunits could not be ruled out.

The PFK isolated from rabbit muscle (RM-PFK) is a tetramer composed of four identical 85 kDa subunits. The active tetramer can be dissociated by conditions such as low protein concentration, low pH, and low temperature. At neutral pH and low protein concentration (0.15 mg/ml), the enzyme activators tend to stabilize the tetramer while the inhibitors have the opposite effect (Lad et al., 1973).

RM-PFK is the only mammalian PFK for which a complete amino acid sequence is known at the present time. Poorman et al. (1984) were able to determine 96% of this sequence by the method of peptide sequencing. The uncertain positions and a gap of 30 residues in this amino acid
sequence were deduced by Lee et al. (1987) from the nucleotide sequence of the gene encoding RM-PFK.

2) Kinetics of Rabbit PFK Isozymes

The kinetics of the rabbit PFK isozymes are perhaps the best studied among all the PFK's. Reviews including this subject have been made by Hofmann (1976) and Uyeda (1979). The large size of the mammalian PFK isozymes (more than twice that of the prokaryotic PFK's) reflects the complex allosteric behavior required to control the energy balance in specialized eukaryotic cells. The allosteric repertoires of the mammalian PFK isozymes include all the effectors of the prokaryotic PFK's (positive cooperativity with respect to fructose 6-phosphate, activation by ADP, and inhibition by phosphoenolpyruvate). In addition, these isozymes are activated by ammonium ions and fructose bisphosphates (fructose 1,6-bisphosphate and fructose 2,6-bisphosphate) while they are inhibited by ATP and citrate.

The most potent activator of mammalian PFK's, fructose 2,6-bisphosphate, was only discovered recently (van Schaftingen et al., 1980; Claus et al., 1981; Uyeda et al., 1981). This effector controls the balance between glycolysis and gluconeogenesis by simultaneously activating PFK and inhibiting fructose 1,6-bisphosphatase. The level of this critical allosteric ligand is controlled by the bifunctional enzyme 6-phosphofructo-2-kinase / fructose-2,6-bisphosphatase (El-Magharabi et al., 1982; Kountz et al., 1985). A single phosphorylation by cAMP-dependent
protein kinase activates the bisphosphatase and inactivates the kinase functions of this enzyme. The cAMP-dependent protein kinase system thus links the regulation of glycolysis and gluconeogenesis to hormonal control by glucagon (Pilkis et al., 1982). However, the regulation of PFK and fructose-1,6-bisphosphatase is quite complex since one must also consider the roles of insulin (Krupp and Dunaway, 1984), adrenalin (Clark and Patten, 1981), and the anomeric specificities of the two enzymes (Younathan et al., 1981; Kelley et al., 1986).

The other allosteric effectors of the rabbit PFK isozymes are much less potent than fructose 2,6-bisphosphate in their effects. The major inhibitors are ATP (Lardy and Parks, 1956), citrate (Tsai and Kemp, 1974), and phosphoenolpyruvate (Kemp, 1971). However, the inhibition caused by ATP can be relieved by its own breakdown products (ADP, AMP, cAMP, and Pi) as well as by ammonium ions (Abrahams and Younathan, 1971) and fructose 6-phosphate. Mammalian PFK's show cooperative kinetics with respect to fructose 6-phosphate and the curve for the concentration of this substrate versus relative velocity is therefore sigmoidal. In general, the activators of RM-PFK increase the apparent affinity of the enzyme for fructose 6-phosphate and shift the sigmoidal saturation curve to the left. On the other hand, the inhibitors decrease this apparent affinity and thus shift the saturation curve to the right.
The various tissues of higher animals require different types of glycolytic regulation. For example, muscle cells must be capable of increasing the rate of glycolysis in order to meet the demand for ATP under anaerobic conditions. Hepatocytes have very different requirements since they must be capable of carrying out gluconeogenesis in order to maintain blood glucose levels. These levels are critical to brain cells since glucose is their only available fuel under non-fasting conditions. Erythrocytes are another unusual case since they are unable to carry out oxidative phosphorylation and therefore rely upon substrate-level phosphorylation for their ATP.

The kinetics of the various PFK isozymes no doubt reflect the metabolic requirements of their source tissues. Foe and Kemp (1985) purified the three isozymic subunits from rabbit brain and allowed them to reassociate in order to obtain homotetramers composed exclusively of A, B, or C subunits. They found that the three isozymes all displayed sigmoidal kinetics with respect to fructose 6-phosphate. The sensitivity displayed toward inhibition by ATP was of the order B>C>A and toward inhibition by citrate was of the order A>C>B. The A and B isozymes displayed equal sensitivity toward activation by AMP and fructose 2,6-bisphosphate. However, the C isozyme was considerably less sensitive than the other two isozymes toward these two activators. Foe and Kemp (1985) also noted that the kinetic properties of PFK heterotetramers are probably
influenced by interactions between the different subunits since their kinetics cannot be predicted from the properties of the homotetramers.

3) Genes Encoding Mammalian PFK Isozymes

Lee et al. (1987) have isolated a genomic clone for RM-PFK and determined the nucleotide sequence of all the coding regions. One-quarter of this coding sequence has been confirmed by a partial cDNA for RM-PFK (Section III of this work, pp. 59-77). The sequence of this cDNA also established the 3'end of the RM-PFK mRNA. However, the genomic clone (Lee et al., 1987) terminates 61 base pairs upstream of the initiation codon of RM-PFK so the 5'end of RM-PFK mRNA has yet to be defined.

Significant progress has been made in cloning the genes for the human PFK isozymes. Valdez et al. (1987) have used the RM-PFK gene to isolate a homologous clone from a human genomic library. Nucleotide sequence analysis of this clone has identified an exon which is identical in length and 87% homologous to the first coding exon of the RM-PFK gene. The deduced amino acid sequences of these two coding regions have an even higher 93% homology. It is therefore likely that the human genomic clone is for human muscle PFK although its strong bias towards adenine and thymine is indicative of a liver isozyme (Newgard et al., 1986).

Vora and co-workers have assigned the genes encoding the human M, L, and P isozymes to chromosomes 1, 21, and 10.
respectively (Vora et al., 1982; Vora and Francke, 1981; Vora et al., 1983). They have also used a RM-PFK cDNA probe (Putney et al., 1983) to isolate a cDNA for human muscle PFK (Vora et al., 1986). This clone contains about 500 base pairs of 3'-untranslated sequence and nearly 1350 base pairs of coding sequence. The authors did not present any sequence data, but they claimed that the amino acid sequence deduced from the coding sequence of this clone was 95% homologous with the published amino acid sequence for RM-PFK (Poorman et al., 1984).

Levanon et al. (1986) recently isolated a genomic clone for human liver PFK by screening a library enriched for chromosome 21 sequences with two synthetic oligonucleotide probes. The sequences of these two probes were based upon peptide sequences of purified human liver PFK. The genomic clone for human liver PFK hybridized a 3.5 kb band in Northern blots of human liver RNA and was capable of selectively enriching liver PFK mRNA by hybrid selection. However, no data concerning the nucleotide sequence of this clone was presented by the authors.

4) Structure and Function of RM-PFK

Upon determining the nearly complete amino acid sequence for RM-PFK, Poorman et al. (1984) noted that the enzyme appeared to be a product of gene duplication and divergence since the two halves of the amino acid sequence shared a 32% homology. This confirmed earlier evidence that each RM-PFK protomer contained both heterogeneity and
duplication (Paetkau et al., 1968). Poorman et al. (1984) also noted that the N-terminal half of the RM-PFK amino acid sequence was 44% homologous to the sequence of *Bs*-PFK, while the C-terminal half was 34% homologous to *Bs*-PFK.

This finding and the evidence that the mammalian tetramer has D2 symmetry (Foe and Trujillo, 1980) led Poorman et al. (1984) to suggest that the structure of an RM-PFK tetramer resembled an end-on-end octomer of *Bs*-PFK. However, equilibrium binding studies indicated that there is only one catalytic site per RM-PFK subunit (Kemp and Krebs, 1967). This and the fact that RM-PFK responds to a greater number of allosteric effectors than *Bs*-PFK suggested that half of the catalytic sites on the hypothetical octomer of *Bs*-PFK had evolved into allosteric sites.

The amino acid sequence homology is greater between *Bs*-PFK and the N-half of RM-PFK than it is between *Bs*-PFK and the C-half. In addition, amino acid residues critical to the catalytic site of *Bs*-PFK (Hellinga and Evans, 1985) are conserved in the N-half but not the C-half of RM-PFK. It therefore appears that the C-half of RM-PFK has differentiated through time to assume an allosteric rather than a catalytic role.

Poorman et al. (1984) proposed a tentative working model for RM-PFK in which each subunit resembles a dimer of *Bs*-PFK. The N-half of each dimer retains the catalytic role while the C-half assumes a regulatory role. Detailed
amino acid sequence comparisons between Bs-PFK and RM-PFK suggested that what had originally been the fructose 6-phosphate binding site in the C-half of each dimer had evolved into an allosteric site for the fructose 1,6- and 2,6-bisphosphates.

Poorman et al. (1984) went on to suggest that what had originally been the ADP activation site in the N-half of each dimer had evolved into an ATP inhibition site. The only evidence in support of this model came from limit digestions of RM-PFK with subtilisin (Gottschalk et al., 1983). These experiments indicated that the fructose 6-phosphate binding site and the ATP inhibition sites were located in the N-half of each RM-PFK subunit while the fructose bisphosphate and ADP activation sites were located in the C-half of each subunit.

The validity of this tentative working model for the tertiary structure of RM-PFK could be tested by the technique of site-specific mutagenesis. This would be accomplished by mutating amino acid residues thought to be important in the binding of various ligands.

Projects aimed at using site-specific mutagenesis to probe the active and allosteric sites of RM-PFK have been slowed by the difficulties associated with expressing the mammalian enzyme in E. coli. First, the length of the RM-PFK mRNA coding region is 2343 base pairs. A cDNA of this size is difficult to synthesize and clone into E. coli using conventional methods. Second, expression of RM-PFK
would require that the cDNA be precisely inserted behind an
*E. coli* promoter and ribosome binding site in a plasmid
expression vector. The complexity of performing such a
manipulation increases with the size of the DNA fragment to
be inserted. Third, the codon usage in the RM-PFK cDNA may
not be suitable for high level expression in *E. coli*.
Finally, there is no guarantee that RM-PFK mRNA or protein
will be stable in *E. coli* once they are expressed.

Considerable progress has been made toward the
formidable task of performing site-specific mutagenesis on
RM-PFK. The genomic clone for RM-PFK isolated by Lee et
al., (1987) has provided the nucleotide sequence for all of
the coding regions. In addition, the cDNA for RM-PFK
cloned by French et al. (Section III of this work, pp. 59-
77) covers one-quarter of the RM-PFK coding sequence from
the 3'-terminal end of the gene. The construction of a
full-length RM-PFK cDNA may require the splicing of several
such partial cDNA's.
SECTION I

Nucleotide sequence of the 6-phosphofructo-1-kinase gene from Bacillus stearothermophilus and comparison with the homologous Escherichia coli gene
Nucleotide sequence of the 6-phosphofructo-1-kinase gene from *Bacillus stearothermophilus* and comparison with the homologous *Escherichia coli* gene

(Recombinant DNA; Southern hybridization; partial genomic phage library; thermophile genes; G + C content; allosteric enzyme)

Brent A. French and Simon H. Chang*

Department of Biochemistry, Louisiana State University, Baton Rouge, LA 70803 (U.S.A.) Tel. (504) 388-5147

* To whom correspondence and reprint requests should be addressed.

Abbreviations: aa, amino acid(s); ADP, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; bp, base pairs(s); Bs-PFK, *Bacillus stearothermophilus* PFK; Bs-pfk, gene coding for Bs-PFK; Bs-TyrTS, *B. stearothermophilus* TyrTS; Bs-tyrTS, gene coding for Bs-TyrTS; C, cytidine; d, deoxyribo; Ec-PFK-1, the major *Escherichia coli* PFK; Ec-pfkA, gene coding for Ec-PFK-1; Ec-TyrTS, *E. coli* TyrTS; Ec-tyrTS, gene coding for Ec-TyrTS; G, guanosine; kb, 1000 bp; LB, Luria broth; nt, nucleotide(s); PFK, phosphofructokinase; pfk, gene coding for PFK; RM-PFK, rabbit muscle PFK; RM-pfk, gene coding for RM-PFK; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl, 0.015 M Na3citrate, pH 7-8; TyrTS, tyrosyl-tRNA synthetase; tyrTS, gene encoding TyrTS.
SUMMARY

The gene (Bs-pfk) for 6-phosphofructo-1-kinase (PFK) from *Bacillus stearothermophilus* has been cloned and sequenced. The deduced amino acid (aa) sequence is nearly identical to the sequence which was previously determined by peptide analysis. The elevated G + C content of Bs-pfk relative to the homologous Ec-pfkA from *Escherichia coli* is consistent with previous observations concerning genes from thermophilic prokaryotes. A significant degree of homology exists when the deduced aa sequence of *B. stearothermophilus* PFK is compared with the corrected sequences of rabbit muscle PFK or *E. coli* PFK-1. The cloning and sequencing of Bs-pfk completes the first step toward using site-specific mutagenesis to investigate the structure-function relationships for this allosteric enzyme.

INTRODUCTION

6-Phosphofructo-1-kinase (PFK; EC 2.7.1.11) catalyzes the ATP-dependent phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate. The activity of this enzyme is controlled by physiological modulators and it thus plays a key role in the regulation of glycolysis (Uyeda, 1979). PFK from *B. stearothermophilus* (Bs-PFK) and the major PFK from *E. coli* (Ec-PFK-1) share many physical and kinetic
properties (Evans et al., 1981). They are homotetrameric enzymes composed of 34-kDa and 35-kDa subunits, respectively. Both display cooperative kinetics with respect to fructose 6-phosphate but not with respect to ATP, and they are subject to allosteric activation by ADP and inhibition by phosphoenolpyruvate. The PFK isolated from rabbit muscle (RM-PFK) is also composed of four identical subunits, but each of these 85-kDa subunits is more than twice the size of the prokaryotic PFK subunit. The allosteric regulation of RM-PFK is more complex in that it is activated by AMP and fructose bisphosphates, while it is inhibited by ATP and citrate.

Poorman et al. (1984) noted internal homology between the N- and C-terminal halves of a nearly complete aa sequence for RM-PFK. They also discovered homology between the aa sequences of Bs-PFK (Kolb et al., 1980) and the two halves of RM-PFK. This indicated that RM-pfk evolved from an ancestor of Bs-pfk by a process of gene duplication and divergence. Upon sequencing the gene for the major PFK from E. coli (Ec-pfkA), Hellinga and Evans (1985) noted significant homology between the deduced aa sequence and the sequences for RM-PFK and Bs-PFK.

This paper describes the cloning and sequencing of Bs-pfk and compares the deduced aa sequence with corrected sequences for Ec-PFK-1 and RM-PFK. The strategy for cloning Bs-pfk was based upon genomic Southern blots which indicated that a 4.7-kb EcoRI fragment of
B. stearothermophilus DNA hybridized with Ec-pfkA. This size-class of EcoRI fragments was isolated from a preparative agarose gel and ligated into a λgt10 vector. Bs-pfk was isolated from the size-enriched phage λ library by plaque hybridization using Ec-pfkA as a probe. Information from Southern blots was then used to subclone restriction fragments of Bs-pfk into M13mp18 and M13mp19 for sequencing.

MATERIALS AND METHODS

(a) Bacterial strains, media, and methods

The source of the genomic DNA for the λ library was B. stearothermophilus strain NCA 1503. This strain was obtained from the American Type Culture Collection (Rockville, MD) and grown in LB medium (Maniatis et al., 1982) at 58°C. The dephosphorylated λgt10 arms and packaging extract used to create the genomic library were obtained from Promega Biotec (Madison, WI) under the trade names of Protoclone and Packagene, respectively. The E. coli host used for plating the λ library was strain K803 obtained from Ross C. Hardison at Pennsylvania State University (University Park, PA) and grown in LB medium supplemented with 0.2% maltose and 10 mM MgCl₂. The M13mp18 and M13mp19 vectors as well as the JM107 and JM109 hosts were obtained from Joachim Messing, presently at Rutgers University (Piscataway, NJ). The following methods
were performed as described by Maniatis et al. (1982) unless otherwise specified.

(b) Preparation of probes

Cloned Ec-pfkA was kindly provided by Philip R. Evans (MRC, Cambridge) as an M13mp8 subclone designated mHE1011 (Hellinga and Evans, 1985). A 1.4-kb HindIII fragment containing Ec-pfkA was subcloned into pUC18 to create pUC18/Ec-pfkA. This recombinant was amplified and restricted with HindIII to release the 1.4-kb fragment containing Ec-pfkA. The restriction digest was fractionated on a 1% agarose gel, the 1.4-kb band excised, and DNA purified by electroelution. The gel-purified fragment containing Ec-pfkA was nick-translated (Rigby et al., 1977) to a specific activity of greater than $5 \times 10^7$ cpm/µg for use in probing the initial genomic Southern blots. pUC18/Ec-pfkA was nick-translated to a specific activity of greater than $5 \times 10^7$ cpm/µg for use in probing subsequent Southern blots and plaque lifts.

(c) Genomic Southern blots

Genomic DNA from B. stearothermophilus strain NCA 1503 was isolated by standard procedure (Schleif and Wensink, 1982) and purified by CsCl centrifugation. The genomic DNA was digested with a variety of restriction endonucleases, fractionated on a 1% agarose gel, and bidirectionally transferred to nitrocellulose. One Southern blot was probed with the nick-translated 1.4-kb HindIII fragment containing Ec-pfkA at an empirically determined
hybridization temperature of 56°C. Nick-translated pUC18/Ec-pfkA was used to probe the duplicate filter. The final and most stringent wash of each blot was done in 6X SSC/0.1% SDS for 5 min at 56°C. The autoradiograms of these two blots showed that the presence of vector sequences in the probe did not appreciably reduce the ratio of desired signal to noise (data not shown).

(d) Construction of a partial genomic library

The genomic Southern blots indicated that Bs-pfk resided on a 4.7-kb EcoRI fragment. Genomic EcoRI fragments of this size were enriched before cloning into the vector λgt10. *B. stearothermophilus* DNA (20 μg) was restricted with EcoRI and fractionated on a preparative low-melting-point agarose gel. The bands from 4-6 kb in length were excised and purified (Schmitt and Cohen, 1983) on an Elutip-d column (Shleicher & Schuell, Keene, NH).

The 4-6 kb EcoRI fragments were then ligated with dephosphorylated λgt10 arms and packaged to form viable phage using the protocols provided by Promega Biotech with their Protoclone and Packagene kits. The library was plated onto host strain K803 and duplicate plaque lifts were probed with nick-translated pUC18/Ec-pfkA at 56°C. Positive signals were obtained at a frequency of about 1 in 500 and six independently positive clones were plaque purified. One of these clones (gt10/Bs-pfk) was amplified by the plate lysate method (Huynh et al., 1985) and Southern blotted along with appropriate controls (Fig. 1).
Fig. 1. Southern blot analysis of genomic and cloned $Bs$-$pfk$. DNA from the following sources was restricted, electrophoresed on a 1% agarose gel, transferred to nitrocellulose, and hybridized with nick-translated pUC18/Ec-$pfkA$. Hybridization was performed at 56°C and the most stringent wash was done in 6X SSC/0.1% SDS for 5 min at 56°C. Lanes: (1 & 18) 4 ng of mHE1011 cut by HindIII + BamHI; (2) 1 µg of E. coli genomic DNA cut by HpaI; (3) 2 µg of $B. stearothermophilus$ genomic DNA cut by EcoRI; (4-17) 100 ng of gti10/Bs-$pfk$ cut by: (4) EcoRI, (5) SalI, (6) PstI, (7) SphI, (8) BamHI, (9) SaciA, (10) XbaI, (11) HincII, (12) HindIII, (13) SstI, (14) KpnI, (15) AccI, (16) SmaI, (17) EcoRI + SmaI. The slight difference in the migration between the genomic and cloned EcoRI fragments containing $Bs$-$pfk$ (lanes 3 & 4) may be due to the 20-fold difference in mass of loaded DNA.
e) **Sequence analysis**

The gt10/Bs-pfk clone was mapped by Southern analysis of single and double restriction enzyme digestions. Restriction fragments of Bs-pfk were isolated from low-melting-point agarose gels, subcloned into either M13mp18 or M13mp19, and sequenced by the dideoxy chain termination method (Sanger et al., 1977). The sequencing strategy for Bs-pfk is shown in Fig. 2 and the completed sequence is presented in Fig. 3.

**RESULTS AND DISCUSSION**

(a) **Nucleotide sequence of Bs-pfk**

The 957-bp coding sequence of Bs-pfk (Fig. 3) predicts an aa sequence which is nearly identical to the revised sequence published by Poorman et al. (1984) from the work of Kolb et al. (1980). The nt sequence codes for an Asp-12 rather than Asn, a Gln-82 rather than Glu, and a Glu-95 rather than Gln. These minor differences can be attributed to limitations of peptide sequencing. The deduced aa sequence of Bs-PFK predicts a subunit Mr of 34,122 which agrees reasonably well with the previous estimate of 33-kDa (Kolb et al., 1980).

The nucleotide sequences of Bs-pfk and Ec-pfkA are compared in Fig. 3. Their coding sequences share 58% nt and 55% aa sequence homologies. These values are quite similar to the values generated by a previous comparison of
Fig. 2. Sequencing strategy for the Bs-pfk gene. The 1530-nt sequence shown in Fig. 3 is represented as a bold line with selected restriction sites. Only those sites used during subcloning are indicated. The arrow above the restriction map indicates the location and direction of the open reading frame encoding Bs-PFK. The first G of the GTG initiation codon corresponds to nt-139 in both Fig. 2 and Fig. 3. The arrows below the map indicate the location and direction in which the sequence data was read. The dideoxy chain termination method of Sanger et al. (1977) as modified by Biggin et al. (1983) for [35S]dATP was used to sequence subclones in M13mp18 and M13mp19 (Norrander et al., 1983). Second-strand verification was obtained for 86% of the coding sequence. In addition to the M13 universal primer, two other sequencing primers were made on an Applied Biosystems Model 380A DNA Synthesizer. The sequence of the first 16-mer was identical to nt 549-565 while the sequence of the second 16-mer was complementary to nt 1292-1308.
Fig. 3. The deduced amino acid sequence of Bs-PFK, and the aligned nucleotide sequences of Bs-pfk and Ec-pfkA (Hellinga and Evans, 1985). The numbers above the sequences refer to the aa of Bs-PFK. The underlined corrections in the Ec-pfkA sequence were kindly provided by Philip R. Evans. The Bionet Resource (Intelligenetics, Inc., Mountain View, CA) was used to assemble, analyze, and translate the nt sequence of Bs-pfk.
the tyrosyl-tRNA synthetase (TyrTS) genes from *B. stearothermophilus* and *E. coli* (Winter et al., 1983). The two synthetase genes have 58% nt and 56% aa sequence homologies. The authors also noted an elevated G+C content of *Bs-tyrTS* relative to *Ec-tyrTS*, particularly in the third position of the codons.

**TABLE I**

G + C content of codons and percentage of codons with G or C in the third position for the *pfk* and *tyrTS* genes from *B. stearothermophilus* and *E. coli*.

<table>
<thead>
<tr>
<th>Gene</th>
<th>G + C Content (%)</th>
<th>Codons with G or C at 3rd position (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bs-pfk</em></td>
<td>58.5</td>
<td>71.3</td>
<td>Fig. 3</td>
</tr>
<tr>
<td><em>Ec-pfkA</em></td>
<td>53.6</td>
<td>57.5</td>
<td>Hellinga &amp; Evans (1985)</td>
</tr>
<tr>
<td><em>Bs-tyrTS</em></td>
<td>55.1</td>
<td>68.0</td>
<td>Winter et al. (1983)</td>
</tr>
<tr>
<td><em>Ec-tyrTS</em></td>
<td>51.6</td>
<td>59.6</td>
<td>Barker et al. (1982)</td>
</tr>
</tbody>
</table>

Table I shows the percentage G + C content and the percentage of codons containing G or C in the third position for the *pfk* and *tyrTS* genes from *B. stearothermophilus* and *E. coli*. It demonstrates that the
G + C content of the two genes from the moderate thermophile is increased relative to that of the genes from the mesophile. The difference in G + C content is primarily due to a preference in *B. stearothermophilus* for G or C in the third position of its codons. This finding supports the idea that higher G + C contents help stabilize nucleic acid interactions in thermophilic organisms (Kagawa et al., 1984). In this context it is interesting to note that codons of the 3-isopropylmalate dehydrogenase genes from *Saccharomyces cerevisiae* (mesophile), *Bacillus coagulans* (facultative thermophile), and *Thermus thermophilus* (extreme thermophile) have third position G + C contents of 39.6, 56.3, and 89.4%, respectively (Sekiguchi et al., 1986). The third position G + C content in thermophilic prokaryotes therefore appears to be positively correlated with optimal growth temperature.

(b) Comparison of three related PFK aa sequences

The deduced aa sequence of *Bs*-PFK has been aligned with the corrected aa sequences of *Ec*-PFK-1 and the two halves of RM-PFK in Fig. 4. The 319-aa residue comparison between *Bs*-PFK and the N-terminal half of RM-PFK has a 44% aa sequence homology, while the 307 residue comparison between *Bs*-PFK and the C-terminal half of RM-PFK has a 35% homology. The homologies show that the three enzymes are clearly related and suggest that RM-PFK evolved by a process of gene duplication and divergence (Poorman et al., 1984).
Fig. 4. Alignment of three related PFK sequences. Ec-PFK-1, Bs-PFK, and the amino (N) and carboxyl (C) halves of RM-PFK have been aligned to demonstrate sequence homologies. A comparison between the aa sequences of Bs-PFK and the two halves of RM-PFK was first done by Poorman et al. (1984). The underlined aa are corrections to the alignment made by Hellinga and Evans (1985). The corrected Ec-PFK-1 sequence was obtained by personal communication from Philip R. Evans (MRC, Cambridge) while the corrected RM-PFK sequence was deduced from the DNA sequence of RM-pfk (Lee et al., 1987).
ACKNOWLEDGEMENTS

This investigation was supported by Public Health Service Grant 5 R01 AM 31676 from the National Institutes of Health. We thank Dr. E. S. Younathan, M. LoMonaco, and M. Shirley for valuable discussions and for critical reading of the manuscript.

REFERENCES


SECTION II

High-level expression of *Bacillus stearothermophilus* 6-phosphofructo-1-kinase in *Escherichia coli*
High-level expression of *Bacillus stearothermophilus* 6-phosphofructo-1-kinase in *Escherichia coli*

(Recombinant DNA; thermophile gene; prokaryotic gene expression; allosteric enzyme; gene complementation)

Brent A. French, Benigno C. Valdez, Ezzat S. Younathan and Simon H. Chang*

Department of Biochemistry, Louisiana State University, Baton Rouge, LA 70803 (U.S.A.) Tel. (504) 388-5147

* To whom correspondence and reprint requests should be addressed.

Abbreviations: aa, amino acid(s); ADP, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; bp, base pairs(s); Bs-PFK, *Bacillus stearothermophilus* PFK; Bs-pfk, gene coding for Bs-PFK; DTT, DL-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; kb, 1000 bp; LB, Luria broth; Mr, molecular weight; nt, nucleotide(s); PFK, 6-phosphofructo-1-kinase; pfk, gene coding for PFK; PMSF, phenylmethylsulfonyl flouride; RM-PFK, rabbit muscle PFK; RM-pfk, gene coding for RM-PFK; SDS, sodium dodecyl sulfate.
SUMMARY

The 6-phosphofructo-1-kinase (PFK) gene from *Bacillus stearothermophilus* has been expressed at high-levels in *Escherichia coli*. This expression has been demonstrated by complementation studies, SDS-PAGE, and PFK assays of cell extracts. A level of *B. stearothermophilus* PFK expression corresponding to 20% of the total extracted protein was calculated from densitometric scans of an SDS-polyacrylamide gel. The high-level of recombinant gene expression will enable this laboratory to determine structure-function relationships in *B. stearothermophilus* PFK by the method of site-specific mutagenesis.

INTRODUCTION

6-Phosphofructo-1-kinase (PFK; EC 2.7.1.11) catalyzes the ATP-dependent phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate. The activity of this enzyme is controlled by physiological modulators and it thus plays a key role in the regulation of glycolysis (Uyeda, 1979). PFK from *B. stearothermophilus* (Bs-PFK) and the major PFK from *E. coli* (Ec-PFK-1) share many physical and kinetic properties (Evans et al., 1981). They are homotetrameric enzymes composed of 34-kDa and 35-kDa subunits, respectively. Both display cooperative kinetics with respect to fructose 6-phosphate but not with respect to
ATP, and they are subject to allosteric activation by ADP and inhibition by phosphoenolpyruvate. Although the PFK isolated from rabbit muscle (RM-PFK) is also composed of four identical subunits, each of these 85-kDa subunits is more than twice the size of the prokaryotic PFK subunit. The allosteric regulation of RM-PFK is more complex in that it is activated by AMP and fructose bisphosphates, while it is inhibited by ATP and citrate.

Poorman et al. (1984) noted internal homology between the N- and C-terminal halves of a nearly complete aa sequence for RM-PFK. They also discovered homology between the aa sequences of Bs-PFK (Kolb et al., 1980) and the two halves of RM-PFK. This indicated that RM-PFK evolved from an ancestor of Bs-PFK by a process of gene duplication and divergence. Lee et al. (1987) subsequently obtained a genomic clone for RM-PFK and determined the sequence for all of the coding exons as well as most of the introns. Their deduced aa sequence identified 30-aa residues which had been left as a gap in the aa sequence determined by Poorman et al. (1984).

Upon sequencing the gene for the major PFK from *E. coli*, Hellinga and Evans (1985) noted significant homology between its deduced aa sequence and the sequences for RM-PFK and Bs-PFK. However, no homology is evident between the deduced aa sequences for the major PFK (*Ec-PFK-1*) and the catalytically distinct minor PFK (*Ec-PFK-2*) from *E. coli* (Daldal, 1984).
Evans and co-workers used X-ray crystallography to
determine the 3-dimensional structures of both the active
(Evans and Hudson, 1979; Evans et al., 1981) and the
inactive conformations (Evans et al., 1986) of Bs-PFK. The
2.4 Å resolution obtained for the active conformation
provided important details concerning the ligand binding
sites of the enzyme.

We recently reported the nucleotide (nt) sequence of
the PFK gene from B. stearothermophilus (French and Chang,
1987). We now report the subcloning of the intact Bs-pfk
gene into the plasmid vector pBR322, the transformation of
a PFK null E. coli host and the high-level of Bs-PFK
expression which results. PFK assays of cell extracts
from this recombinant demonstrate a level of Bs-PFK
specific activity which is 40-fold higher than found in
cell extracts from the wild-type B. stearothermophilus.
The high-level of Bs-PFK expression will enable our
laboratory to use site-specific mutagenesis to investigate
structure-function relationships in this allosteric enzyme.

MATERIALS AND METHODS

(a) Bacterial strains and media

The isolation and characterization of a genomic clone
for Bs-pfk (gt10/Bs-pfk) from a λ library of B.
stearothermophilus DNA has been previously described
(French and Chang, 1987). The DH5α competent cells used
for the primary transformation were obtained from BRL (Bethesda, MD). The complementation studies were done using DF1020 [a recA derivative of DF1010 (Daldal, 1983): pro-82, ΔpfkB201, recA56, Δ(rha-pfkA)200, endA1, hsdR17, supE44, thi-1] on pfk-selective plates. The DF1020 strain was kindly provided by Dr. Barbara Bachmann of the *E. coli* Genetic Stock Center (Yale University School of Medicine, New Haven, CN). The pfk-selective plates were composed of minimal medium 63 (Cohen and Rickenberg, 1956) supplemented with 0.4% mannitol, 80 μg/ml proline, 1 μg/ml thiamine hydrochloride, and 1.5% (w/v) agar (Hellinga and Evans, 1985). Strains DF1010 and DF1020 have zero PFK activity and do not grow on glucose or mannitol although they will grow on glycerol. The DF1010 strain carrying the pFD121 plasmid (DF1010/pFD121) was kindly provided by Dr. Dan G. Fraenkel of the Harvard Medical School (Boston, MA).

Plasmid pFD121 consists of the gene for the minor PFK from *E. coli* (pfkB) cloned between the HindIII and *PstI* sites of pBR322 (Daldal, 1983).

(b) Subcloning of *Bs-pfk*

The *Bs-pfk* gene had previously been isolated from a phage λgt10 genomic library as a clone designated gt10/*Bs-pfk* (French and Chang, 1987). Restriction analysis of this clone indicated that the EcoRI cloning site was located 1.2-kb upstream of the *Bs-pfk* initiation codon. Sequence analysis of the *Bs-pfk* gene identified a ClaI site located 359-nt downstream of the termination codon.
Double digestions with EcoRI and ClalI were performed on both gt10/Bs-pfk and pBR322. The restriction products were fractionated on a 1% SeaPlaque agarose gel (FMC BioProducts, Rockland, ME). The 2.52-kb EcoRI/ClalI fragment containing Bs-pfk and the 4.56-kb EcoRI/ClalI fragment of pBR322 were excised from the gel and ligated directly in the low-melting-point agarose (Struhl, 1985). The ligation products were used to transform DH5α competent cells (BRL, Bethesda, MD) and plasmid mini-preps were performed on two recombinants. Restriction analysis of the mini-preps showed that both recombinants contained the desired Bs-pfk insert. One of the clones was designated pBR322/Bs-pfk and used to transform DF1020.

(c) Transformation of DF1020

The pfk-negative host strain DF1020 was transformed by pBR322 and pBR322/Bs-pfk using the frozen storage buffer (FSB) method of Hanahan (1983). Ampicillin resistant transformants were streaked on LM plates supplemented with 35 μg/ml ampicillin (Hanahan, 1983) to assure that pure clones were obtained. The relatively low transformation efficiency of DF1020 (2-4 x 10^5 colonies/μg plasmid) may explain the failure of previous attempts at the direct selection of Bs-pfk.

(d) Complementation of PFK deficiency in DF1020

Four strains were streaked in parallel upon minimal media 63 agar plates supplemented with proline, thiamine, and mannitol or glycerol: DF1020, DF1020/pBR322,
Fig. 1. Complementation of PFK deficiency in *E. coli* strain DF1020. The following strains were streaked in parallel and incubated for 3-days at 37°C upon agar plates which were nonselective (Glycerol) or selective (Mannitol) for PFK activity (Daldal, 1983). Strains: (1) DF1020, (2) DF1020/pBR322/Bs-pfk, (3) DF1020/pBR322, (4) DF1010/pDF121. Both DF1010 and DF1020 have zero PFK activity, so only the two strains expressing cloned *pfk* genes (2 & 4) were able to grow on the *pfk*-selective plates.
DF1020/pBR322/Bs-pfk, and DF1010/pFD121. The photograph in Fig. 1 was taken after 3-days of growth at 37°C although results were evident after only 2-days. All four strains grew on the glycerol plate; however, only those hosts containing pfk genes grew on the mannitol plate. This experiment provided clear evidence that the Bs-pfk gene complements the PFK deficiency in DF1020.

(e) SDS-PAGE of cell lysates and purified Bs-PFK

Overnight cultures of K-10, DF1020, DF1020/pBR322, DF1020/pBR322/Bs-pfk, and B. stearothermophilus strain NCA 1503 were pelleted by centrifugation, resuspended in sonication buffer (50 mM Tris-HCl, pH 7.4, 1 mM DTT, 1 mM EDTA, 1 mM PMSF), and lysed by sonication. The total protein in each sample was determined with a protein assay kit from Bio-Rad Laboratories (Rockville Centre, NY).

Bs-PFK was then purified from the DF1020/pBR322/Bs-pfk lysate using the same procedure followed by Hengartner and Harris (1975). This procedure includes the use of an AMP-Sepharose 4B affinity column in the final step. The protein concentrations after each step of the purification scheme were determined using the Bio-Rad protein assay kit. Samples of the cell lysates (150 μg) and the purified Bs-PFK (15 μg) were electrophoresed on a 10-15% linear gradient SDS/polyacrylamide gel and stained with Coomassie blue (Fig. 2).
Fig. 2. Coomassie-stained SDS-polyacrylamide gel (10-15% linear gradient) of cell lysates and purified Bs-PFK.

Lanes: (1) Mr markers, (2) E. coli K-10, (3) DF1020, (4) DF1020/pBR322, (5) DF1020/pBR322/Bs-pfk, (6) purified Bs-PFK, (7) B. stearothermophilus NCA 1503. The single most abundant protein in lane (5) co-migrates with purified Bs-PFK and has a Mr of 34 kDa.
(f) PFK activities of cell lysates and purified Bs-PFK

The PFK activities of each cell lysate and the purified Bs-PFK were assayed by the method of Kotlarz and Buc (1982). This method was used by Hellinga and Evans (1985) to assay their recombinant Ec-PFK-1. However, this assay differs significantly from the one used by Hengartner and Harris (1975) to determine the specific activity of authentic Bs-PFK. Table 1 summarizes the results of these assays. The recombinant Bs-PFK which was purified to near homogeneity as judged by SDS-PAGE was found to have a specific activity of 140 U/mg.

RESULTS AND DISCUSSION

The results of complementation studies and assays for PFK activity indicate that plasmid pBR322/Bs-pfk directs the production of active Bs-PFK in an *E. coli* host. SDS-PAGE of cell lysates demonstrate that the single most abundant protein produced by this recombinant has a Mr equivalent to that of Bs-PFK. Densitometric scans indicate that Bs-PFK represents 20% of the total protein extracted from cell lysates of the recombinant. The activity of the enzyme in these cell lysates is demonstrated by assays showing a level of Bs-PFK specific activity which is 40 times greater than that found in cell lysates of wild-type *B. stearothermophilus*. 
TABLE I

PFK expression in various strains and recombinants*

<table>
<thead>
<tr>
<th>Cell lysate or preparation</th>
<th>PFK volume activity (U/ml)</th>
<th>[Protein] (mg/ml)</th>
<th>PFK specific activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli K-10</td>
<td>1.5</td>
<td>7.51</td>
<td>0.2</td>
</tr>
<tr>
<td>E. coli DF1020</td>
<td>0.0</td>
<td>7.49</td>
<td>0.0</td>
</tr>
<tr>
<td>DF1020/pBR322</td>
<td>0.0</td>
<td>9.90</td>
<td>0.0</td>
</tr>
<tr>
<td>DF1020/pBR322/Bs-pfk</td>
<td>463.7</td>
<td>9.21</td>
<td>50.3</td>
</tr>
<tr>
<td>Purified Bs-PFK</td>
<td>96.8</td>
<td>0.69</td>
<td>140.3</td>
</tr>
<tr>
<td>B. stearo. NCA 1503</td>
<td>5.2</td>
<td>4.36</td>
<td>1.2</td>
</tr>
</tbody>
</table>

* Assays were carried out as described in Materials and Methods, section (f). The K-10 strain of E. coli is the wild-type from which DF1020 was derived (Daldal, 1983). The construction of pBR322/Bs-pfk is described in section (b) and the transformation of DF1020 is described in section (c) of Materials and Methods. The purification of Bs-PFK from the DF1020/pBR322/Bs-pfk cell lysate is described in section (e) of Materials and Methods. NCA 1503 is the strain of B. stearothermophilus which was used by Evans and Hudson (1979) for the crystal structures of Bs-PFK and by French and Chang (1987) for cloning the Bs-pfk gene.
Two lines of evidence suggest that this high level of expression is directed from the endogenous Bs-pfk promoter. First, the tyrosyl-tRNA synthetase gene from B. stearothermophilus was expressed at levels representing 10% of total soluble protein when it was cloned into the same pBR322 vector (Barker, 1982). Second, the region immediately upstream of the Bs-pfk coding sequence contains elements which share limited homology with E. coli consensus sequences for transcriptional initiation and ribosome binding. These homologies are detailed in Fig. 3.

The high-level expression of Bs-PFK in E. coli will enable this laboratory to use site-specific mutagenesis to probe the structure-function relationships in this allosteric enzyme. The crystal structures corresponding to the R-state (Evans and Hudson, 1979) and the T-state (Evans et al., 1986) of the enzyme have already been determined. The aa residues which are critical to the function of the enzyme have therefore been identified (Hellinga and Evans, 1985). This wealth of structural data makes it possible to design site-specific mutations for the independent verification of the active and allosteric sites (Zoller and Smith, 1985).

Site-specific mutations are also planned to help characterize the transition of the enzyme between the active R-state and the inactive T-state (Blangy et al., 1968). The 44% aa sequence homology (Poorman et al., 1984) and the conservation of critical aa residues (Hellinga and
Fig. 3. Homologies between the \textit{Bs-pfk} promoter region and \textit{E. coli} consensus sequences for transcriptional initiation and ribosome binding (\textit{Ec-con}). The 5'-flanking region of the \textit{Bs-pfk} gene is shown at top with homologous \textit{E. coli} consensus sequences (Lewin, 1985) aligned beneath it. The matches between the two are marked with asterisks. The numbering of the \textit{Bs-pfk} gene is the same as that in a previous paper (French and Chang, 1987). Transcription of the \textit{Bs-pfk} gene probably initiates at one or more of the positions marked with plus sign. Translation of the gene begins with the GTG initiation codon 5-bp downstream of the Shine-Dalgarno sequence.
Evans, 1985) between Bs-PFK and N-terminal half of RM-PFK suggest that structure-function relationships found in Bs-PFK may well apply to the more complex RM-PFK. The PFK from B. stearothermophilus therefore promises to be an excellent system in which to study the structure-function relationships determining the allosteric nature of enzymes.

ACKNOWLEDGEMENTS

This investigation was supported by Public Health Service Grant 5 R01 AM 31676 from the National Institutes of Health. We thank M. LoMonaco and M. Shirley for valuable discussions and for critical reading of the manuscript.

REFERENCES


SECTION III

Molecular cloning and sequencing of a partial cDNA for rabbit muscle 6-phosphofructo-1-kinase
Molecular cloning and sequencing of a partial cDNA for rabbit muscle 6-phosphofructo-1-kinase

(Recombinant DNA; Northern hybridization; Okayama-Berg cDNA library; allosteric enzyme)

Brent A. French, Ming-Ching Kao and Simon H. Chang*

Department of Biochemistry, Louisiana State University, Baton Rouge, LA 70803 (U.S.A.) Tel. (504) 388-5147

* To whom correspondence and reprint requests should be addressed.

Abbreviations: aa, amino acid(s); ADP, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; bp, base pairs(s); Bs-PFK, Bacillus stearothermophilus PFK; C, cytidine; d, deoxyribo; Ec-PFK-1, the major Escherichia coli PFK; EtBr, ethidium bromide; G, guanosine; kb, 1000 bp; nt, nucleotide(s); PFK, 6-phosphofructo-1-kinase; RM-PFK, rabbit muscle PFK; RM-pfk, the gene for RM-PFK; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl, 0.015 M Na$_3$ citrate, pH 7-8; T, thymidine.
SUMMARY

A partial cDNA for rabbit muscle 6-phosphofructo-1-kinase (RM-PFK) has been cloned and sequenced. The nucleotide (nt) sequence of the cDNA agrees with the previously determined RM-pfk genomic sequence. In addition, the amino acid (aa) sequence deduced from the cDNA is nearly identical to the RM-PFK sequence previously determined by peptide analysis. A significant degree of homology exists when the aa sequence of RM-PFK is compared with the sequences of Bacillus stearothermophilus PFK or Escherichia coli PFK-1. The cloning and sequencing of the RM-PFK cDNA fragment represents significant progress toward the long-term goal of using site-specific mutagenesis to investigate the structure-function relationships in this allosteric enzyme.

INTRODUCTION

6-Phosphofructo-1-kinase (PFK; EC 2.7.1.11) catalyzes the ATP-dependent phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate. The activity of this enzyme is controlled by physiological modulators and it thus plays a key role in the regulation of glycolysis (Uyeda, 1979). PFK from B. stearothermophilus (Bs-PFK) and the major PFK from E. coli (Ec-PFK-1) share many physical and kinetic properties (Evans et al., 1981). They are homotetrameric
enzymes composed of 34-kDa and 35-kDa subunits, respectively. Both display cooperative kinetics with respect to fructose 6-phosphate but not with respect to ATP, and they are subject to allosteric activation by ADP and inhibition by phosphoenolpyruvate. Although the PFK isolated from rabbit muscle (RM-PFK) is also composed of four identical subunits, each of these 85-kDa subunits is more than twice the size of the prokaryotic PFK subunit. The increased size of RM-PFK reflects the complexity of its allosteric regulation. In addition to responding to the same effectors which modulate the prokaryotic PFK's, RM-PFK is activated by AMP and fructose bisphosphates and it is inhibited by ATP and citrate.

Poorman et al. (1984) noted internal homology between the N- and C-terminal halves of a nearly complete aa sequence for RM-PFK. They also discovered homology between the aa sequences of Bs-PFK (Kolb et al., 1980) and the two halves of RM-PFK. This indicated that RM-PFK evolved from an ancestor of Bs-PFK by a process of gene duplication and divergence. Upon sequencing the gene for the major PFK from E. coli, Hellinga and Evans (1985) noted significant homology between the deduced aa sequence and the sequences for RM-PFK and Bs-PFK.

Lee et al. (1987) obtained a genomic clone for RM-PFK and determined the sequence for all of the coding exons as well as most of the introns. Their deduced aa sequence identified 30-aa residues which had been left as a gap in
the aa sequence determined by Poorman et al. (1984). In addition, French and Chang (1987) cloned and sequenced the gene encoding Bs-PFK. This sequence served to correct errors present in previously published versions of the Bs-PFK aa sequence (Kolb et al., 1980, Poorman et al., 1984, Hellinga and Evans, 1985). The aa sequence alignment of RM-PFK, Bs-PFK, and Ec-PFK-1 made by French and Chang (1987) is therefore the most correct version to date.

This paper describes the cloning and sequencing of a partial cDNA for RM-PFK. The cDNA library was prepared from rabbit muscle polyadenylated RNA by the method of Okayama and Berg (1982). A 250-nt cDNA probe for RM-PFK was obtained from Scott Putney (Repligen, Boston, MA) and used to isolate a positive clone from the library by colony hybridization. Sequence analysis of this positive clone showed that it contained one-quarter of the RM-PFK coding sequence and the 3'-untranslated region of RM-PFK mRNA. The cDNA sequence confirms one-quarter of the RM-PFK aa sequence determined by Poorman et al. (1984) and thus verifies the homologies between RM-PFK and the two prokaryotic PFK's.

MATERIALS AND METHODS

(a) Bacterial strains, media, and methods

The 3'-oligo(dT)-tailed pSV7186-derived plasmid primer and the 3'-oligo(dG)-tailed pSV1932-derived HindIII linker
required for cloning cDNA by the method of Okayama and Berg (1982) were obtained from P-L Biochemicals, Division of Pharmacia, Inc. (Milwaukee, WI). The E. coli host strain DH1 was obtained from the American Type Culture Collection, Rockville, MD (ATCC no. 33849) and grown in SOB media (Hanahan, 1983). The M13mp18 and M13mp19 vectors as well as the JM107 and JM109 hosts were obtained from Joachim Messing, presently at Rutgers University (Piscataway, NJ). The DH5α competent cells used for transient transformation by the M13 phage recombinants were obtained from BRL (Bethesda, MD). The following methods were performed as described by Maniatis et al. (1982) unless otherwise specified.

(b) Preparation of a probe for RM-PFK

A 250-nt cDNA probe for RM-PFK was kindly provided by Scott Putney, presently at Repligen (Boston, MA). Dr. Putney had isolated the cDNA probe from a shotgun cDNA library of rabbit muscle polyadenylated RNA cloned into the phage vector M13mp8. Among the 178 randomly selected M13 recombinants whose sequences were determined (Putney et al., 1983), a computer search for RM-PFK coding regions identified a single RM-PFK clone. The sequence of the cDNA insert in this clone encoded 60-aa in the C-terminal region of RM-PFK (Poorman et al. 1984).

The replicative form of the M13 clone was amplified and the DNA restricted with BamHI and HindIII to release the RM-PFK cDNA insert. The restriction digest was treated
with bacterial alkaline phosphatase, fractionated on a 1% agarose gel, and the 250-bp cDNA insert purified by electroelution. The dephosphorylated RM-PFK cDNA insert was kinased with $[^{32}\text{P}]\text{ATP}$ and end-filled with $[^{32}\text{P}]\text{dATP}$ to a specific activity of $1\times10^8$ cpm/µg for screening the cDNA library.

(c) Construction of cDNA library

Polyadenylated RNA was isolated from rabbit muscle by the method of Chirgwin et al. (1979) and used to construct a cDNA library by the method of Okayama and Berg (1982). The following methods represent the only deviations from the published protocols.

(i) Oligodeoxycytidylate [oligo(dC)] addition: The second step of the Okayama-Berg procedure calls for a 5 min addition of oligo(dC) tails by terminal deoxynucleotidyl transferase. However, the rate of the terminal transferase reaction is dependent upon many variables including the concentration of 3'-ends in the reaction mixture (Deng and Wu, 1981). This concentration is difficult to accurately determine during the synthesis of cDNA, so the optimal reaction time for the tailing reaction was determined empirically (Affolter and Anderson, 1985). Aliquots of the tailing reaction were removed at time intervals, terminated by phenol extraction, and used for the remainder of the Okayama-Berg procedure. The transformation efficiencies obtained from each sample were compared and plasmid mini-preps of random clones were assayed for cDNA content by
electrophoresis on agarose gels. This procedure determined that a terminal transferase reaction time of 2.3 min was optimum for this particular preparation of cDNA.

(ii) Transformation of E. coli: Okayama and Berg transformed host strain HB101 with their cDNA libraries using minor modifications of the procedure described by Cohen et al. (1972). This procedure typically yields $1 \times 10^7$ transformants per $\mu$g of supercoiled plasmid pBR322. However, the transformation procedure for strain DH1 described by Hanahan (1983) is about ten times more efficient. The Hanahan method was therefore used to transform strain DH1 with the rabbit muscle cDNA library.

d) Colony hybridization of cDNA library

Host cells made competent by the method of Hanahan (1983) were transformed by the rabbit muscle cDNA library and plated onto BA85 nitrocellulose filters (Schleicher & Schuell, Keene, NH) at a density of 10,000 colonies per 82 mm filter. Two replica filters were made from each of four master filters. The replicas were incubated for 5 hrs on L plates containing 100 $\mu$g/ml ampicillin and transferred to L plates containing 250 $\mu$g/ml chloramphenicol for incubation overnight.

The lysis, prehybridization, hybridization, and washing of the filters were done as per Maniatis et al. (1982) with only one minor addition to the prehybridization protocol. After an initial prehybridization of 2 hrs at $42^\circ$C, the filters were each placed colony side up in a
shallow tray of prehybridization solution and rubbed gently with a gloved finger (Hanahan and Meselson, 1983). This treatment assured the removal of colony debris and thus eliminated the background signals which would have otherwise been present after hybridization.

The hybridization was performed using the \(^{32}\text{P}\)-labeled 250-nt cDNA probe at a specific activity of \(1 \times 10^8 \text{ cpm/µg}\) and a final concentration of \(1.3 \times 10^6 \text{ cpm/ml}\) in the hybridization solution. The most stringent wash of the filters was at 68°C for 2 hrs in 1x SSC / 0.1% SDS. An overnight exposure revealed two strong signals on each of two duplicate filters. However, only one positive clone could be isolated after re-screening the colonies corresponding to the two signals. The single positive clone was designated pOB220/RM-\(pfk\) and analyzed as follows.

e) Southern analysis

Southern blot analysis was used to determine the maximum length of the cDNA insert in pOB220/RM-\(pfk\). The cloned DNA was isolated by the plasmid mini-prep procedure, restricted, and fractionated on a 1% agarose gel. The DNA fragments were bidirectionally transferred to nitrocellulose and probed with kinased oligonucleotides corresponding to known positions in the RM-PFK coding region. The Southern blots shown in Fig. 1 suggested that the cDNA clone encoded no fewer than 96-aa and no more than 267-aa.
Fig. 1. Southern blots of pOB220/RM-pfk. Panel A: EtBr stained 1% agarose gel. Lanes: (1 & 7) 300 ng of λ phage DNA cut by HindIII; (2-5) 400 ng of pOB220/RM-pfk cut by; (2) no enzyme, (3) PstI, (4) PvuII, (5) PstI + PvuII; (6) 1 μg of λ/PPK-1 (Lee et al., 1987) cut by EcoRI. Panel B: Southern blot of agarose gel probed with 32P-labeled PFK 6 oligonucleotide complementary to codons 684-688. Panel C: Southern blot of agarose gel probed with 32P-labeled PFK 5 oligonucleotide corresponding to codons 513-517. The hybridization of pOB220/RM-pfk with PFK 6 but not with PFK 5 indicates that the 5' end of the PFK cDNA lies between codons 518 and 684 of the RM-pfk gene.
f) Restriction analysis

Restriction analysis of pOB220/RM-pfk was performed by standard procedure. Single and double restriction digestions were made of the plasmid DNA and resulting fragments were fractionated on agarose gels. Restriction mapping was facilitated by detailed knowledge of the plasmid vector (Okayama and Berg, 1982) as well as the RM-PFK coding sequence (Lee et al., 1987). A restriction map of the partial RM-PFK cDNA is shown in Fig. 2.

g) Sequence analysis

The restriction map of pOB220/RM-pfk revealed that the entire cDNA insert could be directionally excised from the Okayama-Berg vector by the restriction endonucleases HindIII and PvuII. This fragment was subcloned between the HindIII and Smal sites of the phage vector M13mp19 after making the appropriate restrictions and fractionating the products on a 1% Sea-Plaque agarose gel (FMC BioProducts, Rockland, ME). The desired vector and insert bands were excised from the gel and ligated directly in agarose using the procedure of Struhl (1985).

Competent DH5α cells obtained from BRL (Bethesda, MD) were used as transient hosts for the transformation. The transformation was plated with JM109 lawn cells in order to support the growth of recombinant phage. Template DNA was prepared from white plaques as described by Messing (1983). The PFK 6 oligonucleotide complementary to codons 684-688 of RM-pfk was then used to prime DNA sequencing reactions
Fig. 2. Sequencing strategy for RM-PFK cDNA. The sequence shown in Fig. 3 is represented as a bold line with selected restriction sites and locations. The first C of the oligo(dG)-tailed PstI site is defined as position 1 in both Figures 2 and 3. The arrow above the restriction map indicates the location and orientation of RM-pfk codons 598-781. The arrows below the restriction map indicate the location and direction in which the sequence data was read. The dideoxy chain termination method of Sanger et al. (1977) as modified by Biggin et al. (1983) for [35S]dATP was used to sequence subclones in M13mp18 and M13mp19 (Norrander et al., 1983). In addition to the M13 universal primer, two other oligodeoxynucleotides were made on an Applied Biosystems Model 380A DNA Synthesizer for use as sequencing primers. The sequence of the PFK 6 oligonucleotide is complementary to RM-pfk codons 684-688 while the sequence of PFK 7 corresponds to codons 767-772.
using the dideoxy chain termination method (Sanger et al., 1977). The resulting sequence verified the identity of the partial RM-PFK cDNA clone and indicated that it had a 5'-end corresponding to codon 598 of RM-pfk. Additional M13 clones were generated and sequenced in a similar manner. A summary of the sequencing strategy for the cDNA insert in pOB220/RM-pfk is shown in Fig. 2 and the completed sequence is presented in Fig. 3.

RESULTS AND DISCUSSION

The 549-nt coding sequence for RM-PFK contained in pOB220/RM-pfk predicts an aa sequence which is identical to the corresponding portion of the RM-PFK sequence published by Poorman et al. (1984). In addition, the nucleotide sequence of the cDNA is in agreement with the RM-pfk genomic sequence determined by Lee et al. (1987). These identities confirm that pOB220/RM-pfk contains a partial cDNA for RM-PFK. This cDNA is particularly valuable in that it defines the polyadenylation site of RM-pfk mRNA. The genomic clone obtained by Lee et al. (1987) extended only 151-nt downstream of the termination codon and was therefore uninformative in this regard. A polyadenylation signal of AATTAA is located 607-nt downstream of the termination codon and 23-nt upstream of the poly-A tail in the RM-PFK cDNA clone.
Fig. 3. Nucleotide and deduced amino acid sequences of RM-PFK cDNA.

The first C of the oligo(dG)-tailed PstI site is defined as position 1.
The partial cDNA clone for rabbit muscle PFK represents significant progress toward the long-term goal of using site-specific mutagenesis to investigate the structure-function relationships in this allosteric enzyme. In cases where full-length cDNA's are difficult to obtain directly, it has been necessary to splice partial cDNA's and genomic fragments together in order to construct a full-length clone (Toole et al., 1984). Alternatively, oligonucleotide-directed mutagenesis has been used to delete introns from genomic clones (Wallace et al., 1980).

It may prove necessary to pursue one of these experimental designs in order to obtain a full-length PFK cDNA. In either case, pOB220/RM-pfk represents a significant contribution since it contains one-quarter of the RM-PFK coding sequence. The corresponding region of the genomic clone contains 5 of the 22 known introns.

ACKNOWLEDGEMENTS

This investigation was supported by Public Health Service Grant 5 R01 AM 31676 from the National Institutes of Health. We thank M. LoMonaco and M. Shirley for valuable discussions and for critical reading of the manuscript.
REFERENCES


SUMMARY

The first two sections of this dissertation describe the development of a system for determining the relationships between structure and function in the PFK from *Bacillus stearothermophilus* (Bs-PFK). This system for site-specific mutagenesis was developed by cloning the gene encoding Bs-PFK, determining the nucleotide sequence of the gene, and directing the expression of Bs-PFK in a PFK null strain of *Escherichia coli*. Part I of the Appendix addresses the experimental design and significance of future Bs-PFK research.

The cloning and sequencing of a partial cDNA for rabbit muscle PFK are the subjects of the third section of this dissertation. The cloned cDNA covers one-quarter of the RM-PFK coding sequence starting from the 3'-end of the gene. The significance of this work with respect to the long-term goal of using site-specific mutagenesis to determine structure-function relationships in RM-PFK is addressed Part II of the Appendix.

In summary, the work presented here represents significant progress toward using the techniques of molecular biology to enhance our understanding of the relationships between structure and function in the key regulatory enzyme of the glycolytic pathway.
REFERENCES


Kotlarz, D. and Buc, H.: Phosphofructokinases from


mediator of hormone action at the fructose 6-phosphate/fructose 1,6-bisphosphate substrate cycle.


Schmitt, J.J. and Cohen, B.N.: Quantitative isolation of DNA restriction fragments from low-melting agarose by


Vora, S., Francke, U.: Assignment of the human gene for


Winter, G., Koch, G.L.E., Hartley, B.S. and Barker, D.G.:

Younathan, E.S., Voll, R.J., and Koerner, T.A.W., Jr.:


APPENDIX

Experimental Design and Significance of Future Research

I. Research on Bs-PFK

A. Immediate Goals

The isolation, purification and enzymatic analysis of Bs-PFK are projects which should be undertaken in the near future. This work is important because a rigorous assessment of Bs-PFK allosteric behavior has never been published. The enzymatic analysis is now feasible due to the high level of Bs-PFK produced by the recombinant DF1020/pBR322/Bs-PFK. The present level of Bs-PFK expression corresponding to 20% of the total soluble protein is more than adequate for large-scale Bs-PFK purification. However, the yield of Bs-PFK can be improved by the following lines of investigation.

The yield of Bs-PFK per liter of culture can most easily be increased by improving the nutritional quality of the culture medium. It is estimated that this yield can be improved several-fold simply by growing the cell cultures in TB rather than LB media (Tartof and Hobbs, 1987).

The production of Bs-PFK in cell cultures of DF1020/pBR322/Bs-pfk can be optimized by assaying PFK activity at various points on the growth curve. Four preliminary PFK assays of cell lysates from mid-log, late-log, mid-stationary and late-stationary phases of cell
growth will indicate the culture phase at which the ratio of active PFK to cell mass is greatest. One would expect cell death and the resulting protease activity to reduce this ratio at some point on the growth curve. There is also a small possibility that Bs-PFK might be released from the bacterial cells, so PFK assays should be performed on the culture supernatants as well as the cell pellets.

There are also a number of relatively simple genetic manipulations which should increase production of Bs-PFK. The first class of such manipulations involves the insert fragment carrying the *Bs-pfk* gene. Hellinga and Evans (1985) found that the level of Ec-PFK-1 expression could be increased nearly four-fold by simply deleting 1.5 kb of extraneous DNA flanking the 5'-end of the *Ec-pfkA* gene. The construction pBR322/Bs-pfk contains at least 1 kb of 5'-flanking sequences which can presumably be deleted.

A second class of genetic manipulations involves the vector carrying the *Bs-pfk* gene. Vector copy number plays a major role in the level of Bs-PFK expression. The pBR322 vector which carries the *Bs-pfk* gene has a copy number of about 20 plasmids per bacterial cell. Reducing the size of this vector has been shown to increase the copy number by 1.5- to 3-fold (Maniatis et al., 1982). Subcloning the 2.52 kb *EcoRI* / *Hind III* fragment containing *Bs-pfk* from pBR322/Bs-pfk into the corresponding sites of pUC18 or pUC19 may therefore lead to a substantial increase in Bs-PFK expression.
The single-stranded bacteriophage vector M13mp18 has a copy number of approximately 200 phage genomes per cell. A recombinant bacteriophage designated mp18/Bs-pfk has already been constructed by a subcloning procedure analogous to the one which created pBR322/Bs-pfk. Time-course PFK assays should be performed on JM107 cell cultures which have been infected by this recombinant. If mp18/Bs-pfk directs a high level of Bs-PFK expression, one might consider creating a PFK null M13 host strain by transferring the F' episome from JM107 into DF1020. This line of investigation is quite attractive since it may be possible to use the same M13 construction for site-specific mutagenesis and expression of mutant Bs-PFK.

The development of alternative host strains belongs to a third class of genetic manipulations which might lead to an increase in Bs-PFK production. There is a small possibility that E. coli protease activity may be limiting the production of Bs-PFK by DF1020. If evidence supporting this possibility develops, it may prove worthwhile to express Bs-PFK in a lon- strain of E. coli such as Y1088 (Huynh et al., 1985). A significantly higher level of Bs-PFK production in the absence of lon protease would make crossing the lon deletion into DF1020 desirable.

B. Site-specific Mutagenesis of Bs-PFK

The cloning and high-level expression of the Bs-pfk gene has made it possible to determine the structure-function relationships of the allosteric enzyme using site-
specific mutagenesis. The method of choice for making predetermined point mutations in a cloned gene is oligonucleotide-directed mutagenesis (Zoller and Smith, 1983).

This procedure uses in vitro enzymatic reactions to incorporate an oligonucleotide containing the desired base change(s) into a cloned gene. The classical approach involves subcloning the gene of interest into bacteriophage M13 and purifying single-stranded recombinant phage DNA. An oligonucleotide containing the desired mismatch(es) is then annealed to the gene and extended with the Klenow fragment of DNA polymerase I. The nicks in the resulting double strands of DNA are sealed with DNA ligase and the products of the ligation are used to transform an E. coli host.

The yield of mutant phage is often very low, but techniques for screening and selecting mutant phage have been developed. One of these techniques involves generating the single-stranded DNA substrate in dut- ung- mutants of E. coli (Kunkel, 1985). The resulting phage DNA will therefore contain a significant number of uracil substitutions for thymine. After annealing and extending the oligonucleotide, the duplex of DNA used for transformation will have uracil incorporated in the wild-type strand but the synthetic strand containing the mismatched oligonucleotide will be normal in constitution. The wild-type strand will therefore be degraded by uracil-
N-glycosylase after transformation of an ung+ E. coli host, while the strand containing the mutation will replicate and produce progeny phage.

This technique will produce a site-specific mutation frequency approaching 100% (Kunkel, 1985). Progeny phage can always be screened for the desired mutation by hybridization with the original 32P-labeled oligonucleotide containing the mismatch. However, with a high mutation frequency it is more convenient to sequence the regions of interest from several recombinants. Once a mutant clone is identified, it is prudent to sequence the entire gene since other mutations may have been introduced during the in vitro manipulations.

Plans have been formulated to perform oligonucleotide-directed mutagenesis on the Bs-pfk gene using the techniques described above. The construction mp18/Bs-pfk consists of the entire Bs-pfk gene subcloned into the single-stranded phage vector M13mp18. A dut- ung- strain of E. coli will be infected by this recombinant and uracil-containing single-stranded DNA purified from the progeny phage. The protocol described above will then be performed using four different mutagenic oligonucleotides.

The mutant Bs-pfk genes which result may well direct adequate levels of Bs-PFK expression from the M13 vectors. If this is not the case, the 2.52 kb EcoRI/HindIII fragments containing the mutant Bs-pfk genes will have to be subcloned into the corresponding sites of pBR322.
Once a suitable level of expression is obtained, the kinetics of each of the four mutant Bs-PFK's will be compared with those of the cloned wild-type Bs-PFK. Similar kinetic constants will indicate that the amino acid substitution had little effect on the function of the enzyme. This in turn will argue that the mutated amino acid was not critical to catalytic or allosteric function. However, a difference in kinetic constants between the standard and experimental Bs-PFK's will indicate that the mutated amino acid played a critical role in the function of the enzyme. Furthermore, the nature of the difference in kinetic constants will yield valuable information concerning the function of the amino acid under study.

Four preliminary mutations of Bs-PFK have been chosen which will enhance our understanding of this allosteric enzyme. The first three mutations will verify the locations of the three ligand-binding sites in Bs-PFK while the fourth will probe the conformational transition of the enzyme between the active R-state and the less active T-state.

The immediate goal will be to verify the positions of the three ligand binding sites which have been identified from the crystal structures of Bs-PFK. There is little doubt that these binding sites are correct, but oligonucleotide-directed mutagenesis will confirm that the ligand-binding behavior of the enzyme in solution is the same as its behavior in the crystalline state. The
following three mutations were designed to alter the kinetics of the enzyme without perturbing the tertiary structure or destroying catalytic activity. These mutations also involve amino acids which are absolutely conserved among Bs-PFK, Ec-PFK-1, and the catalytic N-terminal half of RM-PFK.

The nature of the fructose 6-phosphate binding site will be verified by substituting an Ala for Glu-222. This mutation amounts to the loss of the methylene carboxylic acid group which is responsible for hydrogen-bonding the O4 of fructose 6-phosphate. The mutation should decrease the affinity of the enzyme for fructose 6-phosphate and thus reduce the enzymatic activity of Bs-PFK.

The ATP binding site will be verified by substituting a Cys for Ser-9. This Ser contacts the ribose of ATP, so changing the hydroxyl group of Ser-9 to a sulfhydryl group should cause a subtle change in the Km of the enzyme for ATP. While it is difficult to predict the result of this change in geometry and decrease in hydrogen-bonding capacity, the mutation should produce a detectable change in the kinetics of the mutant enzyme.

The location of the effector site will be verified by substituting an Ala for Arg-25. The crystallographic evidence indicates that both the activator ADP and the inhibitor phosphoenolpyruvate bind to this effector site. The Arg-25 hydrogen bonds the alpha- and beta-phosphates of ADP, so removing the ethylene guanidinium group will
decrease the affinity of the enzyme for the activator ADP. The resulting enzyme should therefore require higher concentrations of ADP than the wild-type enzyme to achieve the same level of activation.

When studied at a resolution of 6 Å, the phosphate group of the inhibitor phosphoenolpyruvate appears to bind in the same place as the beta-phosphate group of ADP. The Arg-25 to Ala mutant should therefore require higher concentrations of phosphoenolpyruvate than the wild-type enzyme to achieve the same level of inhibition. The phosphate group of phosphoenolpyruvate is also hydrogen-bonded by Arg-21, so a decrease rather than a total loss of inhibition by phosphoenolpyruvate is expected.

Oligonucleotide-directed mutagenesis will also be used to investigate the allosteric transition of Bs-PFK between the active R-state and the less active T-state. Evans et al. (1981) suggest that this transition involves "the rearrangement of essentially rigid subunits into a new quaternary structure. The binding sites for the substrate fructose 6-phosphate and the allosteric effectors ADP and phosphoenolpyruvate then crosslink the subunits, locking the structure into the preferred conformation."

Sufficient information is already available to construct a mutant enzyme which will favor the less active T-state. The Arg-252 residue of the fructose 6-phosphate binding site not only hydrogen bonds the P6, O2, and O5 of this substrate, but it is also central to a network of
hydrogen bonds linking fructose 6-phosphate to the adjacent subunit. The loss of the ethylene guanidinium group will not only reduce the enzyme's affinity for fructose 6-phosphate, but it will also interfere with the change in conformation to the more active R-state brought about by the binding of fructose 6-phosphate. It is predicted that the \textit{Bs-PFK Arg-252 to Ala} mutant enzyme will have little or no catalytic activity.

C. Significance of \textit{Bs-PFK} Research

The four mutations of \textit{Bs-PFK} described above should give preliminary indications concerning the validity of the 3-D structures proposed by Evans and colleagues at MRC Cambridge, England. Hellinga and Evans (1985) have cataloged 31 critical amino acid residues in \textit{Bs-PFK} and described the roles which they appear to play in the function of the enzyme. It is anticipated that less than a dozen mutations should be necessary to confirm or disprove the structure-function relationships which they advanced based upon the crystal structures of the enzyme (Evans and Hudson, 1979; Evans et al., 1986).

The site-specific mutagenesis of \textit{Bs-PFK} is expected to confirm the structure-function relationships proposed by Hellinga and Evans (1985) and enhance our understanding of this allosteric enzyme. This work will certainly be relevant to the study of \textit{RM-PFK} because of the strong homology between the two enzymes (Poorman et al., 1984).

The understanding of structure-function relationships
in Bs-PFK may also be relevant to other enzymes. For example, the positive cooperativity which results from the interaction between subunits may well be a common theme in nature. The cooperative oxygenation of hemoglobin (Perutz, 1970) and the allosteric cooperativity of aspartate transcarbamoylase (Wente and Schachman, 1987) are notable examples of this phenomenon. Finally, it is interesting to note that proteins as diverse as ATP synthase, ATP/ADP translocase, adenylate kinase, and PFK all have similar adenine nucleotide binding folds in their structures (Walker et al., 1982).

II. RM-PFK Research

A. Immediate Goals

The expression of a eukaryotic gene in E. coli requires that the gene be free of introns. It will therefore be necessary to synthesize or construct a full-length cDNA of RM-PFK before expression can be attempted. In cases where full-length cDNA's have been difficult to obtain directly, it has been necessary to splice partial cDNA's and genomic fragments together in order to construct a full-length clone (Toole et al., 1984). Alternatively, introns have been deleted from genomic clones by oligonucleotide-directed mutagenesis (Wallace et al., 1980). Oligonucleotides may also prove useful as short linkers and structural units of cDNA.

It may prove necessary to pursue one or more of these
experimental designs in order to obtain a full-length RM-PFK cDNA. Regardless of the approach chosen, pOB220/RM-pfk represents a significant contribution since it encompasses one-quarter of the RM-PFK coding sequence. The corresponding region of the genomic clone contains 5 of the 22 known introns.

Once constructed, the full-length cDNA will be inserted into an expression vector under the control of an inducible prokaryotic promoter. The resulting hybrid gene should provide a reasonably high level of RM-PFK expression in E. coli. These manipulations will complete the system necessary for performing site-specific mutagenesis on RM-PFK.

B. Site-specific Mutagenesis of RM-PFK

A number of site-specific mutations have already been designed for RM-PFK. The initial mutations will focus on the locations of the fructose bisphosphate and AMP/ADP activation sites proposed by Poorman et al. (1984). Mutations have also been planned which will verify the function of the connecting peptide, probe the catalytic site, and determine if the N-terminal half of PFK is enzymatically active.

The proposed location of the fructose bisphosphate activation site will be investigated by substituting Asp for Ser-127C. Poorman et al. (1984) have suggested that the relatively small Ser-127 side chain leaves room in the activation site for the fructose 1,6- and 2,6-
bisphosphates. The probable effect of substituting a carboxyl group for the hydroxyl group will be to reduce the enzyme's affinity for these activators due to repulsion between the carboxyl group and P1 or P2 of the fructose bisphosphates.

A series of mutations are planned to investigate the location of the ATP inhibition site. The mutations involve substituting Ala for Lys-211C, Lys-213C, Arg-21N, and Arg-25N. These residues hydrogen bond the phosphates of ATP, so the mutations should reduce the enzyme's affinity for this inhibitor. The effect should be cumulative for the double, triple, and quadruple mutants. The sensitivity to ATP inhibition displayed by the multiple mutants should therefore be inversely proportional to the number of mutations at this site.

The techniques of oligonucleotide-directed mutagenesis and deletion mutagenesis will be combined to investigate the function of the connecting peptide in RM-PFK. The basic premise of the model proposed by Poorman et al. (1984) is that the N- and C-terminal halves of each RM-PFK subunit associate much like a dimer of Bs-PFK. The peptide connecting these two halves provides the flexibility necessary for such an association.

The 30 amino acids from Lys-320N to Ala-5C form the connecting peptide of RM-PFK. In the first step of the procedure, a HindIII site will be introduced at Lys-320 using oligonucleotide-directed mutagenesis. The only other
HindIII site in the RM-PFK cDNA is located at Lys-334N. A HindIII digestion of this mutant will therefore release a 42 bp fragment encoding 14 amino acids of the connecting peptide. This fragment will then be replaced with double-stranded oligonucleotides of various lengths. These experiments will determine the minimal length of connecting peptide necessary for the proper tertiary structure and function of RM-PFK.

The identity of the catalytic Asp-127N residue will be verified by substituting Ser for Asp. A personal communication from Hellinga and Evans at MRC Cambridge, England reported that the corresponding mutation in Ec-PFK-1 caused an 18,000-fold decrease in the kcat of that enzyme. The Asp-127N to Ser mutation in RM-PFK is expected to result in a similar decrease in catalytic activity since the carboxyl of Asp-127N probably serves as a general base in the reaction mechanism of RM-PFK.

Poorman et al. (1984) proposed that the N-terminal half of RM-PFK serves a catalytic function while the C-terminal half has assumed an allosteric role. If this model is valid, there is a small chance that the N-terminal half of the enzyme will retain catalytic activity in the absence of the C-terminal half. This possibility will be investigated by making use of the unique HindIII site in RM-PFK cDNA. The site corresponds to Lys-334N which defines the C-terminal end of the N-terminal half of RM-PFK. A double-stranded oligonucleotide containing a
termination codon will thus be inserted after cleavage at this HindIII site. The resulting mutant RM-PFK will consist of only the N-terminal half of each subunit and will therefore resemble the prokaryotic PFK's. There is a small chance that these mutant subunits will be capable of forming active tetramers. If this is the case and the model of Poorman et al. (1984) is correct, then the mutant tetramers will no longer be sensitive to activation by ADP and the fructose bisphosphates.

C. Significance of RM-PFK Research

It is anticipated that the site-specific mutagenesis of RM-PFK will play a key role in supporting or disproving the model proposed by Poorman et al. (1984). This model for the locations of the fructose bisphosphate activation site and the AMP/ADP activation site is a reasonable one, and the site-specific mutagenesis may well provide evidence in its support. However, the model will be disproven if mutations at the proposed activation sites have no effect upon the kinetics of RM-PFK.
CURRICULUM VITAE

Brent Arthur French

PERSONAL

Born: December 18, 1956 in Urbana, Illinois.
Addresses: Dept. of Biochemistry Home:
322 Choppin Hall
LA State University 2513 Belmont Ave
Baton Rouge, LA 70803 Baton Rouge, LA 70808
Phone: (504) 388-5222 Phone: (504) 383-9163
Status: Two children ages 5 months and 3 years.

EDUCATION

B.Sc. May 1982, LA State University, Biochemistry.
Ph.D. May 1987, LA State University, Biochemistry.
Thesis Title: Molecular Cloning and Sequencing of the Bacillus stearothermophilus 6-Phosphofructo-1-kinase Gene and of a Partial Rabbit Muscle 6-Phosphofructo-1-kinase cDNA.

RESEARCH EXPERIENCE

1982 Undergraduate Research: Analysis of in vivo DNA Alkylation by Two-Dimensional Chromatography.

1985-1987 Doctoral Research: Molecular Cloning, Sequencing and High-Level Expression of the 6-Phosphofructo-1-kinase Gene from *B. stearothermophilus*.

TEACHING EXPERIENCE

1982-1983 Teaching Assistant: Biochemistry Laboratory.

1986 Teaching Assistant: Biochemistry Course.

1987 Teaching Assistant: Biochemistry Laboratory.

AWARDS


PUBLICATIONS: CURRENT AND PENDING


French, B.A. and Chang, S.H.: Nucleotide sequence of the 6-phosphofructo-1-kinase gene from *Bacillus*
stearothermophilus and comparison with the homologous

POSTERS

Chang, S.H., French, B.A., Lee, C-P., Younathan, E.S. and
Putney, S.D.: Molecular cloning and characterization
of the gene of rabbit muscle phosphofructokinase.
ASBC/AAI Annual Meeting: June 3-7, 1984, St. Louis,

Lee, C-P., French, B.A., Putney, S.D., Kao, M-C. and Chang,
S.H.: Molecular cloning and characterization of rabbit
phosphofructokinase genes. 13th International Congress
of Biochemistry: Aug. 25-30, 1985, Amsterdam,
Netherlands. Abstract #TH 050.

REFERENCES

(1) Dr. S.H. Chang, Professor, Department of Biochemistry,
322 Choppin Hall, LA State University, Baton Rouge, LA
70803, Phone: (504) 388-5147.

(2) Dr. R.A. Laine, Chairman, Department of Biochemistry,
322 Choppin Hall, LA State University, Baton Rouge, LA
70803, Phone: (504) 388-1556.

(3) Dr. H.D. Braymer, Professor, Department of Micro-
biology, 508 Life Sciences Bldg., LA State University,
Baton Rouge, LA 70803, Phone: (504) 388-2601.
AREAS OF EXPERIENCE

Northern and Southern blots  Subcloning
Okayama-Berg cDNA libraries  Oligo synthesis
Plasmid genomic libraries  Oligo purification
Lambda phage genomic libraries  Dideoxy sequencing
Colony and plaque hybridizations  Sequence analysis

AREAS OF INTEREST

The ultimate goal of the Bacillus PFK project is to study the structure-function relationships in the enzyme using site-specific mutagenesis. I am still interested in such structure-function work, but I find the field of gene expression to be even more intriguing. My training in molecular biology has given me the ability to clone and manipulate genes. I am now interested in learning systems by which cloned genes can be transfected into higher organisms. This will enable me to investigate the role of trans-acting factors in the regulation of eukaryotic gene transcription.
Candidate: Brent Arthur French

Major Field: Biochemistry

Title of Dissertation: Molecular Cloning and Sequencing of the Bacillus stea rothermophilus 6-Phosphofructo-1-kinase Gene and of a Partial Rabbit Muscle 6-Phosphofructo-1-kinase cDNA

Approved:

[Signatures]

Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination: April 27, 1987