STUDIES ON THE STRUCTURE OF HUMAN PLATELET PHOSPHOFRUCTOKINASE

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CHAPTER 2
EXPERIMENTAL

Declaration.

I hereby declare that this thesis has been composed by me and that all work is my own unless otherwise stated and that the work of which it is a record has been carried out by me and that it has not been accepted in any previous application for a degree.

Craig J. Simpson

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Firstly I must thank Linda Fothergill-Gilmore for her helpful advice and for getting me started with the project.

Secondly, all the people who have found themselves in lab 327 at different times and the Biochemistry Department as a whole for making life interesting.

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ABBREVIATIONS

AMINO ACID RESIDUES, ABBREVIATED TO ONE AND THREE LETTER CODES, AND NUCLEIC ACIDS ARE ABBREVIATED ACCORDING TO THE RECOMMENDATIONS OF THE NOMENCLATURE COMMITTEE OF IUB AND THE IUPAC-IUB JOINT COMMISSION ON BIOCHEMICAL NOMENCLATURE

a) MISCELLANEOUS

13BPG	1,3-BISPHOSPHOGLYCERATE
2PG	2-PHOSPHOGLYCERATE
3PG	3-PHOSPHOGLYCERATE
23BPG	2,3-DIPHOSPHOGLYCERATE
ADP	ADENOSINE 5' DIPHOSPHATE
AMP	ADENOSINE 5' MONOPHOSPHATE
ATP	ADENOSINE 5' TRIPHOSPHATE
BCIP	5-BROMO-4-CHLORO-3-INDOYL PHOSPHATE
CaM	CALMODULIN
cAMP	3'-5' CYCLIC ADENOSINE MONOPHOSPHATE
cDNA	COPY DEOXYRIBONUCLEIC ACID
cGMP	3'-5' CYCLIC GUANOSINE MONOPHOSPHATE
Da	DALTON
DNA	DEOXYRIBONUCLEIC ACID
DS	DOWN'S SYNDROME
DTT	DITHIOTHRIETOL
F16BP	FRUCTOSE 1,6-BISPHOSPHATE

F26BP	FRUCTOSE 2,6-BISPHOSPHATE
F6P	FRUCTOSE 6-PHOSPHATE
G16BP	GLUCOSE 1,6-BISPHOSPHATE
G6P	GLUCOSE 6-PHOSPHATE
GDP	GUANOSINE DIPHOSPHATE
GSH	REDUCED GLUTATHIONE
GSSG	OXIDISED GLUTATHIONE
HPLC	HIGH PRESSURE LIQUID CHROMATOGRAPHY
kDa	KILODALTON
M	MOLAR
M16BP	MANNOSE 1,6-BISPHOSPHATE
MgADP	MAGNESIUM ADP
MgATP	MAGNESIUM ATP
mg	MILLIGRAMME
ml	MILLILITRE
mM	MILLIMOLAR
mmol	MILLIMOLE
mRNA	MESSENGER RIBONUCLEIC ACID
MWC	MONOD-WYMAN-CHANGEUX
NAD+	NICOTINAMIDE ADENINE DINUCLEOTIDE OXIDISED
NADH	NICOTINAMIDE ADENINE DINUCLEOTIDE REDUCED
NBT	NITROBLUE TETRAZOLIUM CHLORIDE
ng	NANOGRAMME
nmol	NANOMOLE

PCR	POLYMERASE CHAIN REACTION
PEG	POLYETHYLENE GLYCOL
PEP	PHOSPHOENOLPYRUVATE
pg	PICOGRAMME
P_i	INORGANIC PHOSPHATE
PIR	PROTEIN INFORMATION RESOURCE
pmol	PICOMOLE
PAGE	POLYACRYLAMIDE GEL ELECTROPHORESIS
R state	RELAXED HIGH AFFINITY STATE
R15BP	RIBOSE 1,5-BISPHOSPHATE
RBC	RED BLOOD CELL
RNA	RIBONUCLEIC ACID
SDM	SITE DIRECTED MUTAGENESIS
SDS	SODIUM DODECYL SULPHATE
T state	TENSE LOW AFFINITY STATE
TCA	TRICARBOXCYLIC ACID
µg	MICROGRAMME
µl	MICROLITRE
µM	MICROMOLAR
µmol	MICROMOLE

b) ENZYMES

L, M and P refer to liver, muscle and platelet type phosphofructokinases.

ADH	ALCOHOL DEHYDROGENASE [EC 1.1.1.1]
BPFK	Bacillus stearothermophilus PFK

BPGM	BISPHOSPHOGLYCERATE MUTASE [EC 5.4.2.4}
ECPFK	*Escherichia coli* PFK
F16BP aldolase	D-FRUCTOSE 1,6-BISPHOSPHATE-GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE [EC 4.1.2.13]
F16BP'ase	FRUCTOSE 1,6-BISPHOSPHATASE [EC 3.1.13.11]
GAPDH	D-GLYCERALDEHYDE 3-PHOSPHATE:NAD OXIDOREDUCTASE (PHOSPHORYLATING)
HK	HEXOKINASE [EC 2.7.1.1]
HLPFK	HUMAN LIVER PFK
HMPFK	HUMAN MUSCLE PFK
HPPFK	HUMAN PLATELET PFK
HPRT	HYPOXANTHINE PHOSPHORIBOSYL TRANSFERASE [EC 2.4.2.8]
PFK or PFK-1	6-PHOSPHOFRUCTO-1-KINASE, [EC 2.7.1.11]
PFK-2	6-PHOSPHOFRUCTO-2-KINASE/ FRUCTOSE 2,6-BISPHOSPHATASE [EC 2.7.1.105/3.1.3.46]
PGM	D-PHOSPHOGLYCERATE 2,3-PHOSPHOMUTASE [EC 5.4.2.1]
PK	PYRUVATE KINASE [EC 2.7.1.40]
RMPFK	RABBIT MUSCLE PFK
SHPFK	SHEEP HEART PFK
SLPFK	SHEEP LIVER PFK

TIM	TRIOSE PHOSPHATE ISOMERASE [EC 5.3.1.1]
Y1PFK	Saccharomyces cerevisiae PFK SUBUNIT 1
Y2PFK	Saccharomyces cerevisiae PFK SUBUNIT 2

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Phosphofructokinase (ATP: D-fructose 6-phosphate 1-transferase, EC 2.7.1.11) catalyses the phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate, and is a key regulatory enzyme in glycolysis. Sequence conservation between mammalian PFK and PFK from *E.coli, B. stearothermophilus and S.citri,* and between the amino and carboxyl halves of the mammalian enzymes suggests that mammalian PFK has evolved from a prokaryotic progenitor by a process of gene duplication and fusion.

The aim of this project was to obtain detailed structural information describing PFK from humans and from the parasitic filarial nematode *Onchocerca volvulus* and so facilitate the design of inhibitors specific for the filarial enzyme. Five *Onchocerca volvulus* cDNA libraries were probed with oligonucleotides, heterologous DNA from E.coli, rabbit muscle and human muscle PFK. Redundant oligonucleotides were used in a polymerase chain reaction to try to clone part of the parasite's PFK. Antisera to *E.coli* and *Ascaris suum* PFK were used to screen libraries for expression of PFK. The lack of good quality DNA libraries and the unexpected gene structure and codon usage of the parasite did not allow the isolation of DNA encoding *O. volvulus* PFK.

In man, PFK is under the control of three structural loci encoding subunits for muscle, liver and platelet types. They are located on separate chromosomes and are regulated independently. During the course of this project, the muscle and liver type DNA sequences were reported from other laboratories. The partial sequence of the platelet type is reported here, and its chromosomal location is confirmed. The sequence codes for three hundred amino acids and comprises 80% of the carboxyl domain in comparison to the muscle and liver types.

The structure, function and evolution of PFK is discussed, based on detailed crystal structure information for PFK from *E.coli* and *B. stearothermophilus* and the amino acid sequences of PFK from other sources

CHAPTER ONE

INTRODUCTION

1.1 HISTORICAL BACKGROUND

6-Phosphofructo-1-kinase (EC 2.7.1.11) irreversibly catalyses the transfer of a phospho group from ATP to the β anomer of fructose 1-phosphate to form the β anomer of fructose 1,6 bisphosphate.

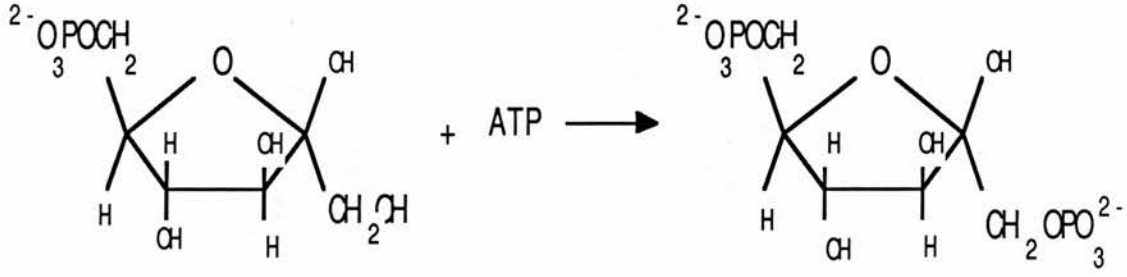

Figure 1. The phosphorylation of fructose 6-phosphate to generate fructose 1,6-bisphosphate

This phosphorylation was first described in erythrocytes by Dische in 1935 and then in muscle by Ostern *et al.* in 1936. Negelein (1936) observed that administration of epinephrine to frog muscle caused the accumulation of hexosemonophosphates and a small increase in lactic acid. He proposed that PFK was a possible regulatory enzyme of glycolysis. Engelhardt &

Sakov (1943) suggested that inactivation of PFK by O_2 was responsible for the Pasteur effect. Aisenberg *et al.* (1957) proposed that an intermediate of oxidative phosphorylation accumulated when glucose was metabolised aerobically which inhibited PFK. Lardy & Parks (1956) noted that ATP had a strong inhibitory effect on PFK and that this might play an important part in carbohydrate metabolism. Since then, PFK has been extensively studied in a variety of organisms and our knowledge, although still incomplete, has been expanded to give a greater appreciation of the properties, structure and regulation of this complex glycolytic enzyme. In the introduction, I will try to describe the complex behaviour of PFK *in vitro* and *in vivo.* There is a wealth of experimental detail and analysis available for PFK, some of which is contradictory. The isoenzymes of mammalian PFK have different attributes and sometimes behave in contradictory ways. Different tissues have varying levels of isoenzymes and vary according to developmental stages.

In general, prokaryotic PFK's are controlled by a small number of effector molecules whereas PFK's from more complex organisms are regulated by a relatively large number of effector molecules. Plant cells also contain a pyrophosphate dependent PFK which shows little homology to PFK-1 except at the catalytic site, and it has not been included in this description. Eukaryotic PFK's undergo association-dissociation reactions and exist in a variety of polymeric states. The structure of PFK from bacteria has been investigated in *E. coli* and *B. stearothermophilus.* It consists of large and small domains which have central cores of β sheet surrounded by α helices as seen in Figure 2.

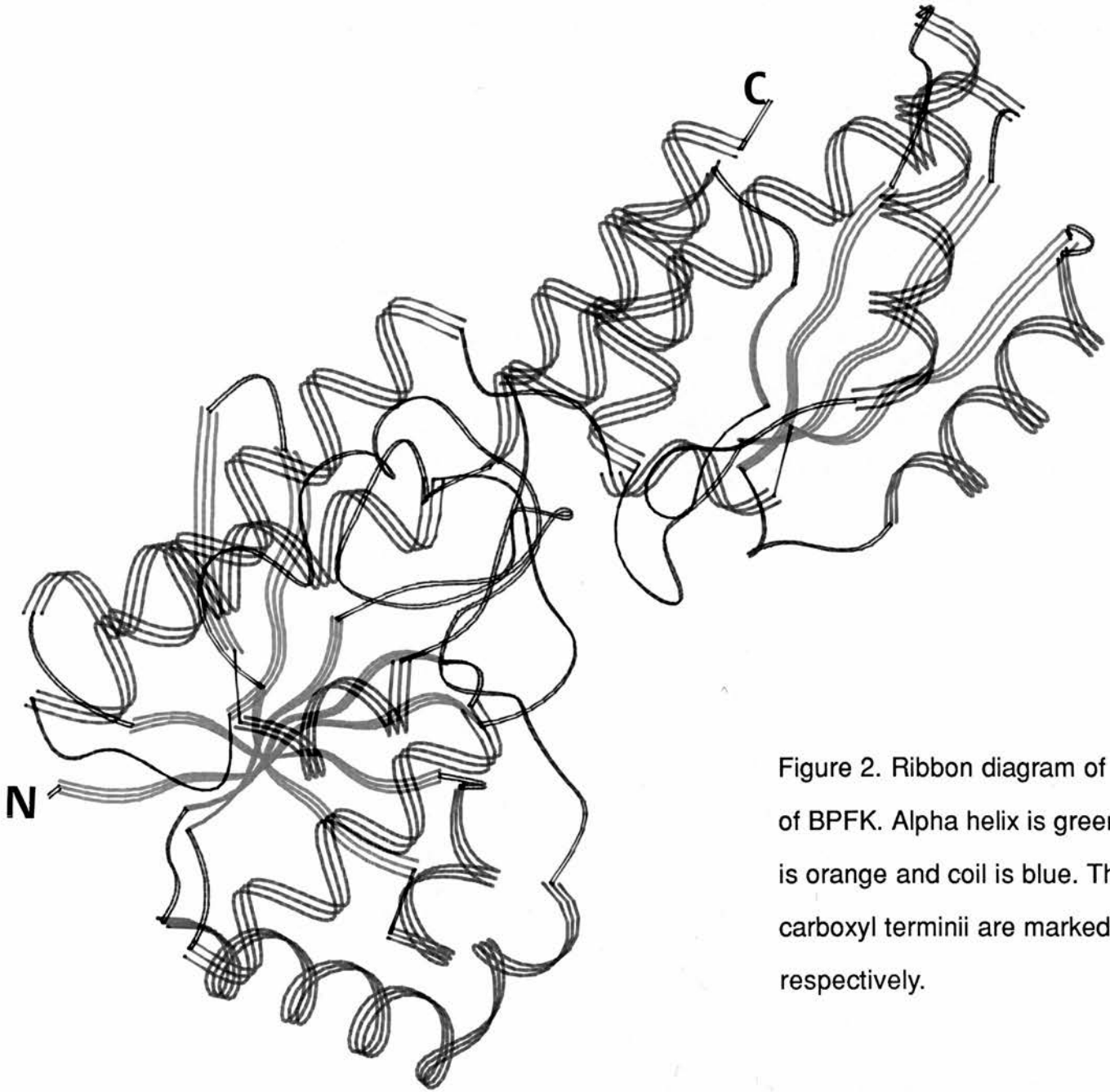

Figure 2. Ribbon diagram of a monomer of BPFK. Alpha helix is green, beta sheet is orange and coil is blue. The amino and carboxyl terminii are marked N and C respectively.

Eukaryotic phosphofructokinases are usually more than twice the size of their bacterial counterparts and appear to be the result of the gene duplication and fusion of a prokaryotic ancestral gene. The amino and carboxyl halves of an eukaryotic PFK have probably the same size and conformation of a bacterial PFK and so an eukaryotic monomer will resemble a prokaryotic dimer. The amino half of the eukaryotic enzymes retains essentially the same function as a prokaryotic enzyme while the carboxyl half has evolved new sites for binding effector molecules (Poormann *et al.,* 1984).

1.2 KNOWN DISTRIBUTION OF PFK

PFK has been isolated from a large number of plant, bacterial verte brate and non verte brate species (Bloxham & Lardy, 1973). DNA sequences for genes encoding PFK are known for *Escherichia coli* (Daldal, 1983; Hellinga & Evans, 1985), *Bacillus stearothermophilus* (French & Chang, 1987), *Thermus thermophilus* (Xu *et al.,* 1991), *Saccharomyces cerevisiae* (Heinisch, 1986; Heinisch *et al.,* 1989), *Spiroplasma citri* (Chevalier *et al.,* 1990), rabbit muscle (Lee *et al.,* 1987), mouse liver (Gehnrich *et al.,* 1988), human muscle (Nakajima *et al.,* 1987) and human liver (Levanon *et al.,* 1989; Elson *et al.,* 1990). Partial amino acid sequences are known for sheep heart and sheep muscle (Sutherland, K. PhD thesis, University of Auckland, 1989). Multiple forms of PFK were found in the rat (Lowry & Passoneau, 1964). De-Faria *et al.* (1976) isolated PFK from several diverse sources in the phylum chordata. These included human, bat, cayman, cobra, turtle, frog and carp. Multiple forms were identified by their electrophoretic mobility. Striated muscle and liver showed mostly single

forms whereas cardiac muscle and brain had a higher frequency of multiple forms. Glycolysis has been thought of as a virtually ubiquitous pathway and it could therefore be assumed that PFK would also be an ubiquitous enzyme. This has been put into doubt by the recent discovery that some archaebacteria do not have the normal complement of glycolytic enzymes (Danson, 1988). *Thermoplasma acidophilum* possesses operational glycolysis, but lacks PFK-1, F1,6BP'ase, F1,6BP Aldolase, glyceraldehyde 3-P dehydrogenase and phosphoglycerate mutase (Budgen & Danson, 1986).

1.3 PFK IN MAMMALS

The description of a new type of glycogen storage disease in skeletal muscle (Tarui *et al.*, 1965; Layzer *et al.,* 1967) led to the idea of isoenzymes of PFK. Tarui's disease is a recessively inherited trait which shows the almost complete absence of PFK in muscle. Half of the normal levels of PFK are present in red blood cells but normal levels are found in white cells. Previous kinetic studies on crude extracts from the rat also indicated the possibility of isoenzymes (Lowry & Passoneau, 1964).

The proposal that erythrocyte and muscle PFK were isoenzymes sharing partial structural identity was put forward to explain this inherited defect (Tarui, 1967; Layzer *et al.*, 1967; Tarui *et al.*, 1969). Normal levels of PFK in other tissues led Layzer & Conway (1970) to propose the existence of multiple isoenzymes composed of three separate types of PFK subunits which are under separate genetic control. Immunochemical studies supported this hypothesis (Tarui *et al.*, 1969; Layzer & Conway, 1970; Layzer, 1971).

The study of PFK from a variety of animal species provided conclusive evidence for the existence of isoenzymes of PFK in the rat (Taylor & Bew,

1970; Tanaka *et al.,* 1971; Kasten *et al.,* 1983; Dunaway & Kasten, 1985; Vora *et al.,* 1985b), rabbit (Tarui *et al.,* 1972; Tsai & Kemp, 1973), chicken (Kono *et al.*, 1973), rabbit, mouse, guinea pig, rat (Gonzalez *et al.*, 1975) and a variety of chordates (de Faria *et al.*, 1976).

1.3.1 Tissue distribution of isoenzymes in humans

Evidence supports the existence of three isoenzymes in humans. The isoenzymes are distinguished as muscle (M), liver (L) and platelet (P) forms. The subunits of these isoenzymes are encoded by three separate structural genes (Vora, 1982).

Anti-PFK antibodies generally tend to be isoenzyme specific, but not species specific. Thus, antibodies raised against homotetramers will completely precipitate that isoenzyme and will also show cross species specificity (Gonzalez *et al.*, 1975; Vora *et al.*, 1982). Anti-liver PFK antibody show no cross reactivity with muscle or platelet homotetramers (Oskam *et al.*, 1985; Vora *et al.*, 1981) and anti-muscle PFK antibody shows virtually no cross reactivity with liver homotetramers (Tsai & Kemp, 1973). Structural similarities between the muscle and platelet subunits is assumed to account for the cross reactivity of anti muscle antibody to platelet PFK (Vora *et al.*, 1981). Subunit specific antibodies have been raised against human PFK isoenzymes (Dunaway *et al.*, 1988) and have been used to study the relative amounts of homo and heterotetramers in human tissues. Only adult muscle showed the presence of a single subunit, the muscle subunit. Other tissues showed various amounts of all three subunits. Table 1 gives the percentage of each subunit present in various tissues determined by densitometric scanning of immunoblots of subunits resolved by SDS-PAGE (Dunaway *et*

al., 1988).

The properties of the native enzymes indicate that the PFK isoenzyme pools in human tissues can be very complex and are not simple combinations of two or three homotetrameric isoenzymes, but complex mixtures of homo and heterotetramers.Dunaway *et al.* (1988) quantified the isoenzymic content of various human tissues by immunotitration with subunit-specific antibodies, stepwise elution from QAE Sephadex, scanning densitometry of silver stained gels and immunoblots.

Table 1 Quantification of the PFK subunits in human tissue

From Dunaway et al. (1988)

	LIVER		MUSCLE		PLATELET	
	STAIN	BLOT	STAIN	BLOT	STAIN	BLOT
TISSUE. SUBUNIT...	%	%	%	%	%	%
SKELETAL MUSCLE	ND	ND	100	100.0	ND	ND
HEART VENTRICLE	3±0.5	2.5	91±7	93.0	7±2	4.5
HEART ATRIA	14±2	15.9	76±3	77.7	10±2	6.4
CEREBRAL CORTEX	15±2	14.2	55±3	58.4	30±4	27.4
LIVER	62±5	61.6	29±3	28.9	10±2	9.5
FIBROBLAST	21±2	21.8	16±3	17.0	63±6	61.2
ERYTHROCYTE	54±4	56.0	43±3	40.3	5±1	3.7
PLATELET	42±3	39.8	19±3	22.5	38±2	37.7
PLACENTA	81±6	83.0	12±2	10.5	8±2	6.5
UMBILICAL CORD	57±4	60.0	16±2	10.0	27±3	30.0

Nakajima *et al.* (1990c) reported the presence of HLPFK mRNA in human muscle, in conflict with the isoenzyme specific antibody work of a variety of authors (Vora, 1982; Dunaway *et al.*, 1988). MLPFK mRNA has also been reported in mouse muscle (Gehnrich *et al.*, 1988). This does not necessarily show that the liver monomer is present in muscle, as the

amount of a gene product (protein), is finely regulated by transcription, translation and post-translational modification.

1.3.2 Structural properties of isoenzymes in humans

The subunit molecular masses of the isoenzymes have been determined by SDS-PAGE. Muscle was reported as 85 kDa (Karadsheh *et al.,* 1977; Cottreau *et al.*, 1979) and 80kDa (Kaur & Layzer, 1977). The amino acid sequence of the muscle enzyme indicates a molecular mass of 85,050 (Nakajima *et al.*, 1987; Sharma *et al.*, 1989). The liver subunit was reported as 80 kDa (Karadshesh *et al.*,1977; Cottreau *et al.*, 1979). The platelet isoenzyme was reported as 85 kDa. The subsequent cloning of the human muscle and liver genes gives a more precise figure for subunit mass. The liver subunit amino acid sequence suggests a Mr of 85,146 (Levanon *et al.,* 1989).

1.3.2.1 Aggregation of isoenzymes

PFK subunits have a tendency to self associate into oligomeric forms larger than the tetramer, which is the lowest active form of the enzyme. This process is influenced by a variety of factors such as pH, enzyme concentration, metabolic activators and inhibitors, and structural cellular components (Paetkau & Lardy, 1967; Layzer *et al.*, 1969; Brand & Soling, 1974; Reinhart & Lardy 1980b; Aaronson & Frieden, 1972).

The liver enzyme was reported to self associate more readily than the muscle enzyme (Kemp, 1971; Trujillo & Deal, 1977; Reinhart & Lardy, 1980b). The rabbit brain isoenzyme, which is 50% platelet form does not aggregate beyond a tetramer at pH 8.0 (Foe & Kemp, 1985).

At low protein concentrations, PFK dissociates into non-active forms smaller than the active tetramer (Layzer *et al.*, 1969; Hulme & Tipton, 1971; Underwood & Newsholme, 1965; Reinhart & Lardy, 1980b). This process

can be reversed and is prevented by the presence of activators such as AMP. Dissociation of the enzyme is promoted by inhibitors such as ATP (Hofer, 1971; Hulme & Tipton, 1971; Reinhart & Lardy, 1980b).

The dissociation constant of large aggregates of rat liver PFK is lower in the presence of saturating F6P than it is in the presence of saturating MgATP. In the absence of either substrate PFK dissociates past the active tetramer with a concomitant loss of activity. MgATP promotes tetramer formation more readily than F6P. This suggests that the monomer-dimer population has a lower affinity for F6P than MgATP, but the opposite is the case for the aggregated population (Ramaiah & Tejwani, 1970, 1973; Reinhart, 1980; Reinhart & Lardy, 1980a,b).

Crystallographic and kinetic studies (Berger & Evans, 1990) indicated that the allosteric and cooperative behaviour of PFK is due to a reversible transition between two states that differ in affinity for F6P by a factor of at least 2,000. Increasing amounts of F6P increases the fraction of the high affinity R state. In the absence of F6P the low affinity T state predominates.

The use of a reactive thiol group to monitor the transitions between R and T states suggested that allosteric transitions were readily made at protein concentrations where the enzyme existed as a tetramer or higher polymer and that dissociation to dimers was not required for the inhibited conformation (Kemp, 1969a).This thiol group has been isolated and sequenced (Simpson *et al.,* 1977). It is a highly reactive cysteine group corresponding to position 88 in RMPFK and position 73 in BPFK. This residue is important for catalytic activity and is conserved in most phosphofructokinases.

1.3.3 PHYSICAL PROPERTIES OF ISOENZYMES

The pH optima have been reported for PFK's from a variety of mammalian species. Most have an optimum at slightly alkaline pH (Layzer *et al.,* 1969; Staal *et al.,* 1972; Dunaway *et al.,* 1972; Massey & Deal, 1973; Balinsky *et al.,* 1979). PFK from Erlich ascites tumour has a lower pH optimum of 7.1 (Sumi & Ui, 1972) which fits in with the high rates of glycolysis and concomitant decrease in pH observed in some tumour cells.

The isoelectric points are reported to be at pH 6.6 for the muscle form, pH 5.0 for the erythrocyte form (M + L subunits) and pH 4.6 for the form in erythrocytes from patients with Taruis disease (L subunits only) (Kaur & Layzer, 1977).

Electron microscopy of pig liver PFK (Foe & Trujillo, 1980) shows that particles ranging in size from tetramers to long flexible chains of tetramers are present. Tetramers are square planar and approximately 110Å on a side. The individual subunits are roughly spherical with a mean radius of 28Å. The chains are formed by end to end association of tetramers and the geometry of association implied a D_2 symmetry (see Section 6.3) with distinct isologous bonding domains for dimer, tetramer and chain formation.

Electron microscopy of rabbit muscle PFK indicated a subunit volume of 101nm^3 and the appearance of a conical shape with rounded off corners and edges of a rectangular prism (Hesterberg *et al.,* 1981). Structural features within the tetramer were interpreted as being due to the four individual subunits being approximately 4 x 6 x 6 nm (tapered to a trapezoidal shape) and arranged with D_2 dihedral symmetry. This is consistent with the observation by Klotz *et al.* (1975) that essentially all tetrameric proteins of known structure display D_2 symmetry.

1.3.4 GENETICS OF ISOENZYMES

The genes for the liver, muscle and platelet type subunits have been assigned to chromosomes 21, 1 and 10, respectively (Weil *et al.*, 1980; Vora & Francke, 1981; Vora *et al.,* 1982; Vora *et al.,* 1983). In addition to the three recognised loci, Ashley *et al.* (1987) identified another possible locus for a PFK gene. They used a mouse PFK cDNA probe to search a human astrocytoma cDNA library to isolate a human PFK cDNA probe which they referred to as human PFKX. They assigned PFKX to chromosome 12 by using the cDNA for slot blot hybridisation to sorted human chromosomes and by Southern blot analysis of DNA from mouse/human somatic cell hybrids. They assigned the homologous mouse gene to chromosome 15, using a panel of hamster/mouse somatic cell hybrids. S1 nuclease protection assays indicated that PFKX is expressed in human fibroblasts and brain. Northern blot analysis of RNA from various adult mouse tissues showed a high level in mouse testes. Further reports on this particular work have not appeared in the literature and the work was presented as an abstract. It may be that this group have isolated the gene for another PFK isoenzyme, such as an embryonic or foetal form, or even a processed pseudogene. Sequence data and perhaps expression of the putative isoenzyme would greatly clarify this work.

Although the three PFKs have similar structures, functions and cellular locations they are not tightly clustered on a specific chromosome. This may be the result of gene duplication followed by translocation and/or early tetraploidisation of the karyotype (Ohno, 1970). HPPFK and HK have been localised to the same region of chromosome 10 in humans (Schwarz *et al.,* 1984) and are considered to be syntenic but later studies suggest otherwise (see Section 6.4). This is of interest as high levels of PFK and HK

are noted in certain tumour cells in addition to an elevated rate of glycolysis. PFK and PK are linked in the mycoplasma *Spiroplasma citri* to form a single transcriptional unit (Chevalier *et al.,* 1990).

1.4 KINETIC AND ALLOSTERIC PROPERTIES OF PFK

PFK activity is affected to the greatest extent by its substrates ATP and F6P, and the products F1,6BP and ADP. More than 30 presumptive regulatory effectors have been reported. The significance of effector regulation *in vivo* is still a subject of controversy. Table 2 lists the known effectors of PFK.

Table 2. EFFECTORS OF PHOSPHOFRUCTOKINASE

INHIBITORS	ACTIVATORS	DEINHIBITORS
ATP[1]	cAMP[6]	cAMP[6]
CITRATE[2]	AMP[6]	AMP[6]
Mg^{2+}[12]	P_i[6]	P_i[6]
Ca^{2+}[5]	ADP[6]	ADP[6]
PHOSPHOCREATINE[15]	NH_4^+[8]	F6P[7]
GLYCERATE 3-P[3]	M1,6BP[12]	M1,6BP[12]
GLYCERATE 2,3BP[3]	K^+[8]	
GLYCERATE 1,3BP[3]	F1,6BP[9]	F1,6BP[9]
GLYCERATE 2-P[3]	F2,6BP[10]	F2,6BP[10]
OLEATE/PALMITATE[14]	R1,5BP[11]	R1,5BP[11]
3',5'-cGMP[12]	G1,6BP[11]	G1,6BP[11]
H^+[4]	OH^-[4]	
GSSG[16]	GSH[16]	
	SO_4^{2-}[17]	
TCA cycle intermediates[2]		

CALMODULIN[5] CALMODULIN[5]

ACETYL CoA(oxidised)[14]

ACTIN[13] ACTIN[13]

(1) Section 1.4.2.1
(2) 1.4.2.2
(3) 1.4.2.4
(4) 1.4.2.3
(5) 1.4.6
(6) 1.4.1.1
(7) 1.1
(8) 1.4.1.2
(9) Section 1.4.1.3
(10) 1.4.3
(11) 1.4.1.4
(12) 1.4.1.5
(13) 1.4.5
(14) Tejwani (1978)
(15) Connet (1989)
(16) Gilbert (1982)
(17) Akkermann, *et al.* (1974)

In general, prokaryotic PFK is controlled by a small number of effector molecules. These are the activators Mg.ADP and Mg.GDP, and the inhibitor PEP (Sols, 1981).

1.4.1.1 Activation of mammalian PFK by adenine nucleotides

PFK is activated by ADP and AMP both of which can also relieve ATP inhibition (Underwood & Newsholme, 1965; Brock, 1969; Kemp, 1971; Tsai & Kemp, 1974). In the absence of Mg^{2+}, ADP binds more tightly than other nucleotides to the activating site, but in the presence of Mg^{2+} the affinity for ADP is greatly reduced (Kemp & Krebs, 1967). The activating site is very specific for the adenine ring (Kemp & Krebs, 1967) but will also bind a variety of phosphorylated derivatives of adenosine besides AMP, cyclic AMP and ADP, e.g. ADP-ribose and NADH (Gottschalk & Kemp, 1981).

In the absence of ADP, the binding of F6P is highly cooperative with a Hill coefficient of 3.8 . The positive homotropic interactions are lowered on the addition of the allosteric effector ADP, with the Hill coefficient dropping to 1.4 at 0.8 mM ADP. (Blangy *et al.*, 1968). ADP thus binds preferentially to

the R state. The effector site (site C) binds ADP and also PEP in *E. coli* PFK (Evans *et al.*, 1981). It lies in a deep cleft formed by the small domains of two subunits. The phosphate residues of these effectors are bound by positively charged side chains from both subunits of the enzyme. (See Section 1.6.1).

Rat liver PFK requires higher concentrations of AMP and ADP to achieve an increase of activity comparable to that observed for the rat muscle enzyme (Kemp, 1971). The relative insensitivity of the liver enzyme to these effectors is due to a higher affinity for ATP at the inhibitory site (Lowry & Passoneau, 1966). Even near saturating concentrations of these effectors fail to increase the affinity of the enzyme for F6P in the presence of 3mM MgATP and thus the enzyme does not appear to function properly at physiological F6P levels (Reinhardt & Lardy, 1980a). It is only when the effects of all the positive effectors (FBP, AMP, and P_i) synergistically combine, that the affinity for F6P appears to approach the level sufficient to account for physiological activity.

The platelet isoenzyme is less sensitive to stimulation by AMP than the muscle and liver enzymes. P_i stimulates the platelet enzyme less than the liver enzyme which is less sensitive than the muscle enzyme (Foe & Kemp, 1985).

cAMP is an activator of PFK at even lower concentrations than AMP or ADP (Kemp 1971; Tsai & Kemp, 1974). The liver enzyme is less sensitive to activation by cAMP than the muscle enzyme (Kemp, 1971).

1.4.1.2 Activation by NH_4^+ and K^+

NH_4^+ and $(NH_4)_2SO_4$ have been found to increase PFK activity and relieve ATP inhibition (Underwood & Newsholme, 1965; Brock, 1969; Kemp, 1971; Tejwani *et al.,* 1973). The concentration of NH_4^+ ions rises in certain

tissues during anoxia (Lowry & Passoneau, 1966). This may be of physiological significance in these tissues. NH_4^+ increases the affinity of the enzyme for F6P (Kemp & Krebs, 1967; Dunaway & Weber, 1974a) but does not change the affinity for ATP or MgATP (Pettigrew & Frieden, 1979a).

Potassium ions are essential for enzyme activity (Uyeda & Racker, 1965; Lowry & Passoneau, 1966; Paetkau & Lardy, 1967; Kemp 1971). The activators K^+ and NH_4^+ increase V_{max} without greatly affecting inhibition by ATP for the human skeletal muscle isoenzyme (Kemp & Foe, 1983), whereas the primary mode of action of the physiological activators (AMP, F16BP and inorganic phosphate) and inhibitors (MgATP, citrate and H^+) in rats is to alter the Km for F6P with little or no effect on V_{max}. Cys-73 in *B.stearothermophilus* may be important for binding of monovalent cations.

1.4.1.3 Activation by F16BP

F16BP activates PFK in the same manner as ADP by decreasing the apparent affinity of the inhibitory site for ATP or MgATP (Pettigrew & Frieden, 1979a), resulting in the loss of ATP inhibition (Bloxham & Lardy, 1973). The synergistic effect of the three activators F16BP, AMP and P_i leads to the assumption that different binding sites exist for the three ligands (Lowry & Passoneau, 1966; Reinhart & Lardy, 1980a). Hers and Hue (1983) have suggested however that F16BP could be considered as an inhibitor at high concentrations.

1.4.1.4 Activation by Ribose 1,5BP

Glycolytic flux in the brain is activated several fold within a few seconds after the initiation of ischemia by decapitation. Analysis of brain samples showed that within 2-5 seconds, F6P decreases by 60%, and F16BP increases 7-fold, consistent with activation of PFK. None of the

known activators of PFK, including F16BP, F26BP, G16BP or AMP, can account for this rapid activation. Ogushi *et al.* (1990) reported that R15BP forms rapidly during the initiation of glycolytic flux and disappears within 20 seconds. Ishikawa *et al.* (1990) found that R15BP was the second most potent activator of rat PFK *in vitro* with F26BP being the most potent, and that R15BP was more potent than F16BP. Other mammalian PFKs show similar affinities for F26BP but vary in their affinities for R15BP. Rat liver PFK had 3-fold less affinity for R15BP than the rat brain enzyme or RMPFK and this may reflect the physiological roles of the isoenzymes. However, R15BP has only been shown to occur in the brain and it is not possible to assess its importance in other cells.

1.4.1.5 Activation by other sugar bisphosphates

Sugar bisphosphates such as mannose 1,6-bisphosphate and glucose 1,6-bisphosphate have been postulated to be regulators of PFK (Rose & Warms, 1974; Uyeda, 1979; Passoneau & Lowry, 1963). More recent work suggests that control of PFK is primarily achieved by variation in the levels of fructose 2,6-bisphosphate, (see Section 1.4.3).

1.4.2 INHIBITION OF PFK

Prokaryotic PFK is inhibited by PEP, but the mammalian enzymes have a large number of inhibitors. Each isoenzyme has a separate response to inhibitors and the responses vary according to the organism (Meinhofer *et al.,* 1980). ATP acts synergistically with other inhibitors (Mathias & Kemp, 1972).

1.4.2.1 Inhibition of PFK by ATP

ATP is a substrate of PFK but will inhibit the enzyme if present at sufficiently high levels (Underwood & Newsholme, 1965). ATP binds to a site distinct from the catalytic site as well as to the catalytic site itself (Colombo

et al., 1975). The human isoenzymes vary in their response to ATP concentration (Dunaway *et al.*, 1988). In the absence of F26BP, the muscle homotetramer is least susceptible to ATP inhibition and the muscle-rich isoenzymes are less susceptible to ATP inhibition than isoenzyme pools from platelets and fibroblasts which contain a high percentage of platelet type subunit. The same trend is seen in the presence of F26BP although F26BP is a potent antagonist of ATP inhibition. Regardless of the presence of F26BP, ATP inhibition is more pronounced as the concentrations of P subunit in a heterotetramer increases and ATP sensitivity may be an intrinsic property of this subunit, (Dunaway *et al.*, 1988)

The degree of inhibition by a fixed concentration of ATP and the concentration at which inhibition occurs is a function of the pH and F6P concentration (Ui, 1966; Brock, 1969; Dunaway & Weber, 1974a; Pettigrew & Frieden, 1979b).

Below pH 7.5, PFK shows a strong sigmoidal dependence on the concentration of F6P and to inhibition by ATP at high concentrations, and also on the allosteric interaction of a number of effectors. At pH 8.0 however, the enzyme displays typical hyperbolic kinetics with respect to F6P and ATP and is not altered by allosteric effectors (Lowry & Passoneau, 1966; Pettigrew & Frieden, 1979b; Uyeda *et al.*, 1983). Decreased sensitivity to ATP occurs at alkaline pH, with the muscle enzyme showing less sensitivity than liver enzyme (Tsai & Kemp, 1974).

ATP inhibition can be relieved by the addition of F6P (Underwood & Newsholme, 1965) but the situation is complicated by the additional effects of F26BP. The liver enzyme requires at least twice as much F6P as the muscle enzyme to give half maximal velocities under any conditions (Kemp, 1971; Tsai & Kemp, 1974).

ATP is thought to lower the affinity of PFK for F6P (Underwood & Newsholme, 1965). It is likely that the liver enzyme has a greater affinity for ATP than the muscle enzyme at the ATP inhibitory site. This would explain why the liver enzyme shows susceptibility to ATP inhibition at a lower ATP concentration than the muscle enzyme (Kemp, 1971).

1.4.2.2 Inhibition by TCA cycle intermediates

PFK is inhibited by a number of TCA cycle intermediates. These are α-oxoglutarate, succinate, malate, acetyl CoA, fumarate and citrate (Underwood & Newsholme, 1965). Citrate is by far the most important inhibitor and acts synergistically with ATP (Underwood & Newsholm, 1965; Uyeda, 1979). In rabbits, the muscle enzyme is more sensitive to citrate inhibition than the platelet form, which in turn is more sensitive than the liver form (Foe & Kemp, 1985). The human platelet enzyme however, is reported as being more sensitive to citrate inhibition than the muscle or liver enzymes (Meinhofer *et al.*, 1980).

Inhibition is believed to occur by reducing the affinity of the enzyme for F6P (Pettigrew & Frieden, 1979b). This would account for F6P relieving inhibition of muscle PFK at lower concentrations than that needed for liver PFK, as muscle PFK has a higher affinity for F6P.

Citrate levels increase when acetyl CoA levels rise. Acetyl CoA condenses with oxaloacetate to form citrate. When glycolysis increases and citric acid cycle intermediates are not consumed as a source of biosynthetic precursors, citrate acts as a negative feedback on glycolysis by inhibiting PFK (Passoneau & Lowry, 1963), and thereby enhances the coordination of glycolysis, ATP production, and TCA cycle intermediate levels (Foe & Kemp, 1985).

1.4.2.3 Inhibition by decreasing pH

Low pH decreases the affinity of PFK for F6P (Trivedi & Danforth, 1966; Reinhart, 1985) as well as causing dissociation below the active tetramer (Paetkau & Lardy, 1967; Hofer & Pette, 1968; Mansour, 1972). The process can be reversed by increasing pH (Hofer & Pette, 1968) and by the addition of ligands (Alpers *et al.*, 1971; Lad *et al.*, 1973) which give an increase in activity and a higher molecular weight of the active enzyme. Inhibition by low pH is greater at higher ATP concentration. This can be counteracted in the human erythrocyte by increasing the concentration of F6P (Layzer, *et al.*, 1969). With frog muscle PFK, increases in F6P concentration displace the pH curve toward the acid side (Trivedi & Danforth, 1966) but this does not occur with HMPFK.

As muscle contracts, the pH rises at first because of hydrolysis of creatine phosphate and this may promote activity of PFK. Later as the concentration of lactic acid increases, pH falls (Karpatkin *et al.*, 1964). This decrease in pH inhibits the activity of PFK so stopping lactate production before the pH becomes low enough to damage the cell (Danforth, 1965).

Bock and Frieden (1974; 1976a; 1976b) proposed a mechanism for pH inactivation and cold lability of PFK based on the alteration of the pK of an ionisable group on the enzyme. The model requires that pH and temperature changes are linked and that different enzyme forms are involved in inactivation and reactivation which proceed by kinetically different pathways. This transition takes place in the pH range 6-7 and so one or more histidines are proposed to be subject to the protonation/deprotonation equilibrium, (Bock *et al.*, 1975). This is supported by the loss of three protons during the formation of a tetramer from monomers between pH 6.0-8.55, since the ionisable residues have an apparent pK_a of 6.9 (Luther & Lee, 1986a).

Different ligands are proposed to bind to protonated or unprotonated forms of the enzyme and shift the apparent pK for the activation or inactivation process (Bock & Frieden, 1976b).

ATP and MgATP increase the pK by 0.1 units whereas F6P decreases it by 0.3 units. At high concentrations of ATP the behaviour is more complex which suggests that more than one ATP binding site is present. The presence of ATP also increases the rate of F6P reactivation. Bock & Frieden's protonation/deprotonation model accords with the Monod, Wyman and Changeux model for allosteric proteins and with the Frieden model of R and T states of allosteric enzymes having two substrates (Pettigrew & Frieden, 1977). Although the protonated form may deactivate to inactive dimeric forms, this will be slow in the absence of effectors which promote dissociation, such as citrate, and may account for the oscillatory behaviour due to PFK seen *in vivo* (Goldhammer & Paradies, 1979).

Positive modulators such as F26BP, G16BP and AMP counteract pH mediated ATP inhibition and stabilise the unprotonated form of the enzyme against citrate inhibition (Dobson *et al.*, 1986). These modulators must reverse pH inhibition of PFK as several cases are reported where PFK is still active when the pH is below that reported to be inhibitory to the enzyme (Newsholme & Crabtree, 1978; Meyer *et al.*, 1982; Bailey & Seymour, 1983).

Decreasing pH increases the K_m for F6P. Studies on rabbit muscle PFK indicated that decreasing pH decreased the apparent dissociation constant for Mg.ATP (Pettigrew & Frieden, 1979a; Kitajima *et al.,* 1983). This may reflect a difference in the allosteric regulation of the L and M isoenzymes (Reinhart, 1985) which agrees with observations that unlike the muscle enzyme, liver PFK exhibits significant cooperativity and MgATP inhibition at high pH (Reinhart & Lardy, 1980a).

1.4.2.4 Inhibition by phosphate esters

The muscle enzyme is inhibited by PEP, glycerate 3-P glycerate 2-P, glycerate 2,3-P and phosphocreatine which act synergistically with ATP to inhibit muscle PFK but have little effect on the liver enzyme (Tsai & Kemp, 1974; Tejwani, 1978). Muscle PFK is inhibited more by glycerate 3-P than by glycerate 2-P but the reverse is true for the liver enzyme. Glycerate 2,3-BP is a more potent inhibitor of the liver enzyme than glycerate 2-P or glycerate 3-P and may be more important than ATP in erythrocytes where it reaches levels high enough to be an inhibitor (Tsai & Kemp, 1973). The erythrocytes contain a heterogenous mixture of five tetrameric isoenzymes resulting from the random association of M and L subunits to form all the possible tetramers ie., M4, M3L, M2L2, ML3 and L4 . The subunits seem to be present at a ratio of 1:1 as is expected from the 50% activity of PFK in erythrocytes in individuals with Tarui's disease who lack the muscle subunit (Vora, 1982).

Glycerate 2,3-BP plays a major role in the regulation of oxygen affinity of haemoglobin in man (Brewer 1974). Binding of glycerate 2,3-BP or ATP decreases the oxygen affinity. The level of glycerate 2,3-BP is governed primarily by its synthesis which depends on the level of its precursor glycerate 1,3-BP (Rose & Warms, 1971; Jacobasch *et al.*, 1974). The synthesis of glycerate 1,3-BP depends in part on overall glycolytic flux and therefore on the key glycolytic enzymes HK, PFK, PK, and BPGM. Major control is exerted by the ratio of PFK/ PK activities; the higher the PFK/PK ratio, the more 23BPG is formed (Jacobasch, *et al.*, 1974; 1980; Gilman, 1981). Patients with inherited PFK deficiency such as in Tarui's disease show the expected decrease in 2,3BPG and ATP levels in the RBC (Lutcher & Bigley, 1974; Oda *et al.*, 1977; Tarui *et al.*, 1978; Vora *et*

al., 1980; 1982; Waterbury & Frenkel, 1972; Miwa, *et al.*, 1972).

Simulations (Connett, 1989) suggest that energy dependent control is linked to the energy charge of the cell as indicated by creatine charge and not on the absolute concentrations of adenine nucleotides. In heart and skeletal muscle, ATP levels are very steady because of the buffering action of creatine kinase (Connett, 1989). A number of studies have suggested that concerted action by several effectors is required to account for the observed glycolytic activity in heart and muscle. The discovery in 1980 that F26BP is a potent regulator of liver PFK (Van Schaftingen *et al.*, 1980b, c) provided a mechanism by which PFK could be controlled, since at physiological F6P concentrations, rat liver PFK is inactive unless activators such as F26BP plus AMP are present (Pilkis *et al.*, 1981b).

1.4.3 THE ROLE OF F26BP

The synergistic action of the effectors F16BP, AMP and P_i was presumed in the early 1980's to be the way in which liver PFK was activated *in vivo* since near saturating concentrations of these individual effectors would fail to activate the enzyme sufficiently to permit it to function at physiological F6P levels (Reinhart & Lardy 1980a). The discovery in 1980 that F26BP is a potent regulator of liver PFK provided direct evidence that notwithstanding *in vitro* evidence, liver PFK could actually function *in vivo*. F26BP is an extremely effective, positive effector of liver and muscle PFK. Its concentration is greatly increased in the liver when glycolysis is active and is decreased by glucagon (Van Schaftingen & Hers, 1980b; Claus *et al.*, 1981). It also inhibits F16BP'ase at micromolar concentrations in liver and muscle (Pilkis *et al.*, 1981b) and appears to be a major regulator of glycolysis and gluconeogensis in the liver (Hue & Rider, 1987).

The presence of F2,6BP has been detected in a variety of human

tissues but not in erythrocytes (Heylin *et al.*, 1982; El-Maghrabi *et al.,* 1986). It has been found in bacteria, fungi, yeast and in higher plants such as mung beans (Sabularse & Anderson, 1981). Nanomolar concentrations of F26BP have been found to stimulate all animal PFK's which have been tested but no effects have been demonstrated on PFK's from these other sources (Sabularse & Anderson, 1981; Cseke *et al.*, 1982; Van Schaftingen & Hers, 1983b).

F26BP is formed by PFK2 from F6P and ATP (Furuya & Uyeda, 1981; El-Maghrabi *et al.*, 1981; Van Schaftingen & Hers, 1981a,b). F26BP is hydrolysed to F6P and Pi by a specific F26BP'ase - FBP'ase 2 (El Maghrabi *et al.*, 1982b). The bisphosphatase and kinase activities are present on a single polypeptide chain (Pilkis *et al.,* 1983b, c; El-Maghrabi *et al.,* 1982a,b).

1.4.3.1 Response of PFK to F26BP

In the absence of activators or in the presence of 1mM ATP, very little PFK activity could be demonstrated for human PFK isoenzyme pools at F6P concentrations less than 1nM or at ATP concentrations greater than 0.5mM (Dunaway *et al.*, 1988). The addition of F26BP gives significant activity when the F6P concentration is less than 1mM and the ATP concentration is greater than 0.5mM. The muscle type homotetramer has the highest affinity for F6P in the absence of F26BP with an apparent Km = 2.0mM, while the liver rich isoenzymes from placenta had apparent Km = 5mM for F6P. The isoenzymes from platelet and fibroblasts with P type isozyme form contents of 40% and 63% respectively, have lower affinities for F6P with apparent Km = 3.5mM for the platelet forms and Km = 4.0mM for the fibroblast forms. The presence of F26BP strongly increases affinities for F6P. The muscle and liver rich isoenzymes from placenta exhibit very similar F6P saturation curves with

apparent Km values of 0.07mM whereas the platelet and fibroblast isozyme pools gave apparent Km values of 0.35 mM and 0.55 mM respectively (Dunaway *et al.,* 1988).

Some mammalian tissues, especially the heart, contain a PFK2/F26BP'ase that acts principally as a kinase (Crepin, *et al.* 1989b), and separate F26BP'ases have not been detected. (F26BP is 50-100 times more effective than F16BP in activating PFK (Pilkis *et al.,* 1981a). The apparent affinity of PFK for F26BP was measured by its ability to promote reassociation, and compared with F16BP. The apparent Kd for F26BP under these conditions was 36μM which is 40-fold lower than the Kd of 1.4mM measured for F16BP (Reinhart, 1983). The concentration of F6P has a positive influence on F26BP concentration. (Hue *et al.*, 1981). F26BP'ase is strongly inhibited by F6P, which decreases V_{max} and increases Km. This inhibition is counteracted by several phosphoric esters, including glycerol-3-P at concentrations normally present in the liver (Van Schaftingen *et al.,* 1982 ; Claus *et al.,* 1982). This might explain the low levels of F26BP seen when glycerol or ethanol is present (Hue *et al.,* 1982).

The apparent affinities of PFK and F26BP'ase for F26BP depend on the concentration of substrates or effectors such as F16BP and AMP. F26BP increases the affinity of PFK for F6P, but has no effect on the maximum activity of the enzyme (Pilkis *et al.,* 1981a; Van Schaftingen & Hers, 1981b; Uyeda *et al.*, 1981). F26BP strongly counteracts inhibition of rat liver PFK by high concentrations of ATP and citrate (Soling *et al.*, 1981). The human isoenzymes have the same response. In the absence of F26BP, M4 showed the least susceptibility to ATP. L-rich enzymes were less sensitive to ATP than were the isoenzyme pools from platelets and fibroblasts. The apparent Km values for ATP of muscle, placenta, platelet and fibroblast forms in the

presence of 1 μM F26BP were 3.3mM, 2.8mM, 0.5mM and ~50nM respectively (Dunaway *et al.,* 1988).

6-Phosphogluconate is an activator of PFK from liver, adipose tissue, kidney and skeletal muscle and acts synergistically with F26BP under most conditions (Sommercorn *et al.* 1984). F26BP will also stabilise liver PFK against the rapid, spontaneous inactivation which occurs in its absence at 37°C (Soling *et al.*, 1981) and 50°C (Uyeda, *et al.*, 1981) and also prevents inactivation by PFK phosphatase and low pH.

F2,6BP will prevent dissociation of PFK even when the enzyme is diluted to concentrations as low as 4 x 10^{-8}M and also promotes reassociation to the tetramer and larger forms from the inactive dimer. It will also inhibit dissociation by MgATP (Reinhart, 1983). The promotion of aggregation of PFK by F26BP may be one of the ways in which F26BP achieves its activating effects *in vivo,* as aggregation increases the activity of the enzyme under physiological conditions.

The efficiency of F26BP in activating PFK is 2 to 3.5 orders of magnitude greater than that of its isomers F16BP and G16BP (Hers & Hue, 1983; Pilkis *et al.*, 1981a). The concentrations of F16BP and F26BP are very similar in the liver and usually change in parallel. It is likely that at least in the liver and probably in other cells, F16BP is not a physiological stimulator of PFK. At high concentrations, F16BP counteracts the positive effect of F26BP on erthrocyte PFK (Heylin *et al.,* 1982) and yeast PFK (Bartrons *et al.,* 1982) and could be considered to be an inhibitor.

Trypanosoma brucei pyruvate kinase, together with PK from the glycosome-containing members of the Kinetoplastida family, i.e. the insect trypanosomatid *Crithidia luciliae,* the human trypanosomatid *Leishmania major* (Van Schaftingen *et al.,* 1985), the parasitic Bodonid *Trypanoplasma*

borelli (Opperdoes *et al.,* 1988), and *Trypanosoma cruzi* (Cazzulo *et al.,* 1989) all differ from their homologous eukaryotic counterparts in that their PFK is not activated by F26BP. However, they contain the only class of PK that is activated by F26BP (van Schaftingen *et al.,* 1987). PFK is sequestered in the glycosome of these organisms and is probably inaccessible to F26BP (Callens *et al.,* 1991).

1.4.3.2. Factors controlling the concentration of F26BP

The major factors that determine the concentration of liver F26BP *in vivo* are the concentrations of F6P and the phosphorylation state of PFK2/F26B'ase. The concentration of F26BP in rat liver is less than 1μM during starvation or diabetes and up to 20μM in the fed state (Pilkis *et al.*, 1986; Claus *et al.,* 1981). In starvation or diabetes, the enzyme is phosphorylated with a 2 to 3 fold increase in Km for F6P (Murray *et al.,* 1984) while there is a decrease in F6P concentration to ~50μM (Hems & Brosnan, 1970; Start & Newsholme, 1986). At this level, the kinase is operating below its Km for F6P and F26BP synthesis is minimal. The enzyme is dephosphorylated in the fed state and has a Km of 50μM for F6P in the presence of P_i (Murray *et al.,* 1984). The enzyme is operating above its Km for F6P as F6P concentrations are 0.1-0.2mM (Start & Newsholme, 1986) giving a high rate of synthesis of F26BP.

1.4.3.3. The effect of F26BP on bisphosphatase activity

The Km of the bisphosphatase (of PFK-2/F26BP'ase) for F26BP is 100nM in the presence of Pi and α-glycerol-P (Stewart *et al.*, 1985) and the concentration of F26BP never falls to below an order of magnitude larger than this and so changes in F26BP concentration will not affect the bisphosphatase activity. There is a strong inhibition of the bisphosphatase activity by F26BP and F6P (El-Maghrabi *et al.,* 1982b; Sakakibara *et al.,*

1984a; Murray *et al.,* 1984; Kitajima *et al.,* 1984; Van Schaftingen *et al.,* 1982b) but this is relieved by phosphorylation of the enzyme (Van Schaftingen *et al.,* 1982b; El Maghrabi *et al.,* 1982a, c; Stewart *et al.,* 1986).

Hormone-induced changes in the level of F26BP and F6BP are achieved by altering the phosphorylation state of the enzyme via cAMP-dependent protein kinase (Pilkis *et al.*, 1983a) or via protein kinase C (Crepin *et al.*, 1989c).

F26BP is an activator of PFK-1 and an inhibitor of F16BP'ase (Van Schaftingen *et al.,* 1981a; Pilkis *et al.,* 1981b). The inhibition is synergistic with AMP for the liver (but not the muscle) enzyme. Liver PFK-1 does not appear to be affected by phosphorylation and these two differences may be a reflection of tissue specificity. The binding sites for F26BP and F16BP may be different or they may overlap (Van Schaftingen, 1987). The hexose bisphosphate binding site has not been ascertained with certainty.

In livers of fed rats producing lactate, the tissue concentrations of AMP (0.1-0.2mM) and F26BP (10μM), should be sufficient to completely inhibit F16BP'ase even at saturating concentrations of substrate. Cycling of metabolites between glucose and triose phosphates does occur however (Clarke et al., 1983; Hue, 1987) indicating that PFK-1 and F16BP'ase are active under these conditions. No reports have demonstrated complete inhibition of F16BP'ase *in vivo,* while in contrast, PFK-1 is completely inactive during gluconeogenesis (Gottschalk *et al.,* 1983). This suggests that F16BP'ase is less inhibited *in vivo* than would be expected from *in vitro* measurements. Kinetic and binding studies indicate that the affinity of F16BP'ase for F26BP is about ten fold less than that for PFK-1 (Kitajima *et al.,* 1983). It may be that control over F16BP'ase is exerted by the level of phosphorylation, although phosphorylation of F16BP'ase by cyclic AMP-

dependent kinase has only been demonstrated for the rat liver enzyme (Ekdahl & Ekman, 1985).

1.4.3.4. Control of glycolytic flux by F26BP

The most obvious role for F26BP is to control glycolysis and gluconeogenesis. This would be of particular importance in the liver where both pathways operate. Gluconeogenesis is stimulated by glucagon and predominates in fasting and diabetes. Upon administration of glucagon or cAMP to isolated hepatocytes, the levels of F26BP decrease rapidly. (Bartrons *et al.,* 1983). This is due to the cAMP-dependent protein kinase mediated phosphorylation of the PFK2/F26BP'ase enzyme which leads to inactivation of the PFK-2 moiety (Garrison & Wagner, 1982; Pilkis *et al.,* 1982b).

Insulin, which acts to counter the effects of glucagon by elevating cAMP levels, opposes the action of glucagon to lower F26BP levels (Terrettaz *et al.,* 1986). The rate of gluconeogenesis is thus accelerated by glucagon and decelerated by insulin, with F26BP as the switching signal. When F26BP levels are high, the activity and flux through PFK-1 is high, F26BP'ase is inhibited and glycolytic flux predominates. When F26BP levels are low, PFK-1 is inhibited, F16BP'ase and flux are enhanced and gluconeogenic flux increases (Pilkis *et al.,* 1988).

The increase in the levels of F16BP increases the activity of pyruvate kinase by acting as a positive effector, and also inhibits phosphorylation of PK-L by cAMP-dependent protein kinase so decreasing its K_m for PEP (El-Maghrabi & Pilkis, 1985; Claus *et al.,* 1979).

Insulin administration increases the synthesis of PFK (Dunaway *et al.,* 1978) and also increases the concentration of a PFK stabilising factor which may be F26BP (Van Schaftingen *et al.,* 1983a). F16Pase activity is

reported to be decreased by insulin administration (Weber *et al.,* 1966).

Increases in glycolysis can occur in the liver and other tissues with no increases in F26BP levels (Hue *et al.*, 1982). This was observed in hepatocytes during anoxia, in muscle during electrical stimulation and in hearts treated with epinephrine. Under these conditions some other factor must be involved in the control of glycolysis.

In long-term starvation or diabetes, the activities of PFK-2/F2,6BP'ase decreased and could be restored by re-feeding or insulin administration and reflected alterations in the level of enzyme protein (Pilkis *et al.*, 1983a; Richards & Uyeda, 1982; Gil *et al.,* 1986). Thyroxine and glucocorticoids increased the total activities and did not involve changes in the phosphorylation state of PFK-2/F26BP'ase. If the steady state concentration of F26BP is determined by the two opposing reactions of the enzyme then an increase in the total amount of enzyme should not change the steady state concentrations of F26BP assuming the phosphorylation state and concentrations of effectors of the enzymic activities do not change. In the starvation/refeeding cycle there appears to be a need for additional enzyme molecules reflecting the need for a lipogenic rather than a gluconeogenic enzyme (Pilkis & El-Maghrabi, 1988).

In the well fed animal, circulating levels of glucose and insulin are elevated and glucose homeostasis is maintained primarily by dietary intake, and when needed, by the breakdown of liver glycogen. The rate of gluconeogenesis is low due to a high rate of substrate cycling at both the F6F/F16BP and the pyruvate/PEP substrate cycles which in turn is due to the high activity of PFK and PK (Hue, 1987). The bifunctional enzyme is dephosphorylated which leads to high levels of F26BP via activation of the kinase and inhibition of the bisphosphatase. PFK is activated by the high

levels of F26BP while F16BP'ase is inhibited. This leads to elevated levels of F16BP which is a strong allosteric activator of PK. The increased allosteric activation and dephosphorylation of PK increases flux through the enzyme and depresses gluconeogenesis (Rognstad & Katz, 1977).

In fasting and longer term starvation the rate of gluconeogenesis is elevated. Insulin levels fall and this increases hepatic levels of cAMP (Claus *et al.*, 1984a). The stimulation of gluconeogenesis is due to inhibition of PFK and activation of F16BP'ase which is effected by lower F26BP levels caused by cAMP-dependent phosphorylation of PFK-2/F26BP'ase-2. This phosphorylation inhibits the kinase and promotes the hydrolysis of F26BP via the bisphosphatase. Lower levels of F26BP and enzyme phosphorylation inhibit PK (Engstrom, 1978). The increase in gluconeogenesis is also caused by an increase in the levels of phosphoenol pyruvate carboxykinase (Hod *et al.,* 1986). In diabetic and starved rat, liver PFK-2 content but not its encoding mRNA, is decreased (Crepin *et al.,* 1988). PFK-2 content can be raised by injection of insulin, and the mRNA content raised by refeeding (Colosia *et al.,* 1988). Thyroid hormone will also increase levels of PFK-2 content and mRNA content in hypothyroid rats (Wall *et al.,* 1989).

F26BP is unlikely to be the only regulator of glycolysis and gluconeogenesis. After refeeding of starved rats, F26BP levels remained low for several hours, rising toward high fed values only when glycogen levels had returned to those of the fed state. This is consistent with an elevated rate of gluconeogenesis during the initial stages of glycogen formation but does not explain the enhanced rate of glycolysis (Claus *et al.*, 1984c). When starved rats are fed a diet high in sucrose the bulk of the hepatic glycogen was gluconoegenic in origin despite the fact that F26BP rose rapidly to high

levels (Kuwajima *et al.*; 1986). Castel ijn *et al.* (1986) reported an unknown low molecular weight activator of F1,6BP'ase that overcame the inhibition by F26BP. A F26BP dependent cytosolic protein from liver was identified by Niemeyer et al. (1987) that inhibited hexokinase, and this might also play a role in the regulation of glycogen production.

1.4.4 COVALENT MODIFICATION OF PFK BY PHOSPHORYLATION

Several laboratories tried to phosphorylate PFK without success after it was shown in 1965 that PFK was made more stable during purification by the addition of fluoride or ATP (Ling *et al.*, 1965). Subsequently, Brand and Soling (1975) phosphorylated a low pH precipitate fraction of liver PFK, and Hofer & Furst (1976) isolated [^{32}P]PFK from muscle tissue of mice previously injected with [^{32}P] phosphate.

1.4.4.1. Phosphorylation of PFK

PFK from a variety of sources has been phosphorylated *in vitro* and *in vivo*. Yeast PFK (Huse *et al.,* 1988), mouse muscle PFK (Hofer and Furst, 1976; Riquelme *et al.*, 1978a), rabbit skeletal muscle PFK (Hussey *et al.*, 1977; Uyeda, *et al.*, 1978), rat skeletal muscle PFK (Uyeda *et al.*, 1978) and rat liver PFK (Kagimoto & Uyeda, 1979) are all partially phosphorylated *in vivo*. They can all be phosphorylated *in vitro* by cAMP-dependent protein kinase (Riquelme *et al.*, 1978b; Sorenson-Ziganke & Hofer, 1979; Riquelme & Kemp, 1980; Claus *et al.,* 1982, 1985; Pilkis *et al.,* 1982b; Mieskes *et al.*, 1987). The Ca^{2+}-calmodulin dependent protein kinase phosphorylates rabbit liver, but not rabbit muscle PFK, at a slightly lower rate than cAMP-dependent protein kinase (Mieskes *et al.*, 1987), and protein kinase C phosphorylates skeletal muscle PFK *in vitro* (Hofer *et al.*, 1985; Mieskes *et al.*, 1987). All three isoenzymes from mammals are reported to be capable of

being phosphorylated (Kemp & Foe, 1983), and possible phosphorylation sites on the three rabbit enzymes have been described (Valiatis *et al.,* 1989), (see section 1.4.4.3).

The extent of phosphorylation of PFK *in vivo* is dependent on the tissue and on its metabolic state (Sorensen-Ziganke & Hofer, 1979; Hofer, 1983). The extent of phosphorylation of PFK is higher in contracting muscle than resting muscle (Hofer & Sorenson-Ziganke, 1979). Hofer and Furst (1976) calculated a phosphate content of ~0.7 moles of phospho group per mole of protomer. Several groups (Hussey *et al.*; 1977; Uyeda *et al.*, 1978; Riquelme *et al.,* 1978b; Kemp & Foe, 1983) all found levels of covalently bound phospho group in rabbit muscle at levels closer to 0.2 moles phospho group per mole of protomer. Rabbit muscle PFK isolated under three different conditions had very different covalent phosphate contents. This was higher than the levels reported by other groups (Hussey *et al.*, 1977; Uyeda *et al.*, 1978; Riquelme *et al.*, 1978a; Riquelme *et al.*, 1978b). The data suggest more than one site of phosphorylation and this has also been observed for rat liver PFK (Pilkis *et al.*, 1982a) and muscle PFK (Starling *et al.*, 1982).

Other studies indicate that no more than two moles phospho group per tetramer could be incorporated into PFK by Ca^{2+}/Calmodulin protein kinase (Mieskes *et al.,* 1987). Domenech *et al.* (1988) also found no more than two moles phospho group per tetramer in rat liver PFK confirming the results of Brand & Soling (1982). There may be a secondary site of phosphorylation at the high affinity calmodulin binding site (see section 1.4.6).

1.4.4.2 Effects of Phosphorylation

The evidence for a physiologically relevant regulation of the activity of

PFK by phosphorylation is contradictory and controversial, and regulation is thought to be mediated primarily by the concentration of F26BP. Isoenzymes of PFK may vary in their response to phosphorylation. Phosphorylation may favour gluconeogenesis in liver but favour glycolysis in muscle. Phosphorylation does not change the maximum catalytic activity of muscle and liver PFK or their relative mobilities on SDS-PAGE (Sakakibara & Uyeda, 1983). Differences have been observed in the response of PFK phosphorylated by cAMP-dependent protein kinase. Phosphorylated muscle and liver PFK exhibit an increased affinity for ATP at the inhibitory site and decreased affinity for F6P at the active site (Foe & Kemp, 1982; Kitajima *et al.,* 1983; Sakakibara & Uyeda, 1983). These enzymes are also less sensitive to activation by AMP, G16BP and inorganic phosphate (Foe & Kemp, 1982) and so are more sensitive to inhibition by ATP and citrate than less phosphorylated forms.

The high-phosphate form of liver PFK also shows a 1.6 fold greater $K_{0.5}$ for F6P than the low-phosphate form and these differences become greater at lower pH (Sakakibara & Uyeda, 1983).

Rabbit muscle PFK phosphorylated with protein kinase C gives a slight decrease in activity but an increase in activity when phosphorylated with protein kinase A (Hofer *et al.,* 1985).

Contradictory findings have been reported for the interaction of F26BP with phosphorylated or dephosphorylated PFK. Some results suggest that the phosphorylated forms of muscle and liver PFK are less sensitive to activation by F26BP (Furuya & Uyeda, 1980; Foe & Kemp, 1982; Sakakibara & Uyeda, 1983) while no change in sensitivity has been reported by others (Pilkis *et al.,* 1982a; Kitajima *et al.* (1983). If phosphorylation does exert a physiological effect, it may not be a direct

effect on enzyme activity but via some indirect action such as an interaction with cellular organelles or some other cell structural component (see section 1.4.5.) It may be that phosphorylation has a more direct effect on PFK-2/F26BP'ase and PK. Glucagon causes levels of cAMP to rise and the activation of cAMP-dependent protein kinase. This kinase will then catalyse the addition of 1 mol of phosphate per mol of liver PFK-2/F26BP'ase subunit (Murray *et al.,* 1984) inactivating PFK-2. Phosphorylation by cAMP dependent protein kinase C can also inactivate PK. Liver PK can also be phosphorylated, and so inhibited, by Ca^{2+}-CaM dependent protein kinase (Schworer *et al.,* 1985). F16BP is a potent inhibitor of both phosphorylations (Claus & Pilkis, 1981).

Sale & Denton (1985a, b) describe the activation of rat adipose tissue PFK by phosphorylation *in vitro* and by exposure to insulin and adrenalin*in vivo.* They put forward the view that an increase in cAMP levels in adipose tissue may result in an increase in glycolysis mediated by the phosphorylation of PFK by cAMP-dependent protein kinase.

1.4.4.3 The phosphorylation site

The site of phosphorylation in rabbit muscle PFK is a serine residue, the sixth amino acid from the carboxyl terminus (Riquelme & Kemp, 1980; Kemp *et al.*, 1981). The serine is preceded by the sequence Arg-Lys-Arg which is similar to other known substrates for cAMP-dependent protein kinase (Kemp & Foe, 1983) where basic residues precede the serine undergoing phosphorylation. The same sequence is present in HMPFK. HLPFK and MLPFK have the sequence Arg-Arg-Thr-Leu-Ser. The phosphorylation sites of liver and muscle PFK are susceptible to limited proteolysis which leaves the catalytic properties intact but reduces the Mr by ~2000 (Riquelme & Kemp, 1980; Krystek & Hofer, 1981). This is seen with

trypsin and subtilisin (Sakakibara & Uyeda, 1983; Riquelme & Kemp, 1980; Kemp *et al.,* 1981; Kitajima *et al.*, 1983).

Reports of a second phosphorylation site in the muscle enzyme (Hofer & Sorenson-Ziganke, 1979; Sorenson-Ziganke & Hofer, 1979) have been confirmed by Zhao *et al.* (1991). They note that the site may be in the connecting peptide region and that phosphorylation inhibits RMPFK but not RLPFK or yeast PFK. Protein kinase C phosphorylates one or more separate sites of *Ascaris suum* PFK (Hofer *et al.,* 1985). Protein kinase C and the Ca^{2+}/calmodulin-dependent protein kinase phosphoryate PFK at the same site as cAMP-dependent protein kinase (Hofer *et al.,* 1985; Mieskes *et al.*, 1987; Domenech *et al.*, 1988).

Phosphorylation sites have been reported for the three rabbit isoenzymes (Valiatis *et al.*, 1989). Rabbit muscle and liver forms are composed of the homotetramers M_4 and L_4 respectively (Tsai & Kemp, 1972,1973; Gonzalez *et al.,* 1975). P type subunit is the predominant form in rabbit brain with slightly less M type and a small amount of L type (Foe & Kemp, 1984,1985).

Valiatis *et al.* describe the same phosphorylation site as in previous reports (Riquelme & Kemp, 1980; Kemp *et al.,* 1981). The site of phosphorylation for rabbit liver PFK is identical to that proposed by Gehnrich *et al.* (1988) for mouse liver PFK and for human liver PFK by Levanon *et al.* (1989). The sequence of the labelled peptide from rabbit liver is 80% identical to the human and mouse liver sequences.

The rabbit platelet type sequence is dissimilar to the sequence of the human platelet sequence presented in this thesis (see Chapter 5) but only thirteen residues were reported. The rabbit platelet sequence shows no similarity to PFK from any organism but sequence conservation may not be

strong at the carboxyl terminal tail.

The *in vitro* site of phosphorylation for the rabbit muscle enzyme is identical to the *in vivo* site (Kemp *et al.,* 1981) but it is unknown if this holds true for the liver and platelet sequences.

1.4.4.4 Control of phosphorylation

The elevation of pH or the addition of AMP increases the extent of phosphorylation of the rabbit muscle and rat liver enzymes (Kemp *et al.*, 1981; Pilkis *et al.*, 1982a; Foe & Kemp, 1984; Domenech *et al.*, 1988) while the addition of ATP or citrate inhibits the phosphorylation of both enzymes. F16BP had no effect on the rat liver enzyme but increased the phosphorylation rate of the rabbit muscle enzyme (Kemp *et al.,* 1981). F26BP enhanced the rate of phosphorylation of the rat liver enzyme (Pilkis *et al.*, 1982a; Foe & Kemp, 1984; Domenech *et al.,* 1988).

Phosphorylation appears to inhibit the activity of the liver and muscle enzymes. The active form of the enzyme is a better substrate for the cAMP-dependent protein kinase than the inactive form, and so increased levels of cAMP may inhibit active enzymes in this way.

Glucagon changes the concentration of F26BP thereby changing the activity of PFK (see section 1.4.3.1). It also increases the incorporation of ^{32}P into rat liver PFK *in vivo* (Kagimoto & Uyeda, 1979), isolated hepatocytes (Claus *et al.,* 1980) and isolated perfused liver (Kagimoto & Uyeda, 1980). The phosphorylated enzyme shows a large decrease in activity (Kagimoto & Uyeda, 1979).

Glucose increases the phosphorylation rate of PFK from rat liver (Brand & Soling, 1982; Brand *et al.*, 1983; Claus *et al.,* 1982) but this is dependent on the fed state of the animal (Brand *et al.*, 1983). In starved animals, glucose or glucagon can separately increase the rate of

incorporation of ^{32}P, and together they increase phosphorylation to a maximal rate. In the fed animal, both glucose and glucagon are necessary to achieve incorporation of ^{32}P into liver PFK (Brand & Soling, 1982; Claus *et al.*, 1982).

1.4.4.5 Phosphorylation of Ascaris suum and Fasciola hepatica PFK.

This project was not only concerned with characterising human PFK. We were also interested in characterising PFK from the human parasitic filarial nematode *Onchocerca volvulus,* and examining any differences in structure and control of PFK between the two species (see section 1.10).

Phosphorylation of mammalian PFK by cAMP-dependent protein kinase does not appear to result in any significant change of the catalytic and regulatory properties. Hofer *et al.* (1982) and Daum *et al.* (1986) noted that purified PFK from the pig intestinal parasitic nematode *Ascaris suum* was effectively phosphorylated and activated by a protein kinase purified from *Ascaris suum* muscle, and that the activation could be reversed by the action of a phosphatase purified from the same tissue. This protein kinase, and the cAMP dependent protein kinase both catalyse the incorporation of 1 mol of phosphate per subunit of tetramer, but cAMP-dependent protein kinase does not activate *Ascaris suum* PFK. This suggests that the muscle protein kinase is phosphorylating a residue distinct from that phosphorylated by cAMP-dependent protein kinase. PFK from the liver fluke flatworm *Fasciola hepatica* is also activated by phosphorylation (Kamemoto & Mansour, 1986). The Mr of PFK from *F. hepatica* is ~83,000 which is comparable to the mammalian isoenzymes. It would be interesting to know

if there is any structural diversity between the enzymes to account for this difference in response to phosphorylation. The *Ascaris suum* phosphorylation site has been sequenced (Kulkarni *et al.,* 1987) and it bears no relationship to the proposed phosphorylation sites of the mammalian enzymes. It may have a relationship to the effector site of *E. coli* PFK however (see Section 6.4).

It would be interesting to know if this situation occurs in *O.volvulus* as it might then be possible to target phosphorylation in the parasite as a possible site for drug therapy. This assumes of course that the *Ascaris* muscle protein kinase does not have a counterpart in the mammalian host which is responsible for activating PFK by phosphorylation. The significance of this activation by phosphorylation is discussed in section 6.4

1.4.5 Interaction of PFK with Actin

Evidence has accumulated which suggests that some of the glycolytic enzymes are actin binding proteins (Masters, 1984; Clarke *et al.*, 1983). PFK, aldolase, glyceraldehyde 3-P-dehydrogenase and PK bind to paracrystalline actin bundles (Clarke *et al.*, 1983; Roberts & Somero, 1987). High concentrations of PFK form a precipitate with actin A. The addition of F-actin to a solution of rabbit muscle PFK partially reverses the inhibition seen at high ATP concentrations, and increases the affinity of the enzyme for F6P with a slight effect on V_{max} (Liou and Anderson 1980). F-actin also augments activation by AMP and partially counters inhibition by citrate. Luther and Lee (1986b), proposed that in contracting muscle, the need for energy is high and that localising the glycolytic enzymes at the point where energy is needed would increase the efficiency of the system. If the glycolytic enzymes are in the muscle matrix where ATP is utilised, then the ATP produced can be more efficiently delivered and used. If PFK is considered to be the key

regulatory enzyme in glycolysis then its location needs to be controlled and this could be determined by phosphorylation status. There may be a time lag between phosphorylation and the formation of a PFK-actin complex. The kinetic behaviour of phosphorylated PFK not complexed with actin is more sigmoidal than the dephosphorylated form, and so its activity at the same F6P concentration is lower than the dephosphorylated form. In resting muscle, energy is still needed and a dephosphorylated PFK not complexed with actin will give higher activity at the same concentration of substrate.

Phosphorylated PFK has a higher affinity for F-actin than the dephosphorylated enzyme. F-actin acts as a positive effector of the phosphorylated form but does not significantly affect the kinetic properties of the dephosphorylated form (Luther & Lee, 1986b). Once the complex is formed, the enzymic activity is increased so that glycolytic flux increases to meet the needs of the contracting muscle.

Actin stabilises the active tetrameric form of PFK and it is possible that this is the cause of the kinetic activation (Roberts & Somero, 1987). Polyethylene glycols have been found to prevent or decrease the rate of spontaneous deactivation of rat liver PFK which is due to subunit dissociation. The K_m for F6P at pH 7 in the presence of 3mM MgATP was dramatically lowered by increasing PEG concentration (Reinhart, 1980).

Tropomyosin and troponin are thin filament proteins associated with actin, and they reduce the binding of PFK to actin. ATP and ADP also inhibit binding but not F6P or F26BP. It is possible that actin binds to PFK at the adenosine activating site (Roberts & Somero, 1989) and that the phosphorylation of PFK could lead to a change in conformation which is too minor or distant to affect access of ATP or ADP but modifies the ability of the enzyme to interact with actin.

Binding sites for GAPDH, aldolase and PFK were localised on the actin monomer in microfilaments (Mejean *et al.,* 1989). The locations were investigated by measuring the competition between each enzyme and anti-actin antibodies directed against known, limited portions of the actin sequence. GAPDH and aldolase gave specific reactivity to certain parts of the actin microfilament, but PFK modified the reactivity of all antibodies tested suggesting that the interface between PFK and actin is large with ill-defined boundaries.

1.4.6 Interaction of PFK with Calmodulin

Calmodulin (CaM) is a ubiquitous intracellular Ca^{2+} receptor, which plays a part in the majority of the Ca^{2+}-regulated processes that have been studied in eukaryotic cells. Phosphofructokinase has been shown to be a calmodulin binding protein (Mayr & Heilmeyer, 1983). The high affinity interaction of CaM with PFK could be attributed to binding of CaM to the inactive dimeric form of the enzyme (Buschmeier *et al.*, 1987).

There are two CaM binding sites per subunit of rabbit skeletal muscle PFK each with different affinities for CaM. The high and low affinity sites have Kd values of 1μM and 3mM, respectively. The high affinity site can bind CaM when PFK is a dimer or a monomer but not a tetramer (Mayr, 1984b). The weaker binding site is in the C-terminal part of the enzyme and contains the site which is phosphorylated by the cAMP-dependent protein kinase (Kemp *et al.*, 1981). The high affinity site is in the 'connecting peptide region' (Poorman *et al.*, 1984). This region is also proposed to make contact between dimers in the tetrameric state, a view supported by the inability of CaM to bind to the high affinity site in the tetramer. Its resistance to digestion with sub tilisin in the tetramer also supports this conclusion (Buschmeier *et al.*, 1987)).

Under certain conditions, Ca^{2+}-calmodulin (Ca^{2+}-CaM) will accelerate the rate of dissociation of PFK from the active tetramer to inactive dimeric and monomeric forms, and will oppose the effect of regulatory activators (Mayr, 1984a, 1984b). On the one hand, CaM acts as a Ca^{2+}-dependent hysteretic inhibitor of the active tetramer inducing the shift to the inactive dimeric state which is stabilised by CaM so shifting the equilibrium state to dissociated inactive forms. On the other hand, CaM can also allosterically activate tetrameric PFK under certain conditions. The hysteretic inactivation implies that the dimer can no longer be reconstituted to the active tetramer if CaM dissociates when the concentration of calcium falls and that the dimers have undergone a conformational change. Large inactive polymers are formed under these conditions (Mayr & Heilmeyer, 1983; Mayr, 1984a). If Ca^{2+}CaM is present in high concentrations then the oligomerisation of dimers is suppressed and a slow depolymerisation to inactive oligomers can occur. The Ca^{2+} CaM deactivated enzyme isomer can be back isomerised by Mg^{2+} ATP and dimers can be best reassociated to the active tetramer by F26BP, although CaM is still required for this process (Mayr, 1984a). High concentrations of Ca^{2+}CaM modulate the association state of PFK in a way resembling that of allosteric effectors such as F26BP. The apparent allosteric activation of dilute PFK by CaM is in fact a slowdown of tetramer dissociation.

The binding of CaM can prevent phosphorylation, and vice versa. The unknown secondary phosphorylation site for PFK may be present at the CaM high affinity site (see Section 1.4.4.1) or may be involved at the ATP inhibition site (see section 6.4). The serine residue at position 377 in RMPFK at the amino end of the high affinity CaM site, which is preceded by Lys-Leu-Arg-Gly-Arg has three basic residues and is partially consistent with the

sequence of other cAMP-dependent phosphorylation sites (Kemp & Foe, 1983). This is not the case with the liver enzymes. It has been suggested that CaM and cAMP-dependent protein kinase act on common sequences in proteins, subject to dual control by Ca^{2+} and cAMP, and that modification of certain CaM binding sites is one of the functions of the cAMP-dependent protein kinases (Malencik & Anderson, 1982, 1983; Anderson & Malencik, 1986).

Another activity of Ca^{2+}-CaM dependent protein kinase is the phosphorylation of liver (but not muscle) PK at two sites (Schworer *et al.,* 1985). One site is the same serine residue phosphorylated by cAMP dependent protein kinase while the second site is an unique threonyl residue close to this serine. Phosphorylation inhibits PK and enhances flux towards gluconeogenesis. It is possible that Ca^{2+}-CaM dependent phosphorylation of PFK is modulated by Ca^{2+} linked hormones such as vasopressin and angiotensin in a manner similar to PK.

Sheep heart PFK is reputed to be phosphorylated by Ca^{2+}-CaM protein kinase at two sites distinct from those which are phosphorylated by cyclic AMP dependent protein kinase (Mahrenholz *et al.,* 1991). Inactivation of RMPFK by other proteins has been noted by Zhao *et al.* (1991). In decreasing order of effectiveness these proteins are, skeletal muscle troponin, >troponin, the two smooth muscle myosin light chains, α-actin and (the calcium binding protein) bovine brain S-100>>parvalbumin and soybean trypsin inhibitor. Troponin and Ca^{2+}-CaM-induced phosphorylation is strongly inhibited by F26BP and/or F16BP. Binding to or phosphorylation of a site in the connecting peptide of PFK may result in the formation of inactive dimers. Rabbit liver and yeast PFK do not show any appreciable

loss of activity in the presence of Ca^{2+}-CaM or troponin

The connecting peptide serine residue which is thought to be phosphorylated is also found in HMPFK, SHPFK, SLPFK and HLPFK. In resting muscle, PFK may be inactivated in a dimeric form. This could be mediated by Ca^{2+} concentration for example, via troponin or myosin or perhaps by phosphorylation. Activation of PFK in active muscle could perhaps be achieved by rising levels of F16BP and F26BP induced by dephosphorylation of F16BP'ase and F26BP'ase.

1.5.1 Regulation of PFK *In Vivo*

The isoenzymes of PFK are found in differing amounts depending on the tissue being examined (see Section 1.3.1). The isoenzymes are kinetically distinct and do not simply exist in homotetrameric forms but can combine to form heterotetramers. The kinetic and regulatory properties of various isoenzyme pools are dependent on subunit composition, and contribute significantly to the diversities of glycolytic and gluconeogenic rates which have been observed for different tissues (Dunaway, 1983).

1.5.1.1 Regulation of Muscle PFK *In Vivo*

Adult muscle is the only tissue so far to show a homotetrameric enzyme composed of four identical muscle PFK subunits. When muscle contracts, it has an immediate requirement for ATP and this is met by glycolysis which utilises blood glucose, creatine phosphate and muscle glycogen. Creatine phosphate is the first metabolite to be exhausted in stressed muscle (Hearse & Chain, 1972). Under most conditions, there is an excess of creatine phosphate which ensures that a high ratio of ATP to ADP and AMP exists. Helmreich and Cori (1965) have shown that ATP levels do not vary greatly when muscle is stimulated electrically or when glycolytic flux

is increased greatly. The decrease in creatine phosphate concentrations would relieve the inhibition of PFK, PK (Kemp, 1973) and GAPDH (Oguchi *et al.*, 1973). Regulation of muscle PFK *in vivo* by changing levels of ATP, ADP and AMP may only be significant therefore when energy reserves are severely depleted.

When muscle is metabolising fatty acids, the resulting high concentrations of citrate will cause inhibition of muscle PFK and reduce glycolytic flux. During anaerobosis, decreasing pH inhibits muscle PFK. This effect can be partially countered by positive modulators such as F26BP, G16BP and AMP (Section 1.4.2.3). It is important to note that synthesis of F26BP by PFK-2 is stimulated by cAMP induced phosphorylation, and so adrenalin will stimulate glycolysis in muscle. The reverse is true for the liver enzyme (see Section 1.4.3).

Alternative splicing has been reported for HMPFK mRNA (Nakajima *et al.*, 1990a;1990c). Two RNA's for HMPFK have 5' non coding sequences differing in length due to alternative splicing, while another contained an additional sequence at the 5' end of a non-coding 5' sequence. This suggests that the third type was under the control of another promoter.

1.5.1.2 Regulation of Liver PFK *In Vivo*

Gluconeogenesis is more important than glycolysis in the liver. The reactions catalysed by PFK and F16PBP'ase are important to the regulation of glucose metabolism (Hers, 1976; Weber *et al.*, 1967) If both enzymes are active then the homeostatic regulation of ATP is lessened and glycolytic flux is decreased (Hers & Hue, 1983). A high ratio of F1,6B'ase to PFK activity is needed for gluconeogenesis while F16BP'ase activity must be reduced for the promotion of glycolysis. In the livers of fed rats (Van Schaftingen *et al.*, 1980a) and in isolated hepatocytes (Van Schaftingen *et al.*, 1980b), there is

evidence that PFK and F16BP'ase are both active, as there is a recycling of metabolites between F6P and F16BP. Recycling is highest when the switch is taking place between glycolysis and gluconeogenesis (Clark *et al.,* 1974). F26BP is an inhibitor of F16BP'ase but it seems that the inhibition is incomplete allowing PFK and F16BP'ase to be active simultaneously.

A major source of the raw material for gluconeogenesis in the liver is lactate. The Cori cycle allows contracting skeletal muscle to reduce pyruvate to lactate in anaerobic conditions and so regenerate NAD^+. The NAD^+ allows glycolysis to proceed and so the muscle can shift some of the metabolic burden to the liver. The glucose produced during gluconeogenesis can then be carried to the muscle allowing contraction to proceed.

1.5.1.3 Regulation of Platelet Type PFK *In Vivo*

Platelet PFK is found in association with other PFK isoenzymes. ATP inhibition of platelet-rich isoenzyme pools *in vitro* increases as the proportion of the platelet subunit increases. The effect is the same when F26BP is present (Dunaway *et al.,* 1988). Platelet-rich isoenzymes also show the lowest affinity for F6P *in vitro,* and this is also true when F26BP is present (Dunaway *et al.*, 1988).

The physiological implications of these *in vitro* results may be that the platelet type isoenzyme subunit is present in those tissues which are less important for an organism's survival when glucose is scarce. An important tissue for survival in this regard is muscle, which shows the least sensitivity to ATP, although it is undeniable that brain is an important tissue. Isoenzymes rich in platelet type subunits would also slow down glycolysis more quickly than other types when excess lactate production occurred, so protecting the cell from a dangerous drop in pH.

It is interesting to note that cancerous cells show altered isoenzyme profiles (Vora *et al.*, 1985a,b; Gekakis *et al.*, 1989). Cancerous cells show an increased rate of glycolysis and a decrease in the rate of gluconeogenesis. Several cancerous cell lines have platelet type species as the predominant subunit in the PFK isozymes when compared to their pre-cancerous counterparts. The L-subunit also increases in certain cancerous cell lines and it may be that the L and P isoenzymes are essential to the survival of cancer cells and may provide sensitive targets for the design of selective cancer chemotherapy (Vora *et al.*, 1985b).

Several reports have characterised the platelet enzyme. Meinhofer *et al.* (1980) purified human homotetramers and investigated their kinetics. Maximum cooperativity to F6P was shown by M_4 (Hill coefficient ~3-4) and L_4 (Hill coefficient 1.4-3.4) while positive homotropic interactions were much less pronounced for the platelet homotetramer P_4 (Hill coefficient <1.5). . The main kinetic features were: hyperbolic kinetics for F6P (except at high ATP concentration); absence of activation by AMP and glucose 16BP; slight activation by NH_4^+ and slight inhibition by ATP and citrate. Meinhofer *et al.* (1980) found the same kinetic properties for the enzyme from human fibroblasts, which contain up to 63% platelet type monomers (Dunaway *et al.*, 1988; Kahn *et al.*, 1979, 1980) and from some malignant tissues which can contain up to 85% of platelet type subunits (Vora , 1982).

Meinhofer *et al.* (1980) have described HPPFK as being a virtually non-allosteric enzyme and that it is a poor regulatory enzyme as compared to the other PFK forms.

Foe & Kemp (1985) found rather different kinetic properties for rabbit platelet type enzyme (RPPFK) isolated from rabbit brain. Whereas (at 1mM F6P), the RLPFK was completely inhibited by 0.1mM ATP, RLPFK required

2mM ATP for complete inhibition. RMPFK and RPPFK were completely inhibited at 1mM ATP wherease HMPFK and HPPFK still showed activity at 3mM ATP. Another difference is shown in citrate inhibition. The rabbit muscle homotetramers are inhibited most easily by citrate, with RPPFK being less sensitive while RLPFK requires the most citrate for inhibition giving the order RL>RP>RM. For the human tetramers this order is HP>HL>HM. AMP activation was qualitatively but not quantitatively similar between homotetramers, with L and M PFK requiring the same amount (but less than required for the platelet type) of AMP to achieve activation. The different findings for the kinetic activities of the human and rabbit homotetramers are difficult to explain. First, differences in the methods of purification might have caused one or more preparations to be 'purer' or more denatured than another. Foe and Kemp (1985) noted that either HPPFK may not have fully reassociated during the final stage of purification or that some other intrinsic property of the enzyme would explain that the specific activity of HPPFK was only 70% that of HMPFK with their assay at pH 8.0. Secondly, enzyme assays were done using aldolase, glycerophosphate dehydrogenase, triose phosphate isomerase and NADH but assay conditions were not identical. Thirdly, there may be some real differences between isoenzymes of the two species. Cross species identity and similarity between isoenzymes is ~98%. Similarity and 96% identity between HMPFK and RMPFK and 96% similarity and 93% identity between HLPFK and MLPFK. These small differences may be enough to account for the different observations of the two groups. Vora *et al.* (1985b) reported different inhibition and activation characteristics of rat PFK homo and heterotetramers compared to the other two groups. A better comparison between homotetramers of different species might be made using cloned genes with the same expression system and the same enzyme

assay conditions.

1.5.1.4 Developmental changes of isoenzymes

Dunaway & Kasten (1989) have examined the subunit compositions of PFK isoenzyme pools in foetal, neonatal and postnatal rat heart and skeletal muscle. Whereas adult rat muscle contains only muscle type PFK subunits, foetal muscle isoenzyme pools comprise 60% L type, 36% M type and 4% P type. Adult heart contains 75% M type but foetal heart contains a roughly equal proportion of all three types of subunit. Presumably, heart in this study refers to heart atria. Adult human heart ventricle PFK is about 92% M type whereas adult human atria contains about 77% M type (Dunaway *et al.*, 1988).

The total activity of PFK in foetal muscle is only 4% of that in adult muscle. Foetal heart has about 33% of adult heart PFK activity. The presence of P type subunit imposes a decreased ability for F26BP to promote PFK activity at low levels of F6P and this effect appears to be directly related to the proportion of P type subunit present in an isoenzyme pool.

The low levels of PFK activity in prenatal heart and muscle may increase chances of survival by keeping glycolytic flux low and preventing a build up of lactate. In adult heart and muscle, the increased levels of M type subunit will allow for effective utilisation of glucose due to the higher affinity for F6P and tolerance of high levels of ATP shown by this subunit. As the levels of M type subunit rise, isoenzymes require less F26BP to increase enzyme activity. This may provide an explanation for the low levels of F26BP seen in adult muscle (Uyeda *et al.*, 1981; Hue *et al.*, 1982)

1.5.1.5 Overview of regulation of PFK *in vivo*

Glycolysis is a virtually ubiquitous pathway which usually

consists of ten main enzymes and also has a regulatory enzyme, PFK-2/F26BP'ase. In addition to its catabolic role, it serves an anabolic function by providing C_3 precursors for the synthesis of fatty acids, cholesterol and amino acids and is therefore an amphibolic pathway. In mammalian tissues, the orientation of glycolytic flux (glycolysis or gluconeogenesis) is determined by tissue type and the hormonal and nutritional state of the tissue (Dunaway & Weber, 1974b). It has been an assumption that control of a pathway is located at those enzymes (preferably allosteric) which catalyse reactions far from equilibrium (Newsholme & Start, 1973). Evidence supporting the key role of PFK is based on changes in the concentration of allosteric effectors of PFK when glycolysis is stimulated (Ramaiah, 1974). More quantitative analysis of control of enzyme flux has been developed by Kacser & Burns (1973) and by Heinrich & Rapoport (1974) which allow control coefficients to be distibruted to each step in a pathway.

Application of control analysis to glycolysis in erythrocytes (Rapoport *et al.,* 1976), yeast (Fell, 1984) and a rat liver extract (Torres *et al.,* 1986), indicate that control is mostly distributed between HK and PFK. The most potent activator of PFK is F26BP which is also a strong inhibitor of F16BP'ase. F26BP is present in all mammalian tissues examined (Van Schaftingen, 1987) and it is likely that it plays a pivotal role in the control of glycolytic flux in a variety of these tissues.

F26BP cannot be regarded as the only regulator of flux in all tissues, but it is likely to be the major control element for switching between glycolysis and gluconeogenesis in the liver and so for the control of provision of lipogenesis. The affinity of liver PFK for F26BP is at least one hundred fold greater than for F16BP but only tenfold greater in muscle. F16BP is likely to have a greater stimulatory effect than F26BP when it

exceeds F26BP concentrations e.g. in contracting muscle (Uyeda *et al.*, 1981). Muscle rich isoenzymes are activated by lower levels of F26BP however and also are less inhibited by ATP than liver or platelet rich forms (Dunaway & Kasten, 1989). (See section 1.5.1.4).

Phosphorylation of PFK-1, PFK-2/F26BP'ase, F16BP'ase and PK by cAMP-dependant protein kinase is under the control of short and long term hormonal changes and these changes can be tissue specific. Hormones can also exert tissue specific control over glycolytic flux by affecting the synthesis and degradation of specific glycolytic enzymes.

The regulated import and degradation of F16BP'ase into the yeast vacuole has recently been described (Chiang & Schekman,1991). The loss of F16BP'ase to the vacuole is dependent on glucose. Cells transformed with a multicopy plasmid containing the F16BP'ase gene (expression elevated twenty fold) were transferred during logarithmic growth from a medium containing ethanol as a carbon source, in which F16BP'ase has a half life of ninety hours, to glucose medium, in which F16BP'ase has a half life of thirty minutes. After three hours in glucose medium, F16BP'ase activity was reduced by ninety percent.

Cell fractionation and immunofluorescence studies indicate that F16BP'ase becomes localised to the vacuole fraction after growth on glucose. This may be a way in which the yeast cell prevents the futile cycle of glucose anabolism and catabolism when glucose is available as a carbon source (Chiang & Schekman, 1991).

Apparently direct import of cytosolic proteins into the lysosome have been reported. A large family of cytosolic proteins that bear a Lys-Phe-Glu-Arg-Gln (KFERQ) localising signal are degraded in the lysosome when mammalian cells are deprived of serum (Lamponi *et al.*, 1987; Chiang &

Dice, 1988). The yeast F16BP'ase has two KFERQ like motifs, EKVKQ and QKKLQ (Dice, 1990).

Yeast PFK1 contains two KFERQ like sequences; KFPKQ at position 132 with 80% similarity and 60% identity; KFDTH at position 910 with 80% similarity and 40% identity. Yeast PFK2 contains QLERA at position 700 with 60% similarity and 40% identity.A definition of similarity based on the programs of the GCG computing package is found in section 2.2.15. It seems reasonable to speculate that two different degradatory pathways could function to prevent futile cycles in glycolysis or other systems with one signal, e.g. glucose. This signal could cause degradation or compartmentalisation of an anabolic glycolytic enzyme while its absence could have the same effect on a catabolic enzyme.

A search of the sequences for PFK to find a KFERQ like sequence shows that all sequences have a sequence with 40% identity and 60% similarity to KFERQ. It could be argued however that this is not sufficiently similar to justify the argument for PFK sequences to be involved in the "KFERQ" transport system. RMPFK and SHPFK have sequences which show 80% similarity but only 40% identity. It would be necessary to examine the localisation of PFK during gluconeogenesis to see if such a situation exists in reality.

Another possible control over PFK could result from anomeric specificity. PFK and F16BP'ase act on the β anomer of F6P and the α anomer of F16BP, and are activated by the α anomer of F16BP and the β anomer of F6P respectively. Koerner *et al.* (1977), suggest that PFK and F16BP'ase are allosterically activated by the substrate, and competitively inhibited by the product of the other, and that this could suppress futile cycling between the two enzymes.

1.6 SPECIFIC RESIDUES CONTRIBUTING TO ACTIVITY

The crystal structures of the PFK's from *B. stearothermophilus* (T + R states; Schirmer & Evans, 1990) and *E. coli* (Rypniewski & Evans, 1989) have been determined. Site-directed mutagenesis can be used to examine the role of specific residues necessary for the observed actions of the enzyme. The eukaryotic enzymes arose from a duplication of the prokaryotic gene and are regulated by a wider number of effectors. Some catalytic and allosteric sites in the eukaryotic enzyme are thought to have changed to provide for new functions for the enzyme (Poorman *et al.,* 1984).

1.6.1 Crystallographic studies of bacterial PFK

ECPFK and BSPFK have been crystallised in their high (R) and low (T) affinity states and their structures have been determined by X-ray crystallography to resolutions from 2.4Å to 7Å. (Evans *et al.,* 1986; Rypniewski & Evans, 1989; Evans & Hudson, 1979; Evans *et al.,* 1981; Shirakihara & Evans, 1988; Schirmer & Evans, 1990). Site directed mutagenesis has also been used to investigate key residues in the active site of the *E. coli* enzyme (Hellinga & Evans, 1987; Lau & Fersht, 1989; Berger & Evans, 1990; Lau *et al.,* 1987; Kundrot & Evans, 1991). The subunit of bacterial PFK is divided into two domains. Each domain contains a core of β strands surrounded by α helices.

The active BSPFK is a tetramer of identical subunits of 319 amino acids each. Dimers are formed by two subunits packing together at a large interface and dimers pack together at a small interface to form the tetramer. The large interface within the dimer contains residues which define the allosteric site, which binds the activator and the inhibitor. In the R state the small interface between the dimers contributes residues which bind the 6-phosphate group of a F6P bound in the other dimer. The dimer-dimer

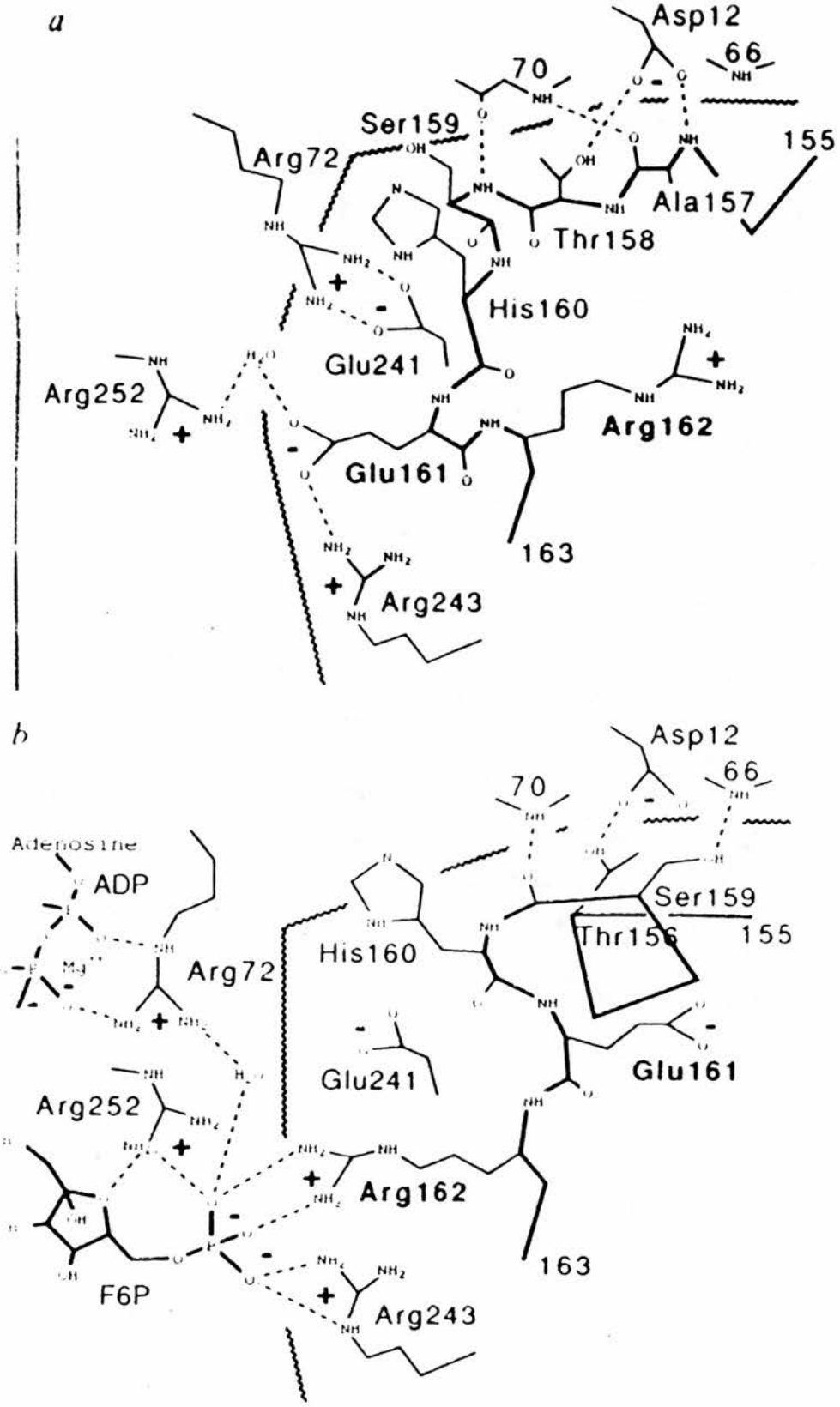

Figure 3.

The catalytic site in a) the T state

and b) the R state of *B. stearothermophilus*

Taken from Schirmer & Evans (1990)

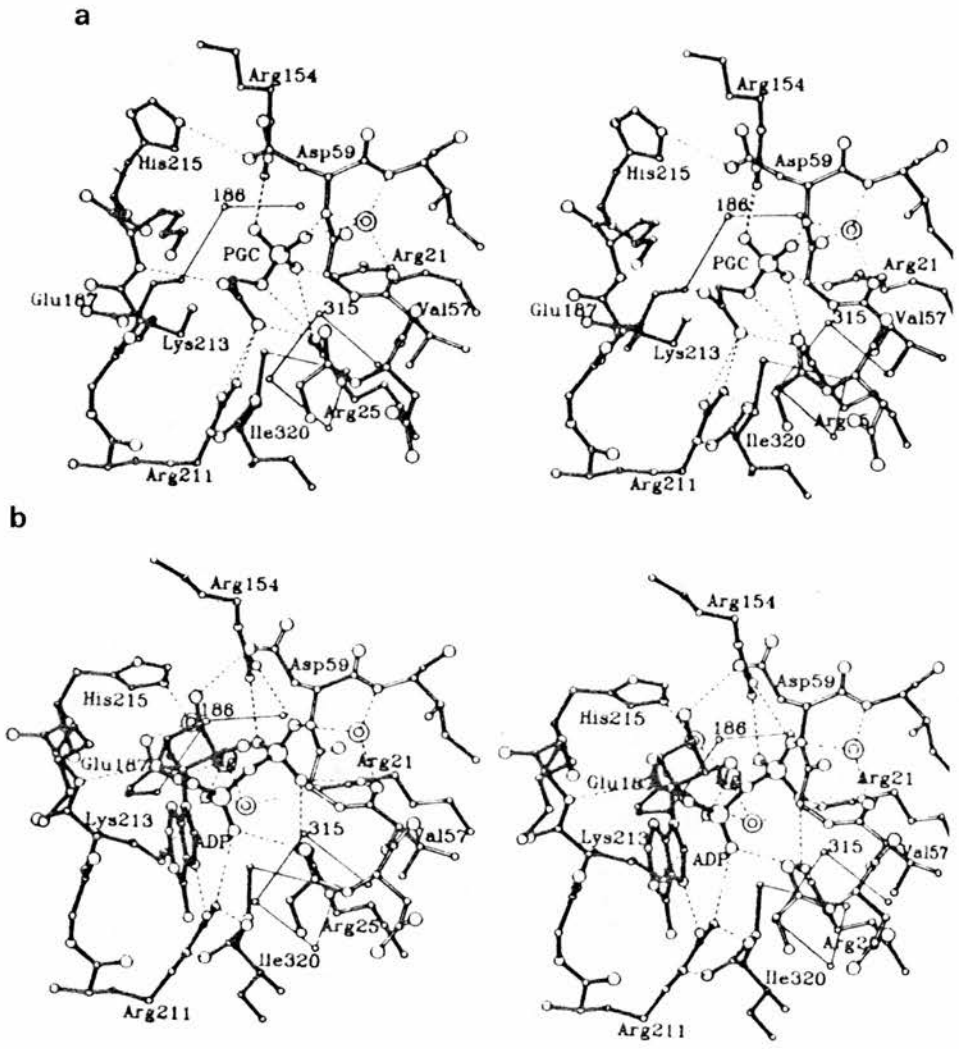

Figure 4.

Stereo view of the effector site of *B. stearothermophilus.*

a) the T state with the PEP analogue 2-phosphoglycolate bound

b) the R state with ADP bound

interface is altered in the transition to the T state by a rotation of about 7 degrees. The significant structural change associated with this rotation is a rearrangement of loops 6-F and 8-H, and of helices 8 and 9.

Figure 3 shows the active site of *B. stearothermophilus* PFK in the R and T states, with F6P and ADP bound at the catalytic site

Figure 4a shows a stereo view of the effector site.The PEP analogue phosphoglycolate is bound. Figure 4b shows the same stereo view of the effector site with ADP bound.In the eukaryotic enzyme these sites are presumed to have mutated to form novel effector sites.The reorganisation of arginines 72 and 162 appears to be theunderlying mechanism for the low F6P affinity seen in the T state

1.6.2 The Active Site.

The binding site for the cooperative substrate F6P consists of residues from both subunits of one dimer and also from the other dimer. The binding site from the other dimer is altered by the change in the 6-F loop. Arg-162 and Arg-243 give a major part of the binding energy for F6P across the subunit interface. This has been demonstrated by mutation of these sites; R162S and R243S (Berger & Evans, 1990). The Km for the binding of F6P for the mutants R162S and R243S were increased by 165 fold and 53 fold, respectively. On reorganisation of the 6-F loop during the R to T state conversion, Arg 162 swings around to bring its guanidinium group close to the main chain end of helix 8. Glu-161 adopts the R state position of Arg-162 and forms a salt bridge with Arg-243 while its carboxylate group overlaps with the old guanidinium position. In this way, the positive charges of Arg-162 and Arg-243 cannot bind the doubly negative charged 6-phosphate of F6P. *E.coli* and the mammalian amino half domains have Gln at position

161 and the charge change is smaller. The sequence of the mammalian carboxyl halves have an Arg at positions 161 and 162 except for the sheep liver sequence which has a Gln at position 161.

The catalytic binding site is composed almost entirely of residues from the large domain. In the change from the R to the T state, Arg 72 forms a salt bridge with Glu 241 and cannot interact with ATP or F6P. In the R state, Arg 72 contacts the beta and gamma phosphates of ATP and also forms a bond via a water molecule to the 6-phosphate of F6P. After transfer of the gamma phosphate of ATP to F6P to form F1,6BP, Arg 72 interacts with the 1-phosphate of F1,6BP. The mutation R72S reduces the catalytic activity 33 fold and also reduces the cooperativity and binding of F6P and F1,6BP (Berger & Evans, 1990). These observations suggest that Arg 72, Arg 243 and Arg 162 perform a critical role in the communication of cooperative and allosteric signals between subunits, particularly Arg-162 and Glu-161 which show the largest movement in the interconversion between the R and the T states.

Asp 127 appears to be the key catalytic residue for the active site (Hellinga & Evans, 1987) and forms hydrogen bonds to the 1-OH group of F6P thus increasing its nucleophilicity for the gamma phosphate of ATP. Asp 127 is conserved in the amino halves of all the eukaryotic enzymes and in all four known prokaryotic PFK sequences. In eukaryotic carboxyl halves however, residue 127 is a serine which may be part of the sugar bisphosphate effector site. Asp-127 is likely to cause electrostatic repulsion of F1,6BP after its formation in the active site, and the removal of the negative charge by replacement with serine makes the the binding of a sugar bisphosphate more favourable.

1.6.2 The Effector Site.

The effector site lies in a deep cleft formed by the residues between the large interface of the dimers and binds both activator ADP and inhibitor PEP. The binding of an effector molecule seems to change the packing of the site itself and the position of the 8H-loop (residues 213-215). The positioning of the 8H-loop is dependent on the bound effector molecule and this movement is somehow coupled to the arrangement of the 6F-loop (residues 155-162) which is responsible for the replacement of Arg 162 with Glu 161 at the active site. The difference in the movement of the 8H-loop and the 6F-loop is presumably a function of the difference in size between ADP and PEP. ADP is larger than PEP and is not totally buried in the effector cleft, as is PEP. It is presumable that the low affinity site can only be formed when the "lips" of the effector site are closed over PEP which does not occur with ADP. The movement of the dimers in the transition from the R to the T state is accommodated by the removal of a layer of water molecules that lie between the dimers and by the rearrangement of the 6F-loop. Lau & Fersht (1989) have also examined the effector binding site of *E.coli* by site directed mutagenesis. They found essentially the same as Schirmer & Evans (1990) with positively charged arginines contacting the activator Mg^{2+}ADP/GDP and the inhibitor PEP. The interesting mutation E187A caused PEP to act as an activator and GDP to act as an inhibitor. E187Q made both regulators into inhibitors. Glu 187 is known from the crystal structure to bind the coordinated Mg^{2+} ion of ADP/GDP and seems to play an essential role in the allosteric transition. Glu 187 may provide a crucial stabilisation of the R state by binding Mg^{2+}. This residue is conserved as an aspartate in the amino and carboxyl halves of all the mammalian enzymes and may fulfil the same function in the amino half and may possibly bind Mg^{2+}ATP in a carboxyl half

inhibitory site. This remains speculative however, until the crystal structure of a mammalian PFK can be examined.

The DNA for PFK from the thermophilic bacterium *T. thermophilus* has been cloned and sequenced (Xu *et al.,* 1991). While almost all the residues contributing to ligand binding sites have been conserved, some differences at the effector site are seen which cause PEP to effect tetramer-dimer interconversion and so inactivate the enzyme.

1.7. Metabolite binding studies

Metabolite binding studies do not give a precise figure for the number of substrate and effector binding sites. The mammalian PFK enzyme, by virtue of its tetrameric association and its larger size when compared to the prokaryotic enzyme is reported to have a variety of binding sites. Three ATP binding sites, one adenine binding site, one or two F6P binding sites, one citrate binding site and one FBP binding site appears to be the general concensus.

Of the three ATP binding sites, one is the catalytic site, one is the inhbitory site and the other is the same as the AMP binding site (Kemp & Krebs, 1967). They differ in their biological actions and ligand specificities. The inhibitory site has a low affinity for ATP (K_D = 100μM), while the catalytic site has a much higher affinity for ATP (K_D = 1μM) (Wolfman *et al.*, 1978; Pettigrew and Frieden, 1979a).

The adenine binding site from sheep heart PFK was compared to the homologous sequences *B. stearothermophilus* (Weng *et al.*, 1980). The *B. stearothermophilus* sequence contained two arginine residues at position 21 and 25 which bound to the phosphates of allosteric effectors (Evans and Hudson, 1979; Hellinga and Evans, 1985). However, when this tryptic fragment is compared to the RM sequence (Lee *et al.*, 1987) and then

compared to the *B. stearothermophilus* sequence it can be seen that the original comparison of the SH fragment to *B. stearothermophilus* was wrong. The comparable part of the *B. stearothermophilus* and *E. coli* enzyme has not been proposed to make any contact to effectors (Hellinga and Evans, 1985).

Riquelme and Kemp (1980) treated muscle PFK with subtilisin and produced a tetrameric enzyme of Mr 74,000 which was inactive. Binding studies showed that only one of the three adenine binding sites was present and that the ATP and F6P binding sites and the ATP inhibitory sites were lost. The enzyme retained the same affinity for cAMP, and binding sites for F16BP and AMP were also present (Gottschalk *et al.*, 1983). It is likely that a separate catalytic site is present as a discrete domain and that several allosteric regulatory sites are present.

There appear to be low and high affinity sites for F6P in SHPFK with K_D's of 11μM and 0.2μM respectively, which are influenced by effectors. F26BP shows the highest affinity while F16BP has a higher affinity than G16BP (Foe *et al.*, 1983). It has been suggested that the fructose/glucose bisphosphate binding site has evolved from a matched F6P binding site in the carboxyl domain (Poorman *et al.*, 1984). Foe *et al.* (1983) also suggested that the binding of F26BP produces a conformational change in PFK which makes it less inhibitable than the conformation produced by F16BP and G16BP.

The citrate binding site can be modified by using pyridoxal phosphate and sodium borohydride (Colombo *et al.*, 1975). The citrate binding site of rabbit skeletal muscle was sequenced by Kemp and co-workers (1987) and found to match the *E. coli* and *Bacillus* PFK allosteric effector site.

1.8 Syndromes Associated with Inherited Deficiencies of PFK Isoenzymes

Vora (1982) describes 25 cases of inherited PFK deficiency occurring in 20 unrelated families. She classed the reported cases into five major groups. Group I - patients with the classic syndrome as described by Tarui *et al.* (1965) and Layzer *et al.* (1967) with both myopathy and haemolysis. Group II - patients with myopathy. Group III - patients with hemolysis. Group IV - asymptomatic patients with partial deficiency of RBC PFK and Group V - patients with progressive fatal myopathy associated with other atypical features.Some Group III cases may belong to Group I but other Group III cases may be genuine (Etiembie *et al.*, 1976; Miwa *et al.*, 1972).

1.8.1 Deficiency of the Muscle Isoenzyme

Group I patients display the classical syndrome resulting from almost a complete lack of muscle PFK corresponding to only 0.5% of normal activity. Erythrocytes have 29-64% of normal activity. When undergoing an ischaemic exercise test, venous lactate and pyruvate fail to increase which implies a block in glycolysis. An increase in the glycolytic intermediates - hexose monophosphates - and a decrease in F16BP and triose phosphates indicate that this block appears to be at the level of PFK (Tarui, 1967; Layzer *et al.*, 1967).

The accumulation of hexose monophosphates can lead to activation of glycogen synthetase resulting in a moderate deposition of glycogen (Agamonolis *et al.*, 1980). The increased F6P concentrations increase the flux through the pentose phosphate pathway. The increased levels of purines and pyrimidines from 5-phosphoribosyl-pyrophosphate (PRPP) in the pentose phosphate shunt may explain the hyperuricemia seen in these

patients (Howell, 1965; 1968). Patients often lose the ability to generate heat by increasing glycolytic flux to maintain body temperature in the cold (Newsholme, 1976).

The possibility that unstable forms of muscle PFK may exist has been investigated but not proven (Vora, 1982). Alternative splicing has been seen for two transcripts encoding HMPFK. One cDNA was lacking sequence encoding exon IX (Sharma *et al.,* 1990). This would result in a HMPFK lacking 31 amino acid residues. Two of these residues have been predicted to be involved in the binding of F6P at positions Arg 243 and Arg 252. On the basis of the bacterial crystal structures the 31 residues are located between the second half of α-helix 9 and all of α-helix 10 and includes β-sheet I. This would presumably lead to a different conformation of the monomer. A homotetramer of this type of subunit would presumably be non-catalytic but it is possible that this subunit could influence the allosteric properties of heterotetramers. An appropriate mammalian expression system would allow investigation of this possiblity. The deletion removes Arg 252 which is implicated as being central to subunit interfaces (Hellinga & Evans, 1985). The deletion of this residue may mean that a subunit containing this deletion may not be able to associate with other subunits.

Nakajima and co-workers (1990b) reported on the nature of the genetic defect associated with group I glycogenosis/ Tarui's disease in a patient. A point mutation at the 5' end of intron 13 changed the normal splice site of CAG:GTATGG to CAG:TTATGG. This caused a cryptic splice site of ACT:GTGAGG to be recognised and spliced in the patient. A silent base transition at position 516 was also seen, ACT-Thr to ACC-Thr. The 75bp in-frame deletion of the mRNA corresponds to a deletion of 25 amino acid residues in the C-terminal half of the peptide just down from the connecting

peptide. This area is considered to contribute in part to the allosteric sites of ADP/AMP activation and sugar bisphosphate activation (Poorman *et al.,* 1984). This deletion would remove residues from the second half of α-helix 1 and all of β-sheet B and α-helix 2 of the C terminal half. This would presumably result in a disadvantageous conformational change but may not completely inactivate the subunit. There may be other mutations of PFK with Tarui disease as several heterogeneous symptoms are shown with this disease (Nakajima *et al.,* 1990b). As with the alternative splicing noted by Sharma *et al.* (1990), site directed mutagenesis of PFK and an appropriate mammalian expression system would allow the investigation of the nature of the physical defects associated with PFK in Tarui's disease and glycogen storage diseases.

1.8.2 Deficiency of the Liver Isoenzyme

Vora *et al.* (1980) speculated that the heterogeneous group of haemolytic syndromes discussed in 1.8.1 may represent a total absence of L subunits or defects in L subunits. No such evidence has been found.

The locus for HLPFK on chromosome 21 has been mapped to band 21q22.3 (Cox *et al.,* 1984; Van Keuren *et al.,* 1986) in the region known to be involved of the phenotype of Down's syndrome (DS). Analysis of the subunit types in erythrocytes of DS patients shows an increase of up to 40% of the L isoenzyme and an absence of M4 (Vora, 1981). Levanon *et* al. (1989) suggested that if a similar alteration occurs in the brain of DS fetuses, it might affect glycolytic flux and the availability of glucose and ATP. Because brain is heavily dependent upon glucose it had been suggested that such an alteration might be linked to the neuropathological changes and their physiological consequences observed in DS.

Levanon *et al.* (1989) observed that LPFK mRNA was present at a relatively high level in normal foetal brain which diminished in adult brain. DS fetal brain showed even higher levels of LPFK mRNA

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1.8.3 Deficiency of the Platelet Isoenzyme

No work has apparently been done on the possible results of a deficiency of the platelet isoenzyme. Dunaway *et al.* (1988) examined the distribution of human isoenzymes. Platelet subunits are most common in fibroblast, platelet and cerebral cortex having 61.2%, 31.7% and 27.4% respectively as determined by Western blotting. They suggest that enhanced sensitivity to inhibitory concentrations of ATP is intrinsic to the platelet subunit. Platelet-type subunit may exert a dampening effect on glycolysis and may also allow other tissues which are relatively more important for immediate survival to compete more effectively for glucose (Dunaway *et al.,* 1988).

HPPFK has previously been assigned to chromosome 10pter-p11.1 (Vora *et al.,* 1983). About 40 cases of 10p duplication on chromosome 10 have been reported. The phenotype represents a relatively specific syndrome (Gonzalez *et al.*, 1983), with severe mental, developmental and growth retardation; characteristic facies; congenital heart disease; CNS, ocular, renal, genital and skeletal abnormalities; congenital hip dysplasia, flexion deformity of hands and fingers and clubfoot.

1.9 Enzymic Evolution

The observation of Poorman *et al.* (1984) that rabbit muscle PFK appeared to be a duplication of the bacterial enzyme raises two problems. It is necessary to understand the molecular basis of this process and also how mutation can affect the duplicated gene.

Most amino acid replacements are the result of single base substitutions. If the mutation is 'conservative' then it is likely not to affect the protein to any great degree. Amino acids can be grouped into such 'conservative' changes. A change such as isoleucine --> valine which could be caused by ATT-->GTT, ATC-->GTC or ATA-->GTA only replaces an aliphatic-hydrophobic amino acid with another. A change in the charge of a residue would be caused by about one third of all point mutations which would be less likely to be tolerated by the protein.

Changes in the amino acid sequence between homologous enzymes or isoenzymes may be tolerated more or less depending on their position in the structure. Catalytic residue changes are much less likely to be tolerated than those on the outside of a protein. Dickerson (1971) and Kimura and Ohta (1973) have shown that these surface residues of mammalian haemoglobin are experiencing ten times more evolutionary change than those in the haem pocket.

If an enzyme is only present as a single copy gene, then such replacements would tend to be conservative at the least, as the scope for change would be severely limited by the need to conserve the structural and catalytic properties of the enzyme. If the gene exists at more than one locus however, or if as seems to be the case with eukaryotic PFK, it has doubled in size then this selective pressure is relaxed. One of the loci or one domain of the duplication event, should be available to allow a much more rapid evolution of the enzyme in a variety of ways. This seems to be the case with mammalian PFK where the amino terminal domain retains much of the original activity of the ancestral protein while the carboxyl domain has evolved to create new effector sites and inter-domain binding sites. The existence of isoenzymic PFK has allowed different tissues to express those

isoenzymes which fulfill the characteristics best suited to the tissue concerned.

1.9.1 Evolution of Glycolysis

The 10 glycolytic enzymes are of central metabolic importance and are virtually ubiquitous. A large number of them have been sequenced and all have known crystal structures (reviewed by Fothergill-Gilmore 1986). Glycolysis is therefore a suitable enzyme system to study in order to understand the evolution of enzymes and pathways.

There are three main theories put forward to describe the evolution of proteins, by divergence.

(1) Those enzymes with a requirement for binding similar ligands such as nucleotides have evolved from a common ancestor. They might also of course have incorporated a domain from such an ancestral enzyme such as the typical NAD^+ binding domain present in dehydrogenases (Rao & Rossmann 1973; Rossmann *et al.*, 1975).

(2) Enzymes catalysing similar reactions eg. kinases, mutases arose as independent groups which have gradually specialised by divergence.

(3) Enzymes which bind the substrates and products have evolved by gene duplication and subsequently diverged to form a pathway of related enzymes.

One of the most striking and elegant domain structures is the $\alpha\beta$ barrel found in TIM and PK. It is also seen in at least 14 other enzymes. A comparison of the amino acid sequences shows no obvious similarity and it appears that $\alpha\beta$ barrels have occurred by convergent evolution. Similarly the mononucleotide binding fold (mnbf) is not proposed to have appeared by divergent evolution on account of the lack of amino acid sequence

similarity. A good example of divergence is that of diphosphoglycerate mutase from phosphoglycerate mutase. They can be considered as isoenzymes although the three reactions they catalyse are at very different rates. They catalyse reactions involving the phospho groups among the carbon atoms of phosphoglycerate (Rose, 1980; Leadlay *et al.,* 1977). The mutase reaction, in which glycerate 3-phosphate goes to glycerate 2-phosphate (reaction 1) is the major reaction catalysed by phosphoglycerate mutase. The cofactor dependant phosphoglycerate mutase can also act as a synthase transferring a phospho group from 1,3BPG to form 2,3BPG (reaction 2), and as a phosphatase, catalysing the removal of a phospho group from 2,3BPG to form 2PG or 3PG (reaction 3). These reactions are at lower rate than the mutase reaction however.

BPGM catalyses reaction 2 at the highest rate and is relatively efficient at catalysing reactions 1 and 3. The BPGM and PGM are about 50% identical in primary structure (Fothergill-Gilmore & Watson, 1989). The different mutases can associate to form heterodimers indicating that they are very similar in tertiary structure and are so an example of divergent evolution.

Similarities are also found between PGM and PFK-2/FBP-2. Residues 250-471 of human liver PFK-2/FBP-2 (Lange & Pilkis, 1990) show 46% similarity and 24% identity to human muscle PGM (Shanske *et al.,* 1987; Tauler *et al.,* 1987). This part of PFK-2/FBP-2 contains the active site histidine of the phosphatase domain of the enzyme and appears to have diverged from an ancestral PGM and undergone a gene fusion with a kinase.

It appears that the glycolytic enzyme pathway has arisen by convergent evolution and random association of enzymes and subsequently

evolved by gene duplication. Gene duplication gives rise to isoenzymes with differing properties and this allows the different metabolic requirments of a variety of tissues to be met.

A striking property of the glycolytic enzymes is their conservation throughout evolution. They are amongst the most conserved enzymes known and are changing at a much slower rate than, for example, the globins (Dayhoff, 1978). With the exception of ADH, which shows an increase and aldolase, which shows a decrease in mutation rates, the glycolytic enzymes are evolving at about the same rate as cytochrome C.

1.9.2 Evolution of PFK

Poorman *et al.* (1984) noted similarities in the amino acid sequences between the amino and carboxyl halves of the 85kDa RM enzyme and the 33 kDa BS enzyme. They suggested that the mammalian enzymes could be considered as two bacterial monomers joined by a connecting peptide. Since then, sequences have been reported for *E. coli*, mouse liver, human muscle, human liver and *S. cerevisiae*, which support the theory of gene duplication. The reported sequences of the two yeast PFK subunits (Heinisch *et al.,* 1989) gives more information on the evolution of PFK. It is proposed that the duplication of the bacterial enzyme occurred in an ancient organism before the split between yeast and the rest of the eukaryotes. A much later duplication event occurred giving rise to the two yeast subunits (Heinisch *et al.,* 1989) The subunits PFK1 and PFK2 of yeast are present on two different chromosomes (Heinisch, *et al.,* 1989) and encode two subunits essential for a functional PFK (Kopperschlager *et al.*, 1977).

Examination of the intron-exon gene structures of HLPFK, HMPFK and RMPFK does not reveal any internal symmetry between the amino and carboxyl halves of the individual enzymes. The amino and carboxyl halves

of the molecule are similar in size but are encoded by different numbers of exons, twelve for the amino half and ten for the carboxyl half.Intron lengths vary while exon lengths remain the same The rabbit muscle and human muscle genes are 40% smaller than the human liver gene but have the same number of exons and introns. This lack of symmetry does not support the idea of a gene duplication and subsequent fusion, but may indicate that the time elapsed since duplicationoccurred has obliterated any evidence of such an event.

1.10 Aims of the project

Onchocerca volvulus is a filarial nematode which infects humans and causes the disease onchocerciasis commonly referred to as river blindness. It is estimated to affect eighteen million people in Africa, and Central and Southern America (WHO, 1987). The vector of the parasite is the blackfly *Simulium damnosum* which transmits infective juvenile forms into the host during a blood meal. These juvenile forms mature to become adult filarioids which migrate to subcutaneous or intramuscular tissues. The adults form nodules in which they mate and the female produces microfilariae which migrate to the circulatory system to be picked up by a feeding blackfly in which they develop into infective juvenile forms to complete the cycle. It is the microfilariae which cause the serious clinical manifestations of onchocerciasis by causing keratitis of the sclerosa.

The overall aim of this project was to obtain detailed structural information on phosphofructokinase from humans and *O. volvulus.* Glycolysis is required for metabolism by the parasite (Barrett, 1983). The inhibition of PFK would provide a potentially effective way of shutting down the glycolytic pathway. If small but important differences existed between the structures of the enzymes between human and *O. volvulus,* then nematode-

specific PFK inhibitors could be designed. Because of the very limited amounts of parasite source material, it was decided that the best way of obtaining the primary sequence of *O. volvulus* PFK would be by means of screening cDNA libraries. Ultimately, the quality of available libraries and the unexpected nature of the parasite's DNA codon bias meant that this was not achieved. Another aim of the project was to characterise PFK from humans. During the project, DNA sequences have been reported for the human muscle and liver isoenzymes. This study has provided a partial sequence of platelet PFK, the third and probably the only other human isoenzyme.

CHAPTER TWO

EXPERIMENTAL

2.1 Materials

2.1.1 E. coli strains.

BHB2688. (N205 recA [limm434 clts b2 red E am S am/l])

BHB2690 (N205 recA [limm434 clts b2 red D am S am/l])

C600hfl (F- thi-1 thr1 leuB6 lacY1 tonA21 supE44 hfl)

DF1020 (D15 (rha-pfkA) DpfkB recA56 HfrC lrelA1 spol pit-10 tonA22 T2r)

ED8654 (supE supF hsdR metB lacY gal trpR)

HB101 (supE44 hsdS20 (rB-mB-) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1

NM531 (supE supF hsdR trpRlacY recA13 metB gal)

TG1 (supE hsdD5 thi D(lac-proAB) F'[traD36 proAB+laclq lacZ D15])

Y1090 (DlacU169 D(lon, araD)139 strA supF (trpC :: Tn10) (pMC9))

2.1.2.1 λ Vectors

EMBL3 (sbh I l1 b189 <polycloning site int29 ninL44 trpE polycloning site> KH54 chiC srl l4O nin5 srl l5O)

gt10 lsrl l1Ob527 srl l3O imm434 (sr134+) srl l4O srl l5O

gt11 llac5 Dshn dIII l2-3 sr l3O clts 857 srl l4O nin5 srll5O Sam100

2.1.2.2 Plasmid vectors

	Source
pK19	Dr. A. Boyd, Edinburgh.
pKS+	Stratagene LTD. Cambridge, U.K.
M13mp8	Amersham International, Amersham, U.K.

M13mp18 and mp19 Bethesda Research, Cambridge, U.K.

2.1.3 Growth media

a) Oxoid LTD, Haverhill, Suffolk. Tryptone, agar No. 1, yeast extract.

b) Difco Laboratories, East Mosely, Suffolk. Bactopeptone.

c) Sigma Chemical Company, Poolem, Dorset. Ampicillin, Gentamycin, Kanamycin, Tetracycline, X-gal, IPTG.

2.1.4 Radiochemicals

Amersham International, Amersham, Buckinghamshire.

Deoxyadenosine 5'-[a-32P]triphosphate, triethylammonium salt, stabilised aqueous solution, 3000Ci/mmol

Deoxyadenosine 5'-[a-35S]thiotriphosphate, triethylammonium salt, stabilised aqueous solution, >400Ci/mmol

ICN LTD, High Wyckombe, Buckinghamshire.

Deoxyadenosine 5'-[-32P]triphosphate, triethylammonium salt, stabilised aqueous solution, >5000Ci/mmol

2.1.5 Enzymes

Sigma. Collagenase.

Northumbria Biologicals LTD, Cramlington, Northumberland.

AvaI, BamHI, BglII, ClaI, EcoRI, HinDIII, KpnI, PstI, SacI, SalI, SmaI, TaqI, XmaI. T4 DNA ligase, T4 DNA polymerase, Klenow enzyme, Taq polymerase

Boehringer Manheim LTD, Lewes, Sussex. RNA'se A, Proteinase K.

2.1.6 Oligonucleotides

OSWEL DNA service, Chemistry Department, University of Edinburgh.

2.1.7 Miscellaneous

a) Amersham; Hybond filters (discs and rolls), Hyperfilm MP X-rayfilm

Sigma; Polaroid 665 and 667 film.

c) Pharmacia; Hexadeoxyribonucleotides, dNTP's.

d) Cambridge Bioscience, Cambridge; Sequenase and sequencing kits.

e) BIORAD LTD, Hemel Hempstead, Berkshire; Goat anti-rabbit IgG(H+L) Alkaline Phosphatase Conjugate

All other chemicals were supplied by Sigma or B.D.H., Poole, Dorset, and were reagent grade or better.

2.2 METHODS

Good laboratory practice was observed at all times. Where work involved the growth of micro-organisms or manipulation of parasite tissue, sterile technique and equipment were used. Plastic ware was incinerated after use and glass and ceramic ware were autoclaved or soaked in hypochlorite bleach prior to washing. Guidelines for the use of radioactive isotopes and other departmental guidelines were strictly adhered to.

Standard experimental techniques were performed as described in Methods In Enzymology (1988) vol. 152, A Guide to Molecular Cloning Techniques.

	Pages
Enzymic manipulation of DNA	91-144
Growth and storage of E. coli	145-151

Extensive use was also made of Maniatis *et al.,* (1982) and Sambrook *et al.,* (1989), the first and second editions respectively of Molecular Cloning: A laboratory manual.

2.2.1 DNA Sequencing

Two methods of DNA sequencing were used. One used Klenow enzyme. The other used a modified form of T7 DNA Polymerase (Sequenase').

Transformation of E.coli.

For a 90mm plate.

1 Add up to 100ng of recombinant M13 DNA to 100µl of competent E.coli containing the F plasmid such as TG1.

2 Incubate on ice for at least 30 minutes, heat shock at 42°C for 3 minutes and put back on ice.

3 Add 40µl each of X-Gal and IPTG and 300µl of fresh TG1 cells. These should be grown first thing in the morning by adding 100µl of an overnight culture to 10ml of fresh broth. These cells form a lawn on the plate and allow the plaques to be seen more easily

4 Heat the tube up to 37°C, add 3ml of top agar (for a 90mm plate) and pour the mixture onto a prewarmed 37°C bottom agar plate.

5 Allow to set and incubate overnight at 37°C.

6 Recombinant plaques will be clear while wild type plaques will be blue.

7 Pick recombinant plaques by spearing with a sterile toothpick. Put the toothpick into a test tube containing 1.5ml of 2XTY. The TY contains a 1:100 dilution of an overnight culture of TG1.

8 Grow at 37°C for 5 hours shaking hard.

9 Spin down the cells at 12,000 r.p.m. for 5 minutes and remove the supernatant to a fresh eppendorf being careful not to pick up any cells.

10 Add 200µl of PEG/NaCl and mix. Leave for 15 minutes.

11 Spin at 12,000 r.p.m. for 2 minutes and remove the supernatant. Spin again and remove the last of the supernatant with a drawn out pasteur

pipette.

12 Add 100µl of TE and then 50µl of phenol.Vortex for 15 seconds and leave for 15 minutes. Vortex for 15 seconds and then spin at 12,000 r.p.m. for 3 minutes.

13 Remove the top aqueous layer to a fresh eppendorf and add 10µl of 3M Na acetate. Add 250µl of ethanol (kept at -20°C). Incubate at -20°C for at least 1 hour and spin at 12,000 r.p.m. for 10 minutes. A pellet should be visible.

14 Redissolve the DNA in 50µl of TE.

Sequencing the DNA

For Klenow enzyme,

single stranded template 5µl

M13 primer @ 1µM 1µl

Klenow reaction buffer 1.5µl

Distilled water 2.5µl

Incubate at 60°C for 1 hour.

Spin the annealed primer/template and add 2µl of [^{35}S]dATPαS (600Ci/mmol)

3 Add 1µl of Klenow fragment (at 1 unit/µl) and mix by pipetting. This is the reaction mix

4 To 4 eppendorf tubes marked A,T,G and C add 2.5µl of the reaction mix.

5 Add 2µl of the relevant dNTP/ddNTP mix to each tube. Place the drop just inside the tube. When all tubes are ready, start the reaction by spinning the tubes which mixes the two solutions.

6 Incubate for 20 minutes at 37°C. Before this time is up add 2µl of the chase

solution just inside the rim of the eppendorf to each tube. After 15 minutes, spin the tubes to mix and incubate for 15 minutes at 37°C.

7 Add 4µl of stop solution/dye to the rim of the eppendorf and spin to stop the reaction at 15 minutes. The samples can be left overnight at -20°C.

8 Heat the reaction tubes to 95°C for 5 minutes and use 3µl of each reaction to load onto the gel.

For Sequenase (Sequenase Protocol Handbook, 1989)

DNA	7µl
Sequencing buffer	2µl
Primer @ 1µM	1µl

Anneal at 65oC for 1 hour

To this mixture add;

DTT 0.1M	1µl
diluted labelling mix	2µl
[a-35S]dATP	0.5µl

3 Mix by pipetting and incubate for 5-10 minutes at room temperature. This is the reaction mix.

4 Put 2.5µl of the relevant termination mix into tubes labelled A,C,G or T. Prewarm the tubes to 37°C. Add 3.5µl of the reaction mix to each tube and spin to start the reaction.

5 Incubate for 5 minutes at 37°C or longer.

6 Add 4µl of stop solution to each tube and store at -20°C.

7 When ready to load the gel, heat the reaction tubes to 95°C for 5 minutes.

Double stranded sequencing

Double stranded sequencing was performed essentially according to the method of Sambrook et al. (1989)

Running the gel

All sequencing reactions were run on a BRL SO gel system.

1 Mix 100ml of acrylamide stock with 200µl of fresh 10% ammonium persulphate and 200µl of TEMED.

2 Pour slowly (avoiding bubbles) into the taped-up glass plates and put in two sharkstooth combs to form a flat surface on the top of the gel. Use two fold-back spring clips to hold the gel at the top. The gel should set after 30 minutes.

3 Untape the gel plates and put it into the gel apparatus. Turn the combs to form sample wells and flush urea out of the wells using a syringe and TBE. Fill the apparatus with TBE. Prerun the gel at 60W for at least 30 minutes. Incubate the samples for two to three minutes at 75-95°C

4 Load the heated samples into the wells. This should be done reasonably quickly. If loading a large number of samples, the gel can be run for 5 minutes to stop any spreading of the samples before loading more.

5 Run the gel at a constant 60W. The bromophenol blue (lower dye) runs at the same position as the first few bases and comes off in about 90 minutes. Gel runs can be varied to see areas further up the gel by loading one sample, running the gel for 2 hours and then loading another 4µl of the same sample and running for another 90 minutes.

6 Take care when dismantling the gel. The lower buffer contains unincorporated ^{35}S. Dismantle the plates, fix for fifteen minutes in 10% acetic acid and drain. The gel is transferred to 3MM paper by inverting the plate and dried on an ATTO gel drier for 1 hour at 75°C.

7 Put the dried gel on 3MM paper into a film cassete with an autoradiography film (Amersham Hyperfilm) and expose overnight at room temperature.

2.2.2 **Maxiprep of plasmid DNA** (Sambrook *et al.*, 1989)

1 Infect 10ml of liquid broth, containing antibiotic if necessary, with a single bacterial colony bearing the required plasmid, and grow overnight.

2 Infect 500ml of broth with antibiotic if necessary with the overnight culture and grow to saturation overnight.

3 Harvest the cells in a Beckman JA14 rotor (or equivalent) at 4,000 r.p.m. for 5 minutes.

4 Resuspend the cells in 8ml of Solution 1 and leave at room temperature for 5 minutes.

5 Transfer the cells to tubes for a Beckman JA20 rotor (or equivalent) and add 16ml of Solution 2. Leave on ice for five minutes with occasional mixing

6 Add 12ml Solution 3 (kept on ice) and mix gently by inversion. Leave on ice for at least 5 minutes and spin in a Beckman JA20 rotor (or equivalent) at 15,000 r.p.m. for 10 minutes. If any precipitate remains, spin again.

7 Transfer the supernatant to a fresh JA20 tube and add an equal volume of propan-2-ol (kept at -20°C). Mix well and store at -20°C for at least ten minutes.

8 Spin as in 6 for ten minutes.

9 Carefully remove the supernatant and wash the pellet in 80% ethanol (kept at -20°C). Dry the pellet in a dessicator.

10 Resuspend the pellet in 4ml of TE

11 Dissolve 4g of CsCl in the sample and add to a tube for a Beckman 70Ti rotor (or equivalent). Add 800µl of ethidium bromide at 10mg/ml and top up the tube with TE containing 1g of CsCl/ml of TE.

12 Seal the tube with a Beckman heat sealer and mix the sample thoroughly. Spin at 45,000 r.p.m. in a Beckman 70Ti rotor for 36 hours. The rotor should be stopped by allowing to coast without the brake after braking

down to ~1000r.p.m.

13 Carefully remove the tube and examine it. A plasmid band is often visible in normal light but may have to be visualised with long wave U.V. transilluminator. The plasmid band is extracted with a 21 gauge needle by first piercing the top of the tube and then removing the plasmid DNA by side puncture. The upper chromosomal band is often not present.

14 Transfer the plasmid band (in about 3ml) to a small test tube and add an equal amount of CsCl saturated propan-2-ol. Mix and remove the upper layer. Repeat until all the ethidium bromide has gone.

15 Transfer the bottom layer to eppendorfs and flush the sample test tube with 2 volumes of water. Add to the sample.

16 Add 1/20th volume of 7.5M NH4 acetate and 1 volume of propan-2-ol (kept at -20°C). Mix and store at -20°C for 30 minutes.

17 Spin at 12,000 r.p.m. for 10 minutes, wash the pellet in 80% ethanol (kept at -20°C). Dry the pellet in a dessicator.

17 Resuspend the pellet in 360µl of TE and add 140µl of 7.5M NH4 acetate and 1ml of propan-2-ol. Leave at room temperature for ten minutes and spin at12,000 r.p.m. for 10 minutes.

18 Resuspend the pellet in 100µl of TE. Concentration estimation, restriction and ligation analysis should then be done on agarose gels. For long term storage, keep at -20°C.

Solution 1 50 mM Glucose, 25 mM Tris.Cl (pH 8.0), 10mM EDTA (pH 8.0)

Solution 2 0.2N NaOH, 1% SDS

Solution 3 5M K acetate 60ml, glacial acetic acid 11.5 ml, H2O 28.5 ml

2.2.3 Minipreps of plasmid DNA

Boiling method (Holmes & Quigley, 1981)

This method was used to prepare small amounts of plasmid DNA for subsequent analysis by restriction enzyme digestion in analysis of constructs, for subcloning of fragments and for preparation of replicative form M13 bacteriophage DNA.

1. Innoculate 10mls. LB. with a single bacterial colony and grow at 37°C overnight.
2. Spin down 2x 1.5 mls. in a 1.5 ml eppendorf tube.
3. Remove the supernatant carefully and resuspend the bacterial pellet in 300 µl. STET + 200 µg. lysozyme and place the tube on ice for one to ten minutes.
4. Put in boilng water bath for two minutes.
5. Spin in cold room microfuge for twenty minutes.
6. Pour off supernatant into fresh tube and add an equal amount of isopropanol. Store in -20°C freeezer for at least twenty minutes. and then spin in cold room microfuge for five minutes.
7. Remove all of supernatant and add 500µl. of 80% ethanol at -20°C. Dislodge pellet by flicking eppendorf, spin down and remove supernatant without dislodging the pellet. Dry under vacuum and resuspend in 50ul. TE. The plasmid DNA should be clean enough to manipulate with most enzymes. If not, then a further precipitation with NH_4 Ac + isopropanol will solve most problems.

If the plasmid is to be cut with restriction enzymes to give fragments of a size less than 500 bp. then treatment with RNase will be useful. Add 1 µl. of RNase (10 mg/ml.) to each digest.

STET = 0.1M NaCl

10mM Tris.Cl (pH8.0)

1mM EDTA (pH8.0)

5% Triton X-100

2.2.4 Radiolabelling of DNA by random priming (Feinberg & Vogelstein, 1983,1984)

DNA fragments can be recovered by electroelution or directly from a low melting point (lma) agarose gel. The reaction can be left overnight at room temperature or for at least 3 hours at 37°C.

Reagents are added in the following order for DNA in TE.

OLB, 3ul. BSA, 1.2µl. DNA, 20ng. [^{32}P]dATP, 3µl. Klenow fragment, 1.2µl.

H_2O to a total of 30µl.

DNA in lma is excised from the gel with a sterile scalpel and weighed. Sterile distilled water is added at a ratio of 1.5ml of water to 1g of DNA in agarose. The DNA is boiled for 7 minutes and equilibrated for 10 minutes before labelling. If the DNA is not to be labelled immediately, it is frozen at -20°C in 20µl aliquots

Labelling DNA in lma agarose

OLB 5ul, BSA 2ul, DNA 20ul, [32P]dATP 3ul, Klenow fragment 2ul

H_2O to 50 µl

It should usually be unnecessary to remove unincorporated [^{32}P]dATP but the easiest way to do this is to make the reaction 2M NH4Ac and add 2 volumes propan-2-ol. A wash in 80% ice cold ethanol followed by dessication and resuspension in 20µl TE should remove the vast majority of unincorporated [^{32}P]dATP.

Reagents.

OLB; Solutions A,B and C in a ratio of 2:5:3 will remain stable for several months at -20°C.

Solution A

625µl 2M Tris.Cl(pH 8.0), 25µl 5M $MgCl_2$, 350µl SDW, 19µl 2-mercaptoethanol. 5µl each of 0.1M; dCTP, dTTP and dGTP, in 3mM Tris (pH8.0) and 0.2mM EDTA (pH 8.0). Store at -20°C

Solution B 2M Hepes pH 6.6 with NaOH. Store at 4oC.

Solution C Hexadeoxyribonucleotides-pD(N)6. These are random hexamers of calf thymus DNA. Pharmacia cat.no. 27-2166-01

Suspend in 3mM Tris, 0.2mM EDTA, pH 7 at 90 O.D units/ml and store at -20°C. BSA 10mg/ml in H2O stored at -20°C

[^{32}P]dATP, Amersham 3000 Ci/mmol, 10µCi/µl

Klenow fragment diluted to 1.5 units/µl

2.2.5 Small scale preparation of lambda. Modified from Sambrook *et al.* (1989)

Isolate a single plaque by spearing it with a sterile pipette tip and blow it into a 50ml. conical flask containg 10ml. of LB. +10mM. $MgSO_4$. Add a drop of plating cells. The cells are best when fresh, but can be used for up to a week after preparation. Shake hard at 37°C overnight.

The next morning, add a drop of chloroform to each lysate and shake gently at 37°C for 10 minutes. The lysate should appear stringy. Spin down lysate for 10 minutes in a Falcon tube and then spin the supernatant at 35,000 r.p.m in a Beckman 45Ti or (equivalent) for at least 1 hour.

Pour off the supernatant and resuspend the glassy pellet in 1ml. of TM. Take

up the resuspended pellet in a 1.5 ml. eppendorf tube and spin at room temperature for 2 minutes at 12,000 r.p.m. Take up 800µl of supernatant in an eppendorf tube and add DNAse and RNase to a final concentration of 50ug/ml. Incubate at 37°C for about 15 minutes.

Add 200ul of TES, mix well and incubate at 70°C for 15 minutes. Cool to room temperature add 150µl of 8M potassium acetate and place on ice for 15 minutes. Spin for 10 minutes at 12,000 r.p.m. and remove 800µl to a new eppendorf. Extract with phenol/chloroform, chloroform and finally with ether. Add 500µl of ice cold propan-2-ol, mix gently and leave at -20°C for at least 15 minutes. A precipitate should form almost immediately. Spin down the precipitate for 10 minutes at 12,000 r.p.m., wash with 80% ice cold ethanol and dry in a vacuum dessicator. The pellet is resuspended in 20µl of TE. For each restriction digest, use 5-10µl, depending on yield. The addition of RNase to a final concentration of 10ug/ml will allow visualisation of small DNA fragments in a gel.

TM 10mM. Tris pH 8, 2mM. MgCl2

TES 300mM Tris pH 9.0, 150mM. EDTA pH 8.0, 1.5% S.D.S

This miniprep is usually good enough to prepare at least 5µg of λ DNA suitable for restriction analysis. A scaled up miniprep of 50ml of lysate can produce up to 50µg of DNA. This is easily sufficient to produce enough insert DNA from a recombinant plaque for subcloning.

2.2.6 Large Scale Preparation of Lambda. Sambrook *et al.* (1989)

1 Use 25ml of a miniprep lysate to infect 5ml of the appropriate E.coli host in $MgSO_4$. Incubate at 37°C with 2mM $MgCl_2$ and 2mM. $MgCl_2$ for 15 minutes. Grow culture with good aeration overnight and treat with chloroform as in minilysate.

2 Spin in Beckman JA14 or equivalent at 10,000 r.p.m. for 5 minutes at 4°C. Put the supernatant into fresh 250ml bottles and add equal amounts of PEG solution. Leave overnight at 4°C.

3 Spin at 3,000 r.p.m. for 5 minutes at 4°C, keep back ~50ml of the supernatant and discard the rest being careful not to lose any of the white precipitate. Use the supernatant to wash down the sides of the centrifuge bottles and pool the supernatant with the white precipitate which contains the PEG and the phage. Spin this down at 5,000 r.p.m. in a Beckman JA20 rotor or equivalent and discard the supernatant. Add solid KCl slowly to a final concentration of 1M, mix gently but thoroughly and leave on ice for 15 minutes. Spin down at 10,000 r.p.m. in a JA20 rotor and discard the pellet .

The phage are then concentrated on a CsCl step gradient. This is done in a Beckman SW27 rotor or equivalent. Pipette the phage suspension into the tube and carefully underlay it with a 1.4g/cm^3 CsCl solution. Then underlay these two phases with a 1.5g/cm^3 solution and finally with a 1.7g/cm^3 solution. Spin at 24,000 r.p.m. for at least two hours. The brake should not be used to slow down the rotor. The phage band should be located between the 1.4g/cm^3 and 1.5g/cm^3 band interface and will be a blue colour depending on the concentration of the phage. Other bands will be present but these are not intact phage. The band is collected with a 26 gauge needle by side puncture of the tube. Make a hole at the top first to allow for pressure equilibration. If there is no band visible, it is probably not worth proceeding

due to the low yield. The DNA is extracted as above with phenol-chloroform. Several methods exist for a large scale lysis procedure. Some demand that the host cells are in mid-log phase when harvested or that a specific ratio of phage to host cells is used. Phage yield will vary according to the procedure and phage used. If a low yield is encountered then try three or four different ratios of phage to host cells in the above procedure and titre the lysate in the morning. It may be possible to increase the yield further by varying the ratio around the best yield.

The step gradient is designed for wild type lambda which has a genome size of 48.5kbp. A smaller phage will have a lower density and vice versa for a larger phage. The phage can also be purified on an equilibrium gradient. This is performed under the same centrifigation conditions, but the phage suspension is mixed with CsCl to a final concentration of 1.5g/g/cm^3. This method is used when preparing less than 1 litre of lysate. Other methods are sometimes used such as glycerol gradients, but the CsCl method is relatively easy to use, quick and gives a reasonably pure preparation.

A large scale liquid lysate is used to produce up to 1mg of DNA from 1000ml of lysate. The lysate is treated with DNase and RNase to release phage from the mass of bacterial DNA in the lysate and then the phage are precipitated using polyethylene glycol and KCl. Phage are concentrated on CsCl step gradients , dialysed and phenol extracted. This method is used when large quantities of highly purified λ DNA is needed such as when making libraries.

2.2.7 Plating lambda bacteriophage to generate plaques.

Sambrook *et al.* (1989)

It is often necessary to generate single plaques of lambda such as

when determining the titre of a library, to separate nonrecombinant from recombinant plaques or to isolate a positive clone during a screening procedure. Although not a complex procedure, it is important to have a good sterile technique and to pay attention to detail.

To generate lambda plaques.

1 Grow up a suitable strain of E.coli overnight to saturation in a suitable broth such as NZCYM + 10mM $MgSO_4$ + 0.2% maltose

The maltose induces production of the lambda receptor (lamB protein) which also allows maltose transport. Magnesium is necessary for the integrity of the phage coat which can therefore be disrupted by divalent cation chelators such as EDTA. This is sometimes utilised in lambda DNA isolation.

2 Spin down the E.coli in an MSE benchtop centrifuge (or equivalent) at 3000 r.p.m. and resuspend in 0.4x the original volume of 10mM $MgSO_4$. The cells are best when fresh but are easily usable for a week if kept at 4°C.

3 Make serial dilutions of the lambda suspension into a sterile test tube and add 100µl of the plating cells for each 90mm plate.

A lambda library will normally have a titre of between 10^8-10^{11} particle forming units (pfu)/ml of library. A single plague in lambda diluent,SM,will contain about $2x10^6$ pfu/ml. It is best therefore to titre a phage suspension giving two scoreable plates. This gives a good estimate of the phage concentration. When the phage are stored at 4°C in the dark with a few drops of chloroform, the titre should remain stable for several months.

4 Add 3ml of top agar (at a temperature of 45°C) such as NZCYM to the bacterial phage suspension and quickly pour onto a prewarmed plate of bottom agar making sure to cover the plate and avoid bubbles. Let set on

a level bench and incubate at the desired temperature.

Many people use many sorts of top and bottom agars. NZCYM contains magnesium and is a rich food allowing quick growth of the E.coli and of the lambda plaques. Most phage are grown at 37°C, but temperature sensitive lysogens are grown as at 32°C while lytic growth is at 42°C. λ gt11 has a temperature sensitive repressor and is usually grown at 37-42°C

5 Incubate the plates at the required temperature for 4 hours in the case of gt11 or overnight if necessary. Count the plaques and estimate the titre of the λ suspension.

Different strains of λ grow into different types of plaques. A large number of plaques with an overcrowded plate gives small plaques and the reverse is true with undercrowding. Some plaques are 'sick' and grow much less well than others. This can be a problem if the insert that you want in a recombinant phage gives a 'sick' plaque. The problem can be further exaggerated in an expression library where a fusion protein can be toxic to the host cell. These problems can be got around by using different host strains and growth conditions for particular phage strains

2.2.8 Heparin Hybridisation Method. Singh & Jones (1984)

1. Prehybridise blot in 4xSSC for at least 15 minutes
2. Heat Hybridisation Solution (HS) to 42°C and prehybridise blot for at least a further 15 minutes. This may sometimes require several hours for a plaque lift or colony blot. Southern transfer blots only require 15 minutes.
3. Boil the labelled probe and add to the hybridisation solution.
4. Incubate overnight at 42°C in a shaking water bath.

5. Wash the filter with 4xSSC at room temperature until most of the activity has been removed.

6. Wash the blot at successively higher temperatures and lower concentrations of SSC until a desired signal is attained. this is seen by autoradiography or by estimating the residual activity by mini-monitor. If the desired stringency of washing is unknown it is best to proceed slowly by increasing the temperature by increments of 5^{O}C, and/or decreasing the concentration of SSC by increments of 0.5.

Stock solutions.

20xSSC: 175.3 g NaCl and 88.2 g Na Citrate per liter H_2O, pH(7.0),

2% Na Pp_i, 10% SDS, Formamide 100% deionised, Heparin 50mg/ml in H_2O.

Hybridisation Solution (HS)

For 10ml HS

2ml 20xSSC, 500ul 2% $NaPp_i$, 5ml Formamide, 40µl Heparin solution, 460µl SDW

2.2.9 Preparation and Transformation of competent cells. Sambrook *et al.*,(1989)

1 Innoculate 10ml of broth with a single colony of the desired E.coli strain, and grow overnight.

2 In the morning, use this culture to innoculate 100ml of broth. Grow until the $O.D._{550}$ is between 0.3 and 0.4. Quickly chill the culture in an ice bath and harvest the cells in a Beckman JA14 rotor (or equivalent) at 4,000 r.p.m. at 4^{O}C.

3 Resuspend the cells in 50ml of 100mM ice cold $CaCl_2$ and leave on ice for

30 minutes.

4 Spin the cells in the JA14 at 3,000 r.p.m. for 5 minutes and resuspend in 4ml of 100mM ice cold $CaCl_2$. The cells should be left on ice for an hour before use. They are best after 24 hours after which they begin to lose transformation efficiency.

Transforming competent cells.

5 Add ~10ng of DNA per transformation reaction. This should be done in precooled test tubes. Use 100µl of cells per reaction. The cells should be treated gently and transferred in a wide bore pipette. If the DNA is from a ligation reaction then up to 100µg can be used.

6 Place the cells on ice for at least 15 minutes and then heat shock the cells for 3 minutes at 42°C. Place the cells back on ice for a few minutes and then add 1ml of LB.

7 Incubate the cells for 1 hour at 37°C without the relevant antibiotic.

8 Spin down the cells at 12,000 r.p.m. for 30 seconds and resuspend in ~150µl of the LB.

9 Pipette the cells onto an agar plate with the relevant antibiotic or selection medium and spread the cells evenly with a glass spreader.

10 Incubate the plates at 37°C overnight. Transformants are picked and miniprepped to check for the relevant plasmid.

A common selection uses the fact that insertion into the polylinker region of some plasmids (pUC,pK,pGEM,M13 phage) disrupts the coding region of lacZ and gives a colour test for recombinants. Wild type plasmid gives a blue colony or plaque with M13 while recombinants are white.

2.2.10 Polyacrylamide gel electrophoresis (PAGE). Sambrook *et al.*

(1989).

1 Wash glass plates in hot Decon and rinse with distilled water. Wipe the plates with ethanol making sure the plates are completely clean.

2 Assemble the plates with vacuum grease, spacers and clips.

3 For a 16x16cm gel, use the following amount of separating solution.

Acrylamide	30%			10%	
concentration	Acrylamide	Buffer	H2O	APS	TEMED
6	6	7.5	16.5	0.05	0.01
7	7	7.5	15.5	0.05	0.01
8	8	7.5	14.5	0.05	0.01
9	9	7.5	13.5	0.05	0.01
10	10	7.5	12.5	0.05	0.01
12	12	7.5	10.5	0.05	0.01
13	13	7.5	9.5	0.05	0.01
15	15	7.5	7.5	0.05	0.01(ml)

The required mixture should be poured immediately in to the gel cast leaving ~3cm for the stacking gel and comb. The gel should be carefully overlayed with distilled water or propan-2-ol.

4 Keep back a little of the mixture to see when the gel has set. Pour off the water or propan-2-ol The stacking gel consists of:

30% acrylamide	buffer	water	10% APS	TEMED	
1.5	2.5	5.97	0.03	0.01	(ml)

2.2.11 Media.

LB per liter, 2xTY per liter NZ per liter

10g. Tryptone	16g. tryptone	10g. casein enzymatic
5g. Yeast Extract	8g. Yeast Extract	5g. NaCl
5g. NaCl	5g. NaCl	MgSO4 - 10mM
pH 7.2		pH. 7.5

Solid Media.

Agar. Add 15g. agar or agarose per liter for bottom agar.

Add 7.5g. agar or agarose per liter for top agar.

2.2.12 Isolation of *Onchocerca gibsonii.*

The worms were a kind gift of Dr. John Comley of Wellcome research laboratories.

Frozen nodules containing the worms were thawed overnight in 70% ethanol at 4°C They were then transferred to PBS and left for 24 hours at 4°C with at least three changes of PBS. They were then transferred to a 0.1% collagenase solution in PBS containing gentamicin at 0.2 mg/ml and incubated at 37°C. The worms could be gently removed from host tissue using a dissecting microscope after about two days. Loose worms were washed in PBS until no host tissue could be observed. Worms were dropped into liquid nitrogen and stored at -70°C until required.

Worm tissue was prepared for SDS-PAGE and subsequent Western blotting by boiling the protein sample for five minutes with SDS and β-mercaptoethanol as described in Sambrook et al. (1989).

Worm DNA was isolated in the presence of SDS, proteinase K and

RNA'se as described by Sambrook et al. (1989), after the tissue had been ground to a fine powder with liquid nitrogen using a mortar and pestle.

2.2.13 Western Blots

SDS polyacrylamide gels were prepared according to Laemlli & Favre (1973). Gels were run on home made plates or on the Hoefer Mighty Tall apparatus. Protein was transferred onto nitrocellulse filters using the LKB semi-dry apparatus. The filters were incubated with TBST for 1 hr with two changes. The filters were then incubated with TBST plus 1° antibody for at least 1hr and then washed thoroughly in TBST three times before incubating with TBST plus 2° antibody for 1 hour. They were then washed at least three times in TBST. The filters were then developed with H_2O_2 and chloronapthol or diaminobenzidine for Horse Radish Peroxidase streptavidin conjugated 2° antibody or NBT and BCIP for Alkaline Phosphatase conjugated 2° antibody. Filters were washed in distilled water, allowed to dry and stored in the dark.

2.2.14 Polymerase Chain Reactions

PCR was done according to the protocol suggested by Promega on a Biorad thermal cycler. Light mineral oil (Sigma) was used as an overlay to prevent evaporation of the sample. An initial 95°C denaturation step was run for five minutes before adding Taq polymerase (2 units) (Promega). The reactions were then run in a water cooled thermal cycler (Biorad), at 55°C for two minutes followed by polymerisation at 72°C for one and a half minutes and denaturation at 95°C for 1 minute. Twenty five cycles were usually performed.

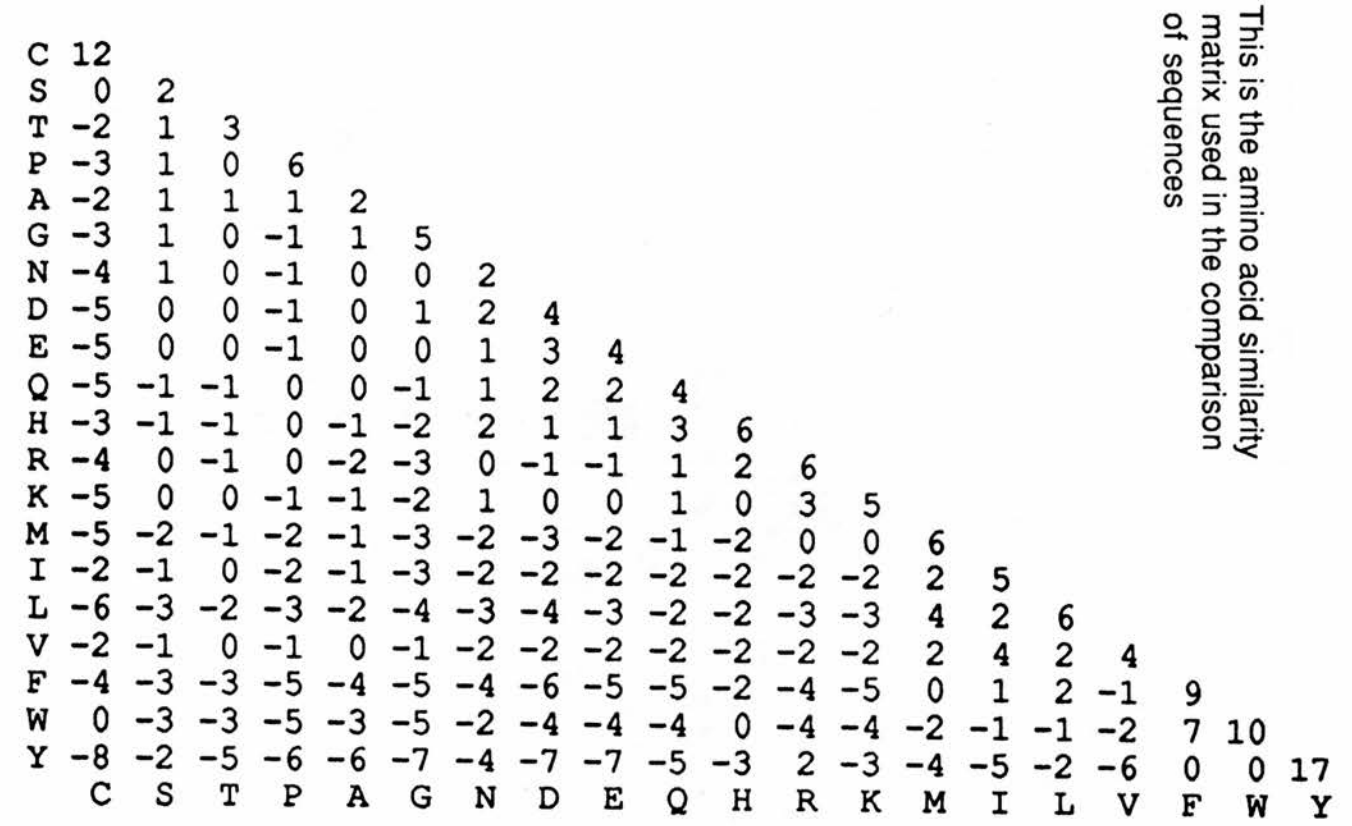


Figure 5. The PAM 250 similarity matrix

Chapter 3. Creation of a human genomic library, and screening of *Onchocerca* cDNA ibraries.

3.1 Creation of a human genomic library.

As a first step towards characterising HMPFK, a human genomic library was created in λ EMBL3 by the method of Zabarovsky & Allikmets (1986). Human DNA extracted from blood was partially digested with Sau3AI to give fragments of an average size of 15kbp. Klenow enzyme was used to partially fill in these digested fragments with dATP and dGTP. They were ligated to EMBL3 which had been digested with SalI and EcoRI and partially filled in with dTTP and dCTPas seen in figure 6. The ligations were packaged using λ packaging extracts prepared by the method of Hohn (1979). This produced 10^4 pfu/μg of ligated DNA when plated on *E. coli* NM538.

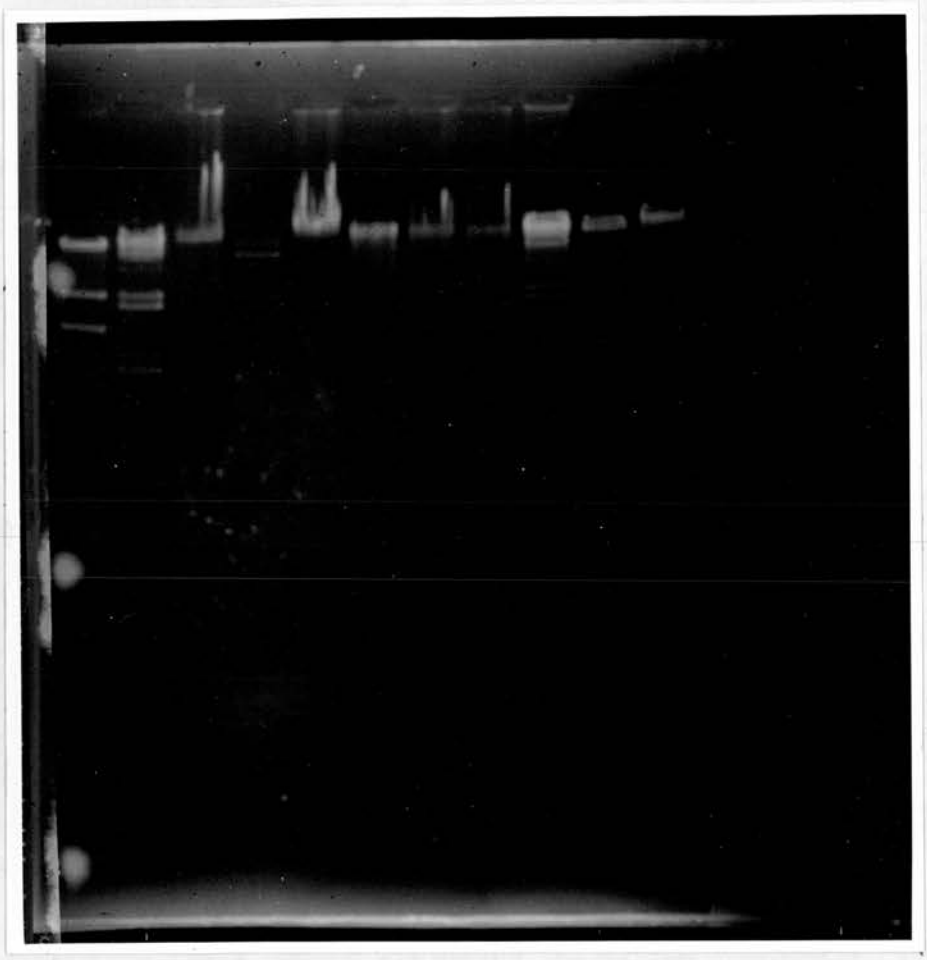


Figure 6. Analysis of the partial filling in and ligation products of human and EMBL3 DNA by electrophoresis in a 0.7% agarose gel

Lane 1. HinDIII digested λ cI*ts*857 molecular weight markers

Lane 2. SalI + EcoRI digested EMBL3

Lane 3. Ligation of the fragments from lane 2

Lane 4. Partially filled in and ligated fragments from lane 2

Lane 5. Human placental genomic DNA

Lane 6. Sau3AI partial digest of DNA from lane 5

Lane 7. Ligation of DNA from lane 5

Lane 8. Partially filled in and ligated DNA from lane 6

Lane 9. Ligation of DNA from lanes 2 and 6

Lane 10. Lamda cI*ts*857

Lane 11. Ligation of partially filled in human and EMBL3 DNA at a 1:1 molar ratio

3.2 Screening of *Onchocerca volvulus* libraries

At this point of the project however, the sequence of genomic HMPFK was published and it was decided to focus the aims of the project towards characterising PFK from *O. volvulus.* We received four cDNA libraries in λgt11 (named Mali, Kumba, Guatemala and Toborou) a kind gift from Dr. John Donelson (University of Iowa). Genomic DNA corresponding to exon 22 of RMPFK was kindly made available by Dr. Simon Chang (Louisiana State University). Six positive clones were isolated from the libraries using the rabbit muscle DNA as a probe. Mini preps of the phage yielded about 5μg of DNA for each clone. The DNA from each positive clone were digested with EcoRI and run on an agarose gel. Only clone 2 contained an insert

Figure 7a. EcoRI digest of positive *onchocerca* clones.
Lanes 1-6 Clones 1-6 Lane 2 contains an insert of ~2.4 kbp.
Lane 9. HinDIII digested λ cI*ts*857 molecular weight markers labelled with Klenow enzyme and [^{32}P]dATP

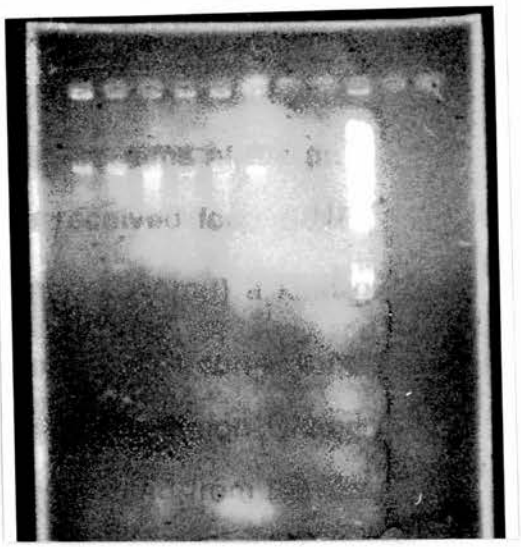


Figure 7b. Southern blot of the gel in Figure 7a probed with DNA corresponding to exon 22 of rabbit muscle PFK labelled with Klenow enzyme and [^{32}P]dATP

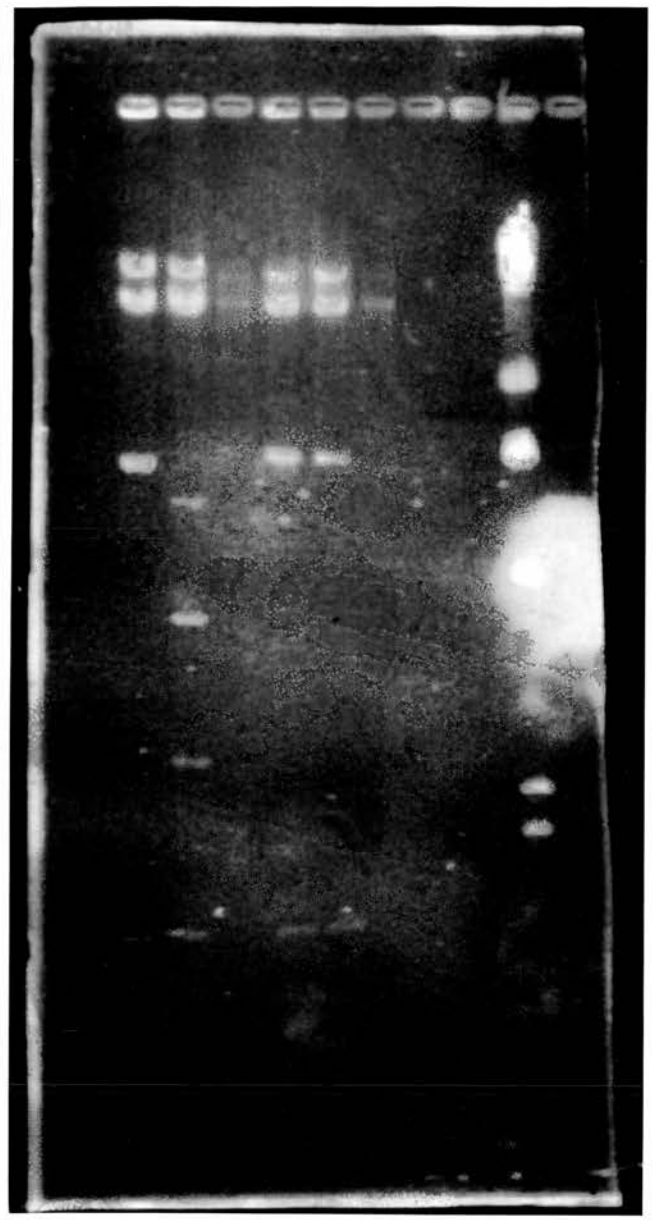


Figure 8a.

Lanes 1-6, KpnI + SacI digests of clones 1-6

Lane 9. HinDIII digested λ c*Its*857 molecular weight markers

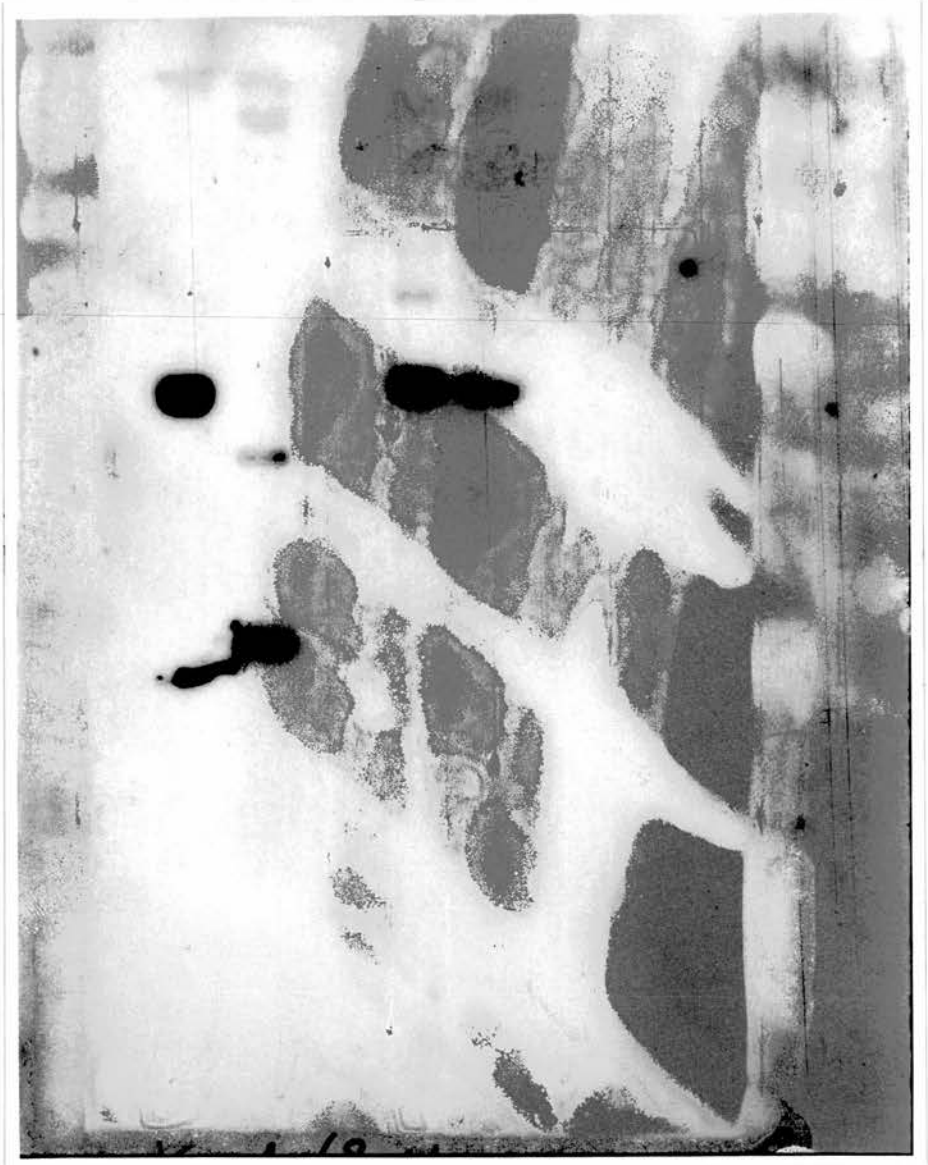


Figure 8b

Southern blot of gel in figure 8a probed with DNA from exon twenty two of RMPFK labelled with [^{32}P] and Klenow enzyme

which was released by EcoRI, (figure- 7a). Digestion with KpnI and SstI produced a variety of fragments which suggested that out of the six, three different clones were present - figure 8a. A Southern of the EcoRI digested DNA showed that RMPFK bound to the insert of clone 2 and to the arms of the digested vector for the other clones - figure 7b. A Southern of the KpnI and SstI digested DNA showed binding of RMPFK in three different patterns - figure 8b. These fragments were electroeluted onto DE81 paper placed in the gel and purified by phenol extraction and ethanol precipitation, before ligation to pUC19 cut with the appropriate restriction enzyme. All attempts to clone these fragments failed. Fragments were extracted from low melting agarose gels, phosphatased vectors and different *E. coli* cells (NM539, TG1, HB101, NM522) were used, but the inserts were refractory to subcloning.

After some time, inserts were digested with Sau3AI and ligated to M13mp18 and M13mp19 digested with BamHI. White plaques were picked and single stranded DNA was extracted. The DNA was sequenced according to the method of Sanger *et al.* (1977). Almost immediately, it was found that the inserts were made up of random fragments of pBR322 and lambda DNA.

The cells in which gt11 are grown (*E. coli* Y1090) contain the plasmid pMC9 which can recombine with lambda DNA in the cell. Although the RMPFK probe was excised from pBR322 and purified on a low melting point agarose gel, it must have contained some contaminating pBR322 DNA and it was this which gave a signal with the positive clones. In attempting to minimise such problems, the Southern which gave a signal for the EcoRI digested clones was probed with pBR322 and did not produce a signal. It seems however that this Hybond-N filter had been treated with alkali to remove the RMPFK probe, but had been left in alkali solution for too long

and been allowed to dry out. Also, the pBR322 DNA used as a positive control for the hybridisation of radiolabelled pBR322 was placed on a fresh filter and yielded a positive result. Obviously, if pBR322 had been on the same filter as clones 1-6, then this problem would have been eliminated earlier.

Two oligonucleotides were designed to allow PCR of fragments inserted into the multiple cloning site of pUC type plasmids. At this time, DNA representing exon I of genomic RMPFK was made available by Dr. Simon Chang and an *O. volvulus* cDNA library was kindly made available by Dr. Tom Unnasch (Case Western University, Ohio). Both RMPFK fragments were produced using PCR, cut with EcoRI and eluted from low melting point agarose gels. About 10^6 phage from each library were screened with both PCR products. Several possible positive clones were produced but none proved positive after secondary screening even at low stringencies. Several attempts to determine the conditions for screening by probing Southern blots of *Onchocerca* DNA proved fruitless and it seems that either the probes were too dissimilar to an *Onchocerca* PFK or that *Onchocerca* PFK sequences are not present in the libraries. *Onchocerca* DNA was never of good quality, being extracted from frozen nodules containing adult *onchocerca gibsonni* which had been kept at -70^0 for over two years. These nodules had also been transported from Australia under unknown conditions. A tubulin clone and a putative hexokinase clone were identified from genomic libraries constructed from DNA from these nodule worms in this laboratory. These clones were used to probe genomic *onchocerca gibsonni* Southern blots and gave very poor quality results. Bands were often badly smeared and did not give a good signal. The Southern blots for these hybridisations are seen in figures 9a and 9b.

Figure 9a.

Southern blot of human placenta and *Onchocerca gibsonni* DNA probed with a putative hexokinase clone from *Onchocerca gibsonni*.

Lane 1. Human + EcoRI

Lane 2. Human + HinDIII

Lane 3. *O. gibsonni* + EcoRI

Lane 4. *O. gibsonni* + HinDIII

Lane 5. λcI*ts*857 HinDIII molecular weight markers

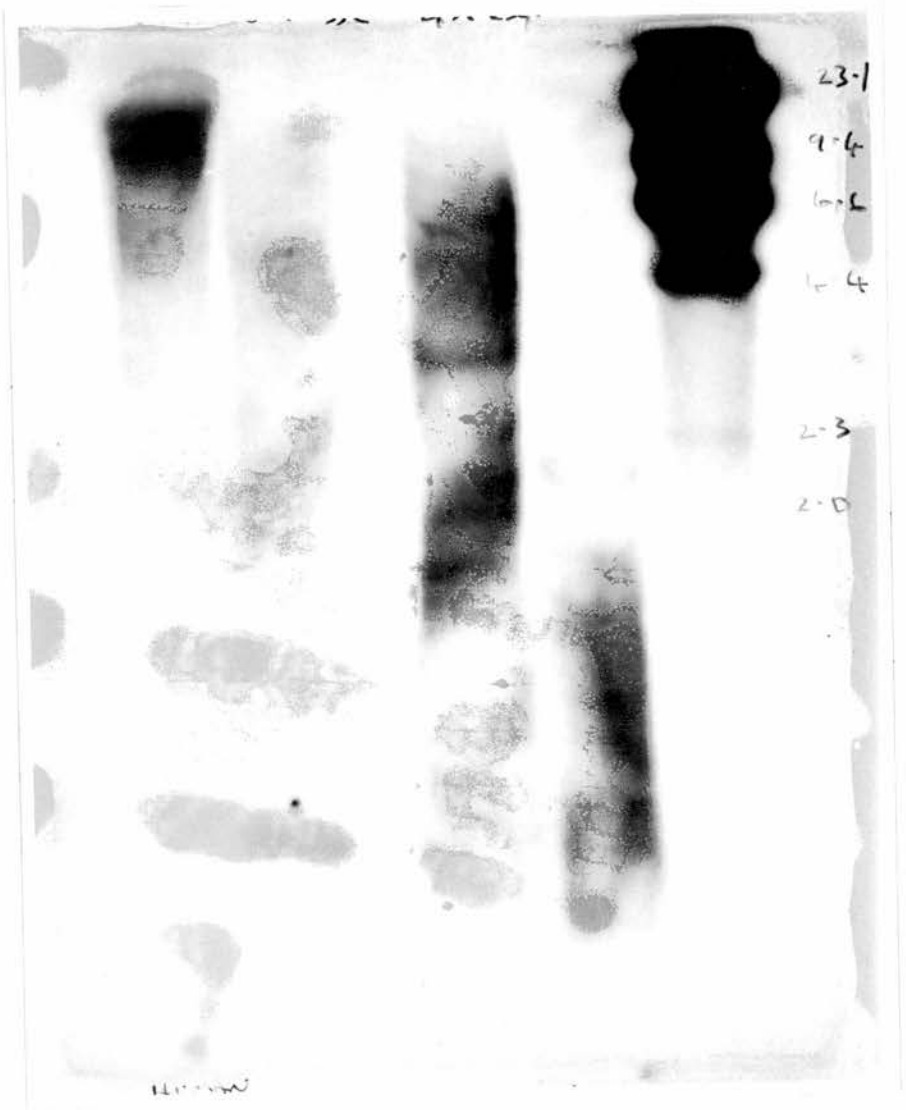


Figure 9b.

Southern blot of human placenta and *Onchocerca gibsonni* DNA probed with a tubulin clone from *Onchocerca gibsonni* .

Lane 1. Human + EcoRI

Lane 2. Human + HinDIII

Lane 3. *O. gibsonni* + EcoRI

Lane 4. *O. gibsonni* + HinDIII

Lane 5. λcI*ts*857 HinDIII labelled with [^{32}P] and Klenow enzyme

3.3 Screening of libraries with anti *Ascaris* PFK polyclonal serum.

HPLC purified *Ascaris suum* PFK was a kind gift of Dr. Werner Hofer (University of Konstanz). One mg. of this PFK was emulsified with 0.5 ml. of Freund's complete adjuvant (Sigma) and injected into the dorsal subcutaneous tissue of a New Zealand White rabbit. Booster injections containing 0.5 mg. of *Ascaris suum* PFK in Freund's incomplete adjuvant (Sigma) were similarly administered at three weekly intervals on three occasions. One week after the third booster injection, the animal was bled and serum purified by allowing the blood to clot at room temperature for one hour and then overnight at 4^0. The clot was removed and cellular debris was removed by centrifugation at 20,000 r.p.m. in a JA-20 rotor for ten minutes. Serum was aliquoted and frozen at -70^oC.

A. suum, *E. coli*, rat muscle and *O.guttorosa* protein was separated on a 6% SDS-PAGE gel (Laemmli & Favre, 1973; Sambrook *et al.,* 1989), and transferred to nitrocellulose on a LKB semi-dry electrophoresis unit (model 7112) by the method supplied by the manufacturer. These blots were incubated with varying dilutions of rabbit serum from a 1:500 to a 1:500,000 dilution. The serum only produced a signal specific for *Ascaris* PFK. The blot was developed using anti-rabbit IgG coupled to alkaline phosphatase (BIO-RAD) and BCIP with NBT by the method of Sambrook *et al.* (1989).

No convincing signal could be obtained for rat or *onchocerca* protein using this anti *ascaris* PFK serum. A representative Western blot is shown in Figure 10. While signal of a diffuse nature was found for *E.coli* protein at a serum dilution of 1:200, the *ascaris* PFK signal could be generated at a 1:500,000 dilution indicating the specificity of the antibody. Because *Onchocerca* material was available in limited quantities, only 100ng was

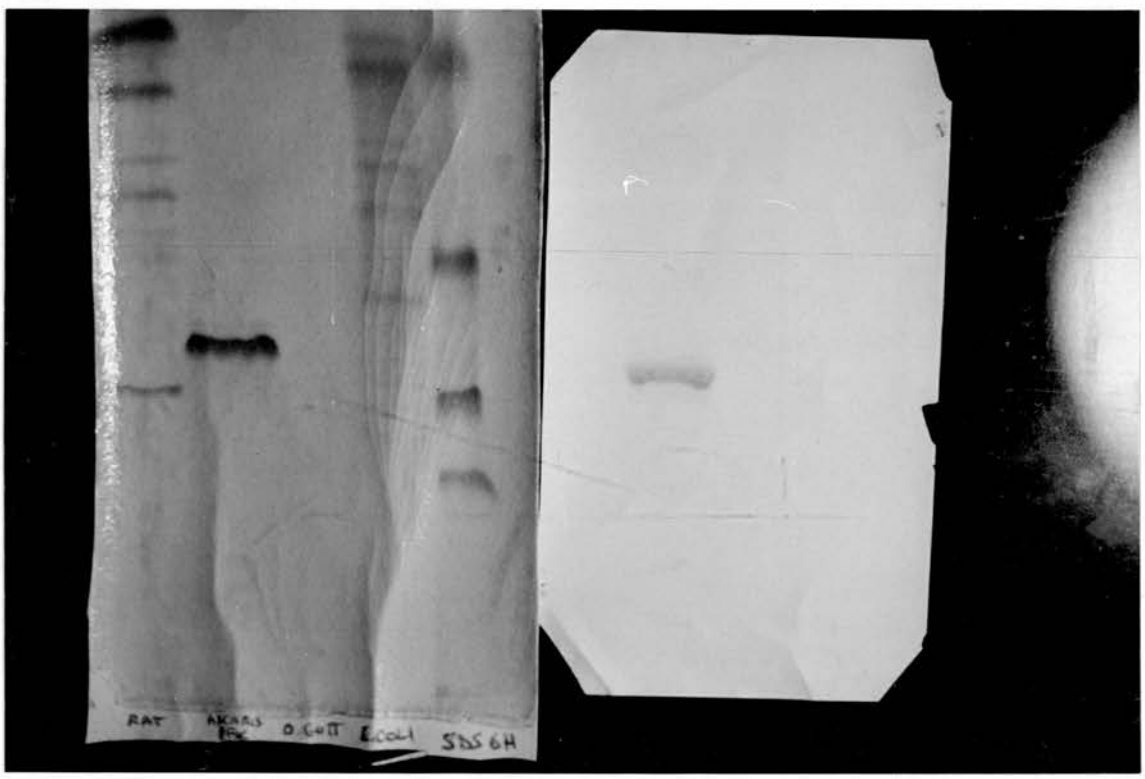


Figure 10.

SDS-PAGE gel and Western blot of rat muscle, *Onchocerca gutturosa, Ascaris suum* PFK and *E. coli* probed with anti *Ascaris suum* polyclonal serum

Lane 1. Rat muscle, ~500µg

Lane 2. *Ascaris suum* PFK, ~1mg

Lane 3. *Onchocerca gutturosa,* ~50µg (gel) ~500µg (Western)

Lane 4. *E. coli*, ~500µg

Lane 5. Molecular weight markers

loaded onto the gel stained with coomassie blue. The gel intended for Western blotting was loaded with ~500ng of sample. Although no signal was seen for Western blots directed against the *Onchocerca material,* the libraries were screened using serum at a dilution of 1:2000. Approximately 10^9 plaques were screened from each of the *onchocerca* libraries, but no positive plaques were found after a secondary screening.
Method - (Mierendorf et al., 1987)

3.4 Complementation studies on PFK deficient *E. coli*

Bacteriophage lambda gt11 carries the mutation cI857 of the cI gene. Lysogens of gt11 may be grown at 32-34^{O}C and the prophage can be induced by growth above 37^{O}C. The *E. coli* strain DF1020 which lacks both the major and minor forms of PFK (*recA pro*82 Δ*pfkB*201 *recA*56 Δ(*rha*pfkA) *endA*1 *hsdR*17 *supE*44) cannot grow on mannitol but it can grow gluconeogenically (Hellinga & Evans, 1985). It was possible even though unlikely, that *Onchocerca volvulus* PFK might be able to complement this deficiency as a lysogen containing the PFK sequence. The procedure for creating a lysogen was that of Huynh *et al. (*1985) except that cells infected with gt11 were grown at 32^{O}C on mannitol plates supplemented with proline and thiamine hydrochloride (Hellinga & Evans, 1985). Over 10^9 plaque forming units from each of the five gt11 libraries were used to infect DF1020 but no viable bacteria were seen on the mannitol plates.

The failure to complement DF1020 could be due to a variety of factors. The most likely explanation is that a full length clone coding for *Onchocerca volvulus* PFK in the correct reading frame, may not be present in any of the libraries. It may not be physiologically possible for *O. volvulus* PFK to complement DF1020 or DF1020 may not be capable of forming a

lysogen with gt11.

3.5 Use of an AT rich oligonucleotide to probe *Onchocerca* libraries

The majority of clones from *Onchocerca volvulus* identified and sequenced to date derive from surface antigen genes and include a heat shock protein (Rothstein *et al.,* 1989), myosin (Erondu & Donelson, 1990), paramyosin (Limberger & McReynolds, 1990), major sperm protein (Scott *et al.,* 1989), ribosomal genes (Gill *et al.,* 1988). They have usually been isolated by screening expression libraries with antisera often taken from immune adults in endemic malarious areas. This procedure is not suited to identifying genes coding for cytosolic proteins which do not normally stimulate a significant immune response in the host. There have been successes in the isolation of HPRT and actin genes from *P. falciparum* (King & Melton, 1987; Wesseling *et al.,* 1988) by the use of radiolabelled heterologous hybridisation probes but this method has not proved to be very useful. *P. falciparum* is the eukaryote with the most A + T rich genome (81%) yet characterised (Goman *et al.,* 1982) and exhibits this most strongly in the choice of the third base for a particular codon, but there is a dearth of probes derived from A + T rich organisms. The majority have been derived from higher eukaryotic genes. The large evolutionary distance between *P. falciparum* and higher eukaryotes increases the likelihood that the divergence between the heterologous probe and the target sequence will be too great to allow successful hybridisation. The RMPFK gene has a G + C richness of 58%. It seemed possible that the corresponding PFK from *onchocerca* might also have an AT rich sequence and this would reduce the usefulness of the rabbit gene as a probe. Rothstein *et al.* (1988) have

estimated the A+T content of the filarial nematodes *Brugia malayi, Brugia pahangi* and *Dirofilaria immitis to be* 72%-74%. They also estimated the A+T content of the free living nematode to be 64%. They used histone, actin and myosin probes from *C. elegans* to probe genomic Southern blots of filarial nematodes and found essentially no hybridisation. They also found that codon usage in the filarial nematodes was heavily biased to A or T in the third codon position. Hyde *et al.* (1989) constructed a table using 10,000 codons from *P. falciparum* to take into account the probability that the choice of codon for a particular amino acid will result in a perfect match and also the probabilities of 2 and 1 base pair matches occurring if the codon selected is wrong. A 51 mer oligonucleotide was designed according to this table which corresponded to a PFK sequence and contains amino acids which have a low number of codon usage possibilities and which is identical for all mammalian PFK's. The conserved peptide has the sequence His-Gln-Arg-Thr-Phe-Val-Leu-Glu-Val-Met-Gly-Arg-His-Cys-Gly-Tyr-Leu which begins at position 160. The reverse complement of this peptide was synthesised and has the sequence, T A A A T A T C C A C A A T G A C G T C C C A T T A C T T C T A G T A C A A A T G T A C G T T G A T G. The oligonucleotide was radiolabelled with γ^{32}P ATP by polynucleotide kinase (Sambrook *et al.,* !989) and used to probe the onchocerca libraries from Mali and the library from Tom Unnasch. Plaque lift filters were incubated overnight in 6xSSC-0.2% SDS, at 50°C. Incorporation of γ^{32}P ATP was ascertained by ammonium formate chromatography on DE81 paper (Rigby *et al.,* 1977). Filters were washed in 6xSSC-0.2%NaP_{pi} for fifteen minutes four times at room temperature, and then for thirty minutes at 50°C. Several positives were isolated from the onchocerca libraries but none proved positive in a

secondary screen.

Recently a tubulin genomic clone has been isolated in this laboratory by Dr. B.M. Pinto using the *P. falciparum* tubulin gene as a probe. This clone is 60% A + T rich in the coding region and 74.5% of the codons possess A or T in the third position, whilst in the noncoding region, the A + T richness is elevated to 80%,. I have composed a codon usage table on the basis of this tubulin clone and used it to backtranslate the sequences of known PFK's from different organisms. Gapping of the oligomer to these backtranslated sequences shows that if *O. volvulus* PFK is identical to the conserved sequence in this region and has a codon usage consistent with that of tubulin then the 51mer oligomer would have 84% identity to all mammalian sequences. The 51mer oligomer shows 67% and 74.5% identity to the enzymes from *E. coli* and *B. stearothermophilus* respectively.

PFK from *Onchocerca* may not have the same codon usage as tubulin and might also differ in its amino acid sequence to the other known sequences. Codon usage for oncho tubulin was used to backtranslate a variety of PFK's. The RMPFK genomic clone (exon twenty two) used as a probe was then compared to the backtranslated PFKs. The identities are shown in Table 3.

If *Onchocercal* PFK has a similar codon usage the that of the tubulin clone, then it is probable that using RMPFK as a probe would decrease the likelihood of detecting PFK in the *onchocerca* libraries.

	% SIMILARITY TO BACKTRANSLATED SEQUENCE	% ACTUAL SIMILARITY
HMPFK	70%	89%
RMPFK	71%	100%
SHPFK	66%	_
SLPFK	54%	_
HLPFK	53%	68%
MLPFK	54%	69%
HPPFK	56%	71%

Table 3

Backtranslated PFK sequences using *Onchocerca gibsonni* tubulin codon usage with comparison to RMPFK

3.6 PCR of *onchocerca* libraries using degenerate oligonucleotides.

Oligos were designed against a conserved area of PFK to see if it was possible to use PCR to amplify a PFK sequence directly from the library.

Oligo I corresponds to the sequence

Ser-Ile-Asp-Asn-Asp-Phe-Cys-Gly-Thr beginning at position 125. The oligo was made with the majority of possible codon usage built in. This oligo was:

TCN ATY GAY AAY GAY TTY TGY GGR ACN G

Oligo 2 corresponds to the sequence

Ser-Arg-Met-Gly-Val-Glu-Ala-Val-Met-Ala

Oligo 2 is GCC ATN ACN GCY TCN ACN CCC ATY CTN C

The reverse complement of this is

GN AGR ATG GGN GTN GAR GCN GTN ATG GC

Key: Y = C or T, R = A or G, N = A, C, G or T.

If *onchocerca* PFK corresponds to the sequence of mammalian PFK and is present in the libraries then a PCR product of ~ 470 bp would be amplified.

The conditions for the reaction were; 10µl oligo 1 ~3µg, 7.5µl of oligo 2 ~3µg, 20µl 5 x amplification buffer, 2µl of each dNTP at 10mM, 2 units of Taq polymerase, 40µl of amplified library. Oligonucleotides were kinased with ATP and polynucleotide kinase before use (Sambrook *et al.,* 1989). The mix was denatured for 10 minutes at 95°C before adding Taq polymerase and was then subjected to 25 cycles of 1 minute at 95°C, 2 minutes at 55°C and 1.5 minutes at 72°C. The Mali library gave the largest yield of a product ~ 500 bp but no single band. The human liver cDNA library, and onchocerca libraries (Toborou & Tom Unnasch) gave a variety of bands below 1kbp, Figures 11a and 11b show a representative sample of these PCR products.

Bands of ~500bp were excised from a low melting agarose gel and phenol chloroform extracted. Initial attempts to subclone these fragments into SmaI cut, phosphatased M13mp8 were unsuccessful so the fragmentswere "polished" with T4 DNA polymerase and Klenow enzyme and dNTP but proved to be unclonable for some reason. There have been reports that Taq polymerase contains a template independent polymerase activity and adds an extra dATP onto the ends of PCR products which will often prove to be unclonable. If this experiment were to be repeated, then the addition of a restriction enzyme site(s) to the 5' end of the oligos might

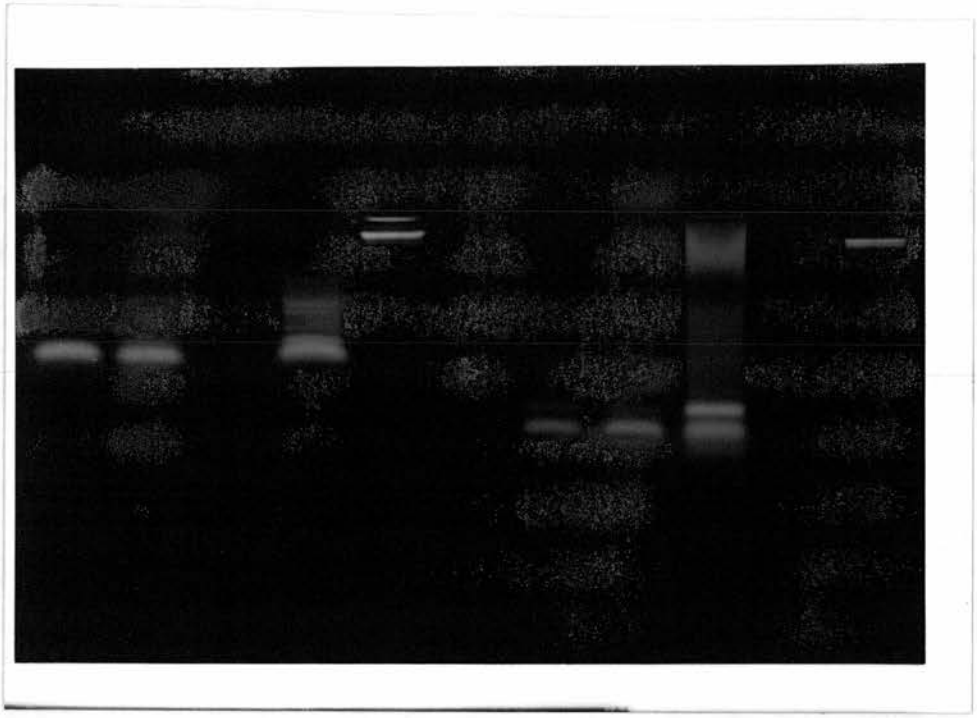


Figure 11a.

Agarose gel electrophoresis of PCR products from human liver cDNA and*Onchocerca volvulus* cDNA libraries and from genomic *Onchocerca guttorosa* DNA generated by using degenerate oligonucleotides corresponding to conserved areas of PFK.

Lane 1. Guatemala library

Lane 2. Human liver library

Lane 3. Kumaba library

Lane 4. Mali library

Lane 5. λcI*ts*857 HinDIII markers

Lane 6. Toborou library

Lane 7. Tom Unnasch library

Lane 8. *Onchocerca gibsonni* genomic DNA

Lane 10. λcI*ts*857 HinDIII markers

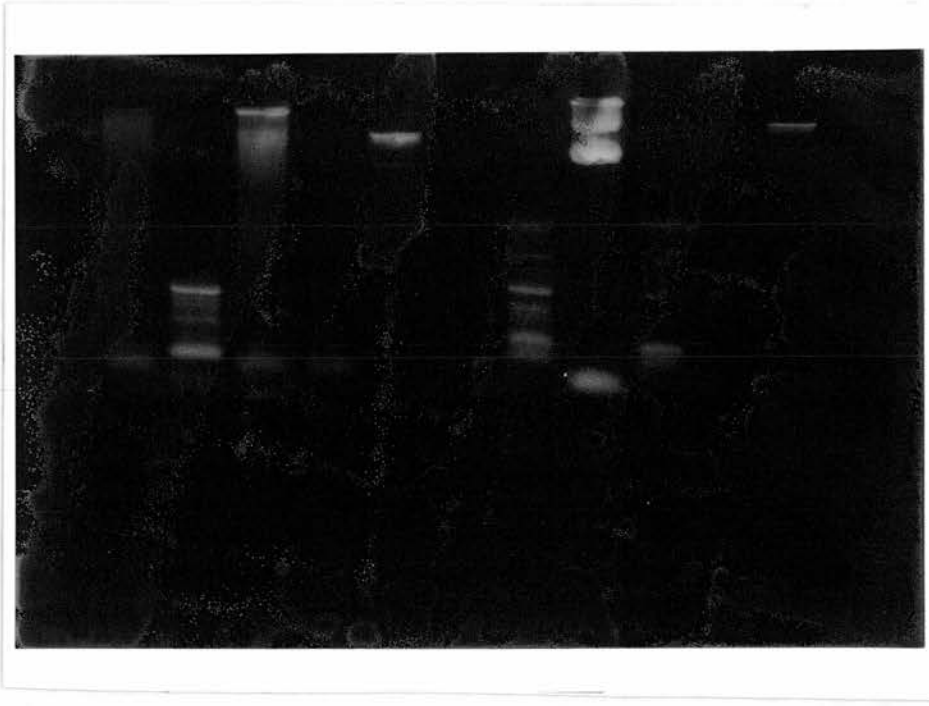


Figure 11b.

Agarose gel electrophoresis of PCR products from human liver cDNA and*Onchocerca volvulus* cDNA libraries and from genomic *Onchocerca gutturosa* DNA generated by degenerate oligonucleotides corresponding to conserved areas of PFK, and of rabbit muscle DNA PCR products generated for use as probes.

Lane 1. *Onchocerca guttorosa* genomic DNA

Lane 2. RMPFK exon one

Lane 3. *Onchocerca gibsonni* genomic DNA

Lane 4. Human liver library

Lane 5. λcI*ts*857 HinDIII markers

Lane 6. Mali library

Lane 7. RMPFK exon twenty two

Lane 8. Tom Unnasch library

Lane 9. 3μg PCR oligo

improve the 'clonability' of these fragments. PCR DNA might also be further purified by isolating bands from an agarose gel and subjecting a small amount of the DNA to a further round of PCR amplification.

Unfortunately the use of these fragments as probes back into the libraries resulted in all plaques giving a very strong signal after a high stringency wash. There may have been contamination of probe DNA by small amounts of λ DNA from the libraries which was present at the 500 bp level as a result of thermal scission or it may be that some part of the λ molecule has been amplified during the PCR reaction.

The fact that 500 bp fragments were generated as expected in some of the libraries makes it probable that thermal scission of λ DNA has taken place giving a background of λ DNA in the PCR product. The 500bp PCR fragment generated from the Mali library hybridises to the PCR reaction oligos indicating that the reverse complement DNA to the oligos is present in the probe DNA (results not shown). This indicates that some component of the library has been amplified.

It is possible that human PFK was amplified, as the libraries are reported to be contaminated by human PFK (John Donelson, personal communication). The Guatemala library is thought to comprise 6% human DNA and the other libraries may comprise about 1% human DNA. By its very nature, PCR would tend to amplify any contaminating human PFK but this must remain speculative as no sequence data is available on these PCR fragments.

CHAPTER FOUR

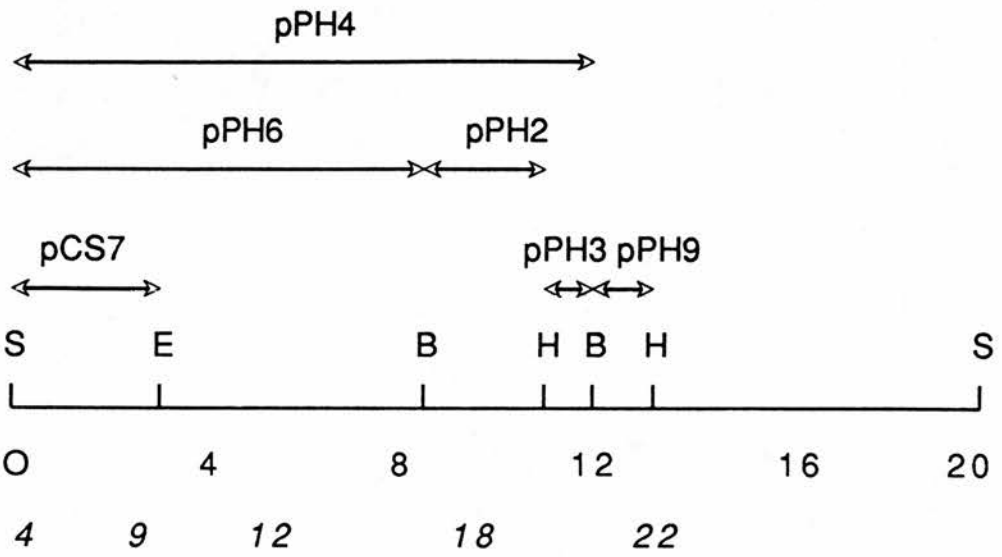
ISOLATION OF A cDNA ENCODING HUMAN MUSCLE PFK

4.1 Use of a genomic clone encoding HMPFK.

A genomic clone of HMPFK was isolated in this laboratory using RMPFK exon twenty two as a probe. The organisation of this clone is shown in figure 12. The clone was 19 kbp long and was thought to contain 7 kbp of PFK sequence. Several plasmids were generated with fragments of the 7 kb region and used as probes in the libraries. No definite positives could be found. After some time it was learned that the orientation of the coding region was incorrect and that previous plasmids contained non-coding sequence DNA. Some other fragments were subcloned and five were sequenced to ascertain the usefulness of the insert DNAs as probes.

The entire fragment containing the HMPFK genomic sequence was subcloned as pPH4, and used as a probe with the human liver gt10 library Ten positives were picked and 2 positives remained after a 4^{o} screen.

One positive clone proved to be a fragment of λ. The other positive, pCS8, contained a fragment which contained two Alu sequences. This was used as a probe against six fragments of the HMPFK genomic clone to find out which contained Alu sequences. A dot blot of the HMPFK genomic subclones probed with pCS8 is shown in figure 13. A signal was obtained for pCS8, pPH2 and pPH6 indicating that two Alu sequences were present in the HMPFK genomic clone or that one Alu sequence spanned the junction of clones pPH2 and pPH6. pCS7, pPH3 and pPH9 did not contain Alu sequences and could be used as probes.



Exon position, 4, 9, 12, 18, 22.

S = SalI, E = EcoRI, B = BamHI, H = HinDIII.

Figure 12. Gene structure of the human genomic clone and names of subclones.

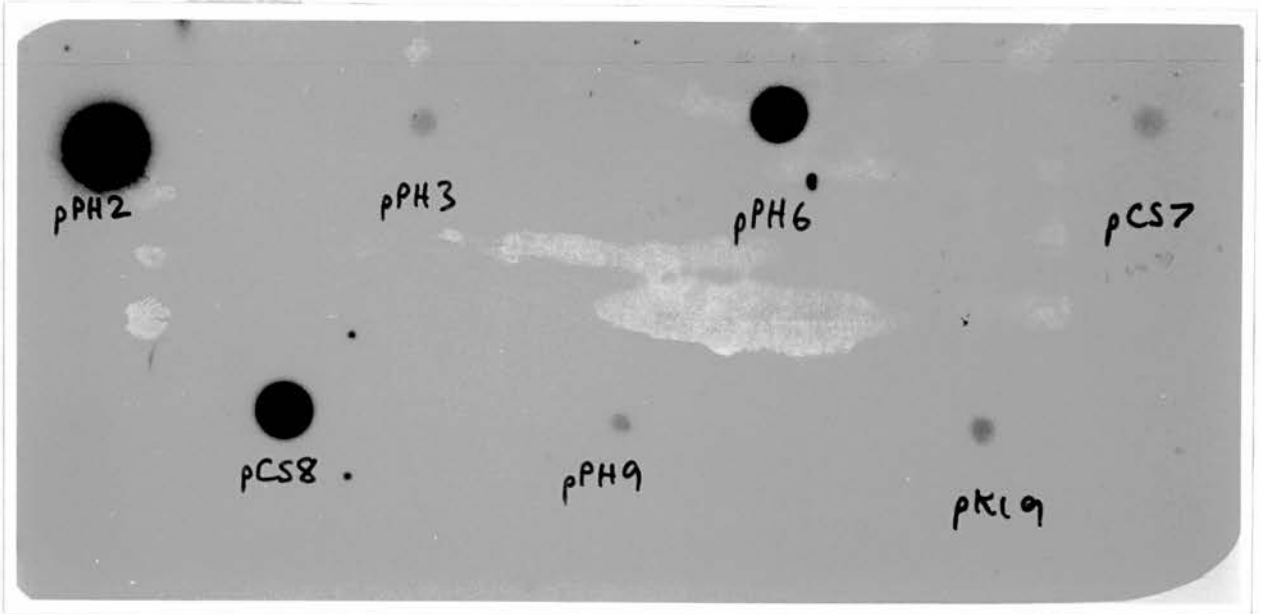


Figure 13

Dot blot of the subcloned fragments of the HMPFK genomic clone inserts from figure 10 probed with pCS8 labelled with Klenow enzyme and [^{32}P]. pPH2, pPH6 and pCS8 contain Alu sequences.

4.2 Isolation of a partial cDNA encoding HMPFK.

An HT1080 tumour cell line of fibrosarcoma in gt11 was a kind gift of Dr. Nigel Spurr (ICRF Clare Hall). This was probed with pPH3 and pPH9. Of four positives, one was HMPFK cDNA from the EcoRI site at 948 bp to 2750bp in the non-coding 3' end. This was designated pCS10.

Chapter FIVE

ISOLATION OF A cDNA ENCODING HUMAN PLATELET PFK

5.1 Isolation of a cDNA clone encoding human platelet PFK

pCS10 was used to probe a Raji B lymphocyte cell library in λgt10 (a kind gift of Dr. Nigel Spurr ICRF Clare Hall) and generated six positives. Two of these gave a strong signal which was not washed off at 1 x SSC, 70°C. The other four were washed off at the higher stringency. These were named R5-R8. R6-R8 proved difficult to sublcone. R5 was subcloned into pK19 and named pCS11. The initial sequence showed 66% identity to MLPFK and 64% identity to HMPFK. Amino acid identities were similar to DNA identities.

5.2 The sequence of the partial cDNA encoding HPPFK.

The sequence corresponded to HMPFK from 1500 bp to its 3' end. It contained a poly A site, a polyadenylation signal, 900bp. of coding region and 238bp. of downstream untranslated sequence. The DNA sequence and inferred amino acid sequence is shown in figure 14.

```
                                                                    60
ATTCCGAAGTACTTGGAAGAGATCGCCACACAGATGCGCACCAGCATCAACGCGCTGCTG
 I  P  K  Y  L  E  E  I  A  T  Q  M  R  T  S  I  N  A  L  L
                                                                   120
ATCATCGGTGGATTCGAGGCCTACCTGGGACTCCTGGAGCTGTCAGCCGCCCGGGAGAAG
 I  I  G  G  F  E  A  Y  L  G  L  L  E  L  S  A  A  R  E  K
                                                                   180
CACGAGGAGTTCTGTGTCCCCATGGTCATGGTTCCCGCTACTGTGTCCAACAATGTGCCG
 H  E  E  F  C  V  P  M  V  M  V  P  A  T  V  S  N  N  V  P
                                                                   240
GGTTCCGATTTCAGCATCGGGGCAGACACCGCCCTGAACACTATCACCGACACCTGCGAC
 G  S  D  F  S  I  G  A  D  T  A  L  N  T  I  T  D  T  C  D
                                                                   300
CGCATCAAGCAGTCCGCCAGCGGAACCAAGCGGCGCGTGTTCATCATCGAGACCATGGGC
 R  I  K  Q  S  A  S  G  T  K  R  R  V  F  I  I  E  T  M  G
                                                                   360
GGCTACTGTGGCTACCTGGCCAACATGGGGGGGCTCGCGGCCGGAGCTGATGCCGCATAC
 G  Y  C  G  Y  L  A  N  M  G  G  L  A  A  G  A  D  A  A  Y
                                                                   420
ATTTTCGAAGAGCCCTTCGACATCAGGGATCTGCAGTCCAACGTGGAGCACCTGACGGAG
 I  F  E  E  P  F  D  I  R  D  L  Q  S  N  V  E  H  L  T  E
                                                                   480
AAAATGAAGACCACCATCCAGAGAGGCCTTGTGCTCAGAAATGAGAGCTGCAGTGAAAAC
 K  M  K  T  T  I  Q  R  G  L  V  L  R  N  E  S  C  S  E  N
                                                                   540
TACACCACCGACTTCATTTACCAGCTGTATTCAGAAGAGGGCAAAGGCGTGTTTGACTGC
 Y  T  T  D  F  I  Y  Q  L  Y  S  E  E  G  K  G  V  F  D  C
                                                                   600
AGGAAGAACGTGCTGGGTCACATGCAGCAGGGTGGGGCACCCTCTCCATTTGATAGAAAC
 R  K  N  V  L  G  H  M  Q  Q  G  G  A  P  S  P  F  D  R  N
                                                                   660
TTTGGAACCAAAATCTCTGCCAGAGCTATGGAGTGGATCACTGAAAAACTCAAGGAGGCC
 F  G  T  K  I  S  A  R  A  M  E  W  I  T  E  K  L  K  E  A
                                                                   720
CGGGGCAGAGGAAAAAAATTTACCACCGATGATTCCATTTGTGTGCTGGGAATAAGCAAA
 R  G  R  G  K  K  F  T  T  D  D  S  I  C  V  L  G  I  S  K
                                                                   780
AGAAACGTTATTTTTCAACCTGTGGCAGAGCTGAAGAAGCAAACGGATTTTGAGCACAGG
 R  N  V  I  F  Q  P  V  A  E  L  K  K  Q  T  D  F  E  H  R
                                                                   840
ATTCCCAAAGAACAGTGGTGGCTCAAGCTACGGCCCCTCATGAAAATCCTGGCCAAGTAC
 I  P  K  E  Q  W  W  L  K  L  R  P  L  M  K  I  L  A  K  Y
                                                                   900
AAGGCCAGCTATGACGTGTCGGACTCAGGCCAGCTGGAACATGTGCAGCCCTGGAGTGTC
 K  A  S  Y  D  V  S  D  S  G  Q  L  E  H  V  Q  P  W  S  V
                                                                   960

tgacccagtcccgcctgcatgtgcctgcagccaccgtggactgtgtctgtttgtaacact
taagttatttatcagcactttatgcacgtattattgacattgaatacctaatcggcgag
tgcccatctgccccaccagctcccagtgcgtgctgtctgtggagtgtgtctcatgctttc
agatgtgatatgagcagaattaattaaacatttgcctatgAn
                        -------
```

Figure 14. Partial DNA sequence and inferred amino acid sequence of human platelet phosphofructokinase

R5-R8 were probed as plaques with pCS11 and pCS10. Only R5 gave a strong signal to pCS11 after washing filters at 0.1 x SSC 0.1% SDS at 65°C. The EcoRI insert of pCS11 was digested with Sau3A1 and subcloned into M13mp18 and M13mp19 for DNA sequencing. Sequencing was also performed on pCS11 directly using forward and reverse sequencing primers originally designed as PCR primers for inserts in pUC type plasmids. The insert was digested with Sma1 to produce two fragments which were subcloned into Bluescript K5+ (Stratagene), designated pCS17 and pCS18. The sequencing strategy is shown in Figure 15.

One oligo was synthesised to sequence across the area covered by the internal Sma1 site, and a Sau3A1 site just upstream of it.

The putative HPPFK sequence begins at position 1417 when compared to the HMPFK DNA sequence and at position 1414 when compared to the HLPFK sequence. The HPPFK sequence is shown in figure 15 along with the comparable sequence from HMPFK and HLPFK. The HPPFK sequence contains an open reading frame of 900 bp followed by a 3' downstream untranslated non-coding region of 238 bp, with the rare polyadenylation signal AATTAAA 12 bp before a poly A tail of 16 residues. HLPFK has a non-coding 3' region of 515 bp with a polyadenylation signal AATAAA 37 bases before the poly (A) tract. HMPFK has a 399 bp untranslated 3' region with the polyadenylation signal AATAAA 18 bp before the poly A tract.

The platelet amino acid sequence is 5 amino acid residues shorter than the muscle PFK and 6 residues shorter than liver PFLK. HPPFK is forecast to have a Mr of 86,500±1,500, HLPFK to have a Mr of 76,700±1,400 and HMPFK to have a Mr of 82,500±1,600 (Dunaway *et al.*, 1988). HMPFK and HPPFK have also been predicted to have Mr values of

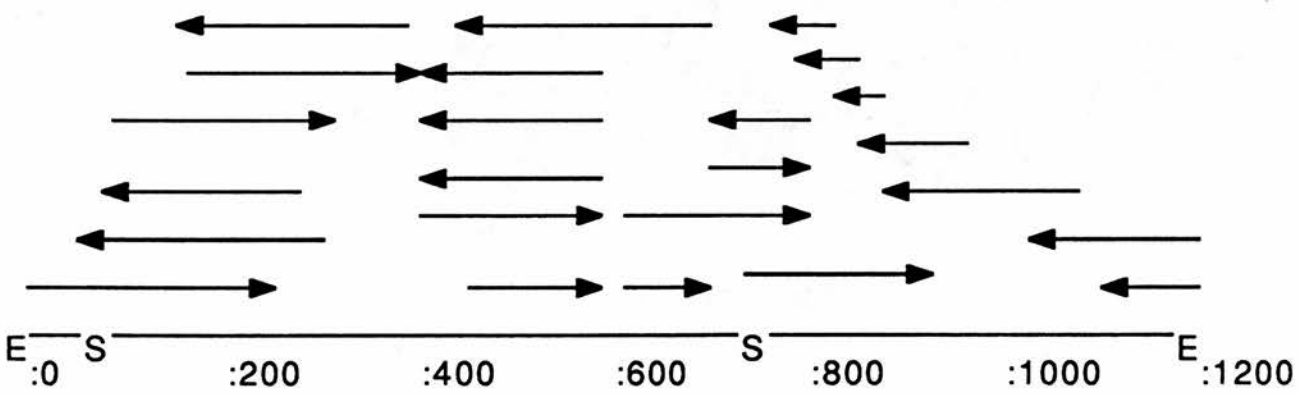
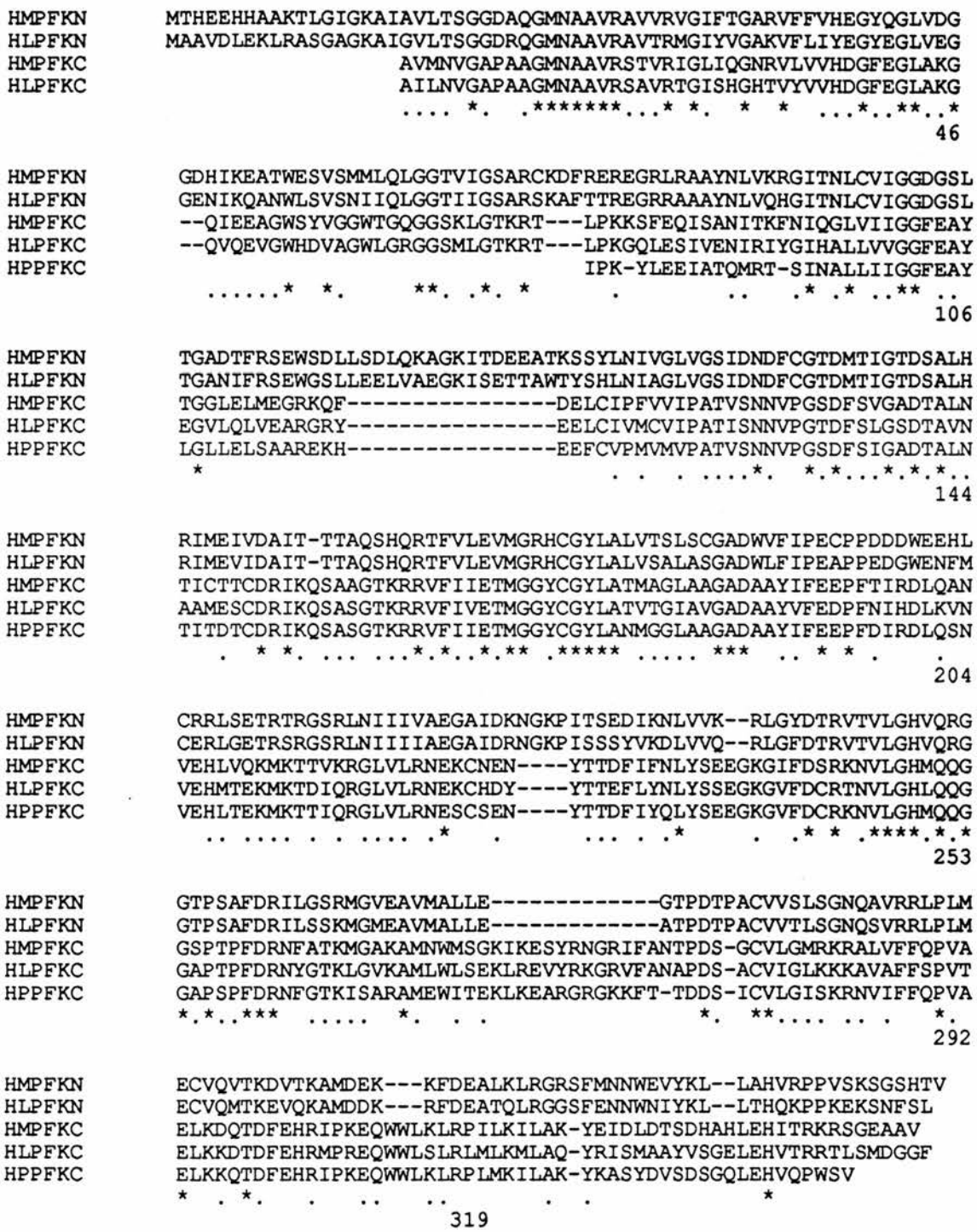


Figure 15. Sequencing strategy for the human platelet clone

E = EcoRI

S = SmaI



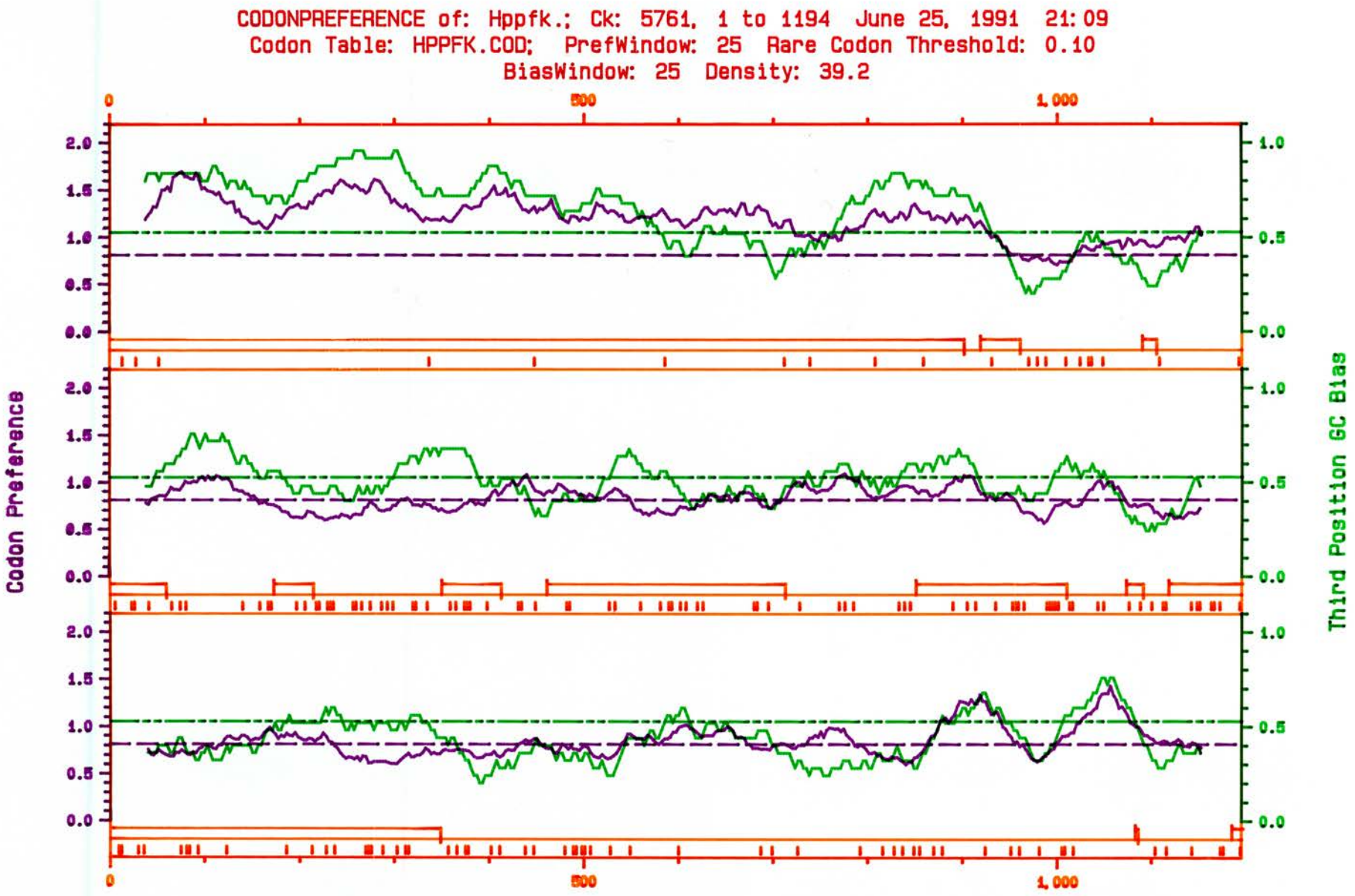
Key.
* = identity
. = similarity
N = AMINO HALF
C = CARBOXYL HALF
HMPFK = HUMAN MUSCLE PFK
HLPFK = HUMAN LIVER PFK
HPPFK = HUMAN PLATELET PFK
Numbering is according to B.stearothermophilus PFK
FIGURE 16. ALIGNMENT OF THE HUMAN PHOSPHOFRUCTOKINASES.

85,000 and HLPFK is given a Mr of 80,000 (Karadsheh *et al.*, 1977; Vora, 1982). The DNA sequence of HLPFK implies a Mr of 85,146 (Levanon *et al.*, 1989) while the DNA sequence for HMPFK implies a Mr of 85,050 (Nakajima *et al.*, 1987).

On the assumption that HPPFK has a similar amino acid sequence to the other human sequences, it is possible to speculate on the Mr of HPPFK. Adding the Mr of the muscle or liver enzyme residues proximal i.e. more amino to the platelet residues to the Mr of the known platelet sequence gives a Mr of 84,147 for HLPFK + HPPFK and 84,464 for HMPFK + HPPFK. This could possibly be interpreted as five to eight residues being missing from the HPPFK sequence. The area around the TGA stop codon was sequenced 4 times on each strand and the 3' sequence shows no obvious similarity to the other PFK DNA sequences or to amino acid sequences in any of the reading frames.

A codon usage table created from the HPPFK sequence by the UWGCG program CODONFREQUENCY was used to scan HPPFK with the UWGCG program CODONPREFERENCE . The output of this program is shown in Figure 16. The presumed open reading frame stops at position 904. A possible open reading frame starts at position 924 and continues for about 40bp. The 20 or so amino acids they represent do not have any similarity to other PFK sequences and it seems likely that the stop codon for HPPFK has been identified correctly.

The partial HPPFK has an isoelectric point of pH 6.98. This compares to isoelectric points of pH 8.68 and pH 7.91 for the partial homologous sequences from HMPFK and HLPFK respectively. The isoelectric points for the complete sequences are pH 7.92 and pH 7.47 for HMPFK and HLPFK respectively. This implies that the amino half of the human enzymes may


CODONPREFERENCE of: Hppfk.; Ck: 5761, 1 to 1194 June 25, 1991 21:09
Codon Table: HPPFK.COD; PrefWindow: 25 Rare Codon Threshold: 0.10
BiasWindow: 25 Density: 39.2
0
500
1,000
2.0
1.5
1.0
0.5
0.0
Codon Preference
Third Position GC Bias

have more acidic residues than the carboxyl halves. HPPFK levels are high in some cancerous cells (Vora, 1982, 1985a,b) and a low isoelectric point might thus be expected for this isoenzyme, given the high rates of glycolysis and low pH levels seen in these cells.

The partial HPPFK sequence has a G + C content of 54%. Sequence analysis shows that 71% of the bases at the third position of codons are either G or C. HLPFK is even more biased in this regard, with a G + C content of 60% and 85% third position G or C (Levanon *et al.,* 1989). Newgard *et al.* (1986) predicted that a high G + C content with G or C in the third codon position, is a hallmark of sequences coding for proteins expressed in muscle tissues. This was based on coding sequences for twenty four liver, and thirteen muscle proteins from human, rat and rabbit sources. Liver sequences were found to contain an average of 51±6% overall and 59±12% in the third codon position whereas muscle sequences contained 58±4% and 80±10% respectively.

If this theory is correct, then PFK sequences (especially HMPFK and HLPFK) would seem to break the rules. Table 4 gives the relative G + C contents of mammalian PFK's.

	CODING SEQUENCE G + C	THIRD CODON POSITION G + C
HMPFK	53%	59%
RMPFK	59%	75%
HPPFK	54%	71%
HLPFK	60%	84%
MLPFK	60%	72%

Berget (1984) has implicated the sequence CAYCG as being important for mRNA polyadenylation and processing. It lies just upstream or downstream of the polyA addition site. HPPFK has the sequence CATTTG which lies just upstream of the polyadenylation signal AATTAAA. HMPFK has the sequence CACTG and HLPFK has the sequence CATCG, both of which lie just upstream from the polyA addition site. The significance of the rare polyadenylation signal AATTAAA is unknown.

CHAPTER SIX.

COMPARISON OF HUMAN PLATELET PFK TO PROKARYOTIC AND EUKARYOTIC PFK

6.1. Similarities between human platelet PFK and other PFK's.

The sequences and tertiary structures of prokaryotic PFK's are known from *E. coli* and *B. stearothermophilus.* The sequences are also known for *S. citri* and *T. thermophilus* PFK. The subunit Mr of the bacterial enzymes is ~35,000 whereas that of the mammalian enzymes are ~78,000 - 86,000. Strong sequence similarity has been observed between the bacterial enzymes and between the amino and carboxyl halves of RMPFK. The pattern of sequence conservation suggests that the mammalian enzyme arose from a prokaryotic progenitor by gene duplication, fusion and divergence (Poorman *et al.,* 1984). The amino halves of mammalian PFK are more similar to the bacterial enzyme than are the carboxyl halves. The partial sequence of HPPFK shows ~30% identity to the bacterial enzymes and ~55% similarity if matched to conserved mutations.

Table 5 gives percentage similarities and percentage identities between all N and C halves of the eukaryotic enzymes. It can be seen that the platelet enzyme is more similar to HMPFK than HLPFK. Different explanations can be given for the differences between the liver and muscle/platelet types. The gene duplications to form three enzymes from one enzyme might have occurred at different times. Thus the platelet or muscle gene might have diverged more recently than the liver gene. It is also possible that the liver enzyme is evolving at a faster rate than the other isoenzymes.

	% SIMILAR	% IDENTICAL
HMC	83.3	70.7
RMC	82.7	69.7
HLC	80.3	63.3
MLC	80.1	63.7
SHC	79.2	64.4
SLC	77.0	62.3
Y2C	60.0	34.7
Y1C	58.5	35.7
Y1N	51.2	26.0
MLN	50.2	27.6
SLN	49.6	29.3
HLN	49.1	27.5
Y2N	49.0	22.6
RMN	48.4	27.2
HMN	47.5	26.9
SHN	45.5	20.9

Table 5. Pairwise comparisons betwen eukaryotic amino and carboxyl halves of PFK against HPPFKC. Alignments were performed using the modified GAP program from UWGCG version. 6.0,. Default gap weight 3.00, default gap length weight 0.10.

The aligned sequences of phosphofructokinase

GAPS ARE REPRESENTED AS --.
UNKNOWN SEQUENCE IS REPRESENTED AS A SPACE.
APPROXIMATELY THE FIRST 200 RESIDUES OF THE YEAST GENES ARE NOT INCLUDED
THE E.COLI SEQUENCE IS FROM FRENCH & CHANG, (1987). THE E.COLI SEQUENCE IN THE SEQUENCE DATABASES IS INCORRECT, PERSONAL COMMUNICATION, EVANS, P.R. (MRC,CAMBRIDGE)

```
                                         !                        !
                               A                 1            B       2
                             bbbbbb      aaaaaaaaaaaaaaaa  bbbbbb aaaaaaa
HUMUSCL-N    MTHEEHHAAKTLGIGKAIAVLTSGGDAQGMNAAVRAVVRVGIFTGARVFFVHEGYQGLVDGG-D
RABMUSCL-N   MTHEEHHAARTLGVGKAIAVLTSGGDAQGMNAAVRAVVRVGIFTGARVFFVHEGYQGLVDGG-D
SHEHEART-N   MTHEEHHEAKTLGIGKAIAVLTSGGDAQGMNAAVRAVVRVGIYTGARVFFVHEGYQGLVDGG-D
SHELIVER-N             TSGAGKAIGVLTSGGDAQGMNAAVRAVTRMGIVTGAKVFLIYEGYEGLVEGG-E
HUMLIVER-N   MAAVDLEKLRASGAGKAIGVLTSGGDRQGMNAAVRAVTRMGIYVGAKVFLIYEGYEGLVEGG-E
MUSLIVER-N   MATVDLEKLRMSGAGKAIGVLTSGGDAQGMNAAVRAVTRMGIYVGAKVFLIYEGYEGLVEGG-E
YEAST1-N                    K-IAVMTSGGDSPGMNAAVRAVVRIGIHFGCDVFAVYEGYEGLLRGG-K
YEAST2-N                    KAIAVMTSGGDAPGMNSNVRAIVRSAIFKGCRAFVVMEGYEGLVRGGPE
HUMUSCL-C                      AVMNVGAPAAGMNAAVRSTVRIGLIQGNRVLVVHDGFEGLAKG---
RABMUSCL-C                     AVMNVGAPAAGMNAAVRSTVRIGLIQGNRVLVVHDGFEGPAKG---
SHEHEART-C                     AVMNVGAPAAGMNAAVRST RIGLIQGNAVLVVHDGFEGLAKG---
SHELIVER-C                      VMNVGAPAAGMNAAVRSAVRSGISQGHTVYVVHDGFEGLAKN---
HUMLIVER-C                     AILNVGAPAAGMNAAVRSAVRTGISHGHTVYVVHDGFEGLAKG---
MUSLIVER-C                     AILNVGAPAAGMNAAVRSAVRTGISEGHTVYIVHDGFEGLAKG---
YEAST1-C                       GIVHVGAPSAALNAATRAATLYCLSHGHKPYAIMNGFSGLIQTG--
YEAST2-C                       AIVNVGAPAGGINSAVYSMATYCMSQGHRPYAIYNGWSGLNRHE--
E.COLI                    MIKKIGVLTSGGDAPGMNAAIRGVVRSALTEGLEVMGIYDGYLGLYE---D
B.ST                       MKRIGVLTSGGDSPGMNAAIRSVVRKAIYHGVEVYGVYHGYAGLIA---G
S.CITRI                   MLKKIGILTSGGDSQGMNAAIAGVIKTAHAKGLETYIIRDGYLGLIN---N
T.THERM                    MKRIGVFTSGGDAPGMNAAIRAVVRQAHALGVEVIGIRRGYAGMIQ---G
                            *  ..   *. . . *..  .        *     .  *. *
                           .1         .12          .25                      .47
```

```
                            !
              C      3                              4            D       5
             bbb  aaaaaaa                     aaaaaaaaaaaaa   bbbbbbb aaaaaaaa
HUMUSCL-N    HIKEATWESVSMMLQLGGTVIGSARCKDFRE-REGRLRAAYNLVKRGITNLCVIGGDGSLTGAD
RABMUSCL-N   HIREATWESVSMMLQLGGTVIGSARCKDFRE-REGRLRAAHNLVKRGITNLCVIGGDGSLTGAD
SHEHEART-N   NIREATWES                CKDFRE-REGRLAAAHNLVK GIGNLCVIGADGSLTGGD
SHELIVER-N   NIR                      CKAFTT-R     AAHNLVKRGITNLCVIGGDASLTGAN
HUMLIVER-N   NIKQANWLSVSNIIQLGGTIIGSARSKAFTT-REGRRAAAYNLVQHGITNLCVIGGDGSLTGAN
MUSLIVER-N   NIKPANWLSVSNIIQLGGTIIGSARCKAFTT-REGRLAAAYNLLQHGITNLCVIGGDGSLTGAN
YEAST1-N     YLKKMAWEDVRGWLSEGGTLIGTARSMEFRKGREGRRQAAGNLISQGIDALVVCGGDGSLTGAD
YEAST2-N     YIKEFHWEDVRGWSAEGGTNIGTARCMEFKK-REGRLLGAEHLIEAGVDALIVCGGDGSLTGAD
HUMUSCL-C    QIDEAGWSYVGGWTGQGGSKLGTKRTLPK-KSFEQ---ISANITKFNIQGLVIIGGFEAYTGGL
RABMUSCL-C   QIEEAGWSYVGGWTGQGGSKLGSKRTLPK-KSFEQ---ISANITKFNIQGLVIIGGFEAYTGGL
SHEHEART-C                            TLPK- SFEE---ITADI
SHELIVER-C   QVQEVSWHDVAGWLGRGGSMLGTKRTLPK-GYMEQ---IVESIRLHNIHALLVIGGFEAYEGVL
HUMLIVER-C   QVQEVGWHDVAGWLGRGGSMLGTKRTLPK-GQLES---IVENIRIYGIHALLVVGGFEAYEGVL
MUSLIVER-C   QVQEVGWHDVAGWLGRGGSMLGTKRTLPK-PHLEA---IVENLRTYNIHALLVIGGFEAYEGVL
HUMPLATE-C                             IPK-KYLEE---IATQMRT-SINALLIIGGFEAYLGLL
YEAST1-C     EVKELSWIDVENWHNLGGSEIGTNRSVASE-DLGT---IAYYFQKNKLDGLIILGGFEGFRSLK
YEAST2-C     SVRSLNWKDMLGWQRSGGSEIGTNRVTPEEADLGM---IAYYFQKYEFDGLIIVGGFEAFESLH
E.COLI       RMVQLDRYSVSDMINRGGTFLGSARCPEFRDE-NIRAVAIENLKKRGIDALVVIGGDGSYMGAM
B.ST         NIKKLEVGDVGDIIHRGGTILYTARCPEFKTE-EGQKKGIEQLKKHGIEGLVVIGGDGSYQGAK
S.CITRI      WIEVVDNNFADSIMLLGGTVIGSARLPEFKDP-EVQKKAVDILKKQEIAALVVIGGDGSYQGAQ
T.THERM      EMVPLGVRDVANIIQRGGTILLTARSQEFLTE-EGRAKAYAKLQAAGIEGLVAIGGDGTFRGAL
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                   .54                .73                                   110
              !                       !                               !
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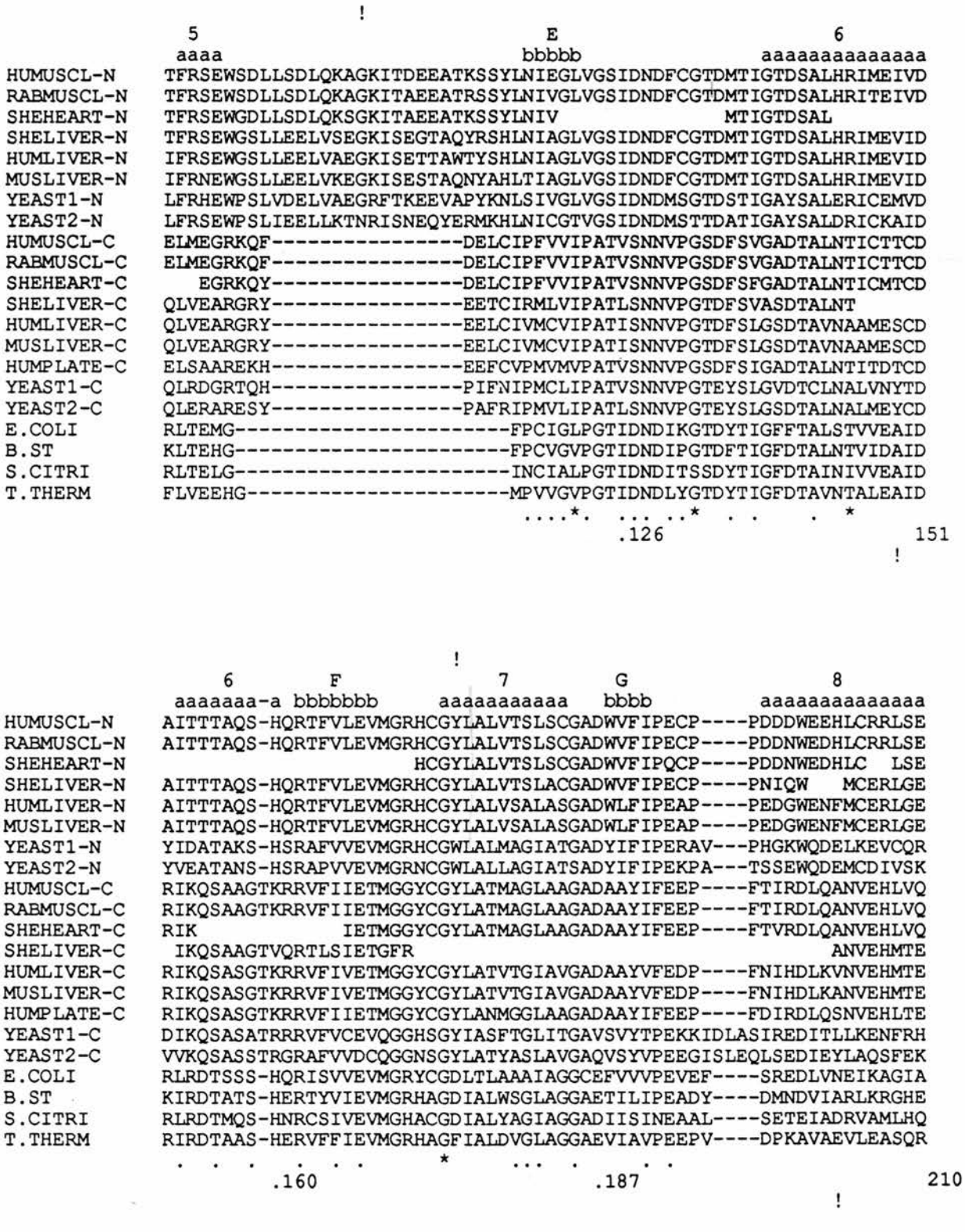
5
E
6
aaaa
bbbbb
aaaaaaaaaaaaaa
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SHEHEART-N TFRSEWGDLLSDLQKSGKITAEEATKSSYLNIV MTIGTDSAL
SHELIVER-N TFRSEWGSLLEELVSEGKISEGTAQYRSHLNIAGLVGSIDNDFCGTDMTIGTDSALHRIMEVID
HUMLIVER-N IFRSEWGSLLEELVAEGKISETTAWTYSHLNIAGLVGSIDNDFCGTDMTIGTDSALHRIMEVID
MUSLIVER-N IFRNEWGSLLEELVKEGKISESTAQNYAHLTIAGLVGSIDNDFCGTDMTIGTDSALHRIMEVID
YEAST1-N LFRHEWPSLVDELVAEGRFTKEEVAPYKNLSIVGLVGSIDNDMSGTDSTIGAYSALERICEMVD
YEAST2-N LFRSEWPSLIEELLKTNRISNEQYERMKHLNICGTVGSIDNDMSTTDATIGAYSALDRICKAID
HUMUSCL-C ELMEGRKQF----------DELCIPFVVIPATVSNNVPGSDFSVGADTALNTICTTCD
RABMUSCL-C ELMEGRKQF----------DELCIPFVVIPATVSNNVPGSDFSVGADTALNTICTTCD
SHEHEART-C EGRKQY----------DELCIPFVVIPATVSNNVPGSDFSFGADTALNTICMTCD
SHELIVER-C QLVEARGRY----------EETCIRMLVIPATLSNNVPGTDFSVASDTALNT
HUMLIVER-C QLVEARGRY----------EELCIVMCVIPATISNNVPGTDFSLGSDTAVNAAMESCD
MUSLIVER-C QLVEARGRY----------EELCIVMCVIPATISNNVPGTDFSLGSDTAVNAAMESCD
HUMPLATE-C ELSAAREKH----------EEFCVPMVMVPATVSNNVPGSDFSIGADTALNTITDTCD
YEAST1-C QLRDGRTQH----------PIFNIPMCLIPATVSNNVPGTEYSLGVDTCLNALVNYTD
YEAST2-C QLERARESY----------PAFRIPMVLIPATLSNNVPGTEYSLGSDTALNALMEYCD
E.COLI RLTEMG----------FPCIGLPGTIDNDIKGTDYTIGFFTALSTVVEAID
B.ST KLTEHG----------FPCVGVPGTIDNDIPGTDFTIGFDTALNTVIDAID
S.CITRI RLTELG----------INCIALPGTIDNDITSSDYTIGFDTAINIVVEAID
T.THERM FLVEEHG----------MPVVGVPGTIDNDLYGTDYTIGFDTAVNTALEAID
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151
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F
7
G
8
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bbbb
aaaaaaaaaaaaaa
HUMUSCL-N AITTTAQS-HQRTFVLEVMGRHCGYLALVTSLSCGADWVFIPECP----PDDDWEEHLCRRLSE
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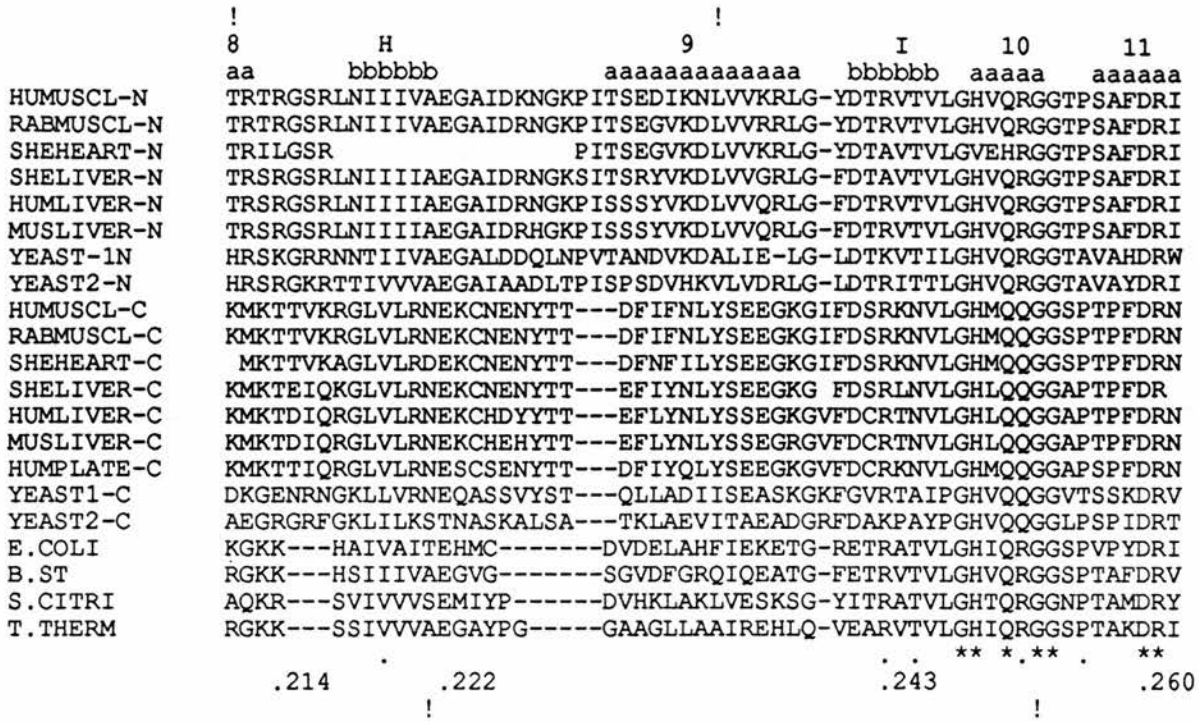
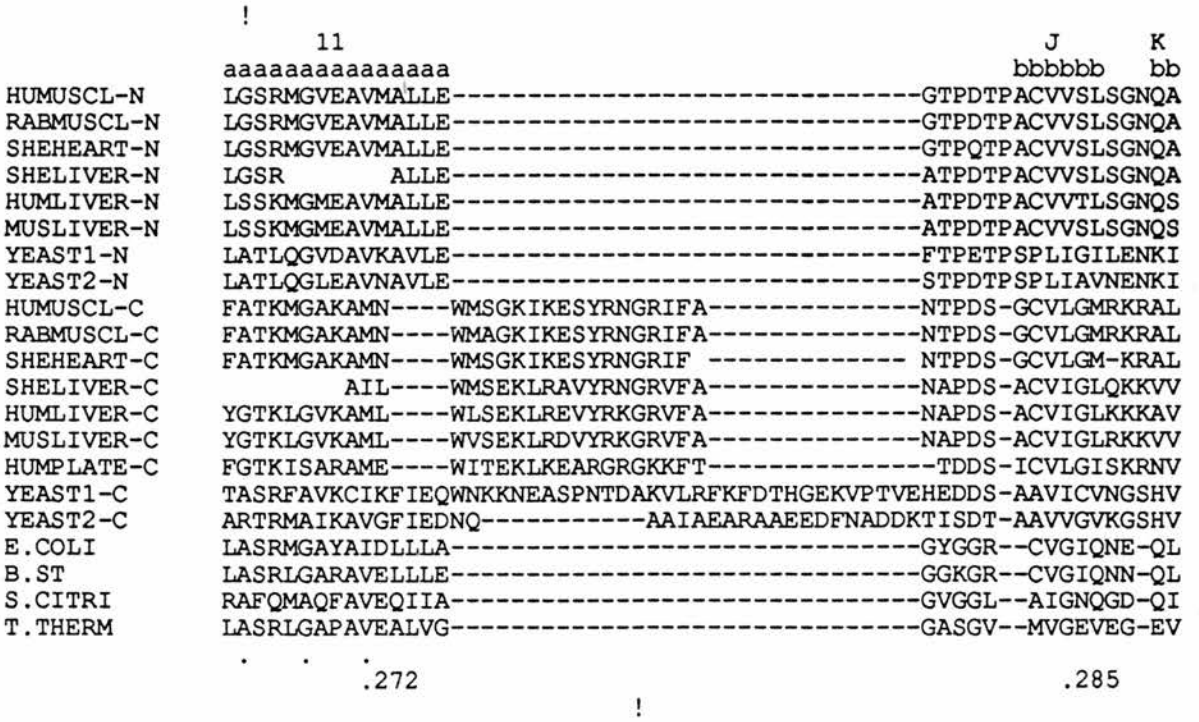
SHEHEART-N HCGYLALVTSLSCGADWVFIPQCP----PDDNWEDHLC LSE
SHELIVER-N AITTTAQS-HQRTFVLEVMGRHCGYLALVTSLACGADWVFIPECP----PNIQW MCERLGE
HUMLIVER-N AITTTAQS-HQRTFVLEVMGRHCGYLALVSALASGADWLFIPEAP----PEDGWENFMCERLGE
MUSLIVER-N AITTTAQS-HQRTFVLEVMGRHCGYLALVSALASGADWLFIPEAP----PEDGWENFMCERLGE
YEAST1-N YIDATAKS-HSRAFVVEVMGRHCGWLALMAGIATGADYIFIPERAV---PHGKWQDELKEVCQR
YEAST2-N YVEATANS-HSRAPVVEVMGRNCGWLALLAGIATSADYIFIPEKPA---TSSEWQDEMCDIVSK
HUMUSCL-C RIKQSAAGTKRRVFIIETMGGYCGYLATMAGLAAGADAAYIFEEP----FTIRDLQANVEHLVQ
RABMUSCL-C RIKQSAAGTKRRVFIIETMGGYCGYLATMAGLAAGADAAYIFEEP----FTIRDLQANVEHLVQ
SHEHEART-C RIK IETMGGYCGYLATMAGLAAGADAAYIFEEP----FTVRDLQANVEHLVQ
SHELIVER-C IKQSAAGTVQRTLSIETGFR ANVEHMTE
HUMLIVER-C RIKQSASGTKRRVFIVETMGGYCGYLATVTGIAVGADAAYVFEDP----FNIHDLKVNVEHMTE
MUSLIVER-C RIKQSASGTKRRVFIVETMGGYCGYLATVTGIAVGADAAYVFEDP----FNIHDLKANVEHMTE
HUMPLATE-C RIKQSASGTKRRVFIIETMGGYCGYLANMGGLAAGADAAYIFEEP----FDIRDLQSNVEHLTE
YEAST1-C DIKQSASATRRRVFVCEVQGGHSGYIASFTGLITGAVSVYTPEKKIDLASIREDITLLKENFRH
YEAST2-C VVKQSASSTRGRAFVVDCQGGNSGYLATYASLAVGAQVSYVPEEGISLEQLSEDIEYLAQSFEK
E.COLI RLRDTSSS-HQRISVVEVMGRYCGDLTLAAAIAGGCEFVVVPEVEF----SREDLVNEIKAGIA
B.ST KIRDTATS-HERTYVIEVMGRHAGDIALWSGLAGGAETILIPEADY----DMNDVIARLKRGHE
S.CITRI RLRDTMQS-HNRCSIVEVMGHACGDIALYAGIAGGADIISINEAAL----SETEIADRVAMLHQ
T.THERM RIRDTAAS-HERVFFIEVMGRHAGFIALDVGLAGGAEVIAVPEEPV----DPKAVAEVLEASQR
.160
.187
210


8 H 9 I 10 11
aa bbbbbb aaaaaaaaaaaaa bbbbbb aaaaa aaaaaa
HUMUSCL-N TRTRGSRLNIIIVAEGAIDKNGKPITSEDIKNLVVKRLG-YDTRVTVLGHVQRGGTPSAFDRI
RABMUSCL-N TRTRGSRLNIIIVAEGAIDRNGKPITSEGVKDLVVRRLG-YDTRVTVLGHVQRGGTPSAFDRI
SHEHEART-N TRILGSR PITSEGVKDLVVKRLG-YDTAVTVLGVEHRGGTPSAFDRI
SHELIVER-N TRSRGSRLNIIIIAEGAIDRNGKSITSRYVKDLVVGRLG-FDTAVTVLGHVQRGGTPSAFDRI
HUMLIVER-N TRSRGSRLNIIIIAEGAIDRNGKPISSSYVKDLVVQRLG-FDTRVTVLGHVQRGGTPSAFDRI
MUSLIVER-N TRSRGSRLNIIIIAEGAIDRHGKPISSSYVKDLVVQRLG-FDTRVTVLGHVQRGGTPSAFDRI
YEAST-1N HRSKGRRNNTIIVAEGALDDQLNPVTANDVKDALIE-LG-LDTKVTILGHVQRGGTAVAHDRW
YEAST2-N HRSRGKRTTIVVVAEGAIAADLTPISPSDVHKVLVDRLG-LDTRITTLGHVQRGGTAVAYDRI
HUMUSCL-C KMKTTVKRGLVLRNEKCNENYTT---DFIFNLYSEEGKGIFDSRKNVLGHMQQGGSPTPFDRN
RABMUSCL-C KMKTTVKRGLVLRNEKCNENYTT---DFIFNLYSEEGKGIFDSRKNVLGHMQQGGSPTPFDRN
SHEHEART-C MKTTVKAGLVLRDEKCNENYTT---DFNFILYSEEGKGIFDSRKNVLGHMQQGGSPTPFDRN
SHELIVER-C KMKTEIQKGLVLRNEKCNENYTT---EFIYNLYSEEGKG FDSRLNVLGHLQQGGAPTPFDR
HUMLIVER-C KMKTDIQRGLVLRNEKCHDYYTT---EFLYNLYSSEGKGVFDCRTNVLGHLQQGGAPTPFDRN
MUSLIVER-C KMKTDIQRGLVLRNEKCHEHYTT---EFLYNLYSSEGRGVFDCRTNVLGHLQQGGAPTPFDRN
HUMPLATE-C KMKTTIQRGLVLRNESCSENYTT---DFIYQLYSEEGKGVFDCRKNVLGHMQQGGAPSPFDRN
YEAST1-C DKGENRNGKLLVRNEQASSVYST---QLLADIISEASKGKFGVRTAIPGHVQQGGVTSSKDRV
YEAST2-C AEGRGRFGKLILKSTNASKALSA---TKLAEVITAEADGRFDAKPAYPGHVQQGGLPSPIDRT
E.COLI KGKK---HAIVAITEHMC-------DVDELAHFIEKETG-RETRATVLGHIQRGGSPVPYDRI
B.ST RGKK---HSIIIVAEGVG-------SGVDFGRQIQEATG-FETRVTVLGHVQRGGSPTAFDRV
S.CITRI AQKR---SVIVVVSEMIYP------DVHKLAKLVESKSG-YITRATVLGHTQRGGNPTAMDRY
T.THERM RGKK---SSIVVVAEGAYPG-----GAAGLLAAIREHLQ-VEARVTVLGHIQRGGSPTAKDRI
.214 .222 .243 .260


11 J K
aaaaaaaaaaaaaaa bbbbbb bb
HUMUSCL-N LGSRMGVEAVMALLE----------GTPDTPACVVSLSGNQA
RABMUSCL-N LGSRMGVEAVMALLE----------GTPDTPACVVSLSGNQA
SHEHEART-N LGSRMGVEAVMALLE----------GTPQTPACVVSLSGNQA
SHELIVER-N LGSR ALLE----------ATPDTPACVVSLSGNQA
HUMLIVER-N LSSKMGMEAVMALLE----------ATPDTPACVVTLSGNQS
MUSLIVER-N LSSKMGMEAVMALLE----------ATPDTPACVVSLSGNQS
YEAST1-N LATLQGVDAVKAVLE----------FTPETPSPLIGILENKI
YEAST2-N LATLQGLEAVNAVLE----------STPDTPSPLIAVNENKI
HUMUSCL-C FATKMGAKAMN----WMSGKIKESYRNGRIFA----------NTPDS-GCVLGMRKRAL
RABMUSCL-C FATKMGAKAMN----WMAGKIKESYRNGRIFA----------NTPDS-GCVLGMRKRAL
SHEHEART-C FATKMGAKAMN----WMSGKIKESYRNGRIF ---------- NTPDS-GCVLGM-KRAL
SHELIVER-C AIL----WMSEKLRAVYRNGRVFA----------NAPDS-ACVIGLQKKVV
HUMLIVER-C YGTKLGVKAML----WLSEKLREVYRKGRVFA----------NAPDS-ACVIGLKKKAV
MUSLIVER-C YGTKLGVKAML----WVSEKLRDVYRKGRVFA----------NAPDS-ACVIGLRKKVV
HUMPLATE-C FGTKISARAME----WITEKLKEARGRGKKFT----------TDDS-ICVLGISKRNV
YEAST1-C TASRFAVKCIKFIEQWNKKNEASPNTDAKVLRFKFDTHGEKVPTVEHEDDS-AAVICVNGSHV
YEAST2-C ARTRMAIKAVGFIEDNQ----------AAIAEARAAEEDFNADDKTISDT-AAVVGVKGSHV
E.COLI LASRMGAYAIDLLLA----------GYGGR--CVGIQNE-QL
B.ST LASRLGARAVELLLE----------GGKGR--CVGIQNN-QL
S.CITRI RAFQMAQFAVEQIIA----------GVGGL--AIGNQGD-QI
T.THERM LASRLGAPAVEALVG----------GASGV--MVGEVEG-EV
.272 .285

```
                          !                    !                    !
                  K   12              13
                 bbbaaaaaaaa    aaaaaaaaaaaa
HUMUSCL-N        VRLPLMECVQVTKDVTKAMDEKKFDEALKLTGRSFMNNWEVYKLLAHVRPPVSKSGSHTV
RABMUSCL-N       VRLPLMECVQVTKDVTKAMDEKRFDEAMKLRGRSFMNNWEVYKLLAHIRPPAPKSGSYTV
SHEHEART-N       VALPLMECVQVTKDVTRAMDERRFDEAMKLAGRSFMNNWEVYKLLAHVRPPKSKSGLHTV
SHELIVER-N       VRLPLMECVQMTKEVQKAMDEKRFDEAIQLRGRSFENNWNIYKLLAHKISKENTWNGGAV
HUMLIVER-N       VRLPLMECVQMTKEVQKAMDDKRFDEATQLRGGSFENNWNIYKLLTHQKPPKEKSNFSL
MUSLIVER-N       VRLPLMECVQVTKDVQKAMDEERFDEAIQLRGRSFENNWKIYKLLAHQKVSKEKSNFSL
YEAST1-N         IRMPLVESVKLTKSVATAIENKDFDKAISLRDTEFIELYENFLSTTVKDDGSELLPV
YEAST2-N         VRKPLMESVKLTKAVAIEAKDFKRAMSLRDTEFIEHLNNFMAINSADHNEPKLPKDKRLKI
HUMUSCL-C        VFQPVAELKDQTDFEHRIPKEQWWLKLRPILKILAKYEIDLDTSDHAHLEHITRKRSGEAAV
RABMUSCL-C       VFQPVTELQNQTDFEHRIPKEQWWLKLRPILKILAKYEIDLDTSEHAHLEHISRKRSGEATV
SHEHEART-C       LFQPVTELQEQTDFEHRIPKEQWWLK RPILK LAKYEIDLDTSEHAHLEHITRKASGEADI
SHELIVER-C       AFSPVTELQEQTDFEHR   EQWWLNLR    MLAHYRISMADYVSGELEHVTRR
HUMLIVER-C       AFSPVTELKKDTDFEHRMPREQWWLSLRLMLKMLAQYRISMAAYVSGELEHVTRRTLSMDKGF
MUSLIVER-C       AFSPVTELKKETDFEHRMPREQWWLNLRLMLKMLAHYRISMADYVSGELEHVTRRTLSIDKGF
HUMPLATE-C       IFQPVAELKKQTDFEHRIPKEQWWLKLRPLMKILAKYKASYDVSDSGQLEHVQPWSV
YEAST1-C         SFKPIANLWENETNVELRKGFEVHWAEYNKIGDILSGRLKLRAEVAALAAENK
YEAST2-C         VYNSIRQLYDYETEVSMRMPKVIHWQATRLIADHLVGRKRVD
E.COLI           VHHDIIDAIENMKRPFKGDWLDCAEKMY
B.ST             VDHDIAEALANKHTIDQRMYALSKELSI
S.CITRI          IARPIMEALSIPRSSRKEIWAKFDQLNQNIYQKS
T.THERM          DLTPLKEAVERRKDINRALLRLSQVLAL
                 .                          .319
                                !
```

Key:

Identity: *

Conservative: .

HUMUSCL HUMAN MUSCLE PFK
RABMUSCL RABBIT MUSCLE PFK
SHEHEART SHEEP HEART PFK
SHELIVER SHEEP LIVER PFK
HUMLIVER HUMAN LIVER PFK
MUSLIVER MOUSE LIVER PFK
YEAST1 SACCHAROMYCES CEREVISIAE PFK SUBUNIT 1
YEAST2 SACCHAROMYCES CEREVISIAE PFK SUBUNIT 2
HUMPLATE HUMAN PLATELET PFK
E.COLI ESCHERICHIA COLI PFK
B.ST BACILLUS STEAROTHERMOPHILUS PFK
S.CITRI SPIROPLASMA CITRI PFK
T.THERM THERMUS THERMOPHILUS PFK
! INDICATES THE POSITION OF INTRONS IN MAMMALIAN PFK.
aaaaa = alpha helix
bbbbb = beta sheet

Figure 18. The aligned sequences of phosphofructokinase

Similarities between muscle and liver C halves and HPPFK-C are ~80%. The muscle enzymes retain 70% identity to HPPFKC but the liver enzymes only retain 63% identity. This would imply that the platelet enzyme is more closely related to the muscle enzymes and that the liver enzymes have evolved further than muscle and platelet and have accumulated more conservative mutations.

6.2 Differences between eukaryotic and prokaryotic PFK's.

An alignment of the known sequences of PFK is shown in figure 18 and the known secondary structural positions are indicated.Because of insertions and deletions the numbering of residues is according to BPFK. This means that an extra number of residues will be referred to as n+the extra number of residues. Table 6 gives the number of insertions and deletions when the eukaryotic and prokaryotic enzymes are compared.

Whereas the amino half residues of the mammalian enzymes at the various catalytic and effector sites are virtually all identical to those found in the bacterial enzyme, these residues are often different in a mammalian carboxyl half. The bacterial and amino halves of the enzyme have Asp-103 and Gly-104. Asp 103-N coordinates a Mg^{2+} ion and Gly 104-N forms hydrogen bonds to the β-phosphate and '1'-oxygen of ATP which leaves no room for a side chain in this position (Hellinga & Evans, 1985). The carboxyl half of the mammalian enzymes have phenylalanine and glutamate at 103C and 104C respectively. This will probably mean that ATP cannot be bound by the carboxyl half and has changed its function from that of a catalytic to a regulatory site.

Relationship to B. Stearotherm-ophilus secondary structure amino half of enzymes	No. of. Residues	Probable Effects/Conformation
N-Terminus	+14	Exterior Structure
Between α2 & βC	+2	Exterior Loop
Between α–5 & βE	+23	Exterior Structure
Between α8 – βH	+3	Exterior Loop at effector site
Between βH & α9	+7	Exterior Loop
Proposed Hinge Segment	+30-33	Exterior secondary structure at end of α13
Carboxyl half of enzymes		
1 residue proximal to α4	-1	Exterior, unknown as gapping is poor here and may not be significant, might shorten exterior loop.
In α4	-3	"
1 Residue proximal of α4 in HPPFK	-1	"
Between α5 & βE	+7	Exterior Loop
Distal end of α6	+1	Close to residue 162-C (FBP binding site) and residue 154-C (ATP inhibition site)
Between α8 & βH	+3	Exterior Loop at ATP inhibition site
Between βH & α9	+5	Exterior Loop
Between α11 & βJ	+17-18	Exterior Loop
1 Residue proximal to βJ	+1	Unknown
C terminus	+17-23	Exterior secondary structure

Table 6: Insertions and Deletions of Amino Acids For Mammalian PFK Compared to B. Stearothermophilus.

Possible nature of insertions and deletions of residues in mammalian PFK compared to bacterial PFK.

N-half: Three extra residues are present in mammalian PFK-N between α8 and βH at positions 214+1, +2 and +3. Lys 213-N, Lys 214-N and His 215-N are proposed to be residues involved at the effector site of *B. stearothermophilus* PFK (Schirmer & Evans, 1990). The extra three residues may account for the lack of inhibition of the mammalian enzymes by PEP. The 8H loop at position 213-216 shows the only major movement at the allosteric effector site in the change from the T to the R state. This is coupled to a rearrangement of the 6F loop which determines the quaternary structure of the T or R states.

The seven extra residues between βH and α9 are close to Glu-222 which is involved with binding F6P.

Carboxyl half: One extra residue is present in mammalian enzymes at position 159+1. This area is crucial for the binding of F6P and is proposed to be a major part of the FBP activation site for the carboxyl half. It is also proposed to form part of the citrate binding site. Three extra residues are found between α8 and βH as is the case for the amino half and are presumably important for any movement of the 8H loop between the R and T states.

Four extra residues are present between βH and α9 whereas seven extra residues are present in the amino half compared to the bacterial enzymes. These residues may also contribute to the FBP binding site or may take part in changes to quaternary structure during inhibition or activation.

6.3.1 Major differences of residues between mammalian PFK and human platelet PFK.

An alignment of mammalian PFK is shown in figure 13. Numbering of residues is according to BSPFK.

It is important to notice major changes of charge e.g. between an arginine and a glutamate, or similarities such as between isoleucine and leucine. Residues may exhibit different properties in the microenvironment of a folded protein chain than those expected of an isolated amino acid. Bordo & Argos (1991) have compiled matrices for the preferred amino acid substitutions of fifty five sequences from nine groups of proteins including haemoglobin, immunoglobulins and serine proteases. The intention of these matrices is to allow the design of "safe" residue substitutions in site directed mutagenesis experiments. They are equally valid to use in comparing those residues which are the result of *in vivo* mutagenesis in enzymes such as mammalian phosphofructokinases. Preferred and non preferred substitutions vary according to whether the residue is buried or exposed. Buried residues are more constrained in their choice of replacement than exposed residues. On this basis, the differences between HPPFK and the other mammalian enzymes is shown in Table 7

HPC	HMC	RMC	SHC	SLC	HLC	MLC	POSITION
92 Residue not present in HPPFK							end of α4
113 S	M	M		V	V	V	end of α5
277 + 10 R	Y	Y	Y	Y	Y	Y	between α11 and βJ
277 + 15 K	I	I	I	V	V	V	between α11 and βJ
278 Residue not present in HPPFK.							
	N	N	N	N	N	N	between α11 and βJ
280 D	P	P	P	P	P	P	between α11 and βJ
282 + 1 I	G	G	G	A	A	A	start of βJ
319 + 1 Y	L	L	L	M	M	M	C terminus
319 + 13 Y	L	L	L	M	M	M	C terminus
319 + 25 Q	T	S	T	T	T	T	C terminus
319 + 26 P	R	R	R	R	R	R	C terminus
319 + 27 W	K	K	K	R	R	R	C terminus
316 + 28 S	R	R	A		T	T	C terminus
319 + 29 V	S	S	S		L	K	C terminus

Table 7. The differences between HPPFK and the other mammalian enzymes The majority of these changes occur in those residues which may continue alpha helix 11, and in the carboxyl tail. They may form part of an ATP inhibition site, or they may be involved in tetramer aggregation. A helical extension of α11 would probably project out towards the amino and carboxyl terminii, which may be involved in tetramer-tetramer association.

Acceptable residue changes.

Table 8 gives "acceptable" mutations.

On the assumption that a negative figure is an "unacceptable" mutation.

A	=	S, T, P, V, Q, E, D, N, G.
C	=	W, S.
D	=	K, H, Q, S, T, A, G, N, E.
E	=	S, T, A, G, N, D, K, H, Q.
F	=	M, I, L, Y, W.
G	=	S, T, A, E, D, N.
H	=	P, N, D, E, Q, W, K, R.
I	=	T, M, F, V, L.
K	=	S, T, N, D, E, Q, W, H, R.
L	=	M, I, F, V.
M	=	R, K, F, V, L, I.
N	=	S, T, A, G, K, R, H, Q, E, D.
P	=	S, T, R, H, Q, A.
R	=	Y, M, K, S, P, N, Q, H.
S	=	C, K, R, E, D, N, G, A, P, T.
T	=	S, V, I, K, E, D, N, G, A, P.
V	=	T, A, M, I, L.
W	=	, C, H, F.
Y	=	F, W.

This gives similarities to HPPFK of

	HMC	**RMC**	**SHC**	**SLC**	**HLC**	**MLC**
Similarity	78%	78%	77%	71%	77%	77%
Total No. of	82	82	78	86	103	103

residue changes

Yet another description of acceptable mutations of residues is described by the programme Simplify, available in the UWGCG suite. Residues which are similar are:

P, A, G, S + T	-	neutral, weakly hydrophobic
Q, N, E + D	-	hydrophilic, acid amine
H, K + R	-	hydrophilic, basic
I, L, V + M	-	hydrophobic
F, Y + W	-	hydrophobic, aromatic

Cysteine is considered to be unique.

This gives similarities to HPPFK of

	HMC	**RMC**	**SHC**	**SLC**	**HLC**	**MLC**
Acceptable	85%	85%	82%	79%	83%	84%

Whichever method is used to look at the similarity of the HPPFK amino acid sequence, to other mammalian PFK carboxyl halves, it is apparent that they share about 80% similarity. If all the other mammalian PFK carboxyl halves have similar residues but the HPPFK has a non-conserved residue (assuming a correct sequence and sequence lineup) at the same position, then there are fourteen differences. All occur between those residues predicted to form regular secondary structural components. Three major differences occur at the carboxy terminals. One of the more interesting differences is at position 280 where HPPFK has an aspartate residue whereas a proline is present in both the amino and carboxyl mammalian halves of the mammalian enzymes. A proline is also present in the amino halves of both yeast sequences. HPPFK has an aspartate residue

at this position as does YPFK1-C sequence, whereas YPFK2-C has a serine residue. This residue is just before βJ in the bacillus structure where the loop from helix eleven makes a sharp turn around beta sheet E. The 17 extra residues in the carboxy halves of the other mammalian sequences may be forced to make a sharp turn at position Pro 280-C

6.3.2 Secondary structural predictions for mammalian residues not directly comparable to bacterial PFK.

The ELIAS program is a modified version of Eliopoulos *et al.,* (1982) which includes the procedures of McLachlan (1978) and Kabat & Wu (1973). A reference for the modified version appears in Sawyer *et al.* (1987). Using the ELIAS program, the muscle, liver and platelet enzymes are predicted to continue α-11 to about position 277 + 10. The muscle and liver enzymes are then predicted to have a turn or coil followed by a β sheet then a turn or coil followed by βJ. The platelet prediction is similar but the probability for a β sheet is lower and is shown in figure 19.

These residues are on the exterior of the enzyme near the proposed ATP inhibition site (Poorman *et al.,* 1984). Lys 266-C is thought to be part of this site and the elongation of alpha helix 11 may be important for this site. The muscle and liver sequences are more alike between α 11 and βJ. The difference in sequence and number of residues may be important for the increased sensitivity of HPPFK to inhibition by ATP. Differences in the sequence and length of the carboxyl terminus after residue 319 may also explain variations between isoenzymes. The program is reasonably correct in its prediction of the 2° structure of BPFK when compared to crystal structure data. It must be borne in mind that the sequence alignment of the

mammalian enzymes may be incorrect in relation to the bacterial enzymes, but these predictions have a reasonable correlation with the bacterial crystal structure. The extra residues at the amino terminus of the mammalian enzymes may contribute to the ATP inhibition site. They are presumably capable of contacting those residues at the carboxyl terminus identified as being essential for ATP inhibition (Valiatis *et al.,* 1989). The first thirty five residues of BPFK were examined with ELIAS and the prediction agrees with the crystal structure data. The fourteen extra residues of HMPFK and HLPFK are predicted to contain an α helix before the first β strand. HMPFK contains two histidines and a lysine in this region, while HLPFK contains a lysine and and an arginine which may be capable of binding to ATP.

The partial HPPFK amino acid sequence appears to have evolved from a common ancestor PFK and to be more similar to the muscle than the liver enzyme. Comparing HPPFK to the carboxyl halves of HMPFK and HLPFK gives 71% and 63% identity respectively. If conserved mutations are also scored as matching however, these figures increase to 83% and 80% respectively. HMPFK compared to HLPFK gives an identity of 68% and 83% similarity if conservative replacements are allowed as matches. The muscle and platelet forms are therefore more similar to each other (and equidistant) in comparison to the liver enzyme

6.4.1 Comparison of residues contributing to catalytic and effector sites of PFK

Residues thought to contribute to the catalytic and effector sites in the bacterial enzyme can be identified by aligning the mammalian sequences with them as shown in figure 17. Usually the mammalian amino half residues are identical or similar to the bacterial ones. The two yeast sequences are included here (Heinisch *et al.,* 1989). Approximately the first

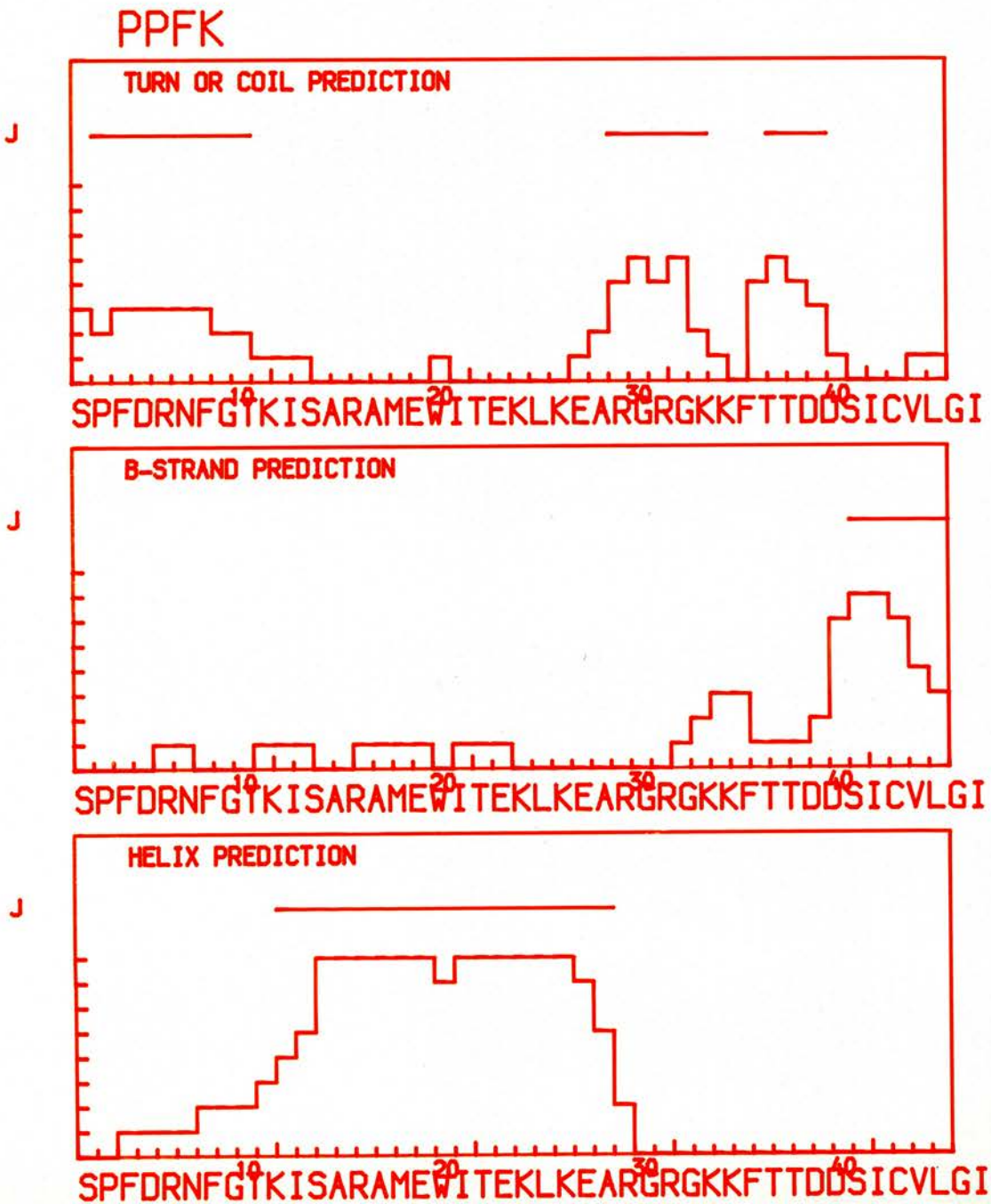


Figure 19. Secondary structure prediction for HPPFK in the area of alpha helix eleven.

two hundred residues of the yeast sequence are omitted as they have no equivalence with the other sequences. The secondary structure of the bacterial enzymes is from Shirakihara & Evans (1988).

TABLE 9. Comparison of catalytic residues.

S. citri and *T.thermophilus* are not included. N or C refers to amino or carboxyl domains. Where no differences are noted, residues are conserved across all species and domains. Residues are taken from Schirmer & Evans (1990).

EFFECTOR SITE

Arg 21 H-bond to β phosphate of ADP

Arg 25 H-bond to α and β phosphates

Val 57 H-bond to β phosphate of ADP via water molecule

Asp 59 main-chain amide H-bond to β phosphate of ADP via water molecule. Side chain H-bond to ribose O-3.

Met in HMPFK-N and RMPFK-N. Asn in HLPFK-N, MLPFK-N and YPFK1-C. Gly in other sequences.

Arg 154 H-bond to β phosphate of ADP

Thr in mammalian amino sequences, Asp in YPFK1-N, Glu in YPFK2-N, Lys for all carboxyl sequences.

Glu 187 Coordinated to Mg^{2+} of ADP and H-bond to His 215 via water molecule in R state only

Asp in all sequences except for Val in YPFK1-C and Gln in YPFK2-C

Arg 211 side-chain H-bonds to α phosphate of ADP and C-terminal residue

Thr in mammalian amino sequences. His in yeast amino sequences. Lys in mammalian carboxyl sequences. Asp in YPFK1-C and Ala in YPFK2-C.

Lys 213 H-bond to α phosphate of ADP

Lys 214 H-bond to O'_4 of ADP ribose
His 215 H-bond to Mg^{2+} of ADP via water

Gapping of sequences may be incorrect around this area. Three arginine residues are available in mammalian amino sequences to form H-bonds. The carboxyl sequences contain several basic residues in this region and as with the amino domain, have more residues than the bacterial enzymes at this point which may extend alpha helix 8.

ACTIVE SITE

Fructose 6-phosphate binding

Arg 72 H-bond to γ phosphate of ATP or 6-phosphate of F16BP

Asp 127 Catalytic residue. Increases nucleophilicity of 1-OH of F6P towards γ phosphate of ATP. H-bond to 3-OH of F6P

Asp in all amino sequences, Asn in all carboxyl sequences

Arg 162 H-bonds to 6-phosphate of F6P

Arg 243 H-bonds to 6-phosphate of F6P

Arg in all sequences except Lys in YPFK1-N and YPFK2-C

Arg 252 H-bond to O of F6P and H-bond to 6-phosphate of F6P

Arg in all amino sequences, Gln in all carboxyl sequences

ATP binding site

Arg 72 H-bond to γ phosphate of ATP or 6-phosphate of F16BP

Gly 11 Main chain amide H-bond to γ phosphate of ATP

Gly in all amino sequences, Ala in all carboxyl sequences

Cys 73 Main chain amide and carbonyl H-bonds to ATP ribose ring

HLPFK-N is Ser but this may be a sequencing error. The genomic sequence (exon 4) does not show complete identity in this area but both have a Ser at this point.

Asp 103 H-bonds to Mg^{2+} and β phosphate of ATP

Asp in all amino sequences, Phe in all carboxyl sequences

Gly 104 H-bonds to β phosphate of ATP

Gly in all amino sequences, Glu in all carboxyl sequences

Ser 105 H-bond to β phosphate of ATP

Ser in all amino sequences, Ala in all carboxyl sequences except for Ala in YPFK2-C

Thr 125 H-bond to β phosphate of ATP

Ser in all amino sequences, Thr in all carboxyl sequences

The mammalian residues assumed to participate in ligand binding are virtually always conserved across species and isoenzymes. Differences are more often found in carboxyl half residues which have mutated from the amino type. Twenty two residues are thought to be involved at catalytic and effector sites in the N and C halves, giving forty four residues in all from ~ 780 residues in a mammalian subunit (Hellinga, & Evans, 1987; Schirmer & Evans, 1990). The N half residues of the mammalian enzymes are identical to the *coli* or *bacillus* enzyme ten times , and the mammalian enzymes have the same residues but differ from the bacterial case once at position Thr-125. At this position, the carboxyl domain residues are identical to the bacterial enzymes. Where there are differences between the mammalian enzymes, it is usually a split between isoenzymes across species where the liver type is distinct from the muscle or platelet type. Differences caused by the SHPFK and SLPFK sequences might be possibly discounted as the sequence is

often different to the other enzymes at the ends of the peptide digests. The sheep sequences were both described by amino acid sequencing. Only one of the twenty two residues of the eukaryotic amino half shows a difference between the bacterial and eukaryotic enymes. Asp-59 which gives two hydrogen bonds to ADP, is methionine in HMPFK-N and RMPFK-N, asparagine in HLPFK-N MLPFK-N and YPFK1-C and glycine (except for unknown sequence for SHPFK-N and SLPFK-N) in all the other enzymes. Eleven residues are conserved between all the mammalian N and C halves and BPFK. Residues are conserved between the mammalian amino half and BPFK at seventeen locations. Differences in residue type between muscle and liver isoenzymes occurs once at position Asp-59. This residue (in the effector site) is proposed to make a hydrogen bond to ADP via a water molecule and a hydrogen bond to the ribose ring of ADP. The residue is methionine in HMPFK-N and RMPFK-N, and asparagine in MLPFK-N and HLPFK-N. Another possible change is seen at Cys-73 which forms hydrogen bonds with ATP in the active site. HLPFK-N is serine at this position but this may be a sequencing error as although the cDNA and genomic sequences have serine at this point, there are differences between the two sequences in exon 4 as a whole.

6.4.2 Contribution of domains and subunits to catalytic and effector sites in the mammalian tetramer.

The mammalian tetramer is known to possess D_2 symmetry (Hesterberg *et al.*,1981) which agrees with the observation of Klotz *et al.* (1975) that all known tetrameric proteins possess D_2 symmetry. Poorman *et al.* (1984) proposed a model for the quaternary structural organisation of RMPFK based on this symmetry arrangement and on its structural relationship to BSPFK.

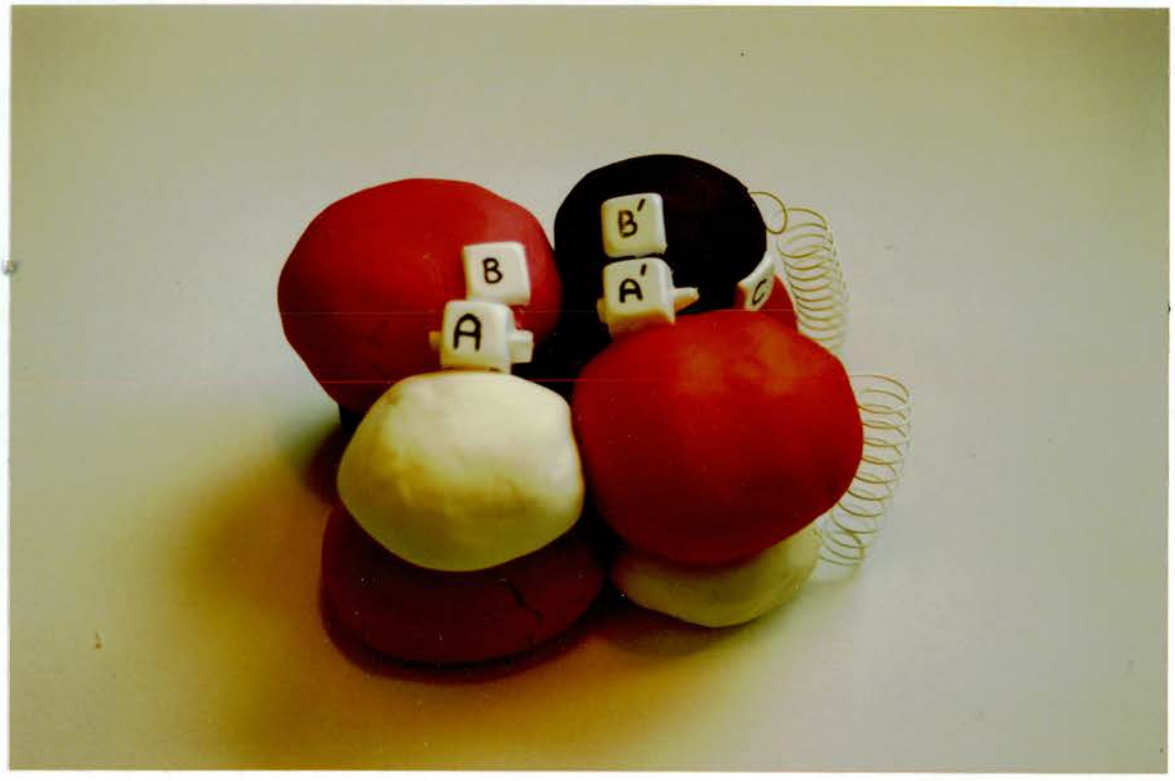


Figure 20a. A model of the mammalian dimer showing the F6P binding site A, the ATP catalytic binding site B, the proposed hexose bisphosphate binding site A', the equivalent site for ATP binding B' and the ADP effector site C.

The dark red ball represents the large domain of the amino half of a subunit and the white or purple balls represent a small domain. The orange ball represents the large domain of a carboxyl half.

Figure 20b. A model of the mammalian dimer showing the F6P binding site A, the ATP catalytic binding site B, the proposed hexose bisphosphate binding site A', the equivalent site for ATP binding B' and the proposed ATP inhibitor site C'.

The dark red ball represents the large domain of the amino half of a subunit and the white or purple balls represent a small domain. The orange ball represents the large domain of a carboxyl half.

Figure 20c. A model of the mammalian tetramer. The connecting peptides are represented by white coils.

Using crystal structure information from the bacterial enzymes (Schirmer & Evans, 1990) as a guide, close examination of the model shows that the catalytic F6P binding site and proposed F26BP site will be composed of residues from dimers from two subunits. This can be seen most readily by examining a model based on the structure of bacterial PFK, comparative sequence alignments and the result of limited digests of mammalian PFK with a variety of proteases.The model is shown in figures 20a,b and c

Table 10 gives the probable contribution of subunits to the various ligand binding sites of mammalian PFK

	F6P binding site			F26BP binding site	
			Subunit		Subunit
Arg-72	Large domain.	amino half.	1	carboxyl half	1
Thr-125	"	".	1	"	1
Asp-127	"	"	1	"	1
Arg-252	"	"	1	"	1
Arg-162	Small domain.	carboxyl half.	2	amino half	2
Arg-243	"	"	2	"	2
Gly-170	Between domains.		1	Between domains	1
Glu-222	"		1	"	1
His-249	"		1	"	1

The ATP catalytic site is likely to be composed of residues from the amino half of subunit one. The residues involved are:
Arg-72, Gly-11, Tyr-41, Phe-73. Asp-103, Gly-104, Ser-105. It is not clear if

the ATP catalytic site has mutated to form a new effector site in subunit two.

The ADP effector site is composed of residues from subunit one whereas subunit two may have residues contributing to the ATP inhibition site.

		ADP activation site		ATP inhibition site	
			Subunit		Subunit
Arg-21	Large domain	carboxyl half	1	amino half	2
Arg-25	"	"	1	"	2
Ser-58	"	"	1	"	2
Asp-59	"	"	1	"	2
Arg-154	Small domain	amino half	1	carboxyl half	2
Gly-185	"	"	1	"	2
Glu-187	"	"	1	"	2
Lys-211	"	"	1	"	2
Lys-213	"	"	1	"	2
Lys-214	"	"	1	"	2

Table 10. Contribution of mammalian subunits to ligand binding sites

Of the other residues identified by Hellinga & Evans (1987) as being potentially important for PFK activity, HPPFK differs from all the other subunits at one conserved position, 107C. Residue 107 is proposed to make a hydrophobic contact to the adenine ring in the ATP catalytic site in the bacterial enzymes. This residue is a methionine in *E. coli* and a glutamine in *B.stearothermophilus.* Residue 107N in all mammalian PFK sequences is a threonine. Residue 107C is threonine for HMPFK-C and RMPFK-C, glutamate for the liver carboxyl sequences while SH 107C is unknown. This

residue is a leucine in HPPFK-C. The HPPFK DNA sequence has been sequenced on both strands at this position. The ATP catalytic binding site may perform no function in the carboxyl half of the eukaryotic enzyme, although it may still be part of a nucleotide binding site Residues are conserved between N and C halves of the mammalian enzymes, but differ from the bacterial enzymes at position 187. Glu 187 is an aspartate in the mammalian enzymes but this is essentially a conservative change. Asp 187 is one of the few residues in the effector site to show a conformational change between the R and T states (Schirmer & Evans, 1990). In the R state, Glu 187 is coordinated to the Mg^{2+} ion of ADP, and is rotated away from the ligand in the T structure. The side chain adopts a new position between the central β sheet of the small domain and helix 9. This movement might presumably be part of the mechanism which changes the conformation of the active site.

The carboxyl terminal residue is a valine for muscle and platelet enzymes, phenylalanine for human liver and mouse liver, isoleucine for sheep heart and arginine for sheep liver.

6.5 The carboxyl terminal tail

When mammalian PFK amino and carboxyl halves are aligned with the bacterial enzymes there are two stretches at their carboxyl tails which 'overhang' by about 30 residues. The amino half overhang is considered to be a connecting peptide between the two halves which are equivalent to a bacterial dimer. This connecting peptide appears to be buried within a mammalian tetramer at the dimer-dimer interface (Poorman *et al.*, 1984). This idea is also favoured by model building studies. The carboxyl half

overhang is 30-36 residues and is thought to contribute to the ATP inhibition site not present in the bacterial enzymes and also to be the site of phosphorylation. Gottschalk *et al.* (1983) partially digested RMPFK with subtilisn and assumed that N-terminal regions contained an ATP inhibition site which was a mutated ADP activation site, as amino terminal fragments were released. Vlahatis *et al.* (1987) briefly exposed RMPFK to *S.aureus* V8 protease. This resulted in an enzyme which exhibited markedly reduced inhibition by ATP. This treatment resulted in the release of two carboxyl terminal peptides that comprised the last 17 residues of the enzyme. Limited proteolysis by trypsin which removes the last seven or eight residues of the carboxyl terminus had no significant effect on ATP inhibition. This implies that the RMPFK sequence His-Ala-His-Leu-Glu-His-Ile-Ser-Gln is the important site of ATP inhibition. Limited digestion of RMPFK with subtilisin removes 50-60 residues from the N-terminus and removes the ability to bind ATP at the inhibition site (Poorman *et al.,* 1984). Previous studies have indicated that three histidines were implicated in the binding of ATP to the inhibitory site of SHPFK on the basis of modification by diethyl pyrocarbonate. Three histidines are present in the RM, HM and SH sequences at this point but only one is present in the HP, HL, SL and ML sequences. The observed inhibition of human PFK by ATP is highest for isoenzymes containing large amounts of platelet type subunit. Liver type tetramers show less inhibition, with muscle type PFK being least inhibited by ATP. The platelet sequence is five residues shorter than the muscle sequences and six residues shorter than the liver sequences. The missing residues contain a serine which is believed to be the site of phosphorylation. Foe and Kemp (1984) reported that phosphorylation of brain PFK was possible. Although brain does contain the platelet type subunit, it also

consists of 14% liver, and 58% muscle type subunits (Dunaway *et al.,* 1988). The cloning and expression of a full length cDNA platelet clone would permit *in vitro* studies on phosphorylation of platelet PFK. Sequences have been reported with possible phosphorylation sites for all three rabbit enzymes (Valiatis *et al.,* 1989).

It is interesting to speculate on the role of phosphorylation, and the binding of actin, calmodulin and ATP to mammalian PFK. The carboxy terminal tail contains the low affinity binding site for calmodulin (Buschmeier *et al.* 1987) and is also reported to contain the site of phosphorylation. It also contributes residues to the ATP inhibition site and shows sequence conservation of several residues in mammalian PFK. Phosphorylation inhibits calmodulin binding and vice versa. A low affinity calmodulin binding site has only been reported for the muscle enzyme and it is unknown if it is present in the other isoenzymes.

It may be possible that high concentrations of calmodulin in contracting muscle stabilise the active tetramer and may also promote the formation of active polymeric PFK, although it has been reported that PFK in contracting muscle is phosphorylated to a greater extent than in resting muscle (Hofer & Sorenson-Ziganke, 1979). PFK bound to actin may form part of a glycolytic complex of enzymes on the cytoskeleton and it is interesting that active polymers of muscle PFK and actin bound PFK are less sensitive to inhibition by ATP. Calmodulin and/or actin binding may prevent access of ATP to the ATP inhibitory site and may also prevent citrate inactivation. The citrate inhibition site might contact the carboxyl terminus as citrate and ATP inhibition act synergistically to inhibit PFK.

It has been shown that PFK has a tendency to self-associate to oligomeric forms higher than the tetramer (Uyeda, 1979). High Mr

aggregates have a higher affinity for F6P than for MgATP. The reverse is true for monomers and dimers (Reinhart & Lardy, 1980b). The quaternary structure of pig liver PFK has been studied by electron microscopy (Foe & Trujillo, 1980), and particles ranging from tetramers to long flexible chains have been observed. The geometry of association of the pig liver enzyme implied that chains are formed by end to end association of tetramers rather than by tetramer stacking. It is likely from examination of the model that the most terminal amino and carboxyl residues of PFK are important for polymer chain formation. Liver PFK self associates to a larger extent than muscle PFK under comparable conditions (Trujillo & Deal, 1977; Reinhart & Lardy, 1980b) while HPPFK does not self-associate beyond the active tetramer (Foe & Kemp, 1985). Differences in the length and sequence of the carboxyl terminal tail between isoenzymes may be responsible for these effects and it is notable that HPPFK has a shorter carboxyl terminal tail than the other isoenzymes.

The availability of a cloned and expressed HPPFK would be useful to investigate its aggregation characteristics as a homotetramer, and in association with the other two subunits as it has been reported that the platelet enzyme does not aggregate beyond the active tetramer (Foe & Kemp, 1985).

It is possible to make structural predictions for the carboxyl tail of the platelet enzyme using the Elias program and the peptide structure program of UWGCG. Beginning at 282C (*B. stearothermophilus* numbering) the prediction is a continuation of α-helix to 319 + 10 folllowed by a β-sheet and then a turn or coil. The human muscle and liver enzymes have the same predicted structure (not shown). Figure 21 shows the prediction for the carboxyl terminal residues of HPPFK.This prediction of the platelet structure

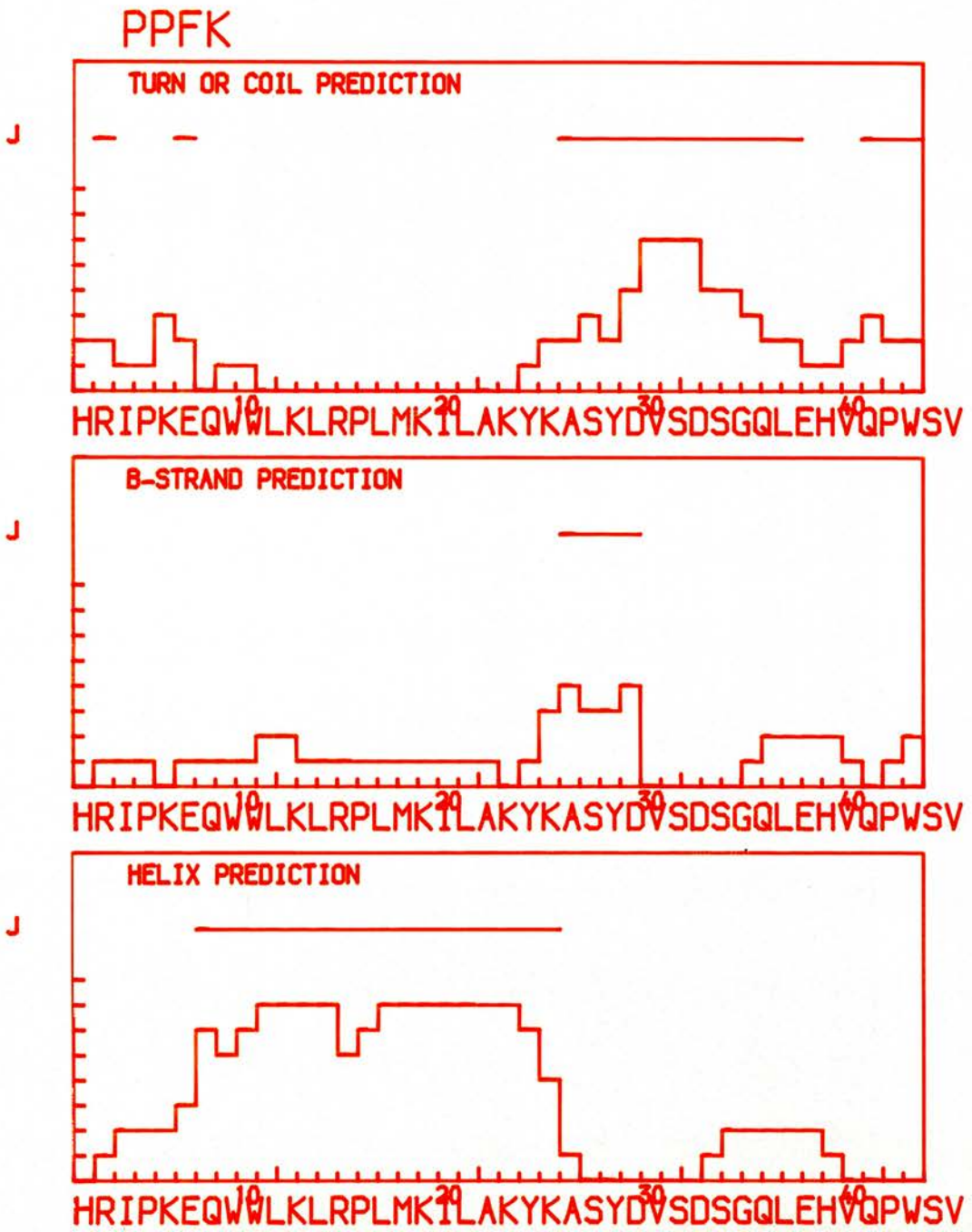


Figure 21. Secondary structure prediction for HPPFK in the area of the carboxyl terminal tail

is in complete agreement with the crystal structure of the bacterial enzyme. The last 10 residues of the platelet enzyme are not predicted to have any strong 2° structure. It is perfectly feasible for the C-terminal tail to contact the ADP effector site of the C half and that this site has mutated to form the ATP inhibition site. The C-terminal tail might also interact with the citrate binding site. This was identified as around the sequence 154C-161C. The sequence of all the mammalian PFK's is highly conserved in the citrate binding region.

The HPPFK sequence is

Arg-Ile-Lys-Gln-Ser-Ala-Ser-Gly-Thr-Lys-Arg-Arg

\+ + + + +

\+ = positively charged residues

This sequence is identical to HLPFK and MLPFK. HMPFK and RMPFK replace the second serine residue with an alanine residue. These residues are present at the C-terminus of α-6 which is very close to the ADP effector site of the bacterial enzyme and could be contacted by the C-terminal tail of the mammalian enzyme.

It has already been noted (Section 1.4) that phosphorylation does not appear to markedly influence the activity of mammalian PFK but that phosphorylation activates PFK from *Ascaris suum* and *Fasciola hepatica.* The phosphorylation site in *Ascaris suum* PFK has been isolated and sequenced as Ala Lys Gly Arg Ser Asp Ser(P) Ile Val Pro Thr (Kamemoto & Mansour, 1986). It is quite different from the phosphorylation site derived from RMPFK.

A search of the NBRF protein database reveals that the *Ascaris* sequence is similar to part of the bacterial enzymes beginning at position

210.

Ascaris	A K G R S D S I V P T
E. coli	A K G K K H A I V A I
Bacillus	E R G K K H S I I I V

This area is proposed by Schirmer & Evans (1990), to be part of the ADP effector site of the bacterial enzymes. By implication, Poorman *et al.* (1984) have proposed that this has mutated to become an ATP inhibition site in the carboxyl half of the eukaryotic enzymes. The alignment of the eukaryotic and bacterial enzymes introduces an extra three residues between Lys-214 and His-215. Examination of the crystal structures of the bacterial enzymes shows that residue 216 is virtually buried in the enzyme and is unlikely to be accessible to a protein kinase. The three extra residues in the eukaryotic enzyme may change the position of this residue however. The phosphorylation of this residue in the parasitic enzymes might be responsible for preventing the access of ATP to this area and/or inducing a conformational change which favours the R state. Residue 216 of the bacterial enzymes is part of the 8H loop which shows the only major conformational change in response to the change of ligand in the effector site and the transition between the R and the T states (Schirmer & Evans, 1990).

6.6 The nucleoside binding site.

Kemp *et al.* (1987) proposed the position of the citrate binding site. They also sequenced a peptide from SHPFK which was not involved in citrate binding but may be part of a nucleoside phosphate binding site. The RMPFK sequence is from 266 C ---> 280C and has the sequence Met Gly Ala Lys Ala Met Asn Trp Met Ala Gly Lys. In the carboxyl half, residue 266 is an arginine proposed to be part of the ATP inhibition site (Poorman *et al.*,

1984). This stretch of residues is present from the middle to the end of α-11.

The lysine which corresponds to the inhibition site arginine, present just before Met-266-C is conserved throughout the mammalian C halves.

6.7 The Calmodulin binding sites

Buschmeier *et al.* (1987) found two binding sites for calmodulin after digesting RMPFK with cyanogen bromide. A high affinity site is located in the connecting peptide of the subunit where two dimers are proposed to make contacts if associating to form an active tetramer. A low affinity site is present at the C-terminus and contains the site which is phosphorylated by cAMP-dependent protein kinase. Binding of CaM is known to prevent phosphorylation and vice versa. The connecting peptide is not present in this partial sequence of HPPFK and awaits discovery.The muscle and heart enzymes are similar and the liver enzymes are similar to each other.

```
HM    N N W E V Y K L L A H V R P P V S K S G S H T V A V M
                  *       *   *         *       *

RM                          I       A P         Y
SH                                  K         L
                                    *
HL          N I         T   Q K     K E     N - F S L   I L
                              *     *
ML          K               Q K V S K E     N   F S L   I L
            *                 *     *
SL          K                 K I S K E N T W N G A V
            *                 *     *
```

Positively charged residues are marked with *.

The low affinity binding site was identified as the residues from Arg-288-C to the carboxyl terminus. This portion of RMPFK contains sixteen basic residues out of a total of sixty seven. The calmodulin binding domains appear to require a tryptophan residue and the low affinity site possesses two tryptophans in an area of particularly high sequence conservation of all the mammalian enzymes.

The percentage of basic residues in the proposed low affinity binding site of mammalian PFK sequences is shown in table 12.

HM	RM	SH	SL	HL	ML	HP
25%	24%	22%	19%	22%	22%	21%

Table 12. Percentage of basic residues in the low affinity mammalian PFK calmodulin binding site

6.8 The hexose bisphosphate binding site.

It seems probable that the doubling of the bacterial structure should give a mammalian PFK tetramer with eight allosteric sites. Comparison of the N half residues with the bacterial enzyme shows that conservation is almost complete in those residues proposed by Schirmer and Evans (1990) to be necessary for binding of ligands.The use of this paper and a pair of stereo viewing glasses makes the sites much more accessible to interpretation. The F6P binding site for the bacterial enzyme consists of residues from both the small and large domains and from the other dimer. Four differences can be seen, three of these are in the sheep heart and liver sequences and may be due to incorrect sequencing. Position 241 in the bacterial enzymes is Glu while the mammalian enzymes have Asp. The function of Glu 241 in the bacterial enzymes is to form a salt bridge with Arg

72 from another subunit, in the T state. This Arg 72 bridges the two substrates F6P and ATP in the R state. This change of Glu-241 --> Asp-241 is relatively conservative and presumably reflects a very slight difference in the mammalian catalytic site.

The C half of the mammalian enzymes shows several differences at these residues. The main difference is at the catalytic residue Asp-127 in bacterial and mammalian N half. This is a serine in the C half of all the mammalian enzymes. The aspartate is thought to increase the nucleophilicity of the '0'-1 hydroxyl of F6P for attack on the γ phosphate of ATP and then repulsion of F16BP. Serine has half the negative charge and is not likely to repel FBP.

This site is a likely candidate for the binding of sugar bisphosphates. It may be the binding site of F26BP, R15BP, F16BP and G16BP. R15BP has only been found to be an *in vivo* activator of rat brain PFK and may have a certain affinity for the platelet isoenzyme which is the major component (~62%) of the rat brain isoenzyme pool. Twelve residues are reported as being important to the FBP binding site (Ishikawa *et al.,* 1990). The C-terminal partial sequence includes all of these residues except for the unsequenced Asp-12-N which is Pro-12-C in all other mammalian PFK's. If the platelet isoenzyme is particularly responsive to R15BP then perhaps some other long range structural difference might account for this. The *in vitro* expression of full length isoenzyme clones might explain a difference, if any, between the isoenzymes in their response to these effectors.

6.9 Subunit interactions

Six deletions from mammalian PFK can be seen in the gapped alignment to bacterial PFK. These occur at positions 81-C, 86-C, 87-C and

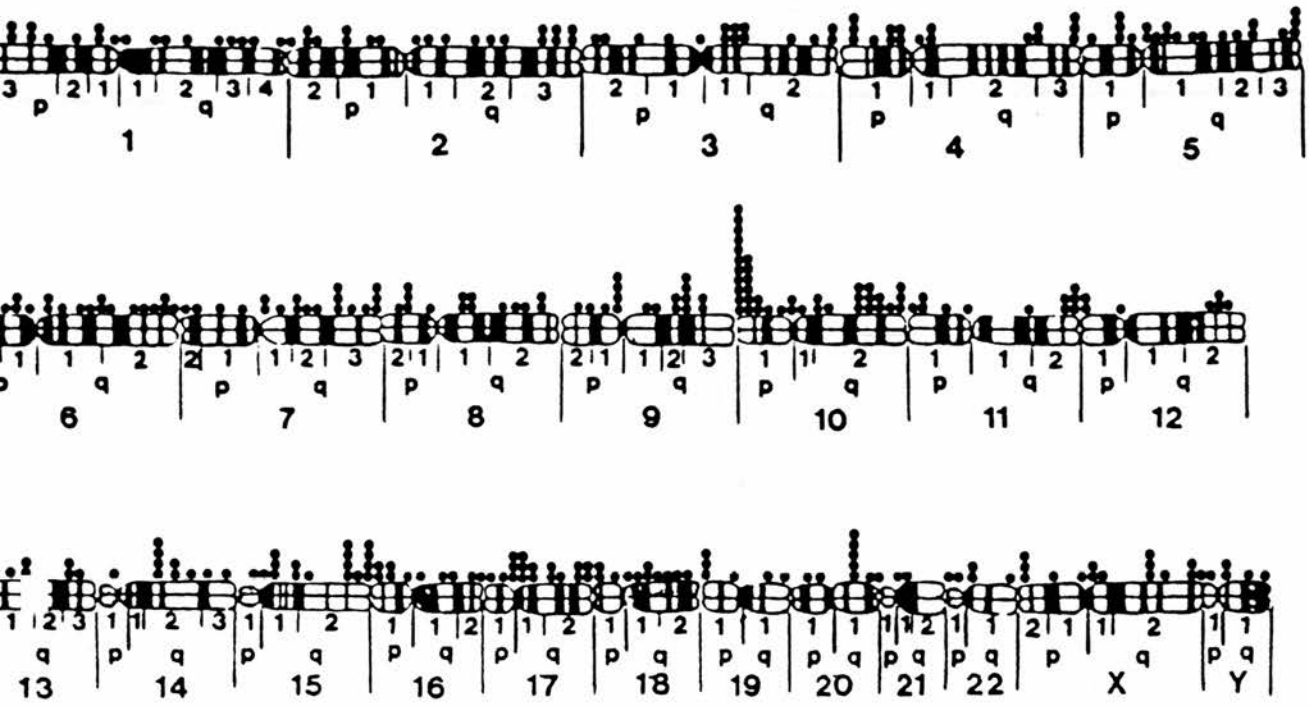
271-C but not in the amino half of the mammalian enzymes. An additional two deletions are present in the platelet sequence at 91-C and 92-C. Many additional residues are present in the mammalian enzymes, the majority being in the amino half. It may be possible that some of these additional residues contribute to further subunit interactions. Arg 252 which is central to the subunit interface in the bacterial enzymes lies in α-10 which is in an area showing a high degree of conservation between mammalian and bacterial enzymes. This residue is a glutamine in all of the carboxyl halves and lies in an area of particularly high sequence conservation. The meaning of such conservation must await the availability of crystal structure data.

6.10 Chromosomal localisation of the platelet clone.

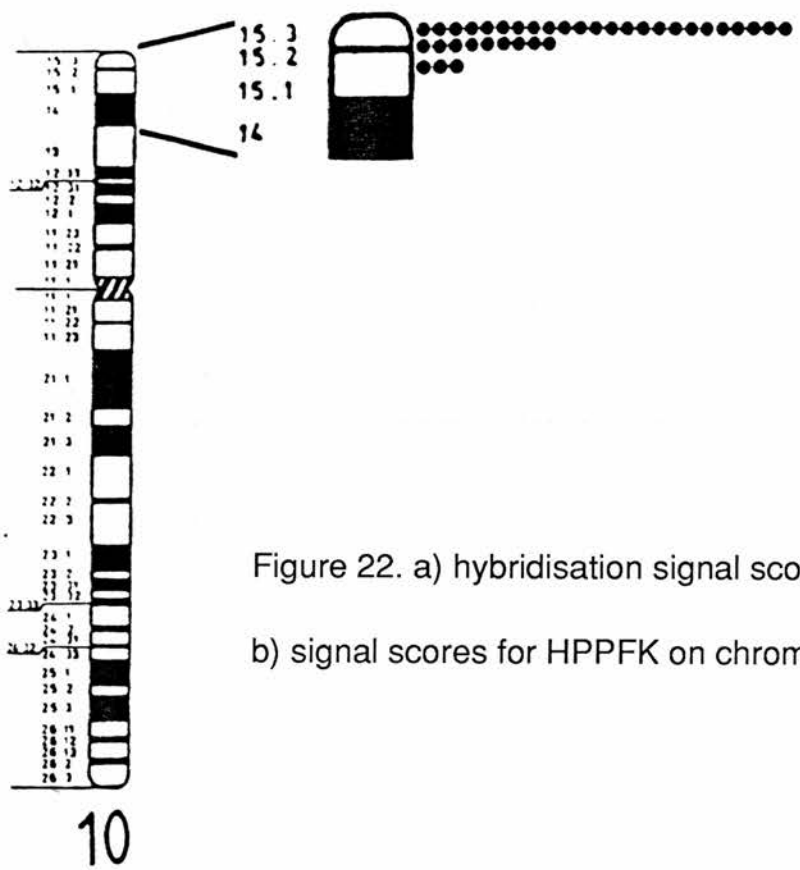
The fragment containing HPPFK DNA in pCS11 was subcloned into pKS+ and named pCS16. This was due to problems with background in hybridisation experiments when pCS11 was used as a probe. Attempts to further verify the position of human muscle PFK using the insert of pCS10 as a probe ran into the same problem and this work was not succesful. The biotin hybridisation work was kindly done by Norma Morrison of the Duncan Guthrie Institute for Medical Genetics, Yorkhill, Glasgow. pCS16 was labelled with biotin-11-dUTP and used for *in-situ* hybridisation on human chromosome spreads. Eighty eight metaphases were scored following hybridisation and the positions of three hundred and thirty six hybridisation signals recorded as shown in Figure 22a. Of these, a highly significant ($P<0.005$) thirty nine signals (11.6%) were located on chromosome ten with seventeen (5.1%) comprising a single peak on 10p15. For confirmation and refinement, the probe was hybrised to longer chromosomes (example Figure 23) obtained from human lymphocyte cultures synchronised with the

mitogen phytohaemagglutinin. The positions of thirty six signals which fell within 10p15 and which could be assigned with confidence to a single sub-band are shown in Figure 22b. Thirty three signals (91.7%) were located at 10p15.2-p15.3 with twenty four signals (66.7%) at 10p15.3. Vora *et al.* (1983) used a variety of human-rodent somatic cell hybrids to localise HPPFK to chromosome 10p using polyspecific rodent antisera.

The localisation of this clone to 10p15.3 supports the contention that it represents a sequence for HPPFK although it cannot be denied that it might represent a processed pseudogene lying close to the gene for HPPFK. The cloning and expression of a full length cDNA for HPPFK would resolve this problem.



a



b

Figure 22. a) hybridisation signal scores for HPPFK

b) signal scores for HPPFK on chromosome ten

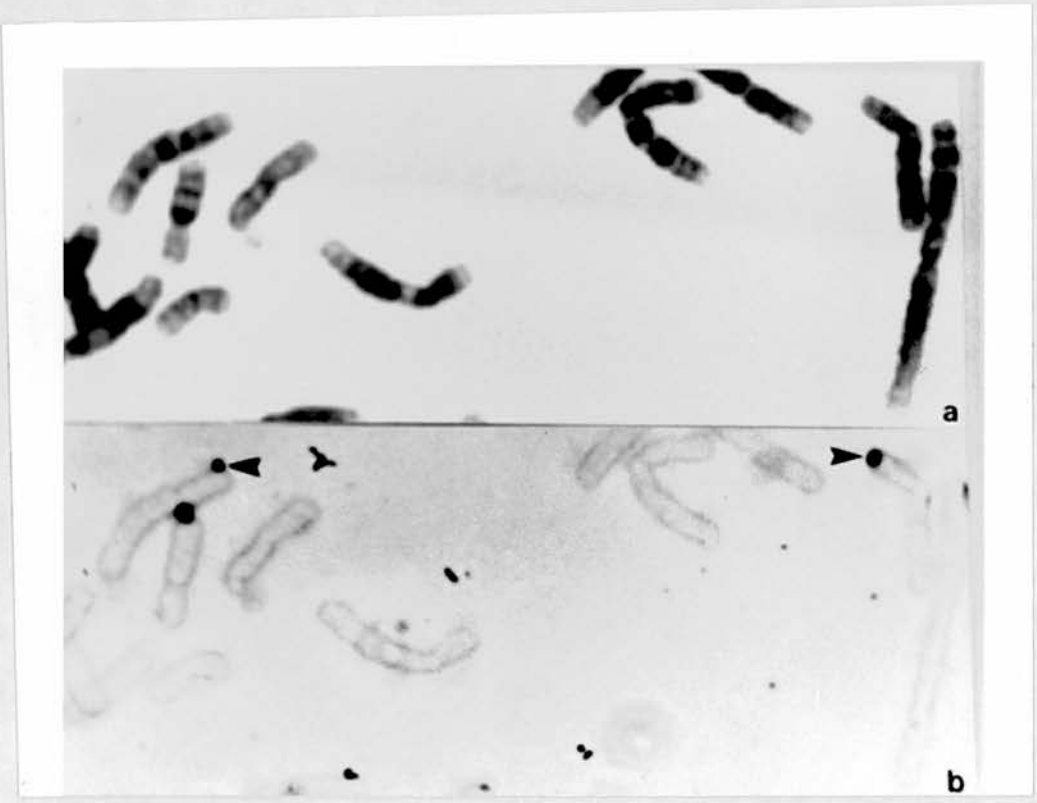


Figure 23. Chromosomal assignment of HPPFK to human chromosome ten, a) before and b) after hybridisation

SUMMMARY

Several methods were used to try to characterise PFK from *Onchocerca volvulus.* Progress was not made due to the quality of the *onchocerca* libraries, the lack of good quality heterologous probes and the unexpected nature of *onchocerca* gene structure and sequence characteristics. The use of a DNA probe from an organism with a similar DNA structure and better quality *onchocerca* libraries might prove more succesful in characterising genes from this parasite. The availability of *onchocerca* tubulin as a probe will give an indication of the quality of any library made available for screening.

A partial genomic clone of human muscle PFK was isolated using a partial genomic clone of rabbit muscle PFK as a probe. This human genomic clone was used to isolate a partial cDNA for human muscle PFK. This cDNA clone was used to isolate a partial cDNA for human platelet PFK. The identity of the clone was further established by localising its position to human chromosome ten. The isolation of a complete clone encoding human platelet PFK would allow further characterisation of the isoenzymes of human PFK as the full length muscle and liver sequences have already been reported. The availability of crystal structure data for the bacterial enzyme makes mammalian phosphofructokinase an interesting target for site directed mutagenesis.

Appendix.

The sequence of the putative human platelet phosphofructokinase has been submitted to the EMBL/GENBANK database under the accession number M64784 and is reported in Simpson & Fothergill-Gilmore (1991) Biochem. Biophys. Res. Comm. (in preparation). The chromosomal localisation of the putative human platelet phosphofructokinase clone has been submitted to Human Genetics (Morrison *et al.,* 1991). This work has been supported by a grant from the World Health Organisation, grant number 08/181/230.

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