Identification of Allosteric Sites in Rabbit Phosphofructo-1-kinase†

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ABSTRACT: Earlier studies indicated an evolutionary relationship between bacterial and mammalian phosphofructo-1-kinases (PFKs) that suggests duplication, tandem fusion, and divergence of catalytic and effector binding sites of a prokaryotic ancestor to yield in eukaryotes a total of six organic ligand binding sites. The identities of residues involved in the four binding sites for allosteric ligands in mammalian PFK have been inferred from this assumed relationship. In the current study of the C isozyme of rabbit PFK, two arginine residues that can be aligned with important residues in the catalytic and allosteric binding sites of bacterial PFK and that are conserved in all eukaryotic PFKs were mutated. Arg-48 was suggested previously to be part of either the ATP inhibitory or the adenine nucleotide activating site. However, the mutant enzyme showed only slightly less sensitivity to ATP inhibition and was fully activatable by adenine nucleotides. On the other hand, sensitivity to citrate and 3-phosphoglycerate inhibition was lost, indicating an important role for Arg-48 in the binding of these allosteric effectors. Mutation of Arg-481, homologous to an active site residue in bacterial PFK, prevented binding and allosteric activation by fructose 2,6-bisphosphate. A new relationship between the allosteric sites of mammalian PFK and bacterial PFK is proposed.

Eukaryotic phosphofructo-1-kinases are more than twice the size of prokaryotic PFKs[1] and are under regulatory control by a wider array of effectors than that seen with the simpler bacterial PFKs. Sequencing data have suggested an evolutionary relationship between the two types of PFK that indicates duplication, tandem fusion, and divergence of catalytic and effector binding sites to yield in eukaryotes a total of six organic ligand binding sites: ATP and Fru 6-P catalytic sites, activator sites for adenine nucleotides and for Fru 2,6-bisP, and inhibitor sites for ATP and for citrate and 3-P-glyceric acid (*1*). Based on the strict conservation of active site residues in the N-terminal half of the eukaryotic enzyme with those of the bacterial PFKs, it is assumed that the active site of the eukaryotic PFK is located in the N-terminus half (*1*). On the other hand, the appropriate identification of the allosteric sites remains to be established. On the basis of earlier partial proteolytic digestion studies, it was initially deduced that the bacterial allosteric site for ADP and phosphoenolpyruvate evolved into the mammalian ATP inhibitory site (*1*). Later discussions based upon the analyses of additional PFK sequences (*2*−*4*) have assumed the validity of this deduction. However, chemical modification studies have suggested as a citrate binding component Lys567 (*5*), which is homologous with a critical residue in the ADP allosteric site of *Escherichia coli* PFK (*6*). A recent analysis of the homologous relationship of the allosteric sites has assumed that the citrate site has evolved from the ADP allosteric site of the duplicated precursor that contributes the carboxyl-terminal half of the mammalian PFK and includes Lys567 (*5*). It was assumed that the ATP inhibitory site arose from the ATP catalytic site of the ancestral PFK. Nonetheless questions remain. In bacterial PFK, the allosteric site is contributed by two different subunits. Depending upon how one arranges the contributing domains of the presumed citrate binding site, it is quite possible that the contributing domain for half of the citrate site of the mammalian enzyme comes from a region near the amino terminus of the mammalian PFK, not from the carboxyl terminal half as was assumed by Li et al. (*7*). Resolution of this problem can be achieved by analyzing the allosteric properties of an enzyme with a mutation at Arg48, which is analogous with Arg25 of *E. coli* PFK.

Another residue that plays an important role in catalysis in *E. coli* PFK is Arg72, located strategically between the ATP and Fru 6-P sites and neutralizing the charge on the phosphoryl group during catalytic transfer (*8*). Considering its location, the homologous residue in the mammalian enzyme, Arg481, could be involved in the allosteric activation site for sugar bisphosphate or in the interaction of an allosteric effector, either an adenine nucleotide activator or inhibitor or citrate.

In the current analysis, we resolve the conflicting opinions concerning the evolution of the allosteric sites of mammalian PFK. We present evidence that the citrate site of rabbit PFK (C isozyme) has evolved from the prokaryotic ADP/phosphoenolpyruvate allosteric site and includes contributions of at least one residue near the amino terminus. Furthermore, it is demonstrated that the homologue of a

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[1] Abbreviations: PFK, phosphofructo-1-kinase; Fru 6-P, fructose 6-phosphate; Fru 1,6-bisP, fructose 1,6-bisphosphate; DTT, dithiothreitol; IPTG, isopropylthiogalactoside; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Tes, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid.

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catalytically important Arg residue of *E. coli* PFK is involved into the fructose 2,6-bisP binding site and plays no role in ATP binding to the inhibitory site.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis. The cloning of the cDNA for rabbit PFK-C into the pET-3d (Novagen) vector and its expression in the BL21 strain of *E. coli* has been described previously (*7*). Site-directed mutagenesis by PCR was performed by the overlap extension PCR method of Ho et al. (*9*). The two cycles of PCR included flanking primers with designed XbaI an ApaI sites. The resulting fragment was cloned into the pET3d vector previously digested with XbaI and ApaI. Mutants were identified by sequencing with the method of Sanger et al. (*10*). Entire reading frames were sequenced to confirm the lack of additional mutations.

Enzyme Expression and Purification. Expression and purification were carried out as described previously with several modifications (*7*). The plasmids containing wild-type and mutant reading frames were transformed into BL21(DE3) cells which were grown overnight in 500 mL of LB medium plus 200 μg/mL of ampicillin. Cells were collected by centrifugation and resuspended in 2000 mL of M9-ZB medium containing 0.5 M sorbitol, 2.5 mM betaine, additional $MgSO_4$ to a concentration of 2.5 mM, and 100 μg/mL of ampicillin. The cells were grown with shaking at 37 °C to an optical density of 0.6 at 600 nm at which point they were transferred to a 30 °C shaking bath and supplemented to 0.4 mM with isopropylthiogalactoside. The cells were harvested by centrifugation after 3 h. Four liters of cell culture were harvested by centrifugation, and the sediments were frozen in a solid CO_2−ethanol bath and stored at −70 °C. The pellets were suspended in 50 mM Tris/HCl buffer (pH 8.0) containing 5 mM $MgCl_2$, 1 mM ATP, 40 mM KCl, 0.3 mg/mL lysozyme, 2 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. The cells were disrupted by sonication and centrifuged at 14 000 rpm for 10 min. The lysate was centrifuged for 25 min at 15 000 rpm. Ammonium sulfate was added to the supernatant to achieve 50% saturation. The sediment, collected by centrifugation at 14 000 rpm for 30 min, was dissolved in 3 mL of Column Buffer (50 mM Tris/phosphate, pH 7.5, 0.2 mM EDTA, 2 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride) plus 20 mM ammonium sulfate and loaded onto a Sephacryl S300 column (230 mL). The high molecular weight fractions containing significant PFK activity were pooled and loaded onto an 18 mL column of ATP-Sepharose previously washed with Column Buffer plus 0.5 mM $MgCl_2$. The column was washed with Column Buffer plus $MgCl_2$ until the OD_{280} of the flow-through was lower than 0.05, at which point the wild-type PFK C isozyme was eluted with Column Buffer plus 0.5 mM $MgCl_2$, 0.1 mM Fru 6-P, and 0.1 mM ADP. The PFK eluted as a single homogeneous peak. The mutant enzymes were eluted with 0.2 mM Fru 6-P and 0.15 mM ADP. Any *E. coli* PFK that has not been removed previously in the Sephacryl S300 step remains bound to the affinity column under these elution conditions. In some cases, a minor contaminant revealed by SDS-PAGE was removed by repeating the affinity chromatography with a 3 mL ATP-Sepharose column. Elution of the enzymes was accomplished with 0.1 mM concentrations of ADP and Fru 6-P.

Enzyme Assay. Assays for total PFK were performed at pH 8.2 as described previously (*13*). Assays to detect allosteric properties were carried out at pH 7.2 in NaTes buffer containing 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.2 mM NADH, 0.6 unit aldolase, 1 unit triose phosphate isomerase, 0.3 unit glycero-P dehydrogenase, and the indicated concentrations of Fru 6-P and ATP. $MgCl_2$ was present at a concentration 3 mM in excess of that of ATP.

In all assays to detect allosteric properties the concentration of F-6-P used was 0.5 mM and the concentration of ATP was varied. For the inhibition analyses involving citrate and D-(−)3-phosphoglyceric acid, the amount of ATP used was at such concentration to give the maximum activity in the absence of other inhibitors.

For the analysis of activators, the amount of ATP used was at a concentration that brought about a 90% decrease of the peak value in the analysis of ATP inhibition. All the effectors used were at the indicated concentrations.

Equilibrium Binding Studies. Ligand binding to wild-type and mutant PFK was studied by the gel filtration technique of Hummel and Dreyer (*11*) and described by Kemp and Krebs (*12*). A 0.5 × 30 cm column of Sephadex G-50 was equilibrated with 25 mm α-glycerophosphate, 25 mm glycylglycine, 1 mm EDTA, and 0.1 mM dithiothreitol plus the radiolabeled ligand. A solution of 0.2−0.4 mg of PFK was applied, and the column was developed at room temperature with a flow rate of 3 mL per h. Fru 2,6-[2-^{32}P]bisP was synthesized as described by Foe et al. (*13*).

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

Other Methods. The concentration of protein was determined by Bradford's dye binding assay with bovine serum albumin as the standard (*14*). Gel electrophoresis of proteins was carried out using 12% polyacrylamide support according to the system of Laemmli (*15*).

RESULTS AND DISCUSSION

Rationale for Mutagenesis— Arg48 as a Contributor to an Allosteric Site. Evans and his colleagues (*16*−*18*) have examined the R- and T-states of *E. coli* and *Bacillus stearothermophilus* PFKs by X-ray crystallography. The crystal structure shows that the ADP site lies between two subunits and appears to make contact with a number of basic residues of both subunits. The T-state with inhibitor 2-phosphoglycolate bound shows that the inhibitor is found in the same site that binds the activator ADP (*18*). Two of the basic residues, Arg21 and Arg25, found in the allosteric site have been shown by mutagenesis (*19*) to be critical to the allosteric control of *E. coli* PFK. Utilizing data in the Genebank, one can align amino acid sequences of seven prokaryotic PFKs with the amino terminal halves of 14 eukaryotic species and demonstrate complete conservation of basic residues in these two positions. Furthermore, Arg residues are aligned as well at this position with the carboxyl terminal halves of all eukaryotic PFKs. For example, Arg25 of *E. coli* PFK aligns with Arg 48 in the amino terminal half and Arg434 in the carboxyl half of rabbit PFK-C. Conservation of these residues in mammalian PFKs is consistent with their being compo-

nents of the allosteric sites, although which site binds which effector remains to be identified. Arg48, analogous with Arg25 in *E. coli* PFK, was chosen for mutagenesis. If the early assumptions (*2*−*4*) concerning the evolution of the sites are correct, then this residue should represent part of the ATP inhibitory site. However, if the sequence alignment and assignment of sites hypothesized by Li et al. (*7*) is correct, Arg48 will be part of the adenine nucleotide activating site. On the other hand, the model for assembly of the sites suggested by Li et al. (*7*) could have the arrangement of the subunits in the tetramer reversed. In that case, the binding site would be comprised of those residues closest to the amino-terminus plus residues near carboxyl terminus. Arg48 then would be part of the binding site for citrate site, not the adenine nucleotide site since the contributions from the carboxyl terminus include Lys567, which had been previously established by chemical modification studies (*5*). An analysis of a mutation at Arg48 would sort out these conflicting views. Arg48 was mutated to leucine to provide part of the space filling properties while eliminating the charge of the arginine.

Rationale for Mutagenesis—*Arg481*. A second interesting target for mutagenesis is the residue corresponding to Arg72 of *E. coli* PFK. In the bacterial enzyme, the residue is located between the ATP and Fru 6-P sites and has been shown to play an important role in charge neutralization during phosphoryl group transfer (*8*). The homologous residue in the amino terminal half of the mammalian enzyme is Arg97, where it undoubtedly plays a catalytic role similar to that of Arg72 in the active site of the bacterial enzyme. In the carboxyl terminal half of the mammalian enzyme the aligned position is Arg481. Employing the assumption that the active site has evolved into allosteric regulatory sites (*1*), one would predict that this arginine is involved in the binding of the allosteric activator, Fru 2,6-bisP, or in the binding of an allosteric inhibitor, either ATP or citrate. As with Arg48, Arg 481 was mutated to leucine to provide part of the space filling properties while eliminating the charge of the arginine. In addition an Arg481 to aspartic acid mutation was produced as well to reverse the charge at this position to prevent interaction with any of the negatively charged potential ligands. Subsequent kinetic studies with ATP and Fru 2,6-bisP showed no significant differences between the properties of the two mutants. Because the R481D mutant was expressed at higher levels, it was used for more extensive kinetic and binding studies.

General Properties of the Wild-Type and Mutant PFKs. The mutant enzymes were bound by the ATP-Sepharose column and were eluted with ADP/Fru 6-P under similar conditions as seen with wild-type PFK. The specific elution by the dead-end complex suggests that the overall integrity of all of the mutants has been maintained. The only difference was the need for slightly higher concentrations of ADP and Fru 6-P to elute the R48L mutant in the first affinity chromatography step. It should be noted that all mutant enzymes eluted from the Sephacryl S300 column at a volume identical to that of wild-type PFK and in the region that would be expected of a protein with a mass in the range of 300 000 Da. This indicates that the mutations do not disrupt the normal oligomeric form of PFK. SDS-PAGE of the purified mutant and wild-type enzymes indicated no apparent contamination. Circular dichroism spectra of the mutants were compared to wild-type PFK and were found to be virtually superimposable (data not shown). This again indicates the mutation has not produced any global change in the secondary structure of the enzyme. The enzymes were assayed at pH 8.2 under conditions that would achieve maximum activity. No differences were detected with both wild-type and mutant enzymes having specific activities in the range of 180 units/mg.

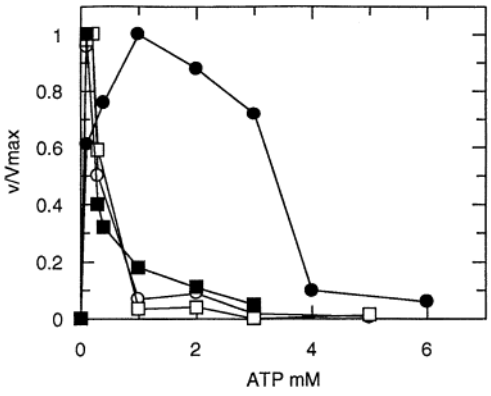

FIGURE 1: ATP inhibition of wild-type PFK (open circles), R48L (solid circles), R481D (open boxes), and R481L (closed boxes). Assays were performed as described in the Methods at pH 7.2 and Fru 6-P at 0.5 mM. Activity is expressed relative to maximal activity at this pH and Fru 6-P concentration, which is achieved in the presence of 0.1 mM ATP.

R48L: ATP Inhibition. When assayed at pH 7.2 and a relatively low concentration of Fru 6-P, PFK was inhibited by its second substrate, ATP. Figure 1 describes the ATP inhibition of wild-type PFK and the R48L mutant. While 50% inhibition of the wild-type enzyme was achieved at approximately 0.4 mM ATP, the R48L mutant was inhibited at a higher concentration; a little over 3 mM ATP was required for 50% inhibition. If indeed Arg48 were important for the binding of ATP to the inhibitory site, one would have expected a much more profound effect of the Arg48L mutation on ATP inhibition. For example, in studies of ATP-dependent PFK from *Bacillus stearothermophilus* and PPi-dependent PFK from *Propionibacterium freudenreichii* (*20*, *21*), mutations of arginine residues in substrate binding sites raised K_m or $S_{0.5}$ by 300−1400-fold. The relatively modest (7−10-fold) increase in the concentration required to achieve inhibition described in Figure 1 suggests that Arg48 plays a relatively small role in the binding of ATP to the inhibitory site or, what is more likely, that the mutation leads to a slight shift in conformation that results in slightly lower affinity for the inhibitor. This slight shift in conformation toward the active form is supported by the data below on adenine nucleotide activation.

R48L: Effects of Adenine Nucleotide Activators. The effects of activators on the activity of PFK are usually determined under conditions where the enzyme is inhibited. When comparing wild-type and mutant enzyme with differing sensitivities to the inhibitor ATP, the problem is complicated. Instead of using a fixed inhibitory concentration of ATP to examine activation, the activation assays were performed with a concentration of ATP that would achieve approximately 90% inhibition in the absence of activators. The data for these experiments are described in Figure 2 where ATP was present at 0.5 mM for the wild-type enzyme and 4 mM for the R48L mutant PFK. Previous binding data

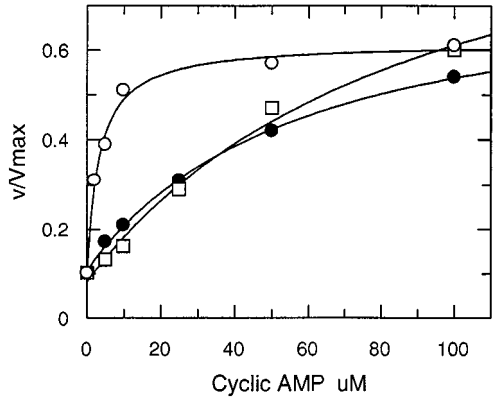

FIGURE 2: Cyclic AMP activation of wild-type PFK (solid circles), R48L (open circles), and R481D (boxes). Assay was carried out as described in the Methods at pH 7.2 and a Fru 6-P of 0.5 mM. The concentration of ATP was 0.5 mM for wild-type PFK and R481D mutant and 4 mM for the R48L mutant.

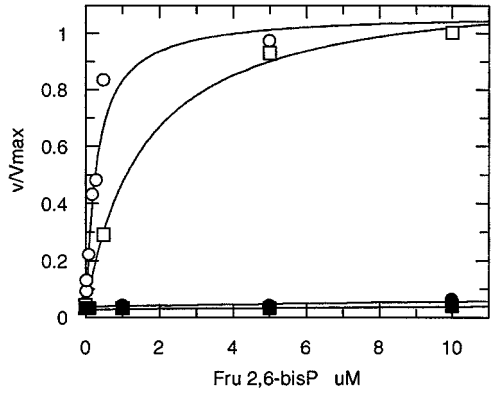

FIGURE 3: Fructose 2,6-bisP activation of wild-type PFK (open boxes), R48L (open circles), R481D (solid circles), and R481L (closed boxes). The concentration of ATP was 0.5 mM for wild-type PFK and the mutants R481D and R481L and 4 mM for the R48L mutant.

have demonstrated that ADP, AMP, and cyclic AMP bind competitively at the same binding site on the enzyme (*12*). The data in the figure are those obtained with cyclic AMP, although AMP and ADP were also shown to activate the enzymes (data not shown). Figure 2 indicates that the mutant R48L may be slightly more sensitive to the activators than wild-type enzyme. These data, as were the ATP inhibition data, are consistent with the suggestion that the conformation of the mutant enzyme may be shifted slightly toward the activated state. In any event, the binding site for the adenine nucleotides is clearly intact in the mutant.

*R48L: Fru 2,6-bisP***.** Figure 3 describes the activation of PFK by Fru 2,6-bisP. Under the conditions of the assay, the half activation concentration of the wild-type enzyme was approximately 0.3 μM. The R48L mutant PFK was fully activated by the sugar bisphosphate. The mutant appears to be slightly more sensitive to the activators. As in the case of adenine nucleotide site, the binding site for Fru 2,6-bisP is fully intact in the R48L mutant enzyme.

R48L: Other Inhibitors. Previous studies have shown the inhibitors of mammalian PFK act synergistically with ATP (*22*). This complicates the comparison of inhibitor action when the wild-type and mutant enzymes have different sensitivities to ATP inhibition. In an attempt to make such comparisons, an ATP concentration was chosen at maximal activity, at a concentration just lower than that required to

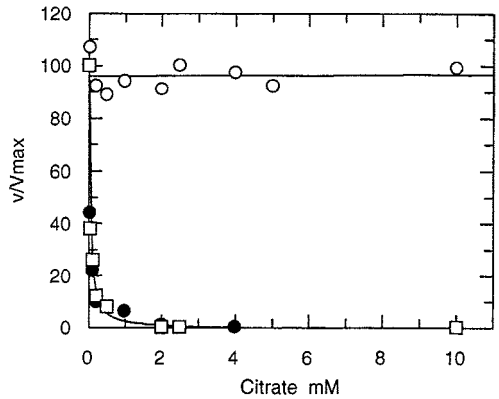

FIGURE 4: Citrate inhibition of wild-type PFK (solid circles), R48L (open circles), and R481D (boxes). Assay was carried out as described in the Methods at pH 7.2 and a Fru 6-P of 0.5 mM. The concentration of ATP was 0.2 mM for wild-type PFK and R481D mutant and 1 mM for the R48L mutant.

produce inhibition. With a Fru 6-P concentration of 0.5 mM, the concentration of ATP was 0.2 mM for the wild-type enzyme and 1.0 mM for the R48L mutant. The citrate inhibition data given in Figure 4 show that 50% inhibition of the wild-type PFK was achieved below 0.25 mM citrate, whereas virtually no inhibition of the mutant was seen at concentrations up to 10 mM. Even if the inhibition were 10% at 10 mM, and further assume that citrate binding by the mutant follows a normal saturation isotherm, then the K_d for citrate binding by the mutant is more than 300 times higher than that of the wild-type PFK. These data strongly suggest that the mutant has lost the ability to effectively bind citrate and that Arg48 is a contributing residue to the citrate binding site.

Earlier binding studies showed that 3-phosphoglyceric acid, another inhibitor of PFK, competes with citrate binding by the enzyme (*22*). The inhibitors share the common feature of a cluster of negative charges that presumably interact with basic residues in the binding site. The distribution of charges is different between the two compounds suggesting that they may not interact with the same residues in the binding pocket. For this reason, the inhibition of the mutant PFK by phosphoglycerate was also examined. The data (not shown) indicate that while the wild-type enzyme was inhibited 50% by 1.5−2.0 mM phosphoglycerate, the mutant enzyme showed no detectable inhibition up to 10 mM. These data confirm the location of Arg48 in the inhibitor binding site of mammalian PFK and indicate the importance of the residue in the binding of both citrate and 3-phosphoglycerate.

The effect is most readily interpreted as a direct effect on citrate or phosphoglycerate binding and not an indirect one through residues at the allosteric interface. Such a situation was observed by Li et al. (*23*) who mutated Gln200 in rabbit muscle PFK. In that case, the mutation presumably lay at the interface between two subunits and changed the interaction of an arginine residue from the adjoining subunit with Fru 6-P.

One might suggest that instead of blocking inhibitor binding, the mutation could change an equilibrium between R- and T-states or block the transition between these states. This is unlikely, however, since a perturbation in the equilibrium or the transition between states would be expected to have similar actions of activators, since these

actions also require a conformational change between active and inactive states. No significant effects were seen on any of the allosteric properties of the enzyme except those associated with the citrate binding site. The assumption of a blocked binding site is based as well on the sequence similarity in the region of the mutation to that of *E. coli* PFK and on the knowledge that the homologous residue of the bacterial enzyme appears to be directly involved in effector binding (*6*).

Allosteric Properties of PFK with Mutations of Arg481. As suggested earlier in this discussion, one of the possible consequences of mutations at Arg481 is the loss of ATP inhibition. As shown in Figure 1, the data for ATP inhibition of wild-type PFK and of the two Arg481 mutants are nearly superimposable, indicating no alteration in nucleotide binding to the inhibitory site. Under the conditions of the assay, 50% inhibition of the wild-type PFK, R481L, and R481D was achieved in the range of 0.4−0.7 mM ATP.

Another way of indirectly examining ATP inhibition is to look at Fru 6-P saturation at a fixed concentration of ATP. The degree of cooperativity in Fru 6-P dependent kinetics is directly dependent upon the concentration of ATP (*24*). When Fru 6-P dependence of wild-type and the two mutant enzymes were compared at a fixed concentration of ATP, the data (not shown) were nearly identical, again suggesting no effect of the mutation on ATP inhibition. It is obvious from these results that Arg481 is not involved in the binding of ATP to the inhibitory allosteric site.

Equilibrium binding of ATP was measured for the wild-type and mutant enzymes using the method of Hummel and Dreyer (*11*) that is based upon gel exclusion through Sephadex G-50 as described in the Materials and Methods. Binding by wild-type PFK and R481D was carried out with 1 μM ATP in the absence of metal ion, a condition under which one sees some contribution to binding by all three nucleotide binding sites (*12*). Under these conditions, no significant difference in ATP binding was observed. The wild-type enzyme bound 0.094 mol of ATP per mol of enzyme subunit, while the R481D mutant bound 0.101 mol of ATP per mol of enzyme. Again these data suggest the integrity of the ATP binding sites.

Citrate inhibition of the Arg481D was tested at 0.2 mM ATP, which provides near maximal activity. The citrate inhibition pattern (Figure 4) was virtually identical to that of the wild-type enzyme, suggesting that the citrate site was uninfluenced by the mutation.

Figure 2 describes the activation of R481D by cyclic AMP. The activation data do not differ significantly from the data obtained with wild-type PFK, suggesting no involvement of Arg481 in the interaction at the nucleotide binding site for activation.

A striking difference is noted when the Fru 2,6-bisP sensitivities of wild-type PFK and R481L and R481D are examined (Figure 3). The mutants were completely insensitive to the activating effects of Fru 2,6-bisP over the range tested. At 100 μM the R481D was activated approximately 10% of the maximum activity achieved with wild-type enzyme. Assuming normal hyperbolic binding, this would translate into a K_a of about 900 μM compared to an approximate K_a of 0.3 μM for the wild-type PFK.

Equilibrium binding of radiolabeled Fru 2,6-bisP was measured for the wild-type and R481D mutant enzymes using gel exclusion through Sephadex G-50 as described in the Materials and Methods. Binding studies of wild-type and R481D PFKs were carried out with 100 nM sugar bisphosphate. Under these conditions, the wild-type enzyme bound 0.041 mol of Fru 2,6-bisP per mol of enzyme subunit, while no significant binding was observed with the R481D mutant under conditions that would have detected 0.002 mol per mol of subunit. It is clear that the Fru 2,6-bisP site has been disrupted by the arginine to aspartic acid mutation.

Table 1: Amino Acid Residues Involved in Ligand Binding[a]

E. coli	PFK-C	
Fru 6-P catalytic		Fru 2,6-P_2 allosteric
D-127	D-175	S-541
R-162	R-576	R-210
M-169	M-217	M-583
E-222	E-273	E-639
R-243	R-664	R-301
H-249	H-307	H-671
R-252	R-310	Q-674
ATP catalytic		ATP inhibitory (or AMP/ADP)
G-11	G-34	A-420
Y-41	Y-64	F-450
R-72	R-97	R-481 (Fru 2,6-bisP)
F-73	S-98	I-482
R-77	R-102	K-486
D-103	D-128	F-509
S-105	S-130	A-511
T-125	S-173	T-538
D-129	D-177	N-543
ADP/PEP	AMP/ADP (or ATP inhibitory)	citrate inhibitory
R-21	R-430	R-44
R-25	R-434	R-48
R-54	W-463	W-79
R-154	M-202	K-567
E-187	D-235	D-601
K-213	K-263	K-627

[a] Residues in the left column are those previously implicated in ligand binding sites of bacterial PFK. Second and third columns indicate those residues of mammalian PFK aligned with the prokaryotic residues and inferred to be in corresponding allosteric ligand binding sites.

Assignments of Ligand Binding Sites. Poorman et al. (*1*) originally suggested that the mammalian PFKs evolved by gene duplication and fusion of an ancestral prokaryotic gene. This proposal, based upon sequence data of the rabbit PFK-A isozyme, indicated the relationship of the three ligand binding sites of the bacterial PFKs and the six organic ligand binding sites of the mammalian enzyme. Subsequent sequencing of a large number of eukaryotic PFKs has supported the duplication-fusion hypothesis. With regard to the identification of residues likely to be involved in substrate and allosteric ligand binding, proposals have been made largely upon knowledge of the bacterial binding sites. The assignment of residues to the citrate binding site is now supported by the data presented in this paper as well as the previous identification of Lys567 by chemical modification with pyridoxal phosphate (*5*). The revised assignments are shown in Table 1. The major difference in assignments between those made by Poorman et al. (*1*), Eto et al. (*4*), Simpson and Fothergill-Gilmore (*3*), and Levanon et al. (*2*) and those indicated in Table 1 is that the ADP/phosphoenolpyruvate sites of the prokaryotic precursor evolved into the citrate site

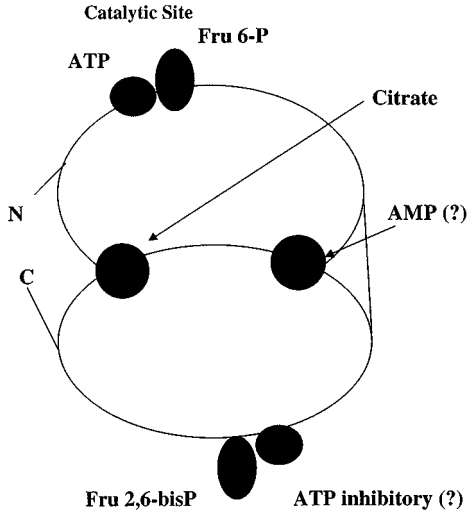

FIGURE 5: Model for the mammalian PFK monomer.

and, probably, the AMP/ADP site. At this point, the AMP/ADP site has not been clearly established. The difference between these assignments and those of Li et al. (*7*) is the placing of residues Arg44, Arg48, and Trp79 in the citrate site instead of the AMP/ADP site. A schematic drawing of the arrangement of the binding sites in the mammalian monomer is shown in Figure 5. This model is based upon the structure of the *E. coli* dimer of Shirakihara and Evans (*17*). A second subunit would be located behind the plane of this figure with the Fru 6-P binding site shared between the subunits. The other mammalian dimer is to the right of this structure with major sites of dimer−dimer interaction being provided by the peptide linking the ancestral monomers to form the mammalian monomer. Note the citrate site can now be assigned with contributions to binding coming from residues near the amino terminus (Arg44, Arg-48, and W-79) and the carboxyl terminus (K-567, D601, and K627).

The Fru 2,6-bisP binding site is now established as the site that evolved from the prokaryotic Fru 6-P active site. This was suggested to the case for yeast PFK, which has a number of similar features to the mammalian enzyme, including evidence for gene duplication and fusion. Mutation of Ser-724, which is homologous to Ser-541 in rabbit PFK (see Table 1), produced an enzyme that was not responsive to Fru 2,6-bisP (25). In the current study a residue was chosen that was homologous to a residue involved in phosphoryl transfer in *E. coli* PFK (Arg72) and thus associated with the ATP binding site (*8*) of that enzyme. The data herein clearly shows that the residue homologous to Arg72 in the carboxyl-terminal half of rabbit PFK is part of the Fru 2,6-bisP site, probably interacting with the phosphate in the 2 position of the sugar phosphate. Furthermore, the data call into question the assignment of the ATP inhibitory site of mammalian PFK as a homologue of the ATP active site of *E. coli* PFK, suggesting that this site could instead be the AMP/ADP site. Assignment of this site remains to be established in further studies.

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