Evolution of the Allosteric Ligand Sites of Mammalian Phosphofructo-1-kinase†

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ABSTRACT: Mammalian phosphofructokinase (PFK) has evolved by a process of tandem gene duplication and fusion to yield a protein that is more than double the size of prokaryotic PFKs. On the basis of complete conservation of active site residues in the N-terminal half of the eukaryotic enzyme with those of the bacterial PFKs, one assumes that the active site of the eukaryotic PFK is located in the N-terminal half. Again using sequence comparisons, the four allosteric ligand sites of mammalian PFK have been thought to arise from the duplicated catalytic and regulatory sites of the ancestral PFK. Previous site-directed mutagenesis studies [Li et al. (1999) *Biochemistry 38*, 16407−16412; Chang and Kemp (2002) *Biochem. Biophys. Res. Commun. 290*, 670−675] have identified the origins of the citrate and fructose 2,6-bisphosphate sites. Here, site-directed mutagenesis of two arginine residues (Arg-433 and Arg-429) of mouse phosphofructokinase is used to identify the ATP inhibitory site, and, by inference, the AMP/ADP site. Mutation of the residues to alanine reduced ATP inhibition in the case of Arg-429 and eliminated ATP inhibition in the instance of Arg-433. The Arg-433 mutant could be inhibited by citrate, and that inhibition could be reversed by fructose 2,6-bisphosphate and cyclic AMP, a high-affinity ligand for the AMP/ADP binding site. It is concluded that the two inhibitors, ATP and citrate, of mammalian PFK interact with sites that have evolved from the duplicated phosphoenolpyruvate/ADP allosteric site of the ancestral PFK. The two sites for activators, fructose 2,6-bisphosphate and AMP or ADP, have evolved from the catalytic site of the ancestral precursor.

Mammalian phosphofructo-1-kinases (PFKs)[1] are more than twice the size of prokaryotic PFKs and are under regulatory control by a wider array of effectors than that seen with the bacterial PFKs, which have a single allosteric site capable of binding both inhibitor and activator. The added complexity in control in mammalian PFK results from the presence of six organic ligand binding sites: ATP and Fru 6-P catalytic sites, activator sites for AMP or ADP and for Fru 2,6-P_2, and inhibitor sites for ATP and for citrate (*1*). Sequencing data have suggested an evolutionary relationship between prokaryotic and eukaryotic PFKs that indicates duplication, tandem fusion, and divergence of catalytic and effector binding sites of a prokaryotic ancestor to yield a doubling of the number of organic ligand binding sites in eukaryotic PFK (*2*).

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-terminal half (*2*). Site-directed mutagenesis (*3*) and chemical modification studies (*4*) have shown that the citrate site is homologous with the phosphoenolpyruvate/ADP allosteric site of *Escherichia coli* PFK (*5*) and that the Fru 2,6-P_2 site is homologous with the Fru 6-P catalytic site of the bacterial enzyme (*6*, *7*). What remain to be resolved are the origins of the ATP inhibitory site and the AMP/ADP activating site of the mammalian enzyme. One can reasonably hypothesize that the binding sites for these two ligands evolved by mutation of the prokaryotic ATP catalytic site and the phosphoenolpyruvate/ADP allosteric site, but which evolved to which remains a question.

In the current analysis, we resolve this final question concerning the origin of the allosteric sites of mammalian PFK by site-directed mutagenesis of the mouse PFK-C isozyme.

EXPERIMENTAL PROCEDURES

Mutagenesis, Expression, and Purification of Mouse PFK-C. The construction of *E. coli* DF1020(DE3) pLysS that is appropriate for transformation with pET20b-PFK-C has been described previously (*6*). This host, constructed from *E. coli* DF1020 [*pro*-82, Δ*pfk*201, *recA*56, Δ(*rha-pfkA*)200, *endA*1, *hsdR*17, *supE 44*] lacking the PFK gene, contains the integrated lambda DE3 prophage and the pET compatible plasmid, (pLysS) (Novagen). RbCl competent cells were transformed with the recombinant plasmid DNA (pET20b-mousePFKC) and selected on LB containing chloramphenicol and ampicillin. Two mutants (R429A and R433A) were constructed from wild-type PFKC in pET20b by the use of the Quick Change XL Site-Directed Mutagenesis Kit (Stratagene). Both sense and antisense primers (39 nucleotides each) were synthesized. For R429A, the mutagenic sense

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[1] Abbreviations: PFK, phosphofructo-1-kinase; Fru 6-P, fructose 6-phosphate; Fru 2,6-P_2, fructose 2,6-bisphosphate; SDS, sodium dodecyl sulfate; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

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primer changed 5′-CGC-3′ to 5′-GCT-3′, whereas in R433A, 5′-CGA-3′ was changed to 5′-GCT3′. The modified PFKC in pET20b was purified and sequenced to verify the mutation using the ThermoSequenase cycle sequencing kit from Amersham. Mutated PFKC in pET20b was then transformed into *E. coli* DF1020(DE3). To confirm the mutation, the entire sequences of both mutants were sequenced in one direction at the Iowa State University sequencing facility.

A single colony of *E. coli* DF1020(DE3) pLysS transformed with either wild-type or mutant pET20b-PFKC was grown in 15 mL of LB culture with chloramphenicol and ampicillin at 37 °C until an OD_{600} of 0.6 was reached. Then 10 mL of this culture was inoculated into 1 L of M9 medium plus 10 g N-Z Amine A (Sigma-Aldrich Chemicals), 5 g of NaCl, 0.5 M sorbitol, and 2.5 mM betaine and antibiotics. Incubation was continued at 37 °C until the OD_{600} reached 0.6. Isopropylthiogalactoside was added to a final concentration of 0.4 mM, and the incubation was continued at 30 °C for 40 h. The cells were harvested by centrifugation at 5000 rpm for 20 min, and the sediment was stored at −80 °C.

The wild-type enzyme and the two mutants were purified by a variation of the method described by Ramadoss et al. (*8*). The sediment was suspended in 50 mM Tris/HCl (pH 8.0), 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol (Buffer 1). The suspension was sonicated in an ice bath using three 10 s bursts on setting 8 with a Fisher Scientific model 550 Sonic Dismembranator. The supernatant, recovered after centrifugation at 17 000 rpm for 20 min at 4 °C, was loaded onto an 8 mL ATP−Sepharose affinity column prepared by the method of Lindberg and Mosbach (*9*) and equilibrated with Buffer 1. The column was washed with 8 column volumes of Buffer 1 and then eluted with 3 column volumes of Buffer 1 containing 0.15 mM fructose 6-P and 0.15 mM ADP. The enzyme was concentrated to greater than 5 mg/mL using a Millipore centrifugal filter device. ATP was added to a final concentration of 0.1 mM. An equal volume of glycerol was added, and the solution was stored at −20 °C. Under these conditions, the wild-type enzyme and the mutants were stable for many months. SDS-gel electrophoresis of these three preparations, carried out using a 10% polyacrylamide support according to the system of Laemmli (*10*), indicated a single band corresponding to a mass of 85 kDa in each case.

Enzyme Assay. Assays for total PFK activity and for the determinations of apparent K_m values under nonallosteric conditions were performed at 30 °C in 50 mM Tris/HCl (pH 8.2), 100 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.2 mM NADH, 0.6 unit of aldolase, 1 unit of triosephosphate isomerase, 0.3 unit of glycerol-P dehydrogenase, and the indicated concentrations of Fru 6-P and ATP. The concentration of $MgCl_2$ was maintained at a concentration 2 mM in excess of that of ATP.

Assays to detect allosteric properties were carried out at pH 7.2 and 30 °C in 50 mM KTes buffer containing 100 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.2 mM NADH, 0.6 unit of aldolase, 1 unit of triosephosphate isomerase, 0.3 unit of glycerol-P dehydrogenase, and the indicated concentrations of Fru 6-P and ATP. $MgCl_2$ was present at a concentration 2 mM in excess of that of ATP. The allosteric behavior was expressed by relating the observed activities to a V_{max} that was determined at pH 7.2 and 0.5 mM Fru 6-P, 0.5 mM ATP, 0.6 mM Fru 2,6-bisP, and other conditions given for the pH 7.2 assay.

RESULTS AND DISCUSSION

Rationale for Selection of Residues for Mutagenesis. The phosphoenolpyruvate/ADP allosteric site of bacterial PFK has been previously indicated by X-ray crystallographic studies of the Evans laboratory (*12*, *13*). Utilizing site-directed mutagenesis, Lau and Ferscht (*13*) identified two residues, Arg-21 and Arg-25, that were particularly important for the binding of both allosteric effectors. The sequence surrounding these two residues is shown below along with the sequence of the two homologous regions of mouse PFK-C. It should be noted that these sequences are strongly conserved in all PFKs.

	21 25	
Escherichia coli PFK	MNAAV**R**GVV**R**SAL	
	43 47	
Mouse PFK-C N-terminal half	MNAAV**R**AVV**R**MGI	
	429 433	
Mouse PFK-C C-terminal half	MNAAV**R**SAV**R**VGI	

Earlier studies of the rabbit PFK-C isozyme showed that mutation of the residue corresponding to Arg-47 eliminated the ability of citrate to inhibit the enzyme, suggesting that the citrate site has evolved from the bacterial phosphoenolpyruvate/ADP site and that the site is composed of residues contributed for the most part by residues from the N-terminal half of mammalian PFK (*3*). These data further suggested that mutation of Arg-429 and Arg-433 from the carboxyl half of the molecule should greatly influence the interaction of either ATP as an inhibitor or AMP as an activator of the enzyme. These residues were mutated, and the allosteric properties were evaluated.

Kinetic Properties of the Mutants. To determine maximum activity and kinetic parameters, the wild-type and mutant preparations of R429A and R433A were measured at pH 8.2 where allosteric properties are not seen. All preparations had maximum activity at pH 8.2 in the range of 135−155 units/mg. The apparent K_ms for Fru 6-P in the presence of 0.5 mM ATP were not influenced by the mutations, with apparent K_ms for all preparations in the range of 0.6−0.8 mM. Similarly, the apparent K_ms for ATP determined in the presence of 5 mM Fru 6-P were unaltered, with values of approximately 0.1 mM for both wild-type and mutant enzymes.

ATP Inhibition. Allosteric properties were determined at pH 7.2, under which conditions the well-documented regulatory properties of PFK are observed. Note that in all assays $MgCl_2$ was present at a concentration 2 mM in excess of that of ATP to ensure that the concentration of free ATP is exceedingly low. Figure 1 describes the ATP inhibition of R429A and R433A. Under the conditions of the assay, the wild-type enzyme was inhibited 50% at an ATP concentration slightly less than 1 mM. It should be noted that as ATP concentration is increased, inhibition begins at relatively low concentrations of the nucleotide and that optimal activity (V_{max} referred to in the figure) can be seen only in the

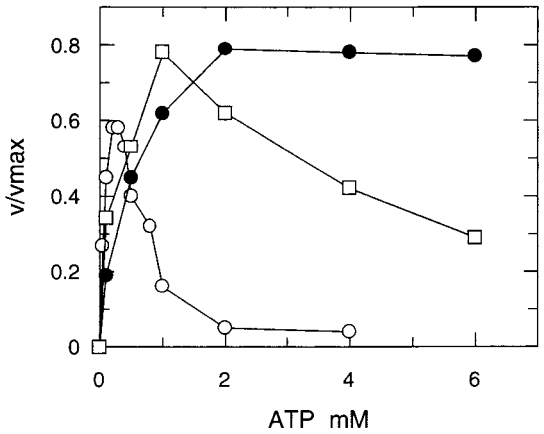

FIGURE 1: ATP inhibition of wild-type PFK (open circles), R433A (solid circles), and R429A (open square). Assays were performed as described under Experimental Procedures at pH 7.2 and Fru 6-P at 0.5 mM. The value for V_{max} used to calculate the numbers on the ordinate was determined at pH 7.2 and 0.5 mM Fru 6-P, 0.5 mM ATP, and 0.6 mM Fru 2,6-bisP. Points are the mean of 3−6 determinations.

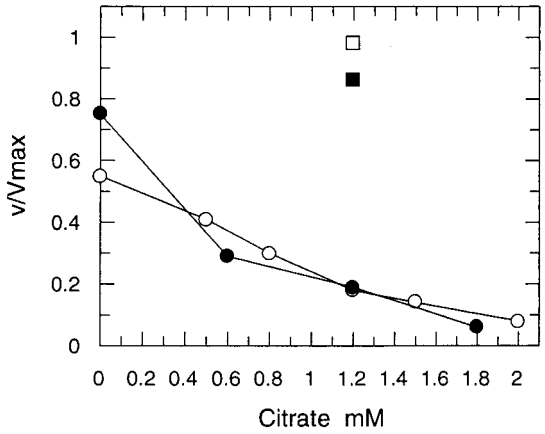

FIGURE 2: Citrate inhibition of wild-type PFK (open circles) and R433A (solid circles). Assay was carried out as described under Experimental Procedures at pH 7.2 and a Fru 6-P concentration of 0.5 mM. The concentration of ATP was 0.3 mM for wild-type PFK and 3 mM for the mutant. The squares (open, wild type; closed, R433A) indicate the effect of 0.6 mM Fru 2,6-bisP on the activity at the indicated concentration of citrate. The value for V_{max} used to calculate the values on the ordinate was determined at pH 7.2 and 0.5 mM Fru 6-P, 0.5 mM ATP, and 0.6 mM Fru 2,6-bisP. All data points are the mean of 2−4 determinations.

presence of Fru 2,6-P_2. Because the data in the figure were obtained in the absence of Fru 2,6-P_2, the maximum activity is seen at about 0.2 mM ATP. On the other hand, the mutant enzyme, R433A, showed no inhibition at concentrations as high as 6 mM ATP and achieved a maximal activity close to that seen in the presence of Fru 2,6-P_2. The second mutant, R429A, was less sensitive to ATP inhibition than the wild-type enzyme, but inhibition was observed with 50% inhibition near 4 mM. The lack of total desensitization to inhibition is not surprising when these data are compared to the study of the mutants of *E. coli* PFK by Lau and Ferscht (*14*). That study showed that whereas the R25A mutation abolished inhibition by phosphoenolpyruvate in *E. coli* PFK, the R21A mutation only weakened the inhibition. On the basis of the data in Figure 1, further studies of the actions of allosteric effectors on the mammalian enzyme were carried out only with R433A, where the most striking effect on ATP inhibition was seen.

Citrate Inhibition. If Arg-433 represents a component of the ATP inhibitory site and not the AMP activating site, then the usual method for studying the action of AMP or ADP, that is reversal of ATP inhibition, is not possible. However, it should be possible to investigate citrate inhibition to see if inhibition by that effector can be reversed by adenosine mono- and diphosphates. Citrate has been shown to act synergistically with ATP as an inhibitor, and it has not been established in kinetic studies that citrate inhibition can be observed in the absence of ATP inhibition (*1*), or whether it functions only in the presence of ATP inhibition. However, some evidence for an independent allosteric effect of citrate was observed in early binding studies that showed that citrate decreased the binding of Fru 6-P in the absence of ATP (*15*), indicating the possibility of inhibition by citrate in kinetic studies that does not require occupancy of the ATP inhibitory site. As shown in Figure 2, citrate does indeed inhibit the Arg-433 mutant of PFK. Under the conditions of the assay, 50% inhibition was observed at citrate concentrations less than 1 mM. It is obvious that the citrate site remains intact in the Arg-433 mutant and that citrate inhibition is exhibited even in the absence of ATP inhibition.

Fru 2,6-P_2 Activation. Shown also in Figure 2 is the effect of Fru 2,6-P_2 on the native and mutant enzyme inhibited by citrate. The mutation of Arg-433 has produced no significant effect on the ability of Fru 2,6-P_2 to activate the enzyme. Activation by Fru 2,6-P_2 is obviously a direct effect and not simply the reversal of ATP inhibition. The ability of citrate to inhibit the mutant and Fru 2,6-P_2 to activate it suggests that the mutation neither alters the two binding sites nor does it affect the ability of the enzyme to undergo allosteric transitions that lead to activated or inhibited enzyme.

Activation by Cyclic AMP. Although cyclic AMP is undoubtedly not a physiologically significant regulator of PFK, it is nonetheless an appropriate, chemically stable indicator of the AMP/ADP site of the enzyme because it binds competitively with both AMP and ADP (*15*). Figure 3 shows that the inhibition of the Arg-433 mutant by citrate can be reversed in the presence of cyclic AMP, demonstrating the integrity of its binding site in the mutant and further demonstrating that the AMP/ADP site is distinct from the ATP inhibitory site.

The foregoing data strongly implicate Arg-433, which is homologous to an Arg residue in the allosteric site of *E. coli* PFK, as a component of the inhibitory site for ATP. It further suggests that the AMP/ADP activating site has most likely evolved from the ATP catalytic site of prokaryotic PFK. It might be argued that the mutation is not involved in the binding of ATP but instead interferes with the transmission of the allosteric signal. This interference would have to be very specific for ATP because the allosteric signal is clearly transmitted in the case of inhibition by citrate and activation by AMP and Fru 2,6-P_2. If one proposes a simple allosteric equilibrium, either preexisting or induced, between active and inactive states, then it is somewhat of a stretch to interpret the data as indicating anything other than a disruption of the binding of ATP. Loss of binding has not been demonstrated by these studies, but certainly it is clear that Arg-433 is a component of the ATP inhibitory site and its integrity is necessary for the allosteric effect of ATP.

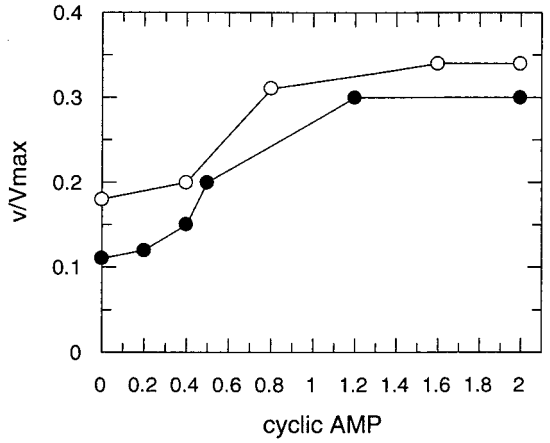

FIGURE 3: Cyclic AMP activation of wild-type PFK (open circles) and R433A (solid circles). Assay was carried out as described under Experimental Procedures at pH 7.2 and a Fru 6-P concentration of 0.5 mM. The concentration of ATP was 0.5 mM for wild-type PFK and 3 mM for the mutant. Citrate concentration was 1.2 mM for both enzymes. The value for V_{max} used to calculate the numbers on the ordinate was determined at pH 7.2 and 0.5 mM Fru 6-P, 0.5 mM ATP, and 0.6 mM Fru 2,6-bisP. Points are the mean of 2−4 determinations.

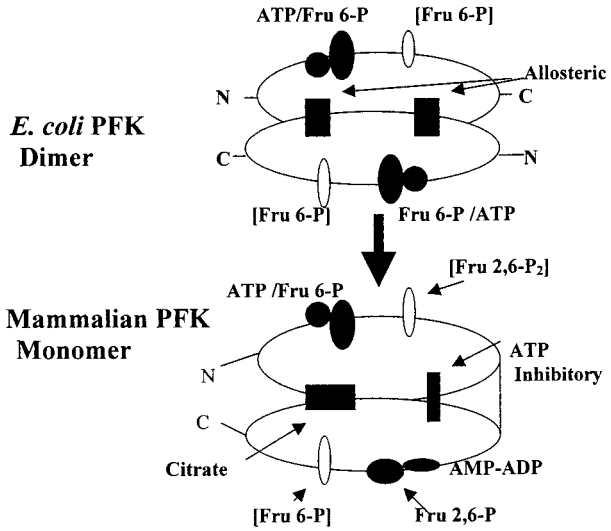

FIGURE 4: Model for the evolution of a monomer of mammalian PFK from a dimer of a prokaryotic ancestor. Allosteric and substrate (ATP/Fru 6-P) sites are indicated. Sites labeled in brackets are contributed largely by the subunits behind the plane of the shown figure.

A Model for the Evolution of the Allosteric Sites of Mammalian PFK. Shown in Figure 4 is a proposed model for the evolution of the ligand binding sites of mammalian PFK based on the current and earlier studies (*2*−*4*). Note that the figure shows a bacterial dimer and a mammalian monomer, whereas the active forms of both enzymes are tetramers. The bacterial model is rooted in the structure of the *E. coli* dimer of Shirakihara and Evans (*13*), which is represented in the upper half of the figure. While the ATP catalytic site is found within the shown subunit, all other ligand sites are shared between subunits. A second dimer would be located behind the plane of this figure with the Fru 6-P binding site shared between the subunits. The unfilled oval represents that part of the Fru 6-P site (indicated in brackets) on the shown subunit that is largely contributed by the subunit behind the plane of the figure. The lower half of the figure shows the proposed mammalian monomer that has been formed by tandem duplication and fusion of the prokaryotic precursor, with mutation of duplicated sites into additional allosteric ligand binding sites. Again, a second monomer would be located behind the plane of this figure with the Fru 6-P and Fru 2,6-P_2 binding sites shared between the subunits. The upper unfilled oval of the mammalian monomer represents part of the Fru 2,6-P_2 site (indicated in brackets) on the shown subunit that is largely contributed by the subunit behind the plane of the figure. The lower unfilled oval of the mammalian enzyme represents part of the Fru 6-P site (indicated in brackets) that is contributed by the subunit behind the plane of the figure. The other mammalian dimer that makes up the mammalian tetramer would be 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.

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

Fru 6-P catalytic		Fru 2,6-P_2
D-127 (16)	D-174	S-539 (7)
R-162 (17)	R-575	R-209
M-169 (18)	M-216	M-582
R-243 (17)	R-664	R-300 (7)
H-249 (19)	H-306	H-670 (7)
R-252 (20)	R-309	Q-673

ATP catalytic		AMP/ADP
Y-41 (21)	Y-63	F-449
R-72 (17)	R-96	R-480 [Fru 2,6-bisP] (3)
F-76 (21)	F-100	G-484
R-77 (21)	R-101	K-485
D-103 (22)	D-127	F-508
S-105 (23)	S-129	A-510
T-125 (22)	S-172	T-537
D-129 (22)	D-176	N-541

ADP/PEP	ATP inhibitory	citrate
R-21 (14)	R-429 (this study)	R-43
R-25 (14)	R-433 (this study)	R-47 (3)
R-54 (5)	W-462	W-78
R-154 (5)	M-201	K-566
E−187 (14)	E−234	D-600
K-213 (14)	K-262	K-626 (4)

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

Table 1 provides a summary of previous structural and mutagenic studies of the allosteric ligand binding sites of *E. coli* and mammalian PFKs. The residues that are described for the catalytic and allosteric sites of *E. coli* PFK have been imputed to be involved by X-ray crystallographic studies (*13*) and have had roles confirmed by site-directed mutagenesis (*5*, *14*, *16*−*23*). The corresponding residues of the mammalian enzyme have been suggested by amino acid alignment. The proposed catalytic site of mammalian PFK, comprised of Fru 6-P and ATP subsites in the amino-terminal half of the molecule, shows complete identity with 14 residues of the active site of *E. coli* PFK with the exception of Thr-125 being substituted by the serine residue in all mammalian PFKs, a very conservative substitution. In the carboxyl-terminal half of the mammalian enzyme, the integrity of Ser-539, Arg-300, and His-670, which align with three Fru 6-P residues of the *E. coli* PFK active site, has

been shown by site-directed mutagenesis to be necessary for activation of the mammalian enzyme by Fru 2,6-bisP (*7*). Mutagenesis studies (*3*) have shown as well that another component of the Fru 2,6-bisP binding site is Arg-480, which is homologous to Arg-72 in *E. coli* PFK that appears to interact with the γ-phosphoryl group of ATP (*24*). The role of Arg-480 in the Fru 2,6-bisP site of mammalian PFK is probably that of interacting with the phosphate in the 2 position of the sugar phosphate and thus contributing to the high-affinity binding of this effector.

Previous studies concluded that the allosteric site for citrate developed from the phosphoenolpyruvate/ADP allosteric site of *Escherichia coli* PFK. Allosteric inhibition by citrate in rabbit PFK-C was completely eliminated by mutagenesis of Arg-47 (*3*), a residue that corresponds to *E. coli* PFK residue Arg-25, which is essential for inhibition of that enzyme by phosphoenolpyruvate (*14*). Modification of Arg-626 in rabbit PFK-A isozyme by pyridoxal phosphate eliminated citrate binding (*4*). Arg-626 corresponds to Lys-213 in *E. coli* PFK, also a residue critical for phosphoenolpyruvate inhibition.

The current studies provide the identification of the ATP inhibitory site as being derived from the ADP/phosphoenolpyruvate site of the prokaryotic ancestor. This site, like the citrate site, is composed of residues from both the amino- and carboxyl-terminal halves of the mammalian enzyme. It should be noted that the two inhibitors of mammalian PFK interact with sites that have evolved from the duplicated allosteric site of the ancestral PFK. The two sites for activators, Fru 2,6-bisP and AMP or ADP, have evolved from the catalytic site of the ancestral precursor.

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