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Identification of the Catalytic Residue in Rabbit Muscle Phosphofructokinase by Site-directed Mutagenesis

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ABSTRACT

Based on primary sequence alignment of rabbit muscle and bacterial phosphofructokinase, the aspartyl 166 residue of rabbit muscle phosphofructokinase (RMPFK) is proposed to be the catalytic residue involved in the abstraction of a proton from the 1-OH group of fructose 6-phosphate (Fru6P) to enhance its nucleophilicity to attack the γ-phosphate of ATP. By site-directed mutagenesis of cloned RMPFK cDNA in *E. coli*, aspartate 166 has been mutated to alanine (DA166), serine (DS166), and histidine (DH166). The mutated enzymes have been purified and analyzed for kinetic properties. Initial results, that the mostly pure mutants, DA166 and DS166, have respectively 1/2 and 1/20 the specific activity of wild type RMPFK, at first seem to refute the original hypothesis. Upon further analysis, an impurity has been detected that also catalyzes NADH oxidation with a $K_{m(Fru6P)}$ value comparable to that of RMPFK under the reaction conditions. This impurity is most likely the constitutively-expressed *E. coli* mannitol 1-phosphate dehydrogenase (Mtl 1-P DH). A study using enzyme assay conditions that differentiate Mtl 1-P DH and RMPFK activity indicates that mutants DA166 and DS166 have little or no RMPFK specific activity and $K_{m(Fru6P)}$ value is unchanged. These preliminary results suggest that aspartate 166 is a key residue involved in rate enhancement, probably by acting as the general base catalyst in RMPFK.

CHAPTER 1

INTRODUCTION

Background

Phosphofructokinase (PFK, ATP:β-D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11) catalyzes a key regulatory step in the glycolytic pathway, namely the MgATP-dependent phosphorylation of fructose 6-phosphate (Fru6P) to produce fructose 1,6-bisphosphate (Fru1,6P_2): MgATP + Fru6P → MgADP + Fru1,6P_2.

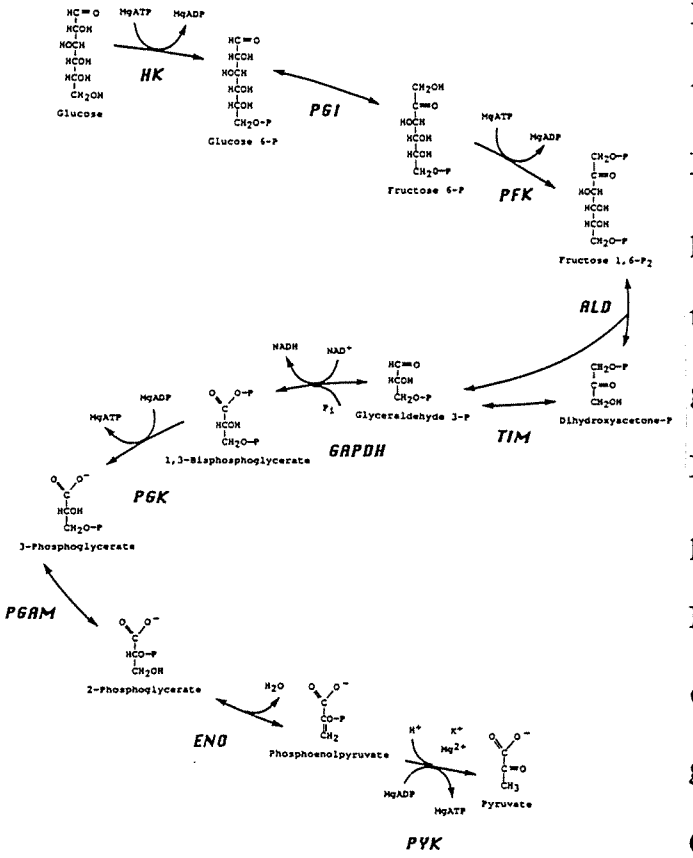

Fig. 1.1 Glycolysis. The abbreviations for the enzymes are as follows: HK, hexokinase; PGI, glucosephosphate isomerase; PFK, phosphofructokinase; ALD, aldolase; TIM, triosephosphate isomerase; GAPDH, glyceraldehyde phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGAM, phosphoglycerate mutase; ENO, enolase; PYK, pyruvate kinase. The letter "P" in the chemical structures represents a phospho group. Figure is taken from Fothergill-Gilmore and Michels, 1993.

Ostern *et al.* first discovered the reaction catalyzed by PFK in muscle (1936). Cori first recognized the rate-limiting role of PFK in frog muscle (1942). The enzyme is characterized by allosteric kinetics, a complex oligomeric structure, and multiple modes of regulation. Mammalian PFK is regulated by a number of ligands including substrates, reaction products, and other cellular metabolites in a pH-dependent manner (Uyeda, 1979; Goldhammer and Paradies, 1979; Kemp and Foe, 1983). It is potently inhibited by high concentrations of ATP and synergistically by citrate, a TCA cycle intermediate. The ATP inhibition can be relieved by P_i, AMP, cAMP, ADP, Fru6P, Fru1,6P_2, and Fru2,6P_2. PFK exhibits both heterotropic regulation by effectors on substrate binding and homotropic cooperativity with regard to its substrate Fru6P (Tsai and Kemp, 1973). In addition, PFK undergoes rapid association and dissociation depending on protein concentration, ionic strength, pH, temperature, and ligand concentration (Hesterberg and Lee, 1981; Luther *et al.*,1983). Rabbit muscle PFK (RMPFK) is stable within the pH range of 6.0 to 10.0. At pH 7.0, the enzyme associates in equilibria simplified as follows: 4M $2M_2$ M_4 $1/4M_{16}$, where M_4 represents the tetramer of molecular weight 340,000 and the smallest active form of PFK (Luther *et al.* 1983). At pH 8.0, the enzyme exists primarily as a tetramer. The reaction mechanism of RMPFK is still in dispute: it may be sequential ordered or sequential random (Uyeda, 1972; Hanson *et al.*, 1973).

Three distinct mammalian PFK isozymes, developmentally and tissue-specifically-controlled, have been isolated (Tsai and Kemp, 1973; Uyeda, 1979). These three subunit types are called M for muscle type, L for liver type, and C for brain or platelet type (Tsai and Kemp, 1974; Thrasher *et al.*, 1981). Each has molecular mass 85,000 ± 5,000. In skeletal muscle, the M isozyme is the predominant form.

In vitro and *in vivo*, PFK is itself a substrate for the cAMP-dependent protein kinase, but evidence for physiologically relevant regulation of PFK and Fru1,6P_2ase (an enzyme that catalyzes the conversion of Fru1,6P_2 to Fru6P with the hydrolytic removal of inorganic phosphate in gluconeogenesis) activity to suppress futile cycling of ATP to ADP by phosphorylation is both contradictory and controversial (Pilkis *et al.*, 1987). Rather, Fru2,6P_2 is a key physiological modulator (Pilkis *et al.*, 1981; Uyeda *et al.*, 1981; Van Schaftingen and Hers, 1981). That is, Fru2,6P_2 is a potent allosteric activator of PFK through the synergistic action with AMP to oppose ATP and citrate inhibition, and it has the opposite effect on the gluconeogenic enzyme. Citrate is a key mediator for intracellular coordination of glycolytic and mitochondrial ATP production (Garland *et al.*, 1963; Parmeggiani and Bowman, 1963).

For the past 60 years, mammalian PFKs and especially RMPFK have been intensely studied in regard to kinetic properties and physiological relevance. But structural properties have remained obscure until the report of the full protein primary sequence of RMPFK by Poorman *et al.* (1984). This study aligning the amino acid sequence of the N- and C- halves of RMPFK with that of PFK from *Bacillus stearothermophilus* has provided evidence that mammalian PFKs, about twice the size of the bacterial enzymes, evolved by duplication of and divergence from a prokaryotic progenitor.

Bacterial PFK

The best understood PFK are those from bacteria, specifically, *Escherichia coli* and *Bacillus stearothermophilus*. Two forms of PFK from *E. coli* exist (Thomas *et al.*, 1972). PFK-1 (EcPFK) is the major form that accounts for approximately 90% of PFK activity found in crude

cell extracts. This enzyme is composed of 319 amino acids, and it exists as a tetramer of four identical subunits, each 35,000 in molecular mass. PFK from *B. stearothermophilus* (BsPFK), of 320 amino acids, is a thermostable tetrameric enzyme of similar molecular weight. This enzyme exhibits 55% sequence homology to EcPFK (French and Chang, 1987). A chimeric bacterial PFK consisting of the ATP-binding domain of BsPFK and the Fru6P-binding domain of EcPFK retains 50% of the catalytic activity of the native enzymes and similar affinities for the substrates (Byrnes *et al.*, 1995). Other bacterial PFKs whose genes have been cloned also show significant sequence homology to EcPFK.

X-ray crystal structures of EcPFK and BsPFK in the presence and absence of substrates, products, activators and inhibitors have been determined (Evans and Hudson, 1979; Evans *et al.*, 1981; Shirakihara and Evans, 1988; Rypniewski and Evans, 1989; Schirmer and Evans, 1990). Structurally, the subunits of these two enzymes share the same secondary elements: their α-carbon traces are nearly superimposable and both enzymes form stable tetramers composed of dimer of rigid dimers with 222 symmetry. Each subunit can be divided into two domains, a large and a small one, each with a core of β-sheet strands surrounded by α-helices, that respectively bind substrates ATP and Fru6P within a cleft between the two domains. In EcPFK, movement of the two domains relative each other opens and closes the active site (Evans, 1992). The effector site for binding activator ADP/GDP or inhibitor phosphoenolpyruvate lies in the large interface between two subunits of the rigid dimer called the regulatory interface. The dimer-dimer interface, called the active interface, forms part of the Fru6P binding site. Thus, a subunit has three ligand binding sites, two of which form the active site to bind substrates Fru6P and ATP as well as Mg^{2+} and the third the effector site that binds both allosteric activators and inhibitors.

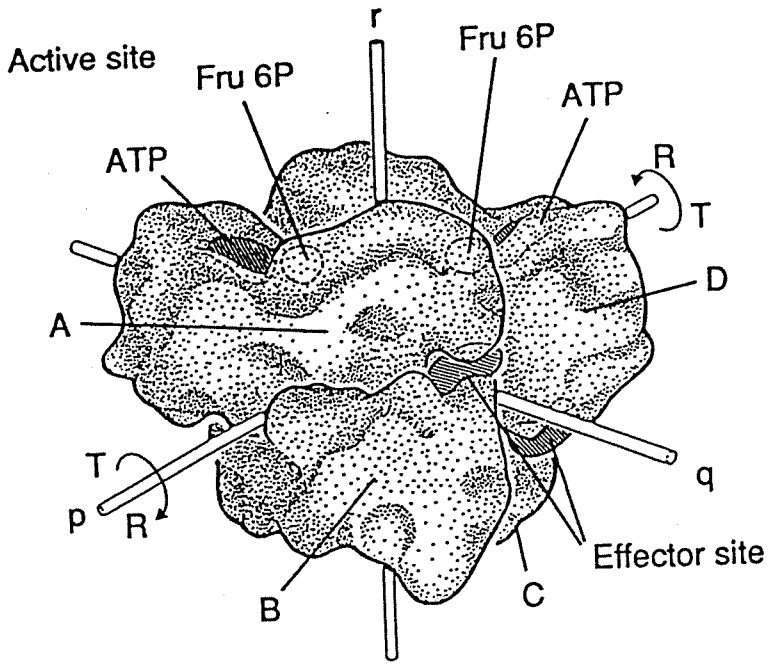

Fig. 1.2 Schematic view of the structure of EcPFK tetramer, R-state substrate complex with activator ADP in the effector site. Ligand binding sites are indicated. Figure is taken from Evans, 1992.

RMPFK structure

Sequence homology analysis of N- and C-halves of RMPFK and BsPFK suggests that the RMPFK tetramer has 4 active sites and 16 allosteric sites, including 4 effector sites derived from ancestor active sites for binding sugar bisphosphate . The homology of the N-half and BsPFK is 44%, C-half and BsPFK is 34% and N-half versus C-half is 32%. From this data, Poorman *et al.* (1984) have proposed that the tertiary structure would be conserved through evolution, that the N- and C-halves of RMPFK share a similar conformation to the BsPFK subunit. The two halves make up two super domains, structurally homologous to the bacterial monomer, linked by a connecting peptide of about 30 residues. In addition, because the N-half of RMPFK has retained the key catalytic residues while the C-half has not, the N-half of RMPFK has been proposed to have retained the binding sites for substrates Fru6P and ATP and also contains the binding site to the allosteric inhibitor citrate, while the C-half carries the other allosteric effector sites, an ATP

inhibitory site, an adenine nucleotide activating site, and a sugar bisphosphate site that binds Fru2,6P_2 and Fru1,6P_2, having evolved from the Fru6P binding site (Kemp and Foe, 1983). Equilibrium binding studies also indicate only four catalytic sites in active RMPFK (Kemp and Krebs, 1967; Hill and Hammes, 1975).

Catalytic mechanism of bacterial PFK

A past study on the stereochemical course of phosphoryl transfer from Fru1,6P_2 to ADP catalyzed by both BsPFK and RMPFK has shown that the reaction involves an inversion of configuration at the phosphorus atom, suggesting that the mechanism of the forward reaction, the phosphorylation of Fru6P, consists basically of a direct, in-line nucleophilic attack of the 1-hydroxyl group of Fru6P on the γ-phosphate of ATP (Jarvest *et al.*, 1981). In bacterial PFK, this reaction occurs in the closed subunit conformation of the enzyme-substrate complex and requires a divalent cation Mg^{2+} or Mn^{2+} as well as a monovalent cation K^+, NH_4^+ or Tl^+. The three-dimensional crystal structure and subsequent site-directed mutagenesis studies have identified Asp 127 of EcPFK as the key catalytic residue that acts as the general base catalyst by abstracting the proton from the 1-OH group of Fru6P to increase its nucleophilicity (Hellinga and Evans, 1987). Mutating Asp 127 to Ser reduces the forward catalytic efficiency 18,000-fold without changing the affinity for substrates and the reverse 3,100-fold while reducing $K_{m(Fru1,6P2)}$ by a factor of 45. pK_a of Asp 127 has been estimated to be 6.7 in free PFK and 6.6 in the complex between PFK and Fru6P, reasonable values for a group acting as the base catalyst. Due to charge repulsion, pK_a of this group is greater than 9.6 in the enzyme complex to Fru1,6P_2 (Laine *et al.*, 1992).

The other groups immediately around the catalytic site are important in stabilizing the negative charges on the phosphates, for positioning the donor and acceptor substrates, and for stabilizing the proposed pentacoordinate transition state. Thr 125, Arg 72, Arg 171, and the peptide NH of Gly 11 probably stabilize the transition state. Asp 103 and Asp 129 are required to position the Mg^{2+} ion that in turn stabilizes the transition state (Berger and Evans, 1992). In the T state, Arg 72 forms a salt bridge to Glu 241 so that it can no longer interact with the transferring phosphate.

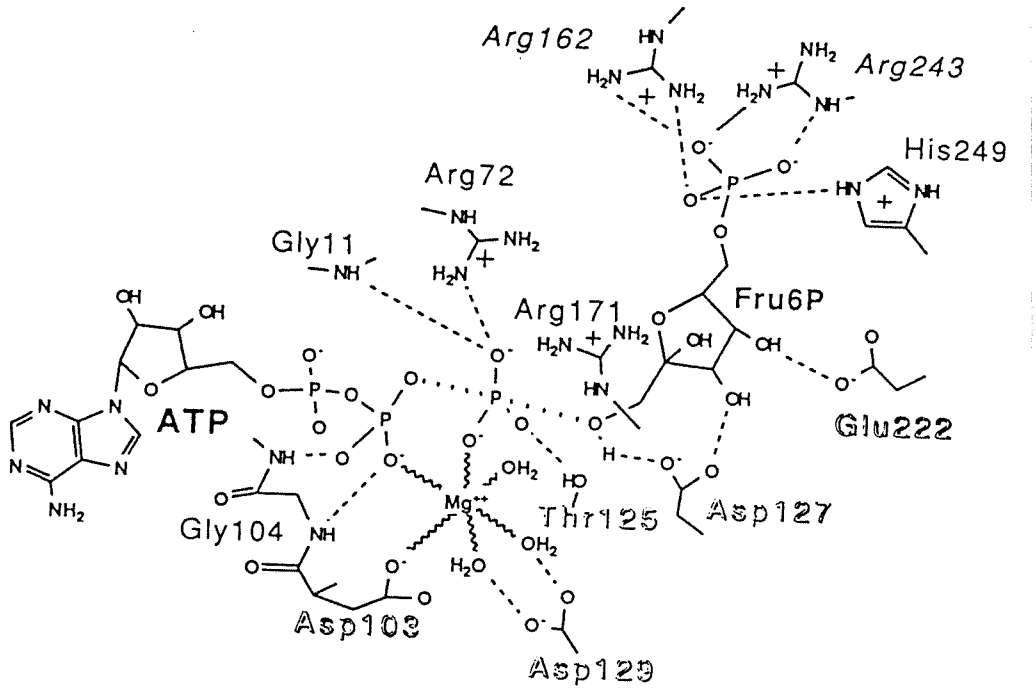

Fig. 1.3 Schematic diagram of the transition state and selected active site residues in EcPFK. Figure is taken from Berger and Evans, 1992.

Proposed catalytic mechanism of RMPFK

Residue Asp 127 of EcPFK is conserved in all prokaryotic PFK and N-half eukaryotic PFK sequences known. By protein primary sequence alignment, the corresponding residue in the N-half of RMPFK is Asp 166.

```
                bbb  ☆          aaaaaaaaaaaaaaaaaaaaa  bbbbbbb     aaaaa
hummus-N        EGLVGSIDNDFCGTDMTIGTDSALHRIMEIVDAITTTAQSH-QRTFVLEVMGRHCGYLAL
rabmus-N        VGLVGSIDNDFCGTDMTIGTDSALHRITEIVDAITTTAQSH-QRTFVLEVMGRHCGYLAL
humliv-N        AGLVGSIDNDFCGTDMTIGTDSALHRIMEVIDAITTTAQSH-QRTFVLEVMGRHCGYLAL
mouliv-N        AGLVGSIDNDFCGTDMTIGTDSALHRIMEVIDAITTTAQSH-QRTFVLEVMGRHCGYLAL
yeaA-N          VGLVGSIDNDMSGTDSTIGAYSALERICEMVDYIDATAKSH-SRAFVVEVMGRHCGWLAL
yeaB-N          CGTVGSIDNDMSTTDATIGAYSALDRICKAIDYVEATANSH-SRAFVVEVMGRNCGWLAL
Eco             IGLPGTIDNDIKGTDYTIGFFTALSTVVEAIDRLRDTSSSH-QRISVVEVMGRYCGDLTL
Bst             VGVPGTIDNDIPGTDFTIGFDTALNTVIDAIDKIRDTATSH-ERTYVIEVMGRHAGDIAL
Sci             IALPGTIDNDITSSDYTIGFDTAINIVVEAIDRLRDTMQSH-NRCSIVEVMGHACGDIAL
```

Fig. 1.4 Protein sequence alignment of a stretch of amino acids around the proposed aspartyl catalytic residue, shown by the large star symbol, in the N-half of several eukaryotic PFKs and in prokaryotic PFKs. a indicates α-helix and b indicates β-strand secondary structures. Figure is modified from Fothergill-Gilmore and Michels, 1993.

In the divergent evolution of PFK from prokaryotes to eukaryotes, an increase in sophistication of the regulatory mechanisms has occurred to accommodate the increasingly complex metabolic environment, in part through the gene duplication-fusion event (reviewed by Gilmore and Michels, 1993). However, despite the wide-ranging diversification of amino acid sequence, most of the amino acids important in binding substrates and catalysis, including Asp 127, Asp 103, Asp 129, Arg 72, and Arg 171 numbered for EcPFK, have been conserved through evolution in all available sequences (N-half PFK for eukaryotes) from mammalian, yeast, and bacterial sources; and the sequence-predicted secondary structures for the catalytic domain are

also the same. These observations and the detection of the same inversion of configuration of the transferred phosphate group occurring in the reaction catalyzed by RMPFK suggest the same catalytic mechanism for RMPFK as for the bacterial enzymes and that Asp 166 of RMPFK could be the base catalyst that abstracts the proton from 1-OH of Fru6P. Further, site-specific mutagenesis has already identified the corresponding Asp in the distantly-related (23% sequence homology to EcPFK) pyrophosphate-dependent phosphofructokinase from *Propionibacterium freudenreichii* as the critical catalytic residue (Green *et al.*, 1993).

Objective of this thesis

Based on the known amino acid sequence of RMPFK, our lab has previously cloned both the gene and cDNA encoding this enzyme (Lee *et al.*, 1987; Li *et al.*, 1990). The full-length cDNA has also been inserted into the plasmid pPL2 that carries a λ P_L promoter and co-transformed with pcI857 encoding the thermolabile λ repressor into the endogenous PFK-deficient *E. coli* strain DF1020 (Li *et al.*, 1993). In this work, by site-directed mutagenesis, we examine the importance of Asp 166 in the catalytic function of RMPFK. In the process, we encounter problems in low yield of soluble recombinant protein and in enzyme purification.

CHAPTER 2

MATERIALS AND METHODS

Materials

Restriction endonucleases, T4 DNA ligase, T4 DNA polymerase, and T4 Polynucleotide kinase were obtained from Bethesda Research Laboratories (BRL) (Bethesda, MD), New England Biolabs, Inc. (NEB) (Beverly, MA), and Promega Biotec Corporation (Madison, WI). A sequenase kit was purchased from United States Biochemicals Corp. (USB) (Cleveland, OH). Altered Sites *in vitro* mutagenesis system was from Promega. RNAse A, aldolase (Type IV), mixture of triose-phosphate isomerase and glycerol 3-phosphate dehydrogenase were ordered from Sigma Chemical Co. (St. Louis, MO).

The expression vector containing the lambda P_L promoter (pPL2) and the pcI857 plasmid containing the cI857ts gene were from Khorana (MIT, Cambridge, MA). Bacterial strains and helper phage R408 that were used in mutagenesis were purchased from Promega. *E. coli* strain DF1020 (*pro*-82, *pfkB*201, *recA*56, (*rha-pfkA*)200, *endA*1, *hsdR*17, *supE*44, *thi*-1) was from Bachmann at the *E. coli* Genetic Stock Center (Yale, New Haven, CT).

[α-^{35}S]-dATP was purchased from New England Nuclear (NEN)/Dupont (Wilmington, DE). Oligonucleotides were ordered from GeneLab (Baton Rouge, LA) or American Synthesis Inc. (Pleasanton, CA).

Bacto-tryptone, bacto-yeast extract, and bacto-agar were purchased from Difco Laboratory (Detroit, MI). Agarose was obtained from BRL or American Research Products Company (AMRESCO) (Solon, OH). Acrylamide stock solution (40 % w/v) (ACRYL-40) and

N,N'-methylene-bisacrylamide stock solution (2% w/v) (BIS-2) were ordered from AMRESCO. Urea, Tris(hydroxymethyl)aminomethane (Tris), ethylenediaminetetraacetic acid (EDTA), nicotinamide adenine dinucleotide (reduced form, NADH), sodium dodecyl sulfate (SDS), polyethyleneglycol (PEG), phenylmethylsulfonyl fluoride (PMSF), fructose 6-phosphate (sodium salt), adenosine triphosphate (sodium salt), dithiothreitol, Cibacron Blue 3GA-Agarose (Type 3000-CL-L), sorbitol, betaine, ampicillin, kanamycin, and tetracycline were from Sigma. DEAE cellulose (DE52) was from Whatman (Hillsboro, OR). Centricon-30 and Centriprep-50 were from Amicon, Inc. (Beverly, MA). Gene Clean kit was from Bio 101, Inc. (Lo Jolla, CA). 3MM Whatman filter papers were ordered from Fisher Scientific. Polaroid film was purchased from Polaroid Co. Films XAR-5 and XRP-5 were from Eastman Kodak Co. (Rochester, NY).

Site-directed mutagenesis

Previously, the full-length RMPFK cDNA was cloned and inserted into the plasmid pPL2, which in turn was co-transformed with pcI857 into *E. coli* strain DF1020. The 941 bp XbaI/BamHI fragment from pPL2/RMPFK coding the NH_2-terminal 310 amino acids was subcloned into pSELECT-1 phagemid that had been pre-digested with XbaI and BamHI. The construct was transformed into *E. coli* strain JM109, and single-stranded DNA template was prepared by infecting the transformed cells with helper phage R408. Mutations were made using this single-stranded template and two mutagenic oligonucleotides, one repairing a defect in the β-lactamase gene of the phagemid and the other converting the Asp 166 codon GAC to Ser AGC, Ala GCC, or His CAC (Ser166 5'GCAGAAGTCATTGCTAATGGAGCCGACC3', Ala166 5'GAAGTCATTGGCAATGGAGC3', and His166 5'GTCATTGTGAATGGAGCCG3'). The

mutant strand was synthesized by T4 DNA polymerase, and its ends were ligated by T4 DNA ligase.

The mutant-wild type heteroduplex DNA was transformed into repair-deficient *E. coli* strain BMH 71-18 mut S. Plasmid DNA isolated from this strain was in turn transformed into *E. coli* JM109. The final transformed JM109 cells were plated on agar plates containing ampicillin and colonies were directly screened for mutation by sequence analysis of mini-prep double-stranded plasmid DNA from randomly picked colonies. Full-length sequencing of the cloned fragment from positive mutants was performed in order to ensure that no spurious mutations were made. The correctly mutagenized XbaI/BamHI RMPFK cDNA fragment was re-ligated into pPL2/RMPFK predigested with the same endonucleases. The mutated pPL2/RMPFK plasmid was co-transformed with pcI857 into DF1020 cells deficient in both EcPFK genes *pfkA* and *pfkB* and rechecked for mutation via DNA sequencing. Transformed DF1020 cells were plated on LB-agar containing 100 μg/ml ampicillin and 50 μg/ml kanamycin and grown at 28°C. Single colonies of mutants and wild type were plated on 0.4% mannitol M63 minimal agar media to view PFK production.

Enzyme purification

Transformed DF1020 cells were plated on LB-agar containing 100 μg/ml ampicillin and 50 μg/ml kanamycin. A single colony was inoculated into 3 ml LB containing 100 μg/ml ampicillin and 50 μg/ml kanamycin (LB-AK) and grown at 28°C with agitation for 20 hours. 0.1 ml of the culture was then inoculated into 100 ml LB-AK and grown at 28°C, 300 rpm for 12 hours. 20 ml of this was inoculated into 1 L LB-AK and grown at 28°C, 270 rpm until $A_{600\ nm}$

reached 0.9 to 1.0 (≈5-6 hrs). 500 ml of LB or LB/1.5 M sorbitol/7.5 mM betaine preheated to 70°C was poured into the cell culture with continuous shaking. Cells were heatshocked and incubated at 42°C, 270 rpm for 3.5 hrs. Cells from a total of 6 L of culture were harvested by centrifugation at 4°C, 12,000 x g for 15 minutes. Pellets were stored at -70°C until ready for use.

Cell pellets were weighed and resuspended at 15 % w/v in sonication buffer (0.1 M Tris-phos pH 8.0, 0.1 mM EDTA, 0.5 mM ATP, 2 mM DTT, 0.1 mM PMSF) (120 ml). The suspension was then sonicated at 0°C for six 2 min intervals at level 7 with 2 min cooling intervals. Cell lysate was centrifuged at 4°C, 15,000 x g for 30 min and supernatant was saved. Cell pellet was resuspended in 60 ml sonication buffer and sonicated at 0°C for 1 min x 6 at level 6 with 2 min cooling intervals. Cell lysate was spun down as above and supernatant combined.

Powdered ammonium sulfate was slowly added to the crude extract to 35% saturation and the suspension was gently stirred at 4°C for 1 hr. This was centrifuged at 4°C, 18,000 x g for 20 min, and the pellet was discarded. The supernatant was brought to 55% $(NH_4)_2SO_4$ saturation while gently stirred at 4°C overnight. The milky suspension was centrifuged at 4°C, 18,000 x g for 40 min and the pellet was stored at 4°C. When ready to use, the pellet was resuspended in 1.5 vols of column buffer (0.1 M Tris-phosphate pH 8.0, 0.1 mM EDTA, 0.1 mM ATP, 1 mM DTT, 0.1 mM PMSF) and dialyzed against 500 ml of column buffer with 2 changes at 4 °C.

Pre-swollen DEAE-cellulose (DE52) (40 g) was suspended in column buffer and packed into a column and equilibrated with 10 vols of column buffer. The protein sample was loaded onto the column and washed with 10 vols of column buffer at a flow rate of 0.25 or 0.5 ml/min at 4°C. RMPFK was eluted with column buffer containing 0.25 M Tris-phos. Eluate fractions were checked for protein concentration using the Bio-Rad Coomassie brilliant blue dye-binding assay

solution with bovine serum albumin as standard and for PFK activity at pH 8.0. Fractions containing the majority of PFK activity were combined and precipitated with 80% saturation of $(NH_4)_2SO_4$ for overnight. The suspension was centrifuged at 16,000 x g for 40 min. The pellet was dissolved in 1.5 vols of column buffer and dialyzed against 1000 fold column buffer. DE52 was regenerated by treatment with 1 N KOH, ddH_2O, 1 N HCl, ddH_2O, 1 M Na-phosphate pH 8.0, and ddH_2O in the listed order.

Cibacron Blue 3GA agarose (1 g, 5 ml) was washed with copious amounts of ddH_2O to get rid of the lactose stabilizer and then suspended in column buffer. The resin was packed in a column and equilibrated with 10 vols of column buffer. The DE52 purified protein sample was loaded on the column and washed with 10 vols of column buffer. Column buffer containing 10 mM Fru6P and 10 mM ATP eluted RMPFK. Eluate fractions were checked for protein concentration and PFK activity. Fractions containing the majority of PFK activity were combined and concentrated using Centricon-30 or Centriprep-50, then dialyzed against 1000 fold column buffer.

The protein sample was further purified with a 1g DE52 column in the same manner as above. Final purified sample was concentrated with Centricon-30 and either dialyzed against 50% glycerol/2 mM ATP/column buffer or added 80% glycerol and 0.1 M ATP to make the same final concentration. The sample was stored at -20°C. Expression level and purity of samples from each step of the purification process were checked by 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using PFK purified from rabbit muscle (Sigma) as a standard. Gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad).

Enzyme activity assays and kinetic studies

To use, the enzyme was dialyzed at 4°C against dialysis buffer (50 mM Tris-HCl, pH 8.0, 1mM DTT, 0.1 mM EDTA) more than 10^7 fold. PFK activity at pH 8.0 was assayed following the method coupling Fru-1,6-P_2 formation with NADH oxidation described by Foe and Kemp (1982). The enzyme, in a final concentration of 0.05-1.2 μg/ml, was assayed at 30°C in a 1 ml reaction mixture containing 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 1 mM DTT, 0.2 mM NADH, 0.8 U/ml aldolase, 1.2 U/ml α-glycerophosphate dehydrogenase, 11 U/ml triose phosphate isomerase, and substrates Fru6P and ATP concentrations as indicated. Under these conditions, RMPFK obeys Michaelis-Menten kinetics. Auxiliary enzymes were dialyzed at 4°C against 50 mM Tris-HCl, pH 8.0 prior to use. Reactions were initiated by adding Fru6P after an initial equilibration of the other components at 30°C for 2 min. Using a thermostatted Hitachi UV-2000 spectrophotometer, the change in absorbance at 340 nm was measured for 70 s after an initial nonlinear phase of 60 s. One unit of PFK activity is defined as the amount that catalyzes the conversion of 1 μmol of Fru6P to Fru1,6P_2 per minute. Mannitol 1-phosphate dehydrogenase (Mtl 1-P DH) activity under the same conditions was measured in the absence of substrate ATP or in the absence of both auxiliary enzymes and ATP.

The kinetic data at pH 8.0 were fit into the Michaelis-Menten kinetics or one-site binding hyperbolic equation that describes the behavior of enzyme with no cooperativity between subunits: $v=Vm[Fru6P]/([Fru6P] + K_{m(Fru6P)})$.

All curve fitting and data analysis were performed using the program GraphPad PRISM version 1.0 for Windows (GraphPad Software, Inc., San Diego, CA).

CHAPTER 3

RESULTS

Site-directed mutagenesis at Asp 166 and expression of mutants

DNA sequencing analysis indicated correct codon replacement for each of the mutants DA166, DS166 and DH166. Full-length sequencing of cDNA insert confirmed that no unintentional mutations were made.

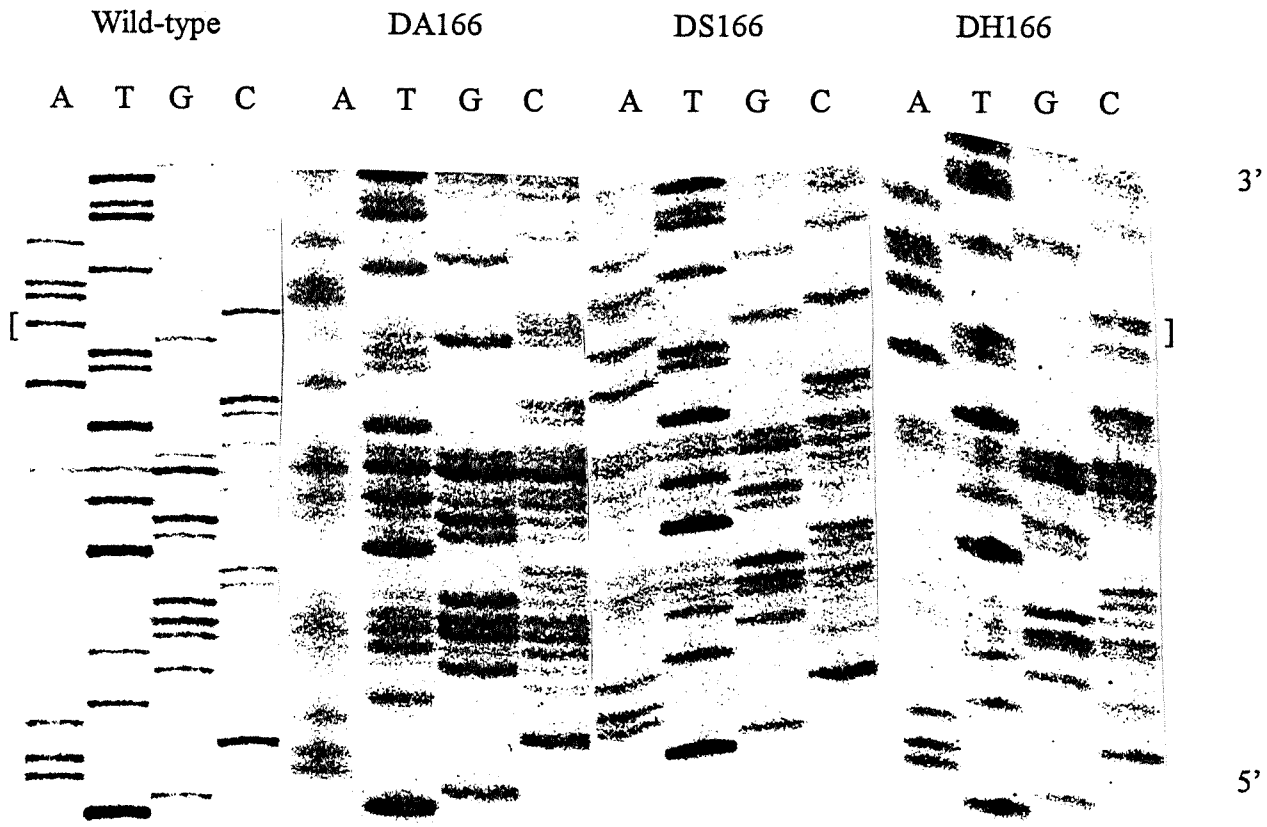

Fig. 3.1 Site-directed mutagenesis at Asp 166. DNA sequencing analysis indicates correct codon replacement for each of the mutants. This figure shows DNA sequences of the mutants and wild type *rmpfk* at the mutation region: wild type with Asp 166 codon GAC, DA166 with Ala codon GCC, DS166 with Ser codon AGC, and DH166 with His codon CAC, marked by bracket.

Selective plating on mannitol/M63 indicated that while *E. coli* DF1020 containing cloned wild-type RMPFK cDNA in pPL2 grew readily at 30°C after 2 days, none of the mutants grew even after one week nor did control DF1020 without plasmid.

Expression and purification of RMPFK mutants

Similar to the wild type, high-level expression of the mutants was achieved by the pPL2-pcI857 expression system in DF1020, as shown by SDS-PAGE. However, most of the expressed recombinant RMPFK remained in the cell sediment after sonication, presumably in the form of insoluble inclusion bodies. Adding sorbitol and betaine to increase the osmotic pressure and facilitate the uptake of the compatible solute glycyl betaine (Blackwell and Horgan 1991) did not appreciably increase the soluble recombinant protein yield but instead probably served to enhance the problem of high level expression of *E. coli* Mtl 1-P DH, discussed in detail later.

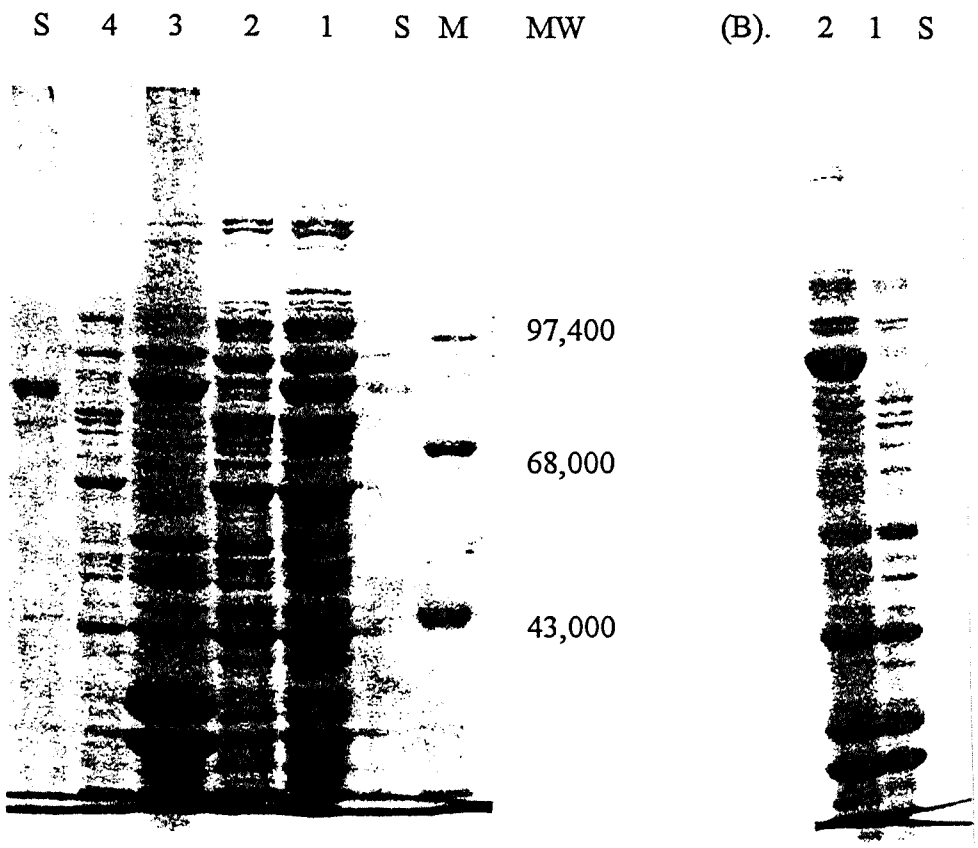

Fig. 3.2 Recombinant protein expression level of the mutants. This figure shows 8% SDS-PAGE analysis of protein extracts from (A) DS166/RMPFK/pPL2/pcI857/DF1020 grown and induced under the conditions described, with the addition of 0.5 M sorbitol and 2.5 mM betaine. Lanes S: Sigma RMPFK; M: protein size markers; 1: cell before sonication; 2: supernatant after sonication; 3: sediment; 4: 35-55% $(NH_4)_2SO_4$ fraction and (B) DA166/RMPFK/pPL2/pcI857/DF1020 grown in the absence of sorbitol and betaine. Lanes S: Sigma RMPFK; 1: supernatant after sonication; 2: sediment.

The mutant RMPFK was purified in 4 steps: ammonium sulfate fractionation (35-55% saturation), DE52 anion-exchange column chromatography, Cibacron blue 3GA agarose column, and DE52 again. The second DE52 column was used due to the presence of impurities in the

enzyme preparation after two columns. However, a second DE52 column used in the same manner as the first did not significantly increase the purity of the sample, nor did a second Cibacron 3GA column when it was used for DA166.

In purifying DS166, after 10 vols of column buffer was passed through the DE52 column to wash the crude sample, PFK activity was starting to be detected in the eluate in the specific activity range of around 100 U/mg. But SDS-PAGE of this sample shows no significant RMPFK band; instead, a major band about 45 kD in size was detected.

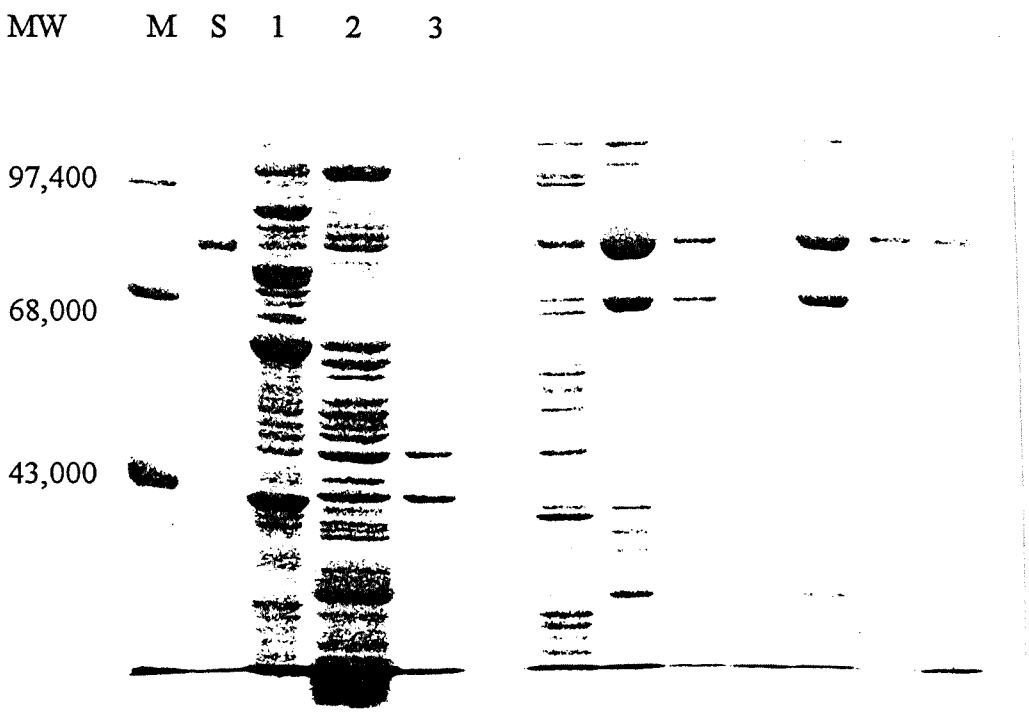

Fig. 3.3 8% SDS-PAGE analysis of purification of DS166 through first DE52 column. Lanes M: protein markers; S: Sigma RMPFK; 1: 35-55% $(NH_4)_2SO_4$ fraction; 2: DE52 column wash eluate; 3: wash eluate after 10 vols. The other lanes are shown in Fig. 4.

In addition, in purifying DH166 and DA166 through the Cibacron 3GA column step, when activity assays were singly used to detect PFK elution, no RMPFK band was detected on SDS-PAGE of the final preparation even though it measured high PFK activity under standard conditions at pH 8.0; however, a minor 45 kD protein band was visible. Measuring $A_{280\ nm}$ was not used due to the presence of ATP in the column buffer, but in later purification schemes for DA166 and DS166, column eluate fractions were checked for protein concentration using the Bio-Rad Coomassie brilliant blue dye-binding assay solution with bovine serum albumin as standard. The final preparations of DA166 and DS166 both still contain this 45 kD protein band since in purifying the enzyme through the DE52 column, only 10 vols of column buffer was used to wash the column, although DS166 has this only as a very minor band as compared to DA166.

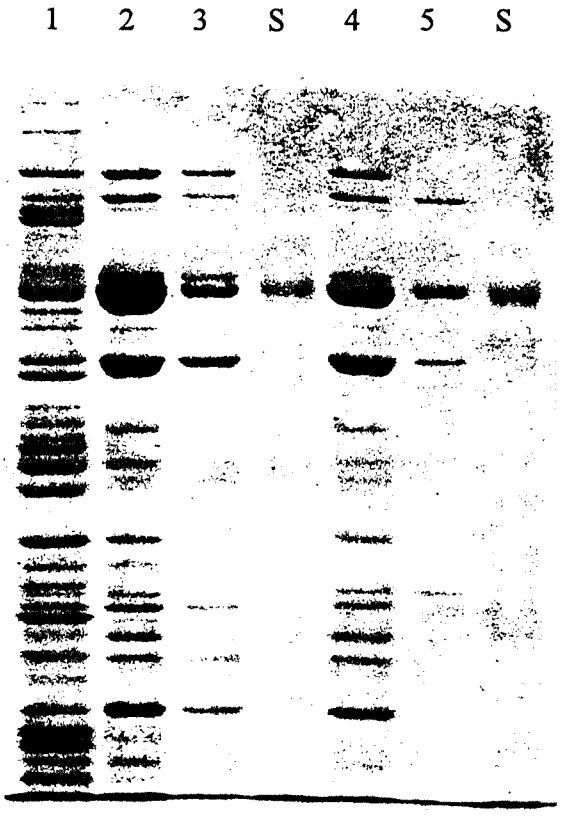

Fig. 3.4 8% SDS-PAGE analysis of purity of DS166 and DA166. Lanes S: Sigma RMPFK; 1: DS166 after first DE52 column; 2 & 3: DS166 after Cibacron Blue 3GA agarose column, concentrated and not concentrated, respectively; 4: DS166 final preparation after second DE52 column; 5: DA166 final preparation after second DE52. No size marker is shown on this figure enlarged from Fig. 3. Note the presence of a ≈45 kD size protein band in the final preparations of both mutants, purified from cells grown and heat-shocked in the presence of sorbitol and betaine.

Kinetic properties of partially-pure mutants DA166 and DS166 at pH 8.0

Table 3.1 shows the data for a study of the kinetic properties of the mutants at pH 8.0 in the presence and absence of ATP and auxiliary enzymes:

(A) Steady-state initial velocities (v, $-dA_{340\ nm}$/min) of partially-pure DA166 RMPFK, 0.10 μg/ml in assay reaction mix, pH 8.0

[Fru6P] mM	no ATP/aux enz	no ATP	1 mM ATP	5 mM ATP
0.00	0.000	0.002	0.002	0.004
0.01	0.001	0.001	0.001	0.002
0.05	0.009	0.009	0.012	0.009
0.20	0.040	0.039	0.037	0.031
0.60	0.061	0.066	0.063	0.061
1.00	0.064	0.069	0.068	0.069
3.00	0.053	0.055	0.055	0.064

(B) Steady-state initial velocities (v, $-dA_{340\ nm}$/min) of partially-pure DS166 RMPFK, 1.2 μg/ml in assay reaction mix, pH 8.0

[Fru6P] mM	no ATP/aux enz	no ATP	1 mM ATP	5 mM ATP
0.00	0.000	0.001	0.001	0.000
0.05	0.022	0.017	0.018	0.014
0.40	0.086	0.085	0.084	0.075
1.00	0.105	0.100	0.107	0.107
3.00	0.088	0.088	0.092	0.103

For DA166, most of the variations of values across a row were due to time factors, i.e., enzymes under low concentrations losing activity over time. For example, 3 mM Fru6P / 5 mM ATP was run 1/2 hour earlier than 3 mM Fru6P / no ATP / no auxiliary enzymes. But for DS166, the values at 3 mM Fru6P across the row were determined in adjacent and duplicate runs, and they indicate a slight increase in activity of the mutant at saturating Fru6P in the presence versus in the absence of ATP. That is, the partially-pure DS166 mutant has some other activity, although very slight, that catalyzes NADH oxidation in the standard PFK activity assay over the

NADH oxidation activity in the absence of ATP, but this does not necessarily have to be PFK activity.

Table 3.2 shows the kinetic parameters—Michaelis constant and specific activity for the two mutants determined from data listed in table 1 under the different reaction conditions and wild type RMPFK at saturating ATP (1 mM)—at pH 8.0:

(A) Partially-pure DA166 RMPFK. PFK activity is determined by subtracting activity in the absence of ATP from that in the presence of 5 mM ATP. Note that the specific activities listed below for both DA166 and DS166, partially-pure, under the different reaction conditions are calculated for PFK activity, as if 1 μmol Fru6P would cause 2 μmol NADH to be oxidized. Multiplying by 2 converts these values to Mtl 1-P DH specific activity.

	no ATP/aux enz	no ATP	1 mM ATP	5 mM ATP	PFK activity
Specific activity (units/mg)	53±6	58±6	56±6	62±6	4±8
$K_{m(Fru6P)}$ (mM)	0.14±0.07	0.16±0.08	0.15±0.07	0.24±0.08	

(B) Partially-pure DS166 RMPFK. PFK activity is determined as above.

	no ATP/aux enz	no ATP	1 mM ATP	5 mM ATP	PFK activity
Specific activity (units/mg)	7.0±0.7	7.0±0.7	7.3±0.8	8.0±0.7	1±1
$K_{m(Fru6P)}$ (mM)	0.12±0.07	0.14±0.08	0.15±0.08	0.23±0.09	

(C). WT RMPFK (Li *et al.*, 1993)

Specific activity (units/mg)	180
$K_{m(Fru6P)}$ (mM)	0.18±0.04

(D). *E. coli* Mtl 1-P DH (Teschner *et al.*,1990)

Specific activity (units/mg)	500
$K_{m(Fru6P)}$ (mM)	0.2 (pH 6.9)-0.3 (pH 8.9)±10%

Since all specific activity and $K_{m(Fru6P)}$ values for the mutants under the different reaction conditions are similar in error overlap, these data show that for the most part, activities of the

mutant enzymes are due to NADH oxidation activity already present in the absence of ATP and auxiliary enzymes. After analysis, this activity is concluded to be due to *E. coli* mannitol 1-phosphate dehydrogenase (Mtl 1-P DH), a monomeric enzyme about 45,000 in size that has a similar $K_{m(Fru6P)}$ value to RMPFK and a specific activity at pH 8.0 80% of the value at pH 7.0, 500 units/mg, with one unit defined as the amount of Mtl 1-P DH that catalyzes in the reverse the conversion of 1 μmol of Fru6P to Mtl 1-P. DS166 preparation, as analyzed by SDS-PAGE in Fig. 4, is less pure but contains a lesser relative amount of Mtl 1-P DH than the DA166 preparation. This observation correlates directly with the lower specific activity in the absence of ATP measured for DS166 than DA166 enzyme preparation. These results in all indicate almost no PFK specific activity for both the mutants DA166 and DS166.

CHAPTER 4

DISCUSSION

The objective of this work has been to investigate the role of Asp 166 in the catalytic function of RMPFK. This residue has been site-specifically mutated to uncharged nonpolar, uncharged polar, and imidazole groups. But due to problems in low yield of soluble recombinant protein and impurity in enzyme preparation, a definite conclusion can not yet be reached as to whether Asp 166 is the general base that interacts with the proton on the 1-OH of Fru6P. Nevertheless, based on preliminary kinetic study results and selective plating on mannitol/M63, Asp 166 appears to be a key residue involved in rate enhancement.

The model accepted for the catalytic transition state of EcPFK indicates that the corresponding Asp 127 acts as a base to abstract the proton on the C-1 OH of Fru6P but does not play a particularly significant role in substrate binding (Hellinga and Evans, 1987). It does, however, stabilize the transition state. The observation that this side chain destabilizes the enzyme-product (EP) complex suggests that Asp 127 also aids in product release. In the C-half of RMPFK, nature has mutated the corresponding residue in the sugar bisphosphate binding site to serine to accommodate the effector. Work on EcPFK has shown that in the EP complex, pKa of Asp 127 is greater than 9.6, so that at the physiological pH, this side chain is uncharged in the EP complex to allow the reverse reaction to occur, thereby satisfying the requirement of microscopic reversibility (Auzat and Garel, 1992). Although uncharged, this side chain does still destabilize $F1,6P_2$ binding by a factor of 45 as compared to the Ser 127 mutant (Hellinga and Evans, 1987). That is, the presence of Asp 127 speeds up both the conversion of enzyme-

substrate to EP and the product release step. Thus, although accepted as the base catalyzing the chemical step of the reaction, this residue may also be important in destabilizing the EP complex to aid product release, especially if the latter is the rate limiting step.

If more refined results in future work reveal that DA166 and DS166 RMPFK mutants have similar reductions in catalytic efficiencies but no change in substrate binding, then Asp 166 should likely play a similar role in catalysis as Asp 127 of EcPFK, in a mechanism similar to that outlined above for the bacterial PFK.

The two problems encountered in this study involve the formation of insoluble inclusion bodies and the impurity Mtl 1-P DH in the enzyme preparations. The pPL2-pcI857 expression system in *E. coli* DF1020 expressed at a high level the recombinant RMPFK, but mostly in the form of insoluble inclusion bodies. Adding sorbitol and betaine did not significantly increase the soluble recombinant protein yield.

Due to over-expression, the production of biologically active recombinant proteins *in E. coli* is often limited by the formation of insoluble inclusion bodies (Marston, 1986). These complexes form as a result of the aggregation of the highly expressed protein with RNA polymerases, ribosomes, nucleic acids, and other cytoplasmic proteins at the site of synthesis in the cell (Hartley and Kane, 1988). For recombinant RMPFK expression, this problem is further augmented by the known tendency of the enzyme to form aggregates under various conditions (Parameggiani *et al.*, 1966; Lad *et al.*, 1973). Blackwell and Horgan's method (1991) adding sorbitol and betaine to cell growth medium involves the use of osmotic stress to facilitate the uptake of the compatible solute glycyl betaine (N,N,N-trimethylglycine). Compatible osmolytes are defined as those that can be accumulated to high intracellular concentrations with minimal

damage to normal metabolism or enzyme function (Higgins *et al.*, 1987). Betaine probably forms a protective shell around the protein, excluding ions that would otherwise induce adverse conformational changes. In this work, however, the addition of sorbitol and betaine does not quantitatively increase the active protein yield, indicating that recombinant RMPFK still aggregates in the presence of the added level of the compatible solute. Past work on the aggregation state of RMPFK has shown that methylamines such as betaine are stabilizers of the enzyme but a threshold concentration of 50-100 mM, with maximal stabilization at 1 M, is needed for the stabilization effect (Hand and Somero, 1982). Hence, increasing the concentration of betaine from 2.5 mM to at l00 mM may increase the active protein yield.

The other problem encountered in this work has also been identified and may also be readily solved. Partially-pure mutant enzyme preparations contained the impurity Mtl 1-P DH—constitutively-expressed in *E. coli* but its level of expression may have been stimulated by the addition of sorbitol to the cell medium—that catalyzes NADH oxidation under the assay conditions. The high specific activity of this contaminant initially masked the low to no activity of the site-specific mutants of RMPFK.

D-sorbitol, an epimer of D-mannitol, is known to induce high level Mtl 1-P DH expression in bacteria (Liss *et al.*, 1962). In the work on expression and purification of PFK C isozyme from rabbit brain, the addition of sorbitol and betaine was also used. Although no problem of Mtl 1-P DH impurity was mentioned, a minor contaminant with molecular mass approximately 45 kD was noted (Li *et al.*, 1994). Most probably, this is *E. coli* Mtl 1-P DH.

The problem of Mtl 1-P DH may be easily addressed by not using sorbitol but salts such as NaCl to induce osmotic stress. Otherwise, if the enzyme expression level is still high, using

purification schemes to solve the problem may be challenging since similar to RMPFK, Mtl 1-P DH binds both nucleotide-analogs and Fru6P and its elution pattern from DEAE-cellulose is only slightly different. The work on the C isozyme used ATP-Sepharose column chromatography in purification and Fru6P/ADP in elution. On the other hand, using NADH to wash the Cibacron affinity column, although costly, may solve the problem since it should elute Mtl 1-P DH.

Detection of the contaminant raises questions on the specificity of the PFK enzyme activity assay. Assays of enzyme activity should be designed to be not only quantitative, but also specific, so that even assays of crude extracts can give valid values of enzyme activity and plotting these against substrate concentration, one can determine the Michaelis constant. However, as found by this work, the activity assay coupling Fru1,6P_2 formation to NADH oxidation through three metabolic enzymes as auxiliaries is not specific. Only if the RMPFK enzyme is purified to homogeneity can one accurately measure the PFK specific activity. Further, use of the other even less specific RMPFK assay that involves the coupling of ADP formation to NADH oxidation, utilizing phosphoenolpyruvate and the enzymes pyruvate kinase and lactate dehydrogenase as auxiliaries still would not solve the problem since it also measures NADH oxidation with Fru6P in reaction mixture. Perhaps instead of α-glycerophosphate dehyderogenase and NADH, the glycolytic enzyme glyceradehyde 3-phosphate dehydrogenase, P_i, and NAD^+ can be used in PFK activity assay coupling Fru1,6P_2 formation to NAD^+ reduction. If practicable and utilized, this assay system should not detect Mtl 1-P DH activity. A more direct but tedious assay system that should also specifically detect PFK activity involves a discontinuous assay utilizing γ-^{32}P labeled ATP as PFK substrate and determining amount of radioactively labeled Fru1,6P_2 formed.

Since the site-directed mutants studied in this work likely have little to no activity (only DH166 may have some activity since the imidazole ring of histidine, with a pK_a of typically 6-7 in proteins, is an ideal group for proton transfer at neutral pH values, can act as a general base catalyst, and does so in a number of enzymes), absolute purification of these enzymes would be and has been difficult. Future work must focus first on purification of wild type RMPFK to homogeneity and subsequently purification of the mutants using the same scheme, using protein concentration assay and SDS-PAGE in detection. The purification difficulty is compounded by the soluble protein yield problem. Unless enough pure mutated enzymes can be obtained, determination of the catalytic efficiency, predicted to be four orders of magnitude lower than the wild type, and the Michaelis constant will not be feasible.

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