

Identification of C-terminal motifs responsible for transmission of inhibition by ATP of mammalian phosphofructokinase, and their contribution to other allosteric effects

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Systematic deletions and point mutations in the C-terminal extension of mammalian PFK (phosphofructokinase) led us to identify Leu-767 and Glu-768 of the M-type isoform (PFK-M) as the motifs responsible for the role of this region in inhibition by MgATP. These amino acids are the only residues of the C-terminus that are conserved in all mammalian isoforms, and were found to have a similar function in the C-type isoenzyme. Both residues in PFK-C and Leu-767 in PFK-M were also observed to be critical for inhibition by citrate, which is synergistic with that by MgATP. Binding studies utilizing titration of intrinsic protein fluorescence indicated that the C-terminal part of the enzyme participates in the signal transduction route from the MgATP inhibitory site to the catalytic site, but does not contribute to the binding of

this inhibitor, whereas it is essential for the binding of citrate. Mutations of the identified structural motifs did not alter either the action of other allosteric effectors that also interact with MgATP, such as the inhibitor phosphoenolpyruvate and the strong activator fructose 2,6-bisphosphate, or the co-operative effect of fructose 6-phosphate. The latter data provide evidence that activation by fructose 2,6-bisphosphate and fructose 6-phosphate co-operativity are not linked to the same allosteric transition as that mediating inhibition by MgATP.

Key words: allosteric regulation, allosteric transition, enzyme engineering, enzyme regulation, glycolysis, phosphofructokinase.

INTRODUCTION

PFK (phosphofructokinase; EC 2.7.1.11) catalyses the MgATP-dependent phosphorylation of Fru-6-*P* (fructose 6-phosphate) to fructose 1,6-bisphosphate. Its activity is highly modulated by the binding of various allosteric effectors at different binding sites, so that PFK is considered to play a basic role in the control of glycolytic flux in most cells [1–3]. Mammalian PFK isoenzymes exist as homo- and hetero-oligomers composed of three types of subunit (C, M and L) that are encoded by distinct genes showing tissue-specific expression [4]. Accordingly, PFK-M is the only form in adult muscle, PFK-L is predominant in the liver, and variable proportions of all three isoenzymes have been detected in other cell types [4–6]. These isoenzymes were reported to differ in their regulatory properties [7–9]. Thus the C isoenzyme is more sensitive to inhibition by MgATP than is PFK-M [7,9], but less sensitive than the L isoform [7]. This is accompanied by a corresponding difference in the apparent affinity for Fru-6-*P*, and was proposed to contribute to the characteristic glycolytic function in particular tissues [7–9]. In the absence of a three-dimensional structure of eukaryotic PFK, assignment of amino acid residues to substrate and effector sites has been made by sequence alignments with the less complex prokaryotic enzyme on the basis of (i) the theory that PFK from eukaryotes appeared after duplication, fusion and divergence of an ancestral prokaryotic gene, hence giving rise to new allosteric sites [10], and (ii) the available X-ray crystal structures of the bacterial enzyme (reviewed in [11]). To date, a few amino acids involved in the response to specific ligands, such as the potent activator Fru-2,6-*P*₂ (fructose 2,6-bisphosphate) [12,13] and the inhibitors citrate [12] and MgATP

[14], have been identified in mammalian PFKs by site-directed mutagenesis.

Allosteric inhibition by MgATP is a key mechanism for the regulation of the flux through PFK in eukaryotic cells. This inhibitory effect interacts with Fru-6-*P* co-operativity (sigmoidal kinetics), increasing the $S_{0.5}$ [2,15,16], and with activation by Fru-2,6-*P*₂, increasing the K_{act} [17], and is synergistic with inhibition by other inhibitors, such as citrate and phosphoenolpyruvate [18], thus allowing a finer modulation of PFK activity. Consequently, identification of the structural elements involved in this regulatory property is a major factor in accounting for the molecular basis of PFK function. Mutation of Arg-429 and Arg-433 of mouse PFK-C led to the proposal that the ATP inhibitory site evolved from the duplicated phosphoenolpyruvate/ADP allosteric site of the ancestral prokaryotic precursor [14]. This site would be located within the sequence region that shows identity between the N- and C-terminal halves of PFK-C, as well as between each of them and the bacterial isoenzymes, and would be composed of residues from both halves. This is similar to the Fru-2,6-*P*₂ binding site, which evolved from the duplicated catalytic site of the prokaryotic enzyme [12,13] and is shared in the interface between two subunits [10,11]. However, in addition to the proposed ATP binding site, earlier work showed that the C-terminal region of the muscle isoenzyme is essential for inhibition by ATP. Thus, from partial proteolysis of rabbit muscle PFK-M, Valaitis et al. [19] identified the sequence H⁷⁶³AHLEHISR as being critical for this property. This sequence is located within the approx. 30-residue C-terminal extension of the muscle enzyme compared with the similar region of bacterial PFK, so the acquisition of this extension during evolution was proposed to provide for this control mechanism

Abbreviations used: PFK, phosphofructokinase; Fru-6-*P*, fructose 6-phosphate; Fru-2,6-*P*₂, fructose 2,6-bisphosphate; HmPFK and AtPFK, phosphofructokinases from human muscle and ascites tumour cells respectively.

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Table 1 Synthetic oligodeoxynucleotides used to generate mutant PFKs

The introduced mutations are in bold face. *SphI* restriction sites are underlined. Stop codons are in bold italic.

Mutation	Sequence
HmPFK-M	
Δ17	5'-GGGGCATGC <u>TTATTA</u> GTCTGAAGTGTCCAAGTCAAT-3'
Δ12	5'-GGGGCATGCGGTACC <u>TTATTA</u> CTCCAGGTGGGCATGGTC-3'
H764A/H766A/H769A	5'-GGGGCATGC <u>TTATTA</u> GACGGCAGCTTCCCGGACCGCTCCGGGATGGCTCCAGGGCGGCAGCGTCTGAAGTGC-3'
L767A	5'-GGGCATGCTCGAG <u>TTATTA</u> GACGGCAGCTTCCCGGACCGCTCCGGGTGATGTGCTCGGCCTGGGCATGGCTGA-3'
E768A	5'-GGGCATGCAGATC <u>TTATTA</u> GACGGCAGCTTCCCGGACCGCTCCGGGTGATGTGAGCCAGGTGGGCATGGCTGA-3'
AtPFK-C	
Δ13	5'-GGGGCATGC <u>TCATCA</u> ATCAGACATGTCATAGCTCGC-3'
Δ4	5'-GGGGCATGC <u>CTACTA</u> GTGCTGCAAGGACTCCAG-3'
H780A	5'-GGGGCATGC <u>TCATCA</u> TAGTTCTTCATGAGCCGCAAGGA-3'
H781A	5'-CATAGTTCTTCAGCGTGCTGCAAGGA-3'
H780A/H781A	5'-GGGGCATGC <u>TCATCA</u> TAGTTCTTCAGCAGCCTGCAAGGA-3'
L775A	5'-GGGGCATGCGAAT <u>TCATCA</u> TAGTTCTTCATGGTGTGCAAGGACTCGGCCTTGCTGAATC-3'
E776A	5'-GGGGCATGCTCGAG <u>TCATCA</u> TAGTTCTTCATGGTGTGCAAGGAAGCCAGCTTGCC-3'
L775A/E776A	5'-GGGGCATGCT <u>CTA</u> GAGTTCTTCATGGTGTGCAAGGAAGCGGCCTTGCTGAATC-3'

[19]. In fact, recently reported allosteric mutants of the non-allosteric PFK from the slime mould *Dictyostelium discoideum*, which lacks the C-terminal nonapeptide described above, still failed to be inhibited by ATP [20]. The cluster of three histidines present in this sequence was then suggested to contribute to the binding of MgATP to the inhibitory site [19], since histidine residues were implicated in this regulatory effect based on effects of diethylpyrocarbonate modification of sheep heart PFK [21], and later the *Ascaris suum* enzyme [22]. Additionally, unidentified histidine residues were proposed to mediate the proton inhibition of animal PFK [23], which is known to modify Fru-6-P affinity [16] and co-operativity [15] and to enhance the binding of MgATP to its inhibitory site [24].

The present work was initiated to investigate the role of C-terminal histidine residues in the inhibition by ATP of mammalian PFK and, eventually, to identify the structural motifs of this region responsible for this important property in two different isoenzymes. Additionally, this led us to investigate the contribution of the identified amino acid residues to the functioning of other allosteric effectors whose mechanisms of action have been connected with that of the inhibitor ATP.

MATERIALS AND METHODS

Materials

All chemical reagents and enzymes used in genetic assays and protein purification were obtained from Roche Molecular Biochemicals, Biotools, Amersham or Sigma. The Sculptor™ *In Vitro* Mutagenesis System for site-directed mutagenesis was purchased from Amersham. Oligonucleotides were synthesized by Isogen. The auxiliary enzymes and biochemicals for the PFK assay were from Sigma. Other reagents were obtained from commercial sources and were of the best grade available.

Strains, media and growth conditions

The *Escherichia coli* strains used for general cloning procedures and amplifications of DNA were DH5α [*SupE44 ΔlacU169* (Φ80 *lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] and TG1 [*supE hsdΔ5 thi Δ(lac-proAB) F'* [*traD36 proAB⁺ lacI^q lacZΔM15*]]. The *Saccharomyces cerevisiae* strain HD152-1D (MATα *pkf1::HIS3 pkf2::HIS3 ura3-52, his3-Δ1 leu2-3,112 trp1-289 MAL2-8c SUC2 GAL*) [9], carrying deletions in both

yeast *PFK* genes, was used for the expression of wild-type and mutant isoforms of PFK-M from human muscle (HmPFK-M) and PFK-C from ascites tumour cells (AtPFK-C). *E. coli* was grown on either liquid or solid LB medium [25], with appropriate antibiotics added when required. Yeast manipulations were as described previously [26].

Genetic manipulations, plasmids and *in vitro* mutagenesis

Point mutations and C-terminal deletions of HmPFK-M and AtPFK-C at the desired positions were introduced into their respective cDNAs by PCR (except for H781A of AtPFK-C; see below) using mutagenic primers that carried an *SphI* site at their 5' end to facilitate subsequent subclonings (Table 1). The double-stranded plasmids p18HmPFK and p18TPC [9], pUC18 derivatives carrying the cDNAs for HmPFK-M and AtPFK-C respectively, were employed as templates. The amplification reaction mixture contained 10–25 ng of template DNA, 0.2 mM each dNTP, 25 pmol of sense (17-mer universal and 16-mer reverse sequencing primers for HmPFK-M and AtPFK-C respectively) and antisense mutagenic primers, 2.5 units of *Taq* DNA polymerase and the buffer provided by the manufacturer in a total volume of 100 μl. PCR was carried out for 30 cycles (94 °C, 1 min; 55 °C, 2 min; 72 °C, 2 min).

To obtain the mutant cDNAs of HmPFK-M downstream of the *PFK2* promoter of *S. cerevisiae* in the expression vector pJH71 [26], the PCR products were first digested with *EspI-SphI*, then the DNA fragments carrying the desired mutations at the 3' end of HmPFK cDNA were isolated by agarose gel electrophoresis and used to replace the corresponding DNA sequence of wild-type *pfk* in pJH71PFK [27]. Mutant cDNAs of AtPFK-C were obtained by cloning the respective *ApaI-SphI*-digested DNA fragments from the mutagenic PCR reaction into *ApaI-SphI*-digested p18TPC. The *Clal-SphI* fragments from the resulting plasmids were used to replace their counterparts in the pJH71-derived plasmid pCHA1 [9], generating the recombinant plasmids in which the mutant *pfk* genes were expressed under the control of the *S. cerevisiae* *PFK2* promoter.

The cDNA for AtPFK-C carrying the H781A mutation was generated by using the Sculptor™ *In Vitro* Mutagenesis System. Previously, the full-length cDNA of AtPFK-C was cloned in M13mp18 as an *EcoRI-KpnI* 2.7 kbp fragment from λZTPC [9], yielding plasmid pMTPC. Mutagenesis was performed as recommended by the manufacturer, using the mutagenic primer

(Table 1) and single-stranded DNA derived from pMTPC as template; the *Clal*–*SphI* DNA fragment from the resulting M13mp18 derivative was finally cloned into pCHA1, as described above. Sequencing of the resulting plasmids by the dideoxynucleotide chain termination method confirmed that only the desired mutations had been introduced.

Expression and purification of recombinant enzymes

Wild-type and mutant isoforms of HmPFK-M and AtPFK-C were functionally expressed in *S. cerevisiae* strain HD152-1D, as yeast transformants with the corresponding plasmids complemented the glucose-negative phenotype of the recipient strain. For purification of the recombinant enzymes, yeast transformants expressing either wild-type or mutant PFK were grown on 1 litre of rich medium containing glucose to early stationary phase, harvested by sedimentation and washed in buffer A (50 mM Tris/phosphate, 0.05 mM fructose 1,6-bisphosphate, 0.1 mM EDTA, 1 mM dithiothreitol, 50 mM NaF, 0.5 mM PMSF and 2.5 µg/ml leupeptin, pH 8.0). Approx. 10–13 g of wet cells were resuspended in 2 vol. of buffer A, mixed with 5 vol. of glass beads and shaken in a refrigerated mill for 5 min. After filtration, the beads were washed with 1 vol. of buffer A. The filtrate was centrifuged at 31 000 g for 30 min at 4 °C. Protamine sulphate dissolved in buffer A was added to the supernatant to a final concentration of 0.2% (w/v). After stirring for 30 min, the mixture was centrifuged as before, and the supernatant fluid was chromatographed on a column (1.9 cm × 7 cm) of Blue Sepharose CL-6B equilibrated with buffer A. The column was washed with 100 ml of buffer A and then with the same buffer containing 0.15 mM ADP. This washing step was omitted in the purification of mutant and wild-type isoforms of HmPFK-M. PFK activity was eluted with buffer A containing 2 mM Fru-6-P and 2 mM ATP. Fractions of 2 ml were collected and those showing PFK activity were pooled and applied to a column (1.9 cm × 7.5 cm) of DEAE-trisacryl equilibrated in buffer B (20 mM Tris/phosphate, 10 mM ammonium sulphate, 10 mM NaF, 10 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM PMSF, 2.5 µg/ml leupeptin, 0.05 mM fructose 1,6-bisphosphate and 0.2 mM ATP, pH 7.5). The column was washed with 100 ml of buffer B, and PFK activity was eluted in 2 ml fractions with a linear gradient of buffer B and the same buffer supplemented with 90 mM ammonium sulphate, 73.5 mM potassium phosphate and 50 mM NaCl [28]. The final preparations were subjected to SDS/PAGE analysis on 10% (w/v) polyacrylamide gels and Coomassie Blue staining as described in [29], and judged to be homogeneous by this criterion (Figure 1). For kinetic studies, the purified preparations were dialysed against 10 mM Hepes, 0.5 mM PMSF, 2.5 µg/ml leupeptin and 20% (v/v) glycerol, pH 7.5. The specific activities of the mutant PFKs (32–58 units/mg) were in the range of those of the recombinant wild-type enzymes [9], as were the amounts of pure protein obtained (0.3–1 mg/litre of culture).

Enzyme activity assay

Total PFK activity was measured in an assay mixture containing 50 mM Tris/HCl, 100 mM KCl, 5 mM MgCl₂, pH 8.2, 5 mM P_i, 1 mM NH₄⁺, 0.1 mM cAMP, 1 µM Fru-2,6-P₂, 0.15 mM NADH, 1 mM MgATP, 1.2 units of aldolase, 10 units of triosephosphate isomerase, 1 unit of glycerol-3-phosphate dehydrogenase, 2–5 µl of the enzyme preparation and 6 mM Fru-6-P in a final volume of 1 ml. 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, 2–5 µl of the purified

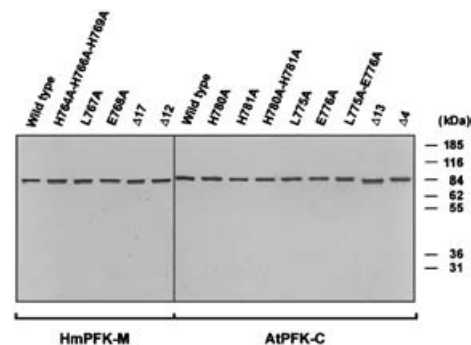


Figure 1 SDS/PAGE of purified mutant and wild-type HmPFK-M and AtPFK-C

Samples containing 2–5 µg of purified enzymes were loaded per lane. The positions and molecular masses of marker proteins are shown on the right.

enzyme, and the indicated concentrations of MgATP, effector and Fru-6-P. In all cases, the reaction was started after a 5-min preincubation by the addition of Fru-6-P, and was followed by measuring the absorbance change at 340 nm at 25 °C. When PFK activity was assayed during purification, glucose 6-phosphate was added to Fru-6-P in proportions of 3:1 (mol/mol). Auxiliary enzymes were desalted as described [20]. One unit of activity is defined as the amount of enzyme catalysing the conversion of 1 µmol of substrate/min under the above conditions.

Fluorescence measurements

Titration of intrinsic PFK fluorescence with MgATP or citrate was measured at 25 °C with a Perkin–Elmer LS50 luminescence spectrometer. The excitation wavelength was set to 294 nm and emission was recorded at 335 nm. The protein concentration was 6–20 µg/ml (within the linear portion of the fluorescence–concentration curve) in 50 mM sodium phosphate, 1 mM EDTA, 25 mM KCl, 0.2 mM dithiothreitol and 10 mM MgCl₂, pH 7.0, and experiments were performed by adding either small increments of ligand or enzyme solution. Background readings were subtracted and corrections were made to compensate for volume changes. Results of the titrations were expressed as the fractional intrinsic fluorescence change F/F_0 , where F_0 and F are the corrected fluorescence intensities in the absence and presence respectively of ligand. Experimental errors in fluorescence measurements were estimated to be less than 1%.

Other methods

Protein concentration was determined by Bradford's dye-binding method [30], using bovine γ -globulin as standard. Protection against thermal denaturation was carried out essentially as described previously [9], except that incubations in the presence of increasing MgATP concentrations were performed at 55 °C for 50 min in 50 mM Hepes, 100 mM KCl and 5 mM MgCl₂, pH 7.0.

RESULTS

Inhibition by MgATP

As shown in Figure 2, the C-terminal nonapeptide H⁷⁶³AHL-EHISR, found to be essential for the inhibitory effect of MgATP on rabbit muscle PFK [19], is highly conserved in all PFK-M

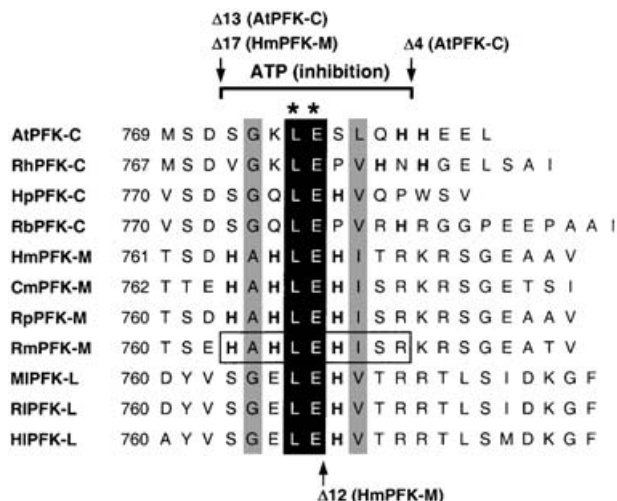


Figure 2 Multiple alignment of the C-termini of mammalian PFKs

Sequences are from: ascites tumour cells (At), rat hypothalamus (Rh), human pancreatic islets (Hp), rabbit brain (Rb), human muscle (Hm), canine muscle (Cm), rat pancreatic islets (Rp), rabbit muscle (Rm), mouse liver (Ml), rat liver (Rl) and human liver (Hl). A nonapeptide reported to be involved in the inhibition of RmPFK-M by ATP [19] is boxed. Histidine residues in this region are in bold face. Black and grey boxes indicate positions in the alignment where the same or a similar amino acid respectively is found in at least 10 out of the 11 listed sequences (plurality 10). Deletions and point mutations introduced in either AtPFK-C or HmPFK-M are indicated by arrows and asterisks respectively. A glutamate residue at position 777 of RbPFK-C was identified by peptide sequencing, whereas the codon GTG for valine (instead of GAG for glutamate) has been reported at the corresponding position in the cDNA sequence [39].

isoenzymes sequenced so far. Of the three histidine residues of this sequence suggested [19] to be important for this effect, only the third one is conserved in all PFK-L isoenzymes and in PFK-C from human pancreatic islets, and none of them is present in other PFK-C isoenzymes, although these latter isoforms contain one or two histidines at nearby positions that could play a similar role. We followed this hypothesis to examine the C-terminal motifs of PFK-C involved in the allosteric effect of MgATP, and thus mutated to alanine residues His-780 and His-781 of AtPFK-C, which has a sequence identical with that of the mouse isoenzyme [9,31] and which was shown to have regulatory properties similar to those of the isoform in normal cells [9]. The corresponding cDNAs were expressed in a PFK-deficient strain of *S. cerevisiae* [9], and enzyme mutants were purified (Figure 1) and characterized. However, neither the double mutant H780A/H781A nor the single mutants H780A or H781A differed in their sensitivity to inhibition by MgATP (Figure 3A) compared with the wild-type enzyme. There were also no differences in Fru-6-P affinity and co-operativity. We then resorted to HmPFK-M (Figure 3B) to test the function of its C-terminal histidines. However, mutation to alanine of the three potential histidines excluded a significant role for these residues, since the sensitivity of the triple mutant H764A/H766A/H769A to inhibition by MgATP scarcely deviated from that of the wild-type enzyme (K_i values of 3.2 and 2.6 mM respectively). Nevertheless, deletion of the 17 C-terminal amino acids ($\Delta 17$) caused a dramatic decrease in inhibition by MgATP (Figure 3B), similar to the results obtained by Valaitis et al. [19], who made a similar truncation of the rabbit muscle enzyme with *Staphylococcus aureus* V8 protease. An equivalent deletion of AtPFK-C, $\Delta 13$ (Figure 3A), also shifted the inhibition curve strikingly to the right, increasing the K_i value by 6-fold (7.8 mM, compared with 1.3 mM for the wild-type enzyme), whereas a $\Delta 4$ deletion had no effect on this property. This indicates that other

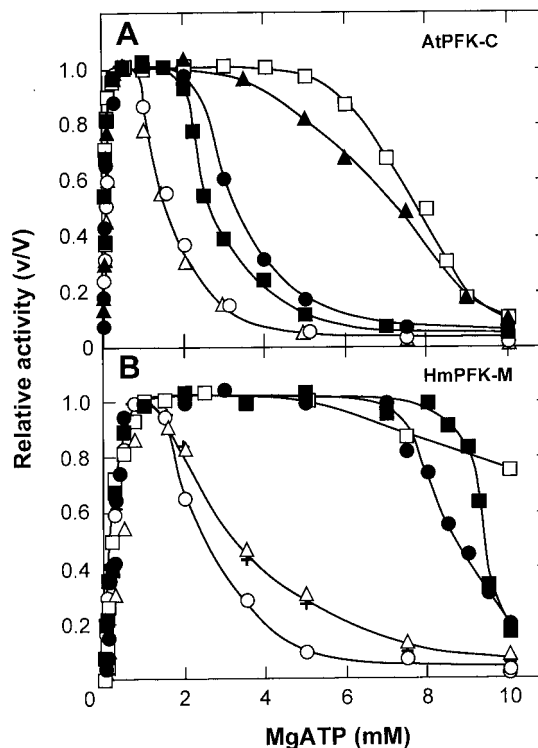


Figure 3 Effects of C-terminal mutations on the inhibition by ATP of AtPFK-C and HmPFK-M

Assays were performed at pH 7.0 with 2 mM Fru-6-P. Activity is expressed relative to the maximal activity obtained under these conditions for each enzyme. (A) AtPFK-C: \circ , wild type; \triangle , H780A/H781A; \square , $\Delta 13$; \blacksquare , L775A; \bullet , E776A; \blacktriangle , L775A/E776A. (B) HmPFK-M: \circ , wild type; \triangle , H764A/H766A/H769A; \square , $\Delta 17$; $+$, $\Delta 12$; \blacksquare , L767A; \bullet , E768A. Mutants H780A, H781A and $\Delta 4$ of AtPFK-C gave MgATP inhibitions patterns that were coincident with that of the wild-type enzyme; these were not included in the Figure for the sake of simplicity. The $\Delta 17$ mutant of HmPFK-M exhibited relative activity values of 0.64 and 0.25 at 11 and 12 mM MgATP respectively.

motifs, different from the selected histidine residues but close to the C-terminal end of PFK-C and located in a region equivalent to that identified in PFK-M, are involved in the inhibitory effect of MgATP on the former isoenzyme as well. A $\Delta 12$ deletion of PFK-M, that eliminated the only histidine residue that is conserved in all M and L isoforms, nevertheless did not affect the response to MgATP (Figure 3B), narrowing the location of the key residues to the pentapeptide H⁷⁶⁴AHLE (Figure 2). Alignments of the C-termini of mammalian PFKs (Figure 2) showed that a leucine and a glutamate located at contiguous positions (767 and 768 in HmPFK-M) are the only amino acids of this region that are conserved in all PFK sequences. Both residues were found to be responsible for the role of the C-terminus on the inhibitory effect of MgATP, since either the L767A mutant or the E768A mutant of HmPFK-M exhibited a marked decrease in this property that was similar to that shown by the $\Delta 17$ mutant (Figure 3B). In the case of AtPFK-C, replacement of both amino acids by alanine was required (L775A/E776A mutant) to reproduce the effect of the $\Delta 13$ deletion (Figure 3A), as each of the single mutants L775A and E776A elicited a relatively mild right-shift of the inhibition curve (K_i values of 2.6 and 3.6 mM respectively, compared with 1.3 mM for the wild-type enzyme). Neither of the described mutations significantly modified the affinity of either isoenzyme for MgATP at the catalytic site (K_m values of PFK-M mutants were 75–97 μ M, compared with 77 μ M for the wild-type

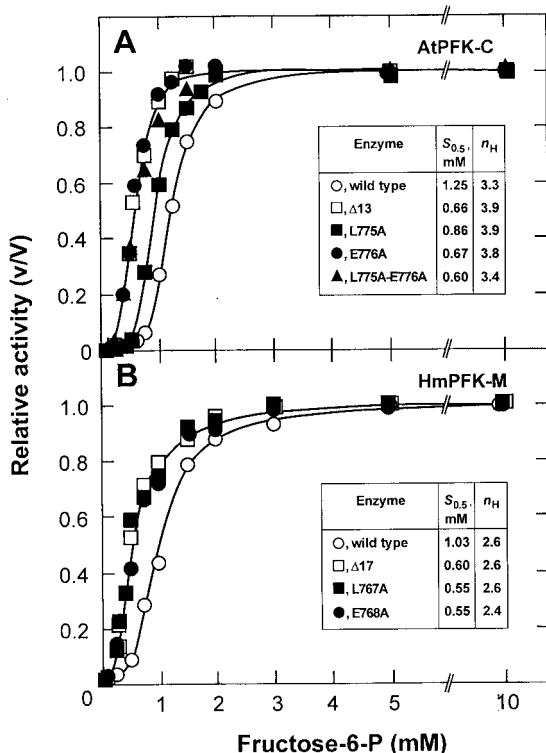


Figure 4 Fru-6-P saturation curves for wild-type and mutant forms of AtPFK-C (A) and HmPFK-M (B)

Assays were carried out at pH 7.0. The concentration of MgATP was fixed at 0.5 and 1 mM for AtPFK-C and HmPFK-M respectively. The insets show $S_{0.5}$ and h (n_H) values calculated from the data. When the assays were performed at 5 mM MgATP with either isoenzyme, the $S_{0.5}$ and h values of the mutant forms did not change significantly, whereas those of wild-type PFK-C were 3.5 mM and 3.8 respectively, and those of wild-type PFK-M were 2.6 mM and 2.9 respectively.

enzyme; those of the PFK-C mutants were 22–34 μ M, compared with 28 μ M for the wild-type enzyme).

C-terminal mutations that desensitized PFK-C and PFK-M to inhibition by MgATP increased moderately the affinity of both isoenzyme types for Fru-6-P at 0.5–1 mM MgATP, but did not significantly decrease the co-operativity of Fru-6-P, as indicated by the corresponding $S_{0.5}$ and h (Hill coefficient) values (Figure 4). For the mutant enzymes, these kinetic properties remained virtually unchanged when the MgATP concentration was raised to 5 mM, a highly inhibitory concentration for the wild-type isoenzymes that markedly diminished their Fru-6-P affinity (see legend to Figure 4). None of the generated mutations modified catalysis noticeably, as shown by the k_{cat} values of the mutants, which varied from 204 to 261 s^{-1} compared with 269 s^{-1} for wild-type PFK-M, and from 179 to 325 s^{-1} compared with 235 s^{-1} for wild-type PFK-C.

Sensitivity to other effectors related to inhibition by MgATP

Citrate is an inhibitor of PFK that functions synergistically with MgATP [18], and its effect was also reported to diminish substantially after C-terminal proteolysis of rabbit muscle PFK-M [19]. Mutation studies indicated that the two effectors bind to separate sites [12,14], but the structural requirements for the synergism between the regulators are unknown. We examined whether the C-terminal motifs found to be critical for the ATP-mediated inhibition of both isoenzymes are involved in the action of

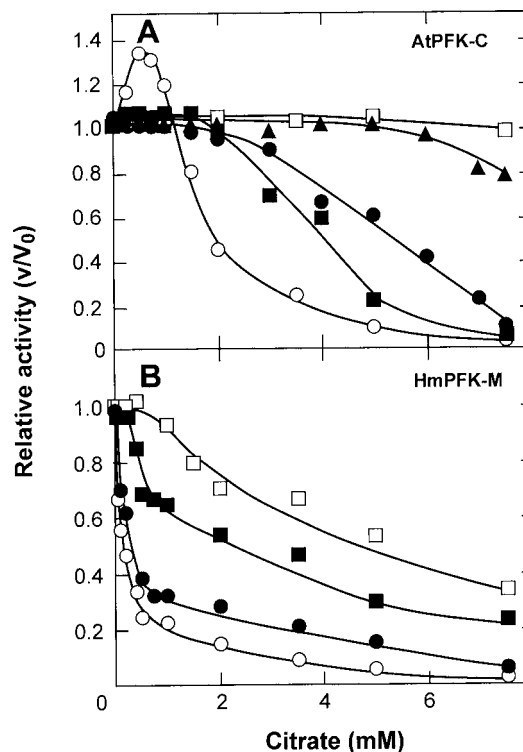


Figure 5 Inhibition by citrate of wild-type and mutant forms of AtPFK-C and HmPFK-M

Assays were carried out at pH 7.0. The Fru-6-P concentration was 2 mM. MgATP concentrations were 0.5 and 1 mM for AtPFK-C and HmPFK-M respectively. Activity is expressed relative to that obtained in the absence of citrate (v_0). (A) AtPFK-C: ○, wild type; □, $\Delta 13$; ■, L775A; ●, E776A; ▲, L775A/E776A. (B) HmPFK-M: ○, wild type; □, $\Delta 17$; ■, L767A; ●, E768A.

citrate (Figure 5). Under the conditions of the assay, PFK-M was substantially more sensitive to inhibition by this metabolite (K_i 0.16 mM) than PFK-C, which was inhibited only at concentrations of citrate above 1 mM (after a small and apparently insignificant activation burst), exhibiting a K_i value of 1.7 mM. Inhibition by citrate was practically abolished in PFK-C after the $\Delta 13$ deletion (Figure 5A), whereas it persisted in the more sensitive M isoenzyme after the equivalent $\Delta 17$ deletion (Figure 5B), although much higher concentrations of citrate were needed, with a 30-fold increase in the K_i value to 5 mM. The C-terminal leucine and glutamate residues responsible for MgATP-mediated inhibition contributed to inhibition of PFK-C by citrate in a similar manner as for the inhibitory effect of MgATP, i.e. both of the single mutants L775A and E776A moderately decreased the action of citrate, whereas the double mutation L775A/E776A virtually mimicked the de-inhibitory effect of the $\Delta 13$ deletion (Figure 5A). In the M-isoenzyme, only the leucine residue appeared to be involved in the action of citrate, as indicated by the marked shift of the inhibition curve to the right shown by the L767A mutant, resulting in a 17-fold higher K_i value (2.77 mM) than for the wild-type enzyme, and the lack of a significant change after the E768A mutation (K_i 0.24 mM) (Figure 5B).

Inhibition of PFK by MgATP is also known to occur synergistically with the effect of the inhibitor phosphoenolpyruvate [18] and to be counteracted by the strong activator Fru-2,6- P_2 [17]. PFK-M was more sensitive to both effectors than PFK-C (Figure 6), with K_i (phosphoenolpyruvate) values of 2.2 and 5.0 mM respectively, and K_{act} (Fru-2,6- P_2) values of 0.3 and 2.7 μ M respectively. However, none of the C-terminal deletions or point

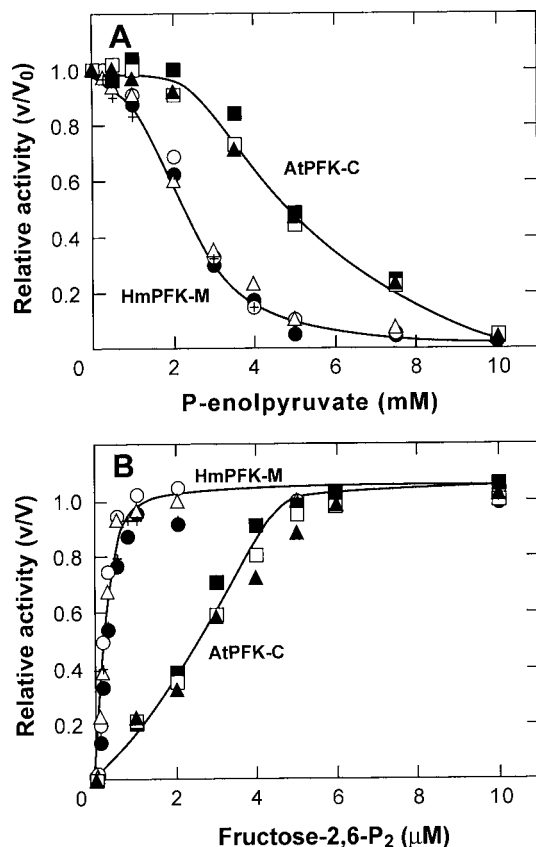


Figure 6 Effects of phosphoenolpyruvate (A) and Fru-2,6- P_2 (B) on wild-type and mutant forms of HmPFK-M and AtPFK-C

Assays were carried out at pH 7.0. Inhibition by phosphoenolpyruvate (P-enolpyruvate) was performed under the conditions described in the legend to Figure 5, and activity is expressed relative to that obtained in the absence of the inhibitor (V_0). Fru-2,6- P_2 activation was performed at a Fru-6- P concentration of 0.8 mM and a MgATP concentration of 5 mM. The indicated mutations did not significantly modify the extension of maximal Fru-2,6- P_2 activation of each isoenzyme. HmPFK-M: ○, wild type; ●, $\Delta 17$; △, L767A; +, E768A. AtPFK-C: □, wild type; ■, $\Delta 13$; ▲, L775A/E776A.

mutations that greatly affected inhibition by MgATP of both isoenzymes were found to modify significantly the effects of phosphoenolpyruvate (Figure 6A) or Fru-2,6- P_2 (Figure 6B) on either PFK type.

Binding of MgATP and citrate

The involvement of the C-terminal region in the binding of MgATP and citrate to PFK-M was examined by titration of intrinsic fluorescence of the $\Delta 17$ mutant and the wild-type enzyme in the presence of increasing concentrations of each of these ligands. The M-type isoform was chosen because it showed more drastic changes in MgATP-mediated inhibition upon manipulation of this region, as well as higher sensitivity to inhibition by citrate. As seen in Figure 7(A), MgATP quenched protein fluorescence and gave a biphasic curve for the $\Delta 17$ mutant that was virtually indistinguishable from that of the wild-type enzyme, with maximum quenching with 10 mM MgATP of 50% and 47% respectively. This type of curve is consistent with the well known binding of MgATP to both the high-affinity catalytic site and the low-affinity inhibitory site [24], and their corresponding apparent dissociation constants calculated from the data were 0.21 μM and 1.13 mM respectively for the mutant, which

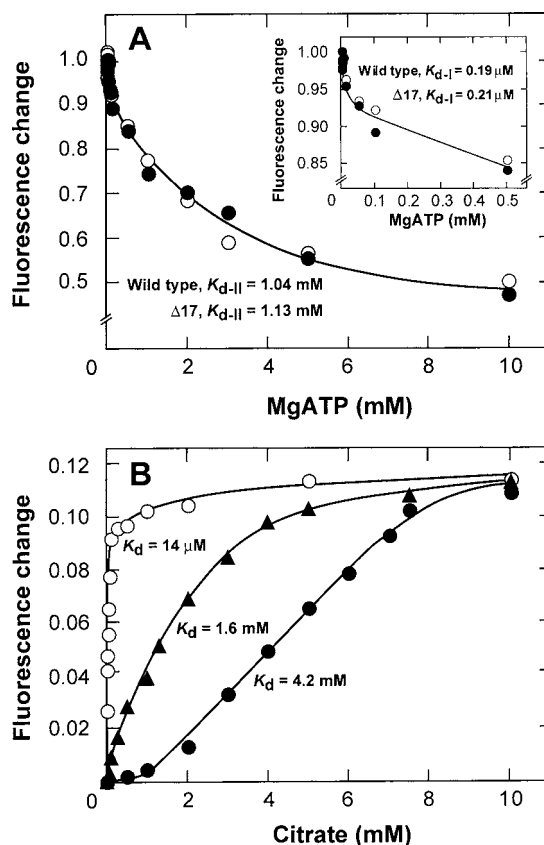


Figure 7 Relative fluorescence intensity of wild-type and mutants $\Delta 17$ and L767A of HmPFK-M as a function of MgATP (A) and citrate (B) concentrations

Conditions were described in the Materials and methods section. ○, wild type; ●, $\Delta 17$; ▲, L767A. The fractional fluorescence change is plotted against increasing ligand concentration. The kinetics of the decrease in fluorescence intensity induced by MgATP was biphasic, as shown in the inset. K_{d-I} and K_{d-II} are the dissociation constants of the catalytic and inhibitory sites for MgATP respectively.

were not significantly different from those of 0.19 μM and 1.04 mM respectively for the wild-type enzyme. Protection by variable concentrations of MgATP against thermal denaturation of the protein at 55 °C for 50 min gave apparent K_d values for the second binding site of 2.0 mM and 1.4 mM for the $\Delta 17$ mutant and the wild-type enzyme respectively (results not shown), i.e. close to the values determined by changes in intrinsic protein fluorescence. These data clearly indicated that, although the C-terminal deletion desensitized PFK to inhibition by MgATP, it did not modify its ability to bind this effector at its inhibitory site.

Figure 7(B) shows that citrate enhanced the fluorescence intensity of PFK-M, suggesting that this ligand induces a different conformation of the protein. The fluorescence changes increased with increasing concentrations of citrate, and yielded a hyperbolic curve that corresponded to an expected single binding site, with a calculated K_d value of 14 μM for the wild-type enzyme. In strong contrast with the observed lack of a role of the C-terminal region of PFK-M in MgATP binding, the binding of citrate was dramatically altered by the $\Delta 17$ deletion, as this mutant exhibited a > 200-fold decrease in the apparent affinity for this ligand with respect to the wild-type enzyme, with a K_d value of 4.2 mM and a sigmoidal binding curve. Furthermore, the L767A mutant also showed a marked decrease in citrate binding (K_d 1.6 mM) that paralleled its effect on inhibition by citrate (Figure 5B). The

fact that this point mutation reduced both phenomena to a somewhat lower degree than the $\Delta 17$ deletion suggests that other, unidentified residues of the C-terminal tail region of PFK-M may also participate in the action of citrate. As also shown in Figure 7(B), maximum fluorescence enhancement (approx. 11%) was not changed significantly by either of these mutations.

DISCUSSION

The series of deletions and single point mutations of PFK-M examined here has led us to identify the contiguous residues Leu-767 and Glu-768 as being responsible for the involvement of the C-terminal region of the enzyme in allosteric inhibition by MgATP, with the replacement of either of these residues being sufficient to desensitize the enzyme to the inhibitor. Although this region exhibits marked variability among PFK sequences from eukaryotic sources, we observed that it performs a similar function in the more inhibitable C-type isoenzyme. The identified amino acids are the only motifs that are conserved in the C-termini of all mammalian PFK sequences, and they were also found to be involved in inhibition by MgATP of PFK-C (positions 775 and 776), although they contribute somewhat differently in this case, since their simultaneous mutation was required for a substantial decrease in the effect of MgATP. The decreased sensitivity to MgATP was not due to a lack of binding of this inhibitor to the allosteric site, as fluorescence titration and protection against irreversible thermal denaturation were not affected by deletion of the final 17 residues of PFK-M (and therefore most probably not by the mutation of the conserved residues of this region either). Neither does desensitization to MgATP appear to be related to a marked increase in Fru-6-P affinity that would reduce MgATP inhibition to such an extent, since none of the insensitive mutants exhibited a substantial change in this property (<50% decrease in their $S_{0.5}$ values for Fru-6-P; Figure 4). The simplest interpretation is that these mutations prevent the conformational change of the enzyme to a less active form induced by binding of MgATP to its inhibitory allosteric site. Leu-775 and Glu-776 of PFK-C, and Leu-767 of PFK-M, are also involved in inhibition by citrate. However, our binding studies with the latter isoenzyme indicate that its C-terminal region, and at least the Leu-767 residue in particular, functions differently in the action of the two allosteric effectors. Thus, whereas this region does not contribute to the binding of MgATP but is involved in the subsequent propagation of the allosteric signal to the catalytic site, it is essential for the binding of citrate (Figure 7). Therefore the two regulators can act together, even involving the same amino acid, without mutual binding exclusion.

Elucidation of the structural interactions exerted by the identified motifs to account for their effects on inhibition by MgATP and citrate awaits determination of the three-dimensional structure of the mammalian enzyme. Nevertheless, the identification of C-terminal residues that are critical for the action of both inhibitors provides a tentative molecular basis for their synergistic operation. Both the ATP inhibitory and the citrate binding sites have been proposed to have developed from the phosphoenolpyruvate/ADP allosteric site of *E. coli* PFK [14], such that the two allosteric sites shared by each subunit pair of the bacterial tetramer [11] evolved to form new specific sites comprising residues from the N- and C-terminal halves of the mammalian enzyme after the process of gene duplication, fusion and mutation [10,32]. Site-directed mutagenesis [12,14] and chemical modification [33] studies suggest that one of the prokaryotic allosteric sites was the origin of the ATP inhibitory site, which would be close to the peptide region connecting the two halves of the mammalian pseudodimeric

subunit, whereas the citrate site is derived from the second prokaryotic allosteric site, and so it would be located at the other end of the eukaryotic subunit near the N- and C-termini. The spatial location of the C-terminal extension of mammalian PFK implied by this model, presumably closer to the citrate site than to the MgATP inhibitory site, agrees with our finding that this region participates in the binding of the former, but not the latter. Whether the involved motifs of the C-termini interact directly with citrate, or induce an optimal binding conformation without being part of the binding site itself, remains to be elucidated.

Although some synergism has been reported [18] between MgATP inhibition and the inhibitor phosphoenolpyruvate, the lack of effect of the C-terminal mutations of PFK-M and PFK-C on the action of phosphoenolpyruvate indicates that it utilizes a different structural pathway. Its antagonistic binding to muscle PFK with regard to citrate [18] suggested that they share a common site. The evolution of the prokaryotic allosteric site also involved a substantial loss of affinity for phosphoenolpyruvate, which became a less physiologically relevant regulator in animal cells in view of its low intracellular concentrations, generally within the micromolar range [34–36], in comparison with the millimolar K_i values of mammalian PFK isoenzymes for this metabolite (Figure 6A).

Mutagenesis of the C-terminal residues found to be critical for inhibition of both PFK-M and PFK-C by MgATP allowed the dissection of this regulatory effect and Fru-6-P co-operativity, in conjunction with Fru-2,6-P₂ activation, into separate phenomena, since desensitization to the inhibitor did not modify the other two properties. These results agree with recent findings obtained by mutations of the non-regulatory PFK from *Dictyostelium discoideum*, which suggested that the action of the inhibitor MgATP is decoupled from Fru-6-P co-operativity, and that stimulation by Fru-2,6-P₂ operates on the same R/T conformational transition that accounts for co-operativity [20]. Nevertheless, the latter observations were made by generating co-operativity and Fru-2,6-P₂ stimulation in a previously non-allosteric PFK, whereas in the present work inhibition by MgATP was eliminated from two normal allosteric isoforms of mammalian PFK without changes in the other two effects. This provides evidence that although MgATP clearly interacts with the action of Fru-6-P [2,15,16] and Fru-2,6-P₂ [17], its inhibitory effect is brought about by an allosteric transition distinct from that involved in Fru-6-P co-operativity and activation by Fru-2,6-P₂. Therefore the operation of these regulatory mechanisms in mammalian PFK does not follow the concerted model of Monod et al. [37], which assumes a unique allosteric transition between two (and only two) conformations.

The high variability of the sequence of the C-terminal extension of eukaryotic PFK appears to serve a variety of functions in different isoenzymes. In yeast, this region has been reported to be necessary for the three-dimensional structure and correct assembly of subunits [38]. In the slime mould *D. discoideum*, this part of the sequence was found to be responsible for the lack of allosteric transitions that characterizes this particular isoform [20]. In mammalian cells, the data reported herein show that the C-terminus contains specific motifs that were critical in the evolutionary process of endowing PFK isoenzymes with sensitivity to new allosteric signals for the regulation of energy metabolism, such as MgATP and citrate.

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