Subunit Interactions and Composition of the Fructose 6-Phosphate Catalytic Site and the Fructose 2,6-Bisphosphate Allosteric Site of Mammalian Phosphofructokinase

Mammalian phosphofructokinase originated by duplication, fusion, and divergence of a primitive prokaryotic gene, with the duplicated fructose 6-phosphate catalytic site in the C-terminal half becoming an allosteric site for the activator fructose 2,6-bisphosphate. It has been suggested that both sites are shared across the interface between subunits aligned in an antiparallel orientation, the N-terminal half of one subunit facing the C-terminal half of the other. The composition of these binding sites and the way in which subunits interact to form the dimer within the tetrameric enzyme have been reexamined by systematic point mutations to alanine of key amino acid residues of human muscle phosphofructokinase. We found that residues His-199, His-298, Arg-201, and Arg-292 contribute to the catalytic site and not to the allosteric site, because their mutation decreased the affinity for fructose 6-phosphate without affecting the activation by fructose 2,6-bisphosphate or its binding affinity. In contrast, residues Arg-566, Arg-655, and His-661 were critical components of the fructose bisphosphate allosteric site, because their mutation strongly reduced the action and affinity of the activator, with no alteration of substrate binding to the active site. Our results suggest that mammalian phosphofructokinase subunits associate with the N-terminal halves facing each other to form the two catalytic sites/dimer and the C-terminal halves forming the allosteric sites. Additionally, mutation of certain residues eliminated activation by fructose 1,6-bisphosphate, but not its binding, with little effect on activation by fructose 2,6-bisphosphate, indicating a divergence in the signal transduction route despite their binding to the same site.

Phosphofructokinase (PFK, EC 2.7.1.11) catalyzes the irreversible MgATP-dependent phosphorylation of fructose 6-phosphate (Fru-6-P) to fructose 1,6-bisphosphate (Fru-1,6-P_2). Extensive literature data have described the complex allosteric response of this enzyme to a number of metabolites, supporting its fundamental role in the control of the glycolytic pathway (1–3). The catalytic site shows positive cooperativity for Fru-6-P, and MgATP works also as a key allosteric inhibitor. Among other regulators, most eukaryotic PFKs are strongly stimulated by Fru-2,6-P_2 and Fru-1,6-P_2. Activation by Fru-2,6-P_2 is particularly important in organisms and tissues with gluconeogenic capability because of its additional action as a potent inhibitor of fructose-1,6-bisphosphatase (4). Fru-1,6-P_2 autocatalytic activation of PFK is specially relevant in skeletal muscle to contribute to the high glycolytic flux during strong exercise (5). The two fructose bisphosphates share a common or partially overlapping allosteric site (6, 7), although they may elicit different conformational changes (8–10). Three distinct subunit isozymes are present in mammalian cells, C, M, and L, which randomly associate into homo- and hetero-tetramers. They are encoded by different genes displaying tissue-specific expression and show individual kinetic properties (7, 9, 11).

Sequence data and the fact that bacterial PFK is about half the size of the more complex eukaryotic enzyme indicated that the latter originated from a process of duplication, fusion, and divergence of an ancestral prokaryotic gene, thus leading to the formation of new regulatory binding sites (12). Accordingly, the Fru-2,6-P_2 allosteric site evolved from the duplicated Fru-6-P catalytic site in the C-terminal half, as supported by mutagenesis studies (10, 13–15). The available crystal structures of bacterial PFKs (reviewed in Ref. 16) and sequence conservation led to the suggestion that both sites are shared across the interface between subunits oriented in an antiparallel way to form each mammalian dimer (12, 16). Most of the bacterial catalytic site (9 of 11 residues that bind Fru-6-P and all 12 residues that bind the adenine nucleotide (17, 18)) is contained within one subunit (Fig. 1). Additionally, the nearly complete sequence conservation between the bacterial and mammalian PFK catalytic sites (10 of 11 residues are identical; only Thr-125 is replaced by Ser (17)) means that the bacterial catalytic site is an excellent
approximation to the architecture of the mammalian catalytic site, as well as to that of the derived allosteric site. On these bases, it has been proposed that residues not only from the C-terminal half but also from the N-terminal half contribute to the Fru-2,6-P₂-binding site and also to the catalytic site (12, 15, 19). Hence, each interface would contain an active site near to the N- and C-ends and an allosteric site closer to the peptide that connects the two PFK domains, as resulting from an interaction between subunits from hereon referred to as “N/C-C/N” (Fig. 2). A reduction of maximum activation by Fru-2,6-P₂ of rabbit muscle PFK after mutation of Arg-292 has been argued to support this proposal (15); nevertheless, the mutant showed high affinity for the effector, and no further information on the binding capacity of the corresponding site was provided. In fact, the locations of both the catalytic and the fructose bisphosphate allosteric sites have not been elucidated. Although the architecture of the enzyme molecule from *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* has been determined by electron and cryoelectron microscopy (20, 21), no x-ray structure of a characteristic eukaryotic PFK has been reported yet, other than that of the *Trypanosoma brucei* enzyme, in which no gene duplication occurred (22). It was recently described (10) that chimeric mammalian PFKs involving exchange of their terminal domains exhibited affinity properties for Fru-6-P and Fru-2,6-P₂ that resembled those of the native isozyme that donated the N-terminal half and the C-terminal half, respectively. This suggested that subunits may pair together in a different way, with each terminal half facing its equivalent one in the adjacent subunit in an N/N-C/C interaction (as also depicted in Fig. 2). In this interaction, the two active sites/dimer would be contributed solely by the N-halfs, whereas the C-halves would provide both allosteric sites. In the current work, we have undertaken to test in which of these two orientations of subunit interactions the mammalian PFK-M tetramer is organized, by systematic mutation of amino acid residues likely to participate in the catalytic or the allosteric site, depending on the way of subunit pairing. Consequently, this investigation led us to resolve the location and composition of the Fru-6-P catalytic site and the fructose bisphosphate allosteric site of mammalian PFK. Additionally, residues specifically involved in the allostery transitions of Fru-2,6-P₂ and Fru-1,6-P₂ have been identified. These findings provide crucial new insight into the structural organization of eukaryotic PFK.

**EXPERIMENTAL PROCEDURES**

**Materials**—All of the chemical reagents and enzymes used in genetic and enzymatic assays and protein purification were obtained from Roche Applied Science, GE Healthcare, Promega, BioLabs, Invitrogen, or Sigma. Other reagents were of the best quality and commercial available.

**Gene Manipulations, Plasmids, and Site-directed Mutagenesis**—Standard procedures for *Escherichia coli* and yeast gene manipulations were as described (23, 24). The cDNAs for human muscle PFK-M carrying the H199A and H298A mutations were generated by using the Sculptor™ *in vitro* mutagenesis system. Previously, the full-length cDNA for this PFK was cloned into M13mp19 as an Xbal-KpnΙ 2.7-kbp fragment from p18HmPFK (9), yielding plasmid m19HmPFK. Mutagenesis was performed as recommended by the manufacturer, using the mutagenic primers indicated in supplemental Table S1 and single-stranded DNAs derived from m19HmPFK as the template. The BamHI fragments from the resulting M13mp19 derivatives were cloned in pJJH71 plasmid (25), generating the recombinant plasmids, in which the mutant *pfk* genes were expressed under the control of the *S. cerevisiae* PFK2 promoter. All other point mutations of PFK-M were introduced by the two-step megaprimer-based PCR mutagenesis strategy (26), using the plasmid p18HmPFK as template and the mutagenic primers containing the desired mutation (supplemental Table S1), and the 17-mer universal and 16-mer reverse sequencing oligonucleotides as flanking primers. A double-stranded mutated fragment was generated in the first PCR that was used as primer in the second PCR to amplify the entire cDNA. The amplification reaction mixtures contained 10–30 ng of template DNA, 0.2 μM each dNTP, 2.5 units of *Taq* DNA polymerase, and the buffer provided by the manufacturer in a total volume of 100 μl. The PCR conditions were 30 cycles of 1 min at 94°C, 2 min at 55°C, and 2 min at 68°C, followed by one cycle of 5 min at 70°C. The final PCR product was cloned in pGEMT-easy vector. To construct the expression plasmids of mutant PFK-M, DNA fragments containing the desired mutations were obtained from the pGEMT-easy derivatives by digestion with appropriate restriction endonucleases and used to replace the corresponding DNA sequence of wild type *pfk* in pJJH71PFK (25). The double mutant expression plasmids were generated by replacing the corresponding

**FIGURE 1.** Schematic view of the catalytic site regions of bacterial PFK, showing the interactions with the products Fru-1,6-P₂ (FBP) and ADP. This view corresponds to subunit B of the crystal structure of the complex PFK from *E. coli* with its reaction products. Arg-162 and Arg-243 belong to another subunit. His-160 is also in the locations of both the catalytic and the fructose bisphosphate allosteric site, as well as to that of the derived allosteric site. On these bases, it has been proposed that residues not only from the C-terminal half but also from the N-terminal half contribute to the Fru-2,6-P₂-binding site and also to the catalytic site (12, 15, 19). Hence, each interface would contain an active site near to the N- and C-ends and an allosteric site closer to the peptide that connects the two PFK domains, as resulting from an interaction between subunits from hereon referred to as “N/C-C/N” (Fig. 2). A reduction of maximum activation by Fru-2,6-P₂ of rabbit muscle PFK after mutation of Arg-292 has been argued to support this proposal (15); nevertheless, the mutant showed high affinity for the effector, and no further information on the binding capacity of the corresponding site was provided. In fact, the locations of both the catalytic and the fructose bisphosphate allosteric sites have not been elucidated. Although the architecture of the enzyme molecule from *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* has been determined by electron and cryoelectron microscopy (20, 21), no x-ray structure of a characteristic eukaryotic PFK has been reported yet, other than that of the *Trypanosoma brucei* enzyme, in which no gene duplication occurred (22). It was recently described (10) that chimeric mammalian PFKs involving exchange of their terminal domains exhibited affinity properties for Fru-6-P and Fru-2,6-P₂ that resembled those of the native isozyme that donated the N-terminal half and the C-terminal half, respectively. This suggested that subunits may pair together in a different way, with each terminal half facing its equivalent one in the adjacent subunit in an N/N-C/C interaction (as also depicted in Fig. 2). In this interaction, the two active sites/dimer would be contributed solely by the N-halfs, whereas the C-halfs would provide both allosteric sites. In the current work, we have undertaken to test in which of these two orientations of subunit interactions the mammalian PFK-M tetramer is organized, by systematic mutation of amino acid residues likely to participate in the catalytic or the allosteric site, depending on the way of subunit pairing. Consequently, this investigation led us to resolve the location and composition of the Fru-6-P catalytic site and the fructose bisphosphate allosteric site of mammalian PFK. Additionally, residues specifically involved in the allostery transitions of Fru-2,6-P₂ and Fru-1,6-P₂ have been identified. These findings provide crucial new insight into the structural organization of eukaryotic PFK.

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Expression and Purification of Recombinant Enzymes—Wild type and mutant forms of PFK-M were expressed in S. cerevisiae strain HD152–1D (carrying deletions in both yeast PFK genes (9)) and purified by chromatographies on blue Sepharose CL-6B and DEAE-trisacryl as described previously (27). The early proposal of Poorman (12) that the Fru-6-P catalytic site and its evolutionarily derived Fru-2,6-P2 allosteric site of mammalian PFK are shared between the dimer subunits implies that some of the structural motifs contributing to these counterparts in the single-mutated pJJH71 derivatives by the respective fragments carrying the second mutation obtained by digestion of either pJJH71- or pGEMT-easy derivatives with appropriate restriction endonucleases. Sequencing of the resulting plasmids by the dideoxynucleotide chain termination method (23) confirmed that only the desired mutations had been introduced.

FIGURE 2. Models for the subunit interactions of the mammalian PFK dimer and the formation of the Fru-6-P catalytic site and the Fru-2,6-P2/Fru-1,6-P2 allosteric site. Locations of the putative Fru-6-P catalytic site (dark gray) and the Fru-2,6-P2/Fru-1,6-P2 allosteric site (light gray), depending on the type of subunit interaction within the dimer, are indicated as predicted by evolution from an ancestral bacterial tetramer (12), which is shown for comparison. N- and C-terminal halves of each mammalian subunit are shown in white and gray, respectively. Bacterial Fru-6-P catalytic sites (as well as mammalian fructose phosphate-binding sites) are displaced to indicate that only a small part of the Fru-6-P-binding site overlaps the adjacent subunit in the vicinity of the fructose bisphosphate allosteric site. The two parts of A and B correspond to front and back views of the same dimer.

Enzyme Assays—Total PFK activity was measured at pH 8.2 with aldolase, glyceraldehyde-3-phosphate dehydrogenase, and triosephosphate isomerase activities as described (27). Assays for kinetic studies were carried out at pH 7.0 in 50 mM Hepes, 100 mM KCl, 5 mM MgCl2, 0.15 mM NADH, auxiliary enzymes, 5–50 μl of the purified enzyme, and the indicated concentrations of MgATP, effector, and Fru-6-P in a final volume of 1 ml. The reaction was started after a 5-min preincubation by the addition of Fru-6-P (27). When mutants H298A and R201A were assayed, the reaction was started after the addition of MgATP, because of their lower activity under the former conditions. Kinetic studies of these mutants and wild type enzyme performed under both conditions gave similar affinity constants for substrates and allosteric effectors. The effect of Fru-1,6-P2 was examined as described (27). Auxiliary enzymes were desalted as described (29). Fructose-1,6-bisphosphatase and fructose-2,6-bisphosphatase activities were measured as described by Aragón et al. (30), except that the pH was 7.0 and Fru-1,6-P2 concentration was 50 μM, and by González-Mateos et al. (31), respectively. One unit of activity is defined as the amount of enzyme that catalyzes the conversion of 1 μmol of substrate/min under the above conditions. Kinetic and binding data are the average of a minimum of three measurements. Standard error did not exceed 10% of the average value. The parameters were determined using the Origin version 7.0 software programmed to fit data to either the Hill or the Michaelis-Menten equation.

Other Methods—Protein concentration was determined by the Bradford dye binding method (32) using bovine γ-globulin as standard. Protection against thermal denaturation was carried out essentially as described previously (9); incubations in the presence of increasing concentrations of the ligand were performed at 55 °C (60 °C in the case of the R201A/R292A mutant) for 10–30 min in 50 mM Hepes, 100 mM KCl, 5 mM MgCl2, pH 7.0. The residual activity remaining after incubation was measured at pH 8.2, as described (27).

RESULTS

Selection of Amino Acid Residues for Site-directed Mutagenesis of PFK-M to Distinguish the Mode of Subunit Interaction—The early proposal of Poorman et al. (12) that the Fru-6-P catalytic site and its evolutionarily derived Fru-2,6-P2 allosteric site of mammalian PFK are shared between the dimer subunits implies that some of the structural motifs contributing to these
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binding sites participate in one or the other of them in a manner that depends on how the subunits interact. Therefore, among the amino acid residues assigned to these sites by sequence alignments with residues pertaining to the bacterial PFK catalytic site (17) (Fig. 3A), we selected those that may allow a distinction between the two possible orientations of subunit interaction schematized in Figs. 2 and 3B. According to this criterion, the N-terminal residues of human PFK-M putatively involved in the binding site closer to the peptide linking the two halves, such as His-199, Arg-201, and Arg-292 (corresponding to His-160, Arg-162 and Arg-243 of E. coli PFK), are the only residues of the Fru-6-P catalytic site located in the adjacent subunit (17, 33) (Fig. 1), would belong to the allosteric site in the N/C-N/C interaction, whereas they would belong to the catalytic site in the N/N-C/N interaction (Fig. 3B). On the other hand, their equivalent C-terminal amino acids, Lys-564, Arg-566, and Arg-655, would be part of the catalytic site in the first type of interaction, whereas in the second one they would belong to the allosteric site. In contrast, other residues are assigned independently of the mode of subunit interaction, such as His-298 (corresponding to the active site His-249 of E. coli PFK (12, 17)) and its C-terminal counterpart His-661, which belong to the catalytic site and the allosteric site, respectively, in both of the proposed orientations. Therefore, we systematically mutated all these residues of PFK-M to alanine to investigate their participation in these binding sites and thus how the enzyme dimer is organized. The corresponding cDNAs were expressed in a PFK-deficient strain of S. cerevisiae (9), and enzyme mutants were purified (supplemental Fig. S1) and characterized. Mutations of His-298 and His-661 were used as positive controls because of their unequivocal location.

Kinetic and Binding Properties of Histidine Mutants—Steady-state kinetic parameters of all mutants and the wild type enzyme related to the functioning of the catalytic site and the MgATP inhibitory and Fru-2,6-P2 activating allosteric sites are shown in Table 1. The later site was also investigated with respect to the activator Fru-1,6-P2 to gain insight into the somewhat different allosteric actions of the two bisphosphates (8–10). Binding parameters for the two activators were analyzed by ligand protection against irreversible thermal denaturation of the enzymes. The H298A and H661A mutants, which were used as positive controls, behaved in agreement with their unequivocal assignment to the catalytic and regulatory sites, respectively. Although affinity for Fru-6-P clearly decreased only in the H298A mutant (S0.5 value of 10.0 mM compared with 4.0 and 2.0 mM for the H661A mutant and the wild type enzyme, respectively), activation by Fru-2,6-P2 was only affected by the H661A mutation, with a 200-fold decrease in affinity (Kd value of 100 μM versus 0.5 μM for the wild type enzyme) (Fig. 4A). A similar change was reported (15) when the corresponding latter mutation was introduced in the rabbit muscle enzyme. Although neither of the two mutants was activated by Fru-1,6-P2 (Fig. 4C), the binding of both Fru-2,6-P2 and Fru-1,6-P2 to the H298A mutant was normal (Fig. 4, B and D), whereas the H661A mutant exhibited a decrease in the apparent affinity for both bisphosphates by 2–3 orders of magnitude, with Kd values of 72 μM and a sigmoidal binding curve (Fig. 4B) and 125 μM for Fru-2,6-P2 and Fru-1,6-P2, respectively, compared with the wild type enzyme (Kd values of 0.2–0.3 μM). These results are consistent with the view that His-298 participates in the Fru-6-P catalytic site and that His-661 is an essential component of the fructose bisphosphate allosteric site.

The H199A mutant exhibited a moderate decrease in the affinity for Fru-6-P (S0.5 value of 8.2 mM compared with 2.0 mM for the wild type enzyme) (Table 1). But in contrast to the previously proposed assignment of this residue to the allosteric site (12), the mutant showed no change in activation by Fru-2,6-P2 (Fig. 4A) nor in binding of the two bisphosphates (Fig. 4, B and D), suggesting no effect on the integrity of this regulatory site. Activation by Fru-1,6-P2 was, however, abolished (Fig. 4C), an effect observed also in the rest of the mutants studied in this work (Table 1). To further investigate the assignment of His-
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TABLE 1

| Steady-state kinetic and binding parameters of wild type and mutant PKFs |
|-----------------------------|-----------------------------|
|                             | $k_{cat}$ $\frac{S_0}{m}$ $\frac{n_H}{m}$ | $K_m$ $\mu M$ | $K_a$ $\mu M$ | $K_m$ $\mu M$ | $K_a$ $\mu M$ | $K_m$ $\mu M$ | $K_a$ $\mu M$ |
| Wild type                   | 357                          | 2.0 (3.0)     | 70 (3.0)      |
| H298A                       | 296 10.0 (3.0)               | 50 (3.0)      |
| H661A                       | 114 4.0 (4.0)                | 180 (4.5)     |
| H199A                       | 190 8.2 (3.0)                | 77 (3.3)      |
| H199A/H298A                 | 106 18.0 (3.0)               | 55 (3.3)      |
| R201A                       | 298 5.0 (4.0)                | 70 (3.5)      |
| R292A                       | 392 7.0 (4.0)                | 70 (3.5)      |
| R201A/R292A                 | ND 10.0 (4.0)                | 75 (5.0)      |
| R566A                       | ND 4.0 (4.0)                 | 100 (1.5)     |
| R655A                       | ND 3.2 (4.0)                 | 100 (2.0)     |
| R566A/R655A                 | ND 4.5 (4.0)                 | 75 (3.5)      |
| K564A                       | 287 2.5 (4.0)                | 100 (5.0)     |

$^a$ $S_0$ and $K_m$: substrate concentration at half-maximal velocity. $S_0$ (Fru-6-P) values were determined at 5 mM MgATP, pH 7.2. $K_m$ (MgATP) values were obtained at Fru-6-P concentrations equal to the corresponding $S_0$ (Fru-6-P) values, pH 7.0.

$^b$ $n_H$: Hill coefficient obtained by fitting the data to Hill equation.

$^c$ $K_a$: MgATP concentration at half-maximal inhibition. The values were obtained at Fru-6-P concentrations equal to the $S_0$ (Fru-6-P) values, pH 7.0.

$^d$ $K_{cat}$: effector concentration at half-maximal activation. These values were obtained at Fru-6-P concentrations equal to the $S_0$ (Fru-6-P) values and 5 mM MgATP, pH 7.0.

$^e$ $K_d$: dissociation constant. These values were determined by enzyme protection against thermal inactivation by increasing concentration of the ligand as described under "Experimental Procedures."

$^f$ ND, not determined.

$^g$ No protection against thermal inactivation was detected by either Fru-2,6-P$_2$ or Fru-1,6-P$_2$ used at concentrations up to 100 mM.

FIGURE 4. Activation (A and C) and protection against thermal inactivation (B and D) by fructose bisphosphates of wild type and histidine mutants of PFK-M. O wild type; A H199A; B H298A; H H661A. A and C assays were performed at pH 7.0 with 5 mM MgATP and the indicated concentrations of either Fru-2,6-P$_2$ (A) or Fru-1,6-P$_2$ (C). The concentrations of Fru-6-P used were equal to the corresponding $S_0$ (Fru-6-P) of each enzyme (Table 1). Activity is expressed relative to the maximal activity obtained under these conditions for each enzyme. Inset in A, $k_{cat}$ values and fold activation calculated from the data. B and D, incubations with increasing concentrations of either Fru-2,6-P$_2$ (B) or Fru-1,6-P$_2$ (D) to examine enzyme protection against thermal denaturation were performed as described under "Experimental Procedures." The residual activity is expressed relative to that obtained before the incubations. Insets in B and D, protection against thermal inactivation of wild type enzyme and the mutants H199A and H298A. $K_d$ values were calculated from the data.

199, a double H199A/H298A mutant was obtained that elicited a stronger dysfunction of the Fru-6-P catalytic site, as indicated by a 9-fold increase in the corresponding $S_0$ value (18.0 mM, compared with 2.0 mM for the wild type enzyme), without important change in the sensitivity to activation by Fru-2,6-P$_2$ and the binding affinity for the two bisphosphates. This clearly supported the interpretation that the allosteric site remained intact after the H199A mutation.

As also shown in Table 1, none of the histidine mutants nor the arginine or lysine mutants described below exhibited noticeable changes in the cooperativity for Fru-6-P ($H_1$ values), affinity of MgATP at the catalytic site ($K_m$ (MgATP) values), allosteric inhibition by MgATP ($K_m$ (MgATP) values), or catalysis ($k_{cat}$ values), compared with the wild type enzyme. Thus, the introduced mutations were suitable to test specifically the response to fructose phosphates.

Kinetic and Binding Properties of the Arginine Mutants—Mutation to alanine of the selected arginine residues (Arg-201, Arg-292, Arg-566, and Arg-655) did not greatly modify the affinity of the Fru-6-P catalytic site (Table 1); the most pronounced changes were exhibited by mutants R201A and R292A with $S_0$ (Fru-6-P) values of 5.0 and 7.0 mM, respectively, compared with 2.0 mM for the wild type enzyme, that were close to those of the H298A and H199A mutants. There was further increase in $S_0$ (Fru-6-P) in the double mutant R201A/ R292A (to 10 mM, five times the value for the wild type enzyme), whereas there was little change when the double mutation was
carried out at residues Arg-566 and Arg-655 (S_{0.5}(Fru-6-P) value of 4.5 mM, compared with 4.0 and 3.2 mM for the corresponding single mutants). In contrast, although activation by Fru-2,6-P₂ and binding of both bisphosphates were not clearly modified in the R201A and R292A mutants (and there was normal binding, though no activation, even in the case of the double mutant R201A/R292A), mutation of either Arg-566 or Arg-655 strongly affected both properties of the enzyme. Thus, the R566A and R655A mutants exhibited a dramatic decrease in sensitivity to Fru-2,6-P₂ with an increase in their $K_{act}$ values by 2 orders of magnitude (50.0 and 45.0 μM, respectively, compared with 0.5 μM for the wild type enzyme), and in addition their activation curves became sigmoidal (Fig. 5A); they showed also a great reduction in the binding of the two regulators, with $K_d$ values increased two orders of magnitude as well (10.0 μM for Fru-2,6-P₂ in both mutants, compared with 0.2 μM for the wild type enzyme and 15.0 and 25.0 μM for Fru-1,6-P₂, respectively, compared with 0.3 μM for the wild type enzyme) (Fig. 5, B and D). Moreover, activation by both bisphosphates and binding of these compounds to the enzyme were abolished by the double mutation R566A/R655A (Table 1 and Figs. 5, B and D). These results demonstrate that the Fru-2,6-P₂/Fru-1,6-P₂ allosteric site was markedly altered after mutations of residues Arg-566 and Arg-655 and that this site was unaffected when the mutated amino acids were Arg-201 or Arg-292.

In contrast to the above mutations, the K564A mutant did not show significant change in the function of either the catalytic site or the fructose bisphosphate allosteric site with respect to the wild type enzyme, except that it was also insensitive to activation by Fru-1,6-P₂ despite normal binding (Table 1).

**DISCUSSION**

To examine the earlier proposal that the Fru-6-P catalytic site and the derived allosteric site for Fru-2,6-P₂/Fru-1,6-P₂ of eukaryotic PFKs were shared across the interface between two subunits oriented in an antiparallel manner (12), we have systematically mutated those amino acid residues of PFK-M initially assigned to these sites (12) whose involvement in one site or the other is dependent on the manner in which subunits associate to form the PFK dimer. We thus sought to locate both binding sites and, consequently, to identify the mode of subunit interaction in the tetrameric enzyme. Binding assays, in addition to kinetic studies, were essential to assess whether the introduced mutations eliciting desensitization to the allosteric regulator affected the integrity of its binding site or the structural route mediating the allosteric transition.

Mutations to alanine of residues His-199, Arg-201, and Arg-292 decreased the affinity of the Fru-6-P catalytic site, with no effect on the function of the Fru-2,6-P₂ allosteric site (Table 1 and Fig. 4). This effect was similar to that observed after mutation of residue His-298 (assigned to the catalytic site in both types of subunit interaction Fig. 3), and it was more apparent in the double mutants H199A/H298A and R201A/R292A. Therefore, these results suggested that residues His-199, Arg-201, and Arg-292 contribute to the catalytic site and not to the allosteric site, although they do not appear to be indispensable for the binding of Fru-6-P, in view of the moderate increase in the $S_{0.5}$ values (not higher than 1 order of magnitude) and the lack of change in Fru-6-P cooperativity elicited by their mutation. The central question on the location of the two binding sites was settled by the striking decrease (of 50–80-fold) in both fructose bisphosphate activation and binding of these effectors to their allosteric site brought about by mutation of either Arg-566 or Arg-655 (Fig. 5), with no change in Fru-6-P affinity (Table 1), because these residues are assigned to this site only in the N/N-C/C interaction (Fig. 3). This resembles the effect of mutating His-661, a residue assigned to the allosteric site independently of the mode of subunit association. Notably there was complete suppression of the function of this regulatory site after the double mutation R566A/R655A (Fig. 5). These results clearly indicate that Arg-566 and Arg-655 are indeed key residues of the fructose bisphosphate allosteric site. Taken
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together, these data strongly suggest that PFK-M subunits associate into dimers containing the Fru-6-P-binding (catalytic) and Fru-2,6-P_2-binding (allosteric) sites in their interface, by apposition of their equivalent terminal halves, that is, in the N/N-C/C interaction (Figs. 2 and 3), instead of in the opposite interaction (N/C-C/N) proposed up to now (12, 15, 19), in which each N-terminal half would face the C-terminal half of the neighbor subunit.

With respect to mutant K564A, its largely normal behavior suggests that, although it is near the allosteric site (because of its proximity to Arg-566), this residue does not participate in the binding of fructose bisphosphates. The lack of activation by Fru-1,6-P_2 showed by this mutant, as well as by all mutants of the active site, without alteration in their ability to bind this compound and to be stimulated by Fru-2,6-P_2, implies that these mutations abolished the particular allosteric transition induced by binding of Fru-1,6-P_2 to the enzyme. Differences in the conformational changes of the mammalian enzyme evoked by the two bisphosphates, despite sharing the same binding site (6), were suggested by the distinct response to other PFK effectors in the presence of each bisphosphate (8), the absence of activation of the Fru-2,6-P_2-sensitive PFK-C by Fru-1,6-P_2 despite binding normally to this isozyme (9), and the restoration of activation after grafting the N terminus of PFK-M onto the C terminus of PFK-C (10). The present work has provided a new step by allowing the dissection of the structural routes involved in the action of each bisphosphate. It is noteworthy that one of the amino acid residues identified as a specific component of the route conveying the signal of Fru-1,6-P_2 to the active site is located in the environment of the allosteric site, namely Lys-564, thus suggesting that this residue is related to the emission of the signal. The other residues involved in this route (His-298, His-199, Arg-201, and Arg-292) are associated with the catalytic site, and therefore, they must participate in the reception of the signal at this site. We speculate that the lack of activation of the R201A/R292A mutant by Fru-2,6-P_2 may be a consequence of the more extensive alteration of the catalytic site caused by the double mutation.

The N/C-C/N interaction of mammalian PFK subunits was first proposed by Poorman et al. (12), based on the removal of N-terminal residues after limit proteolysis of the rabbit muscle enzyme. This suggested to those authors that the N-half of each hairpin-like subunit is pointed outward from the tetramer, so that dimer-dimer interactions would be provided by the peptides connecting the PFK domains derived from the ancestral bacterial monomers (Fig. 2). However, the basis of that proposal is not unambiguous, because in the absence of a crystal structure, the site for dimer interactions as well as the locations of the N-terminal ends are unknown, and therefore, one cannot eliminate the possibility that N termini oriented differently as implied in the N/N-C/C interaction may nevertheless be accessible to proteolysis of the tetramer. In relation also with this point, it was mentioned in the introduction section that Chang and Kemp (15) suggested that residue Arg-292 of rabbit PFK-M is located in the Fru-2,6-P_2 allosteric site, thus favoring the N/C-C/N interaction, because the alanine replacement of this residue limited full activation by this effector, despite normal high affinity. We observed roughly similar kinetic properties in the same mutant of human PFK-M. However, their suggestion is not consistent with our binding studies, which clearly indicated that residue Arg-292 belongs to the catalytic site and not to the allosteric site, as discussed above. Furthermore, a decrease in maximal activation by Fru-2,6-P_2 (rather than its affinity) does not appear to be a reliable indicator of alteration of the regulator-binding site. Thus, such a decrease in maximal activation occurred not only in the R292A mutant (by less than 30%, under our conditions) (Fig. 5A) but also when the mutation affected other active site residues such as His-298 (unequivocally active site) (by 70%) (Fig. 4A), His-199 (by 74%), and Arg-201 (by 55%). The lower maximal stimulation by Fru-2,6-P_2 with some active site mutations may simply reflect a diminished capacity of the active site to respond to the allosteric signal. Moreover, among mutants that exhibited great dysfunction of the allosteric site in terms of reduced binding, decreased full activation by this regulator occurred in the H661A mutant (by 55%), but not at all in R655A and very little in R566A (by 28%), meaning simply that the latter mutants were involved primarily in effector binding.

The results presented here account for previous observations with chimeric PFKs (10), suggesting that the N-terminal half of the mammalian enzyme is responsible for the affinity requirements of the Fru-6-P catalytic site, whereas the C-terminal half is responsible for those of the fructose bisphosphate allosteric site. Other alternative approaches, such as those based on labeling of the terminal regions, or chemical cross-linking of the native enzyme, would not unequivocally distinguish between the two models of subunit association, because as mentioned above, information is lacking on both the location of subunit termini in the tetramer and the way in which dimers interact within the tetramer. Isolation of dimers composed of truncated termini to test subunit association has so far not been practical because of the rapid dissociation of PFK-M into monomers under conditions that destabilize the tetramer (35). Furthermore, production of stable proteins after expression of the N-terminal and the C-terminal halves of the mammalian enzyme has proved unsuccessful (10). Therefore, definite confirmation of the orientation of mammalian PFK subunits will require determination of the three-dimensional structure of the enzyme; however, progress in this area has been hindered by the difficulty in crystallizing a mammalian enzyme.

The N/N-C/C interaction between PFK subunits does not affect the location of other postulated ligand-binding sites of the enzyme, such as the catalytic subsite for MgATP suggested to be confined to a single subunit (12, 16) or the inhibitory sites of MgATP and citrate proposed to derive from the duplicated P-enolpyruvate/ADP allosteric site of the primitive prokaryotic precursor (19) and to be contributed by residues from both halves of a single subunit. The high conservation of the investigated amino acids in eukaryotic PFK suggests that this type of subunit association can be extrapolated to the enzyme from other species of this kingdom.

In summary, we report the distinctive participation of key amino acid residues in the Fru-6-P catalytic site and the Fru-

6 C. Ferreras, O. H. Martinez-Costa, and J. J. Aragón, unpublished observations.
2,6-P$_2$/Fru-1,6-P$_2$ allosteric site of mammalian PFK, and this has led us to suggest a new mode of subunit association to form the enzyme dimer within the tetrameric molecule, with each terminal half of a subunit interacting in an antiparallel manner with the same half of the adjacent subunit (Fig. 2, N/N-C/C interaction). A major consequence of this enzyme organization is that it provides a spatial separation between the catalytic activity and the regulatory function by Fru-2,6-P$_2$/Fru-1,6-P$_2$, with the two catalytic sites restricted to the N terminus, which is more similar to the bacterial enzyme, and the two allosteric sites located in the repeated C terminus. Additionally, the association of identical domains between subunits appears to be a simpler evolutionary way for the formation of the eukaryotic dimer.

Acknowledgments—We thank Dr. Keith Tornheim (Boston University School of Medicine, Boston, MA) for helpful comments and critical review of the manuscript. We also thank V. Sánchez for valuable technical assistance.

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