Identification of Residues of *Escherichia coli* Phosphofructokinase That Contribute to Nucleotide Binding and Specificity†

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ABSTRACT: The apparent affinity of phosphofructo-1-kinase (PFK) of *Escherichia coli* for ATP is at least 10 times higher than for other nucleotides. Mutagenesis was directed toward five residues that may interact with ATP: Y41, F76, R77, R82, and R111. Alanine at position 41 or 76 increased the apparent K_m by 49- and 62-fold, respectively. Position 41 requires the presence of a large hydrophobic residue and is not restricted to aromatic rings. Tryptophan and, to a lesser extent, phenylalanine could substitute at position 76. None of the mutants at 41 or 76 showed a change in the preference for alternative purines, although F76W used CTP 3 times better than the wild type enzyme. Mutations of R77 suggested that the interaction was hydrophobic with no influence on nucleotide preference. Mutation of R82 to alanine or glutamic acid increased the apparent K_m for ATP by more than 20-fold and lowered the k_{cat}/K_m with ATP more than 30-fold. However, these mutants had a higher k_{cat}/K_m than wild type for both GTP and CTP, reflecting a loss of substrate preference. A loss in preference is seen as well with R111A where the k_{cat}/K_m for ATP decreases by only 68%, but the k_{cat}/K_m with GTP increases more than 10-fold. Activities with ITP, CTP, and UTP are also higher than with the wild type enzyme. Arginine residues at positions 82 and 111 are important dictators of nucleoside triphosphate preference.

The ATP-dependent phosphofructo-1-kinase (PFK)[1] of *Escherichia coli* is an allosteric regulatory enzyme that catalyzes the phosphorylation of Fru 6-P to produce Fru 1,6-P_2. With respect to kinetic allosteric properties, the response of the activity to Fru 6-P concentration is sigmoid, and although ADP is an allosteric activator and PEP is an inhibitor, both ligands have been shown to bind to the same site, but apparently providing different conformers. The enzyme is a tetramer with a subunit mass of 35 kDa. The three-dimensional crystal structure has been determined in the presence and absence of allosteric ligands and substrates (*1*−*3*), leading to predictions about the involvement of specific residues in the binding of ligands. Site-directed mutagenesis studies (*4*−*11*) of the allosteric and Fru 6-P binding sites have established critical roles of several residues in these binding sites along with details of the catalytic and regulatory mechanisms.

On the other hand, nothing beyond the predictions based on crystallography has been proposed regarding the role of critical residues in the binding site for ATP, nor have parameters influencing nucleotide specificity been established. No extensive analysis of the nucleotide specificity has been described, although it is generally conceded that PFKs will use all of the common nucleotides. The apparent affinity for ATP is 10 times higher than that for ITP, and the disparity is even greater with other nucleotides (*12*). In this investigation, we examine residues that appear to interact with the nucleoside moiety in the ATP binding site. We identify residues that contribute to the binding of ATP as well as those that have a strong influence on nucleotide specificity.

MATERIALS AND METHODS

Strains. The *E. coli* strains used in these studies were DF 1020 [*pro*-82, Δ*pfk*201, *recA*56, Δ(*rha-pfkA*)200, *endA*1, *hsdR*17, *supE 44*] and XL1-Blue MRF′ supercompetent cells [Δ(*mcrA*)183, Δ(*mcrCB-hsdSMR-mrr*)173, *endA*1, *recA*, *lacI*^{q}*ZDM15*-F′] from Stratagene (La Jolla, CA). The plasmid bearing the PFK gene, pRZ3, has been described previously (*10*).

PCR Mutagenesis. PCR mutagenesis was carried out using a modified two-stepped PCR protocol (*13*). In the first step, two PCRs were performed: one involving a mutagenic antisense primer and a second sense primer that was upstream from the *Hin*dIII site that flanks the 5′ end of the gene, and a second reaction involving a sense mutagenic primer and an antisense primer downstream from the *Bgl*II site within the gene. The two mutagenic primers varied from 18 to 25 bases depending upon the number of nucleotide substitutions required for the change. The reaction in 100 μL included 2.5 units of *Pfu* DNA polymerase (Stratagene), reaction buffer provided by Strategene, 100 ng of pRZ3, 250 ng of flanking primer, 250 ng of mutagenic primer, and each NTP at 0.2 mM. The PCR products were purified with a Prep-

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[1] Abbreviations: PFK, phosphofructo-1-kinase; Fru 6-P, fructose 6-phosphate; Fru 1,6-bisP, fructose 1,6-bisphosphate; PCR, polymerase chain reaction.

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A-Gene DNA purification kit (Bio-Rad, Hercules, CA). The second-round PCR was performed in the same buffer using 100 ng each of the first-round product and 250 ng of each flanking primer. The PCR product was purified as described previously and digested with *Hin*dIII and *Bgl*II. The digested product was again purified with the DNA purification kit and subsequently ligated into pRZ3, and transformed into XL1-Blue MRF′ supercompetent cells. The modified pRZ3 was subsequently purified and sequenced to verify the mutation using the ThermoSequenase cycle sequencing kit from Amersham. For those mutants that showed striking changes in properties, the entire gene was sequenced in one direction at the University of Iowa sequencing facility.

Expression and Purification of the Enzymes. The mutated *E. coli* PFK was expressed in DF 1020 cells. The bacteria were grown in LB media containing 100 ng/mL ampicillin. The wild type and several high-activity mutants were purified to homogeneity by the method of Kotlarz and Buc (*14*) as modified by Banas et al. (*15*) using Blue Sepharose CL-6B affinity chromatography. After loading, the column was washed with 50 mM Tris/acetate, 1 mM EDTA, and 10 mM β-mercaptoethanol (pH 7.4) (buffer A) containing 0.6 M KCl. Elution was achieved by the addition of 1 mM ATP and 2.5 mM $MgCl_2$ in the washing solution. Several mutants that were bound weakly by the column were purified (>95% pure) using Blue Sepharose CL-6B that was washed extensively with buffer A without added KCl and eluted with 1 mM ATP and 2.5 mM $MgCl_2$ in buffer A. In the course of purifying various mutants, it was observed that 1 mM Fru 6-P could be used instead of ATP to elute the enzyme. This was used for several preparations. For the F76A mutant that did not bind to the affinity column at pH 7.4, binding was achieved at pH 8.0 with the addition of 10 mM ammonium sulfate to buffer A. Elution was achieved with 1 mM Fru 6-P. The purified enzymes were stored in the presence of 2 mM ATP and 50% glycerol at − 20 °C. Before being used in kinetic assays, the enzymes were dialyzed against assay buffer. All mutant preparations were judged to be homogeneous by SDS−PAGE.

Enzyme Assay. Enzyme activity for both native and modified *E. coli* PFK was assayed at 30 °C and at pH 7.2 in a Gilford *Response* spectrophotometer. The assay medium contained 150 mM Tes/KOH, 1 mM EDTA, 2.5 mM $MgCl_2$, 0.2 mM NADH, 1 mM GDP, the indicated concentrations of ATP and Fru-6-P, 1 mM dithiothreitol, 0.6 unit of aldolase, and 0.3 unit each of triosephosphate isomerase and glycerophosphate dehydrogenase. Reactions were initiated by the addition of Fru 6-P. All reagents and assay auxiliary enzymes were purchased from Sigma.

For the determination of kinetic values for nucleoside triphosphates and Fru 6-P of both native and modified *E. coli* PFK, the aforementioned assay medium was used with the indicated concentrations of nucleotides or Fru 6-P. For nucleotides, the concentration of Fru 6-P was kept at 1 mM. The magnesium ion concentration was kept 4 mM higher than the concentration of nucleoside triphosphate under all conditions to ensure that virtually all of the nucleotides existed as the magnesium complex. The apparent K_ms for ATP and Fru 6-P were obtained using the Michaelis−Menten equation and the GraFit graphical analysis program. Because the assays were performed in the presence of the allosteric activator, GDP, Fru 6-P saturation curves were hyperbolic.

Determination of Circular Dichroism Spectra. Spectra were determined using a Jasco 700 spectropolarimeter with a 2 mm path at room temperature and a time constant of 1 s at 50 nm/min. Native and modified enzymes were dialyzed against 10 mM potassium phosphate at pH 7.2 prior to being used.

Other Methods. Viewing the model of the active site of PFK took place on a Silicon Graphics 4 Indigo^2 R10,000 workstation running version 96.0516 of Quanta. The Brookhaven Protein Data Bank (PDB file name 1PFK) provided the initial ATP−PFK crystal structure coordinates of the R state complex with Fru 1,6-bisP, ADP, and magnesium ion (*3*). Conversion of the file to the full active site was performed as described previously (*16*). Energy minimizations of mutated structures or structures in the presence of alternative substrates were carried out using the CHARMm force field. Stereo images were generated using the Midas Plus Molecular Interactive Display and Simulation Program.

The concentration of protein was determined with the Bradford's dye binding assay with bovine serum albumin as the standard (*17*). Gel electrophoresis of proteins was carried out using a 14% polyacrylamide support according to the system of Laemmli (*18*).

RESULTS AND DISCUSSION

Strategy for Identifying Candidate Residues. Previously, extensive site-directed mutagenesis studies of the bacterial ATP-dependent PFK have focused on residues involved in Fru 6-P and in allosteric ligand binding (*4*−*11*, *19*, *20*). On the other hand, no residues have been identified that contribute extensively to the binding of MgATP, nor have residues been identified that determine nucleotide specificity. Evans' laboratory (*2*, *3*) has proposed a number of residues that appear from the X-ray crystallographic analysis to be important to some aspects of ATP binding, in particular, those involved in interactions with the phosphoryl groups. Mutations of a few of these residues, R72, D103, T125, D129, and R171, produced little or no change in the apparent K_m for ATP (*4*−*6*).

In this study, residues have been chosen for an analysis of ATP binding by using two approaches. First, we examined the ATP binding site using the coordinates deposited in the Brookhaven Protein Data Bank (PDB file name 1PFK) (*3*). All residues surrounding the nucleotide binding site (actually what is available is the structure containing the product ADP) that appeared to interact with the adenosine were identified. The ADP site is found in a cleft between an α-helix and a 3_{10}-helix with glycine residues 104 and 108 providing space for the adenosine. The main chain of F73 provides hydrogen bonding to the ribose ring. With regard to residues whose side chains could be important, Y41, F76, M107, and the three carbons of the side chain of R77 could provide hydrophobic interactions with the adenine ring. The second approach was to identify among these four residues those that are totally conserved in the binding fold among the sequences of all of the ATP−PFK structures that have been published. M107 and R77 did not seem to be likely candidates on the basis of this criterion. On the other hand, Y41 and F76 were found to be very well-conserved among sequences in the data base. These two aromatic residues are

FIGURE 1: Stereoview of all atoms within 9 Å of N-3 of the adenosine ring of ATP. Note that water molecules are blue. Residues modified in this study are green and yellow. Adenosine is red. The hydrogen atoms have been omitted to simplify viewing.

Table 1: Kinetic Parameters[a] of Tyrosine 41 Mutants

	ATP		ITP		GTP		CTP		UTP	
	K_m	k_{cat}/K_m	K_m	k_{cat}/K_m	K_m	k_{cat}/K_m	K_m	k_{cat}/K_m	K_m	k_{cat}/K_m
wild type	0.21	392	2.2	22	4.3	10	11	5.9	5.1	4.6
Y41A	9.6	0.54	11	0.020	8.8	0.016	13	0.0178	36*	0.0065
Y41F	0.17	595	2.3	25	3.7	16	7.2	7.6	11.6	3.8
Y41W	0.6	30.1	5.3	1.0	5.1	0.74	8.8	0.43	19.5	0.14
Y41L	0.38	226	5.7	8.2	6.4	3.1	8.9	3.5	16.2	1.7

[a] All assays were performed at pH 7.2, 1 mM GDP, and other conditions described in Materials and Methods. Apparent K_ms for nucleotides were determined at 1 mM Fru 6-P. Kinetic constants were derived using the Grafit program. Standard errors did not exceed 15% of the indicated values except where indicated by an asterisk.

aligned in a nearly coplanar manner and at near right angles to the adenosine. Figure 1 provides a stereoview of those atoms within 9 Å of N-3 of the adenosine ring. Not shown are the potential main chain interactions mentioned above. Shown clearly in the figure is the relationship to adenosine of the two aromatic residues: Y41 and F76. They were chosen for mutagenesis.

Four different substitutions were made for the tyrosine at position 41. Substitution of an alanyl residue would remove potential aromatic or hydrophobic interactions. Substitution of phenylalanine or tryptophan would lead to an examination of the specificity for an aromatic residue at this position, whereas substitution of leucine would lead to an examination of whether replacement can be achieved with another hydrophobic residue. Three substitutions were made for F76: alanine, tyrosine, and tryptophan.

Properties of the Mutants at Positions Y41 and F76. During purification, the Blue Sepharose column under conditions used with the wild type enzyme bound all mutants with the exception of F76A. Y41F was purified in a manner exactly like that of the wild type enzyme, while ATP eluted Y41L, Y41W, F76Y, and F76W without added salt. Binding to Blue Sepharose and specific elution by ATP suggest the global integrity of the proteins. Y41A was bound by Blue Sepharose but was eluted by 0.1 M KCl without ATP. F76A was bound to the column at pH 8.0 and eluted with Fru 6-P as described in Materials and Methods. As will be seen in the kinetic analysis, these two mutants, Y41A and F76A, had the lowest apparent affinity for ATP.

The overall structural integrity of mutants Y41A and F76A was examined by circular dichroism as well, which was particularly important for the alanine substitution mutants because of their different behavior during purification and because of their low k_{cat} (see below). The spectra of these two mutants were virtually identical to that of wild type PFK, suggesting no change in the global structure of the mutants.

The enzymes were assayed with five different nucleoside triphosphates: ATP, GTP, ITP, UTP, and CTP. GDP at a concentration of 1 mM was included in the assay to ensure that the enzyme was in the active R state (*12*). It should be noted that the k_{cat} and K_m values for ATP and the other nucleoside triphosphates are only apparent values because they were derived with a single saturating concentration of Fru 6-P (1 mM). However, assays of the mutants at varying Fru 6-P concentrations showed that none exhibited a significant decrease in sugar phosphate affinity. The apparent K_ms for Fru 6-P determined in the presence of 2 mM ATP were in the range of 34−114 μM, whereas that for the wild type enzyme was 107 μM. Clearly, 1 mM Fru 6-P was near saturation in the studies of the nucleoside triphosphate concentration dependence. Furthermore, the Fru 6-P saturation curves for the wild type enzyme and all mutants were nearly hyperbolic as one expects in the presence of 1 mM GDP, which promotes the active R state (*12*).

Values for K_m and k_{cat}/K_m for each of the mutants at position 41 are shown in Table 1. Removal of the bulky, hydrophobic group at position 41 (see Y41A) decreased the apparent affinity for ATP by about 46-fold, whereas substitution with aromatic groups phenylalanine and tryptophan or the hydrophobic residue, leucine, led to little change in the apparent K_m. The magnitude of the decrease in the so-called specificity constant (k_{cat}/K_m) is more than 700-fold for the alanine mutant. The structural requirement at position 41 appears to be restricted to the presence of a large hydrophobic residue and not restricted to aromatic rings because the decrease in k_{cat}/K_m for leucine was only about 40%. It is interesting that phenylalanine at position 41 produces a more efficient enzyme. The specificity constant was about 50% higher for the Y41F mutant, and the K_m was lower. Of the 24 sequences of ATP-dependent PFKs that are available in data bases, all have tyrosine in this position with the single exception of that of *Lactobacillus delbruckeii*, which has phenylalanine and a K_m for ATP that is slightly lower than that seen here with *E. coli* PFK (*21*).

The data in Table 1 show that substitution at position 41 has very little influence on the nucleotide specificity of the

Table 2: Kinetic Parameters[a] of Phenylalanine 76 Mutants

	ATP		ITP		GTP		CTP		UTP	
	K_m	k_{cat}/K_m	K_m	k_{cat}/K_m	K_m	k_{cat}/K_m	K_m	k_{cat}/K_m	K_m	k_{cat}/K_m
wild type	0.21	392	2.2	22	4.3	10	11	5.9	5.1	4.6
F76A	13.1	0.1	7.7	0.015	9.5	0.013	3.6	0.022	32*	0.0049
F76Y	1.14	46	14.3	1.4	10.7	0.81	66*	0.86	27*	0.36
F76W	0.17	224	6.8	4.8	7.1	6.6	1.43	15.8	11.9	1.74

[a] All assays were performed at pH 7.2, 1 mM GDP, and other conditions described in Materials and Methods. Apparent K_ms for nucleotides were determined at 1 mM Fru 6-P. Kinetic constants were derived using the Grafit program. Standard errors did not exceed 15% of the indicated values except where indicated by an asterisk. In those instances, K_m estimate errors can be as high as 50%.

Table 3: Kinetic Parameters[a] of Arginine "Crown" Mutants

	ATP		ITP		GTP		CTP		UTP	
	K_m	k_{cat}/K_m	K_m	k_{cat}/K_m	K_m	k_{cat}/K_m	K_m	k_{cat}/K_m	K_m	k_{cat}/K_m
wild type	0.21	392	2.2	22	4.3	10	11	5.9	5.1	4.6
R77A	0.52	119	19.7	5.5	5.5	1.8	60*	2	21	1.61
R77L	0.52	142	11.9	3.8	8.6	1.77	9.9	0.86	4.4	2.1
R77E	2.2	39	2.6	10.1	6.3	2.3	12.5	1.56	44	1.06
R77D	4.5	3.1	21	0.16	8.5	0.18	34	0.11	19	0.186
R82A	5.5	12.4	14.9	2.9	2.1	14.2	1.64	29	15.0	2.0
R82E	4.5	2.9	6.2	0.31	1.43	1.24	50*	0.2	70*	0.2
R111A	0.43	125	1.65	31	0.62	110	2.2	8.1	7.7	1.18
R111E	1.69	35	5.4	16.8	1.67	32	16.5	1.01	70*	1

[a] All assays were performed at pH 7.2, 1 mM GDP, and other conditions described in Materials and Methods. Apparent K_ms for nucleotides were determined at 1 mM Fru 6-P. Kinetic constants were derived using the Grafit program. Standard errors did not exceed 15% of the indicated values except where indicated by an asterisk. In those instances, K_m estimate errors can be as high as 50%.

enzyme. On the basis of the specificity constant, ATP is used 20−30 times better than ITP, and the order of preference is ATP ≫ ITP > GTP > CTP > UTP.

Mutation of the phenylalanine at position 76 produced results shown in Table 2 that are roughly similar to that for the tyrosine mutation (Table 1). Substitution by alanine increased the apparent K_m for ATP by about 60-fold and decreased the k_{cat}/K_m by 4000-fold. The need for a bulky group at this position is obvious. Either aromatic residue, tyrosine or tryptophan, produces relatively modest changes in substrate affinity with tryptophan substituting better than tyrosine. The specificity constant, k_{cat}/K_m, decreased by about 43% with tryptophan and 88% with tyrosine. With of all of these mutants, ATP remained the best substrate with k_{cat}/K_m values at least 15 times higher than that with any of the other nucleoside triphosphates. However, the order of preference was altered, particularly with the tryptophan mutant. CTP was the second best substrate with a k_{cat}/K_m that was almost 3 times higher than that seen with the wild type enzyme. One can imagine that the bulkier tryptophan may fill the space better with the smaller pyrimidine nucleotide substrate. However, UTP remains the least preferred substrate, indicating that simply filling the space left by the smaller pyrimidine substrate does not provide the full explanation for a change in preference. GTP was slightly preferred over ITP, which reverses the situation seen with the wild type enzyme.

Identifying Candidate Residues for Determining Nucleotide Specificity. Whereas Y41 and F76 were identified as being important for nucleotide binding, these residues do not distinguish among the various bases in terms of binding preference with the exception of the modest changes alluded to in the previous paragraph. In a search for determinants of specificity, residues near N-6 of ATP were identified. In the three-dimensional structure, three arginine residues, R77, R82, and R111, form a "crown" around N-6 with potential interactions directly with N-6 or N-1 of the adenine, or through a water molecule that interacts with N-6. A stereoview of the relationship of the three arginine residues to the adenine ring is illustrated in Figure 1. The three carbons of the R77 side chain can be seen lying across the adenine ring to provide a hydrophobic interaction. The guanidinium groups of the other two arginines, R82 and R111, are shown around the adenine. Shown as well are the water molecules that lie close to N-6 and interact with the guanidinium groups. R82 was particularly interesting because it is conserved in 19 of 24 ATP−PFK sequences in Genbank, with the remaining five bacteria having glutamine in that position. These three residues (R77, R82, and R111) were chosen for mutagenesis and substrate specificity analysis. Arginines at these positions were replaced with either alanine or glutamic acid, the latter providing a reversal of the charge from the wild type enzyme. Two additional mutants were produced at position 77: R77D and R77L. Because the three-dimensional structure of the enzyme suggested that the three carbons of the side chain of R77 could provide hydrophobic interactions with the adenine ring, a leucine substitution was used to test for that interaction.

Properties of the Mutants at Positions R77, R82, and R111. During purification, the Blue Sepharose column bound all of the mutants. The behavior of R82A during purification was identical to that of the wild type enzyme, while the remaining mutants of positions 77, 82, and 111 were eluted by 1 mM ATP or 1 mM Fru 6-P without added salt. In several instances, an additional purification by chromatography on a Mono Q anion exchange column on a FPLC system was carried out as described previously (*10*). The specific elution by ATP of PFK mutants that are bound by Blue Sepharose suggests maintenance of the global integrity of the proteins.

As with the other mutants, the overall structural integrity of the mutants that showed significant changes in k_{cat} (R77D and R82A) was further examined by circular dichroism. The spectra of these two mutants deviated by less than 5% from that seen with wild type PFK, suggesting that the mutations did not produce changes in the global structure.

The enzymes were assayed with five different nucleoside triphosphates (ATP, GTP, ITP, UTP, and CTP), all in the presence of the allosteric activator, GDP. The k_{cat} and K_m values were derived with a single saturating concentration of Fru 6-P (1 mM) and thus are only apparent values. The conclusion that 1 mM Fru 6-P was saturating was based upon determinations of the apparent K_ms for Fru 6-P, which ranged for all mutants of R77, R82, and R111 from 30 to 108 μM in the presence of 2 mM ATP, whereas that for the wild type enzyme was 107 μM.

Table 3 shows the values for the apparent K_m and k_{cat}/K_m for each of the arginine mutants. The changes in kinetic parameters produced by mutations of Arg77 support the hypothesis that the major interaction is a hydrophobic one with little effect on substrate preference at his position. Little change was seen in the apparent affinity for ATP if alanine or leucine was substituted for arginine, whereas increases in K_m of 10- and 20-fold were observed when glutamic and aspartic acids, respectively, were substituted. A drop in the k_{cat}/K_m of slightly more than 60% was seen with the leucine mutant and about 70% with the alanine mutant. A more profound decrease in the k_{cat}/K_m was seen with the aspartate mutant, but the decrease was much smaller with the glutamate substitution. Apparently, the additional carbon in the side chain of glutamate provides some of the hydrophobic interaction that was provided by the side chain of arginine. As was mentioned earlier, an arginine residue at position 77 is not well conserved among the ATP-dependent PFKs that have been sequenced. However, in all cases, residues are found that can provide at least limited hydrophobic interaction by part of the side chain; no Ser, Asp, or Gly residues are found at this position in any of the sequenced PFKs.

With respect to substrate preference, mutations at position 77 had very little effect. The order of substrate preference is roughly the same as that in the wild type. To place a numerical value on substrate preference, it can be defined as the K_m/k_{cat} for ATP divided by the K_m/k_{cat} for any other nucleotide. If anything, several mutants showed an increased preference for ATP. For example, the preference of the wild type enzyme for ATP versus GTP was 38, whereas the values are 66 and 80 for the R77A and R77L mutants.

A mutation at position 82, which is conserved in ATP−PFK structures, has a much more profound effect on the activity of the enzyme. The apparent affinity for ATP decreased by more than 20-fold with either the alanine mutant or the glutamic mutant (Table 3). The k_{cat} value with ATP for the R82A mutant (derived from the data in Table 3) was less strongly influenced. The value for k_{cat}/K_m was lower by more than 30-fold with the alanine mutant and more than 130-fold with the glutamic acid mutant.

What is particularly striking is the apparent role of this residue in substrate preference, and it is clear that the arginine at position 82 contributes very significantly to the preference of *E. coli* PFK for ATP. The data in Table 3 show that substitution of either alanine or glutamic acid for arginine led to a striking suppression of the preference for ATP. R82A had a 40% greater activity (as indicated by the k_{cat}/K_m) than the wild type enzyme with GTP as a substrate and an almost 5 times greater activity with CTP as a substrate. This was due to an apparent increase in the affinity for both GTP and CTP as a result of the substitution of alanine for arginine at position 82. As noted above, position 82 is occupied by glutamine in several bacterial PFKs. Substrate preference has been examined for the PFK of one of these organisms, *Bacillus stearothermophilus* (*22*), which was shown to have a preference (k_{cat}/K_m) for ATP versus GTP of only 3.5-fold as compared to the preference in *E. coli* PFK of 39-fold (Table 3). This supports the suggested role of Arg82 in dictating the preference for ATP.

The consequences of mutation at position R111 (Table 3) are even more interesting with regard to substrate specificity

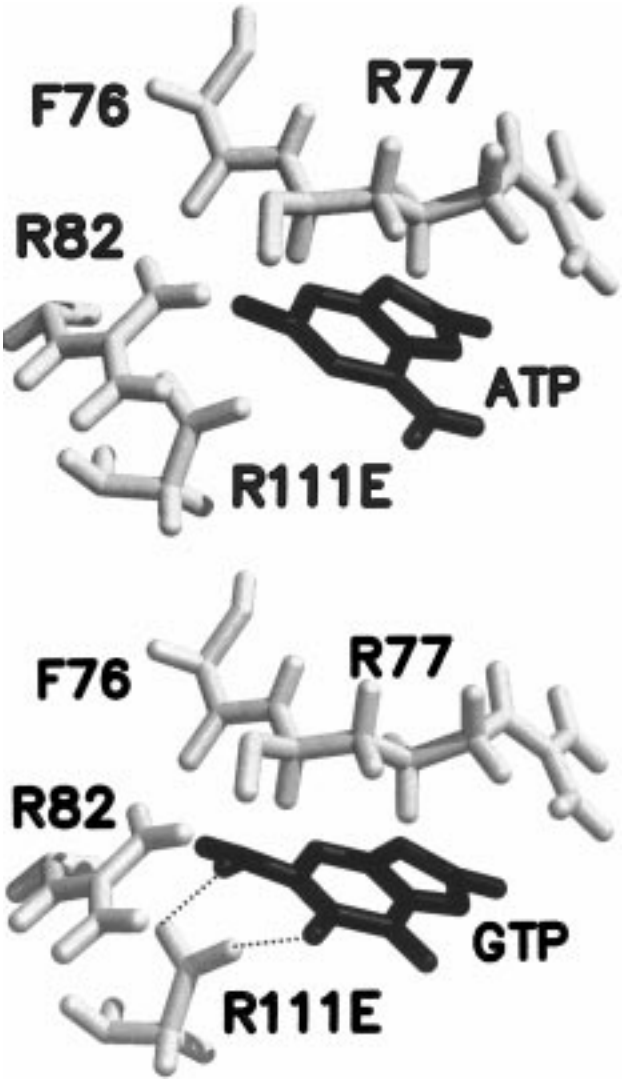

FIGURE 2: Hypothetical relationship between arginine "crown" residues to ATP and GTP in the R111E mutant of PFK. For illustration purposes, several of the residues have been truncated to indicate only those parts of the residues in proximity to the 6 positions of the purine rings. Only the side chain of R111, the guanidinium group of R82, and the backbone atoms of F76 are shown. The Brookhaven Protein Data Bank (PDB file name 1PFK) provided the initial ATP−PFK crystal structure coordinates of the R state complex, which was converted to the full active site as described previously (*16*). Energy minimizations of R111E with ATP or GTP in the active site were carried out using the CHARMm force field.

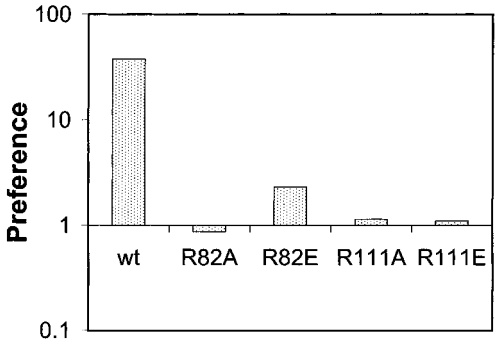

FIGURE 3: Changes in enzyme preference with mutation at R82 and R111. Differences in the height of the histogram columns represent the degree to which substrate preference, calculated as the ratio of $(k_{cat}/K_{m_{ATP}})/(k_{cat}/K_{m_{GTP}})$, is shifted by mutation.

because the mutation did not produce substantial losses in activity but altered substrate specificity significantly. With R111A, the k_{cat}/K_m for ATP decreased by 68%, but the increase in k_{cat}/K_m with GTP as a substrate was more than 10-fold. The activity with the other nucleoside triphosphates was also higher than in the wild type enzyme. Another way to look at the effect of the mutation is to compare the values for the apparent K_m. With the wild type enzyme, the total difference in the apparent affinity from the highest substrate affinity to the lowest substrate affinity was 55-fold, whereas the range seen with the R111A mutant was only about 5-fold. The apparent affinity for GTP for R111A was more than 7-fold greater than that of the wild type.

Substitution of glutamate at position R111 (Table 3) had a greater effect on k_{cat} with ATP as a substrate than that seen with the alanine substitution. With ATP as a substrate, the K_m was about 8 times higher than that of the wild type and k_{cat}/K_m was reduced by 90%. With respect to substrate preference, this mutant used GTP as well as ATP, and 3 times better than did the wild type enzyme.

Using the three-dimensional structure of PFK in the Brookhaven Protein Data Bank, the mutant R111E was generated and energy minimized using the CHARMm force field. The structure was then subjected to additional minimization with either GTP or ATP in the active site. Figure 2 shows the relationship of the bases of ATP and GTP with the arginine "crown" residues of the R111E mutant. Figure 2 depicts additional interactions of the glutamic acid with guanine that may account for the higher affinity of the mutant for GTP than that seen with ATP or with GTP binding to wild type PFK.

Figure 3 further illustrates the loss of substrate preference that results from changes in R82 or R111. In each mutant at these positions, GTP was used nearly as well as ATP. It is interesting that the positive charge at positions 82 and 111 seemed to play little or no role in the binding of nucleotide or in dictating nucleotide preference. Replacement by alanine at these positions showed modest effects on the utilization of ATP. Replacement with a charged residue reduced the extent of ATP utilization by about 90%, but this is relatively moderate when you consider that the charge has been completely reversed. R77 plays a general hydrophobic role in the binding of all nucleotides, while the actions of R82 and R111 seem to be that of providing a hydrogen bond network with the ATP and the water molecules that specifically provide a tight interaction with ATP. It is the details of how that network is constructed that dictate the specificity with regard to the binding and utilization of purines.

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