Site-directed Mutagenesis of Rabbit Muscle Phosphofructokinase cDNA

MUTATIONS AT GLUTAMINE 200 AFFECT THE ALLOSTERIC PROPERTIES OF THE ENZYME*

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‡ This work is dedicated to David W. H. Chang, 1971-1991.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EMBL Data Bank with accession number(s) J02702.

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Due to its central role in the control of glycolytic flux and its allosteric properties, fructose-6-phosphate-1 kinase (PFK, EC 2.7.1.11) has long been a subject of study in terms of both its enzymology and its protein chemistry (reviewed by Uyeda, 1979; Dunaway, 1983). The catalytic and allosteric mechanisms and the structures of Escherichia coli PFK (EcPFK) and Bacillus stearothermophilus PFK (BsPFK) have been elucidated at the crystallographic level (Schirakihara and Evans, 1988; Rypniewski and Evans 1989; Schirmer and Evans, 1990). In contrast, due to the absence of any primary structure, mammalian PFK species have been studied only at the enzymological level until Poorman and co-workers (1984) reported the amino acid sequence of rabbit muscle PFK (RMPFK). Their results showed that the primary sequence of RMPFK (molecular mass 80 kDa) appears essentially to be a direct repeat of bacterial PFK (molecular mass 36 kDa). This duplication pattern has also been observed in many other eukaryotic PFK molecules including that of human liver (Levanon et al., 1989), mouse liver (Gehrlich et al., 1988), and yeast (Heinisch et al., 1989), thus establishing an evolutionary relationship between prokaryotic and eukaryotic PFK. The sequence information of rabbit muscle PFK also promoted the cloning of the gene (Lee et al., 1987) and the full-length cDNA (Li et al., 1990) of this enzyme.

Like the bacterial PFK, mammalian PFK responds to fructose-6-phosphate concentration cooperatively (Passonreaux and Loywer, 1963). However, its activity is subject to much more complex regulation by a variety of effectors and metabolites (reviewed by Dunaway, 1983), including activation by AMP, inorganic phosphate, fructose-6-P, fructose-1,6-P2, and fructose-2,6-P2 (Van Schaftingen et al., 1980; Pilkis et al., 1981; Uyeda et al., 1981) and inhibition by ATP and citrate. The structure and enzymology of mammalian PFK are further complicated by the complex tetramers formed by the assembly of three types of isozymic subunits (Dunaway and Kasten, 1987; Dunaway et al., 1988): M for muscle, L for liver, and C for a third type of subunit. Each of these homo- or heterotetramers not only binds the substrates with different affinity, but also responds to the effectors with diverse sensitivity (Poe and Kemp, 1985; Dunaway et al., 1988). Residues responsible for the binding of...
substrates and effectors have been postulated based on the sequence homology among the NH₂ and COOH halves of rabbit muscle PFK and bacterial PFK, and the crystallographic structure of the latter (Poorman et al., 1984; Hellenga and Evans, 1985). Leading the effort in the identification of these residues, Valaitis and co-workers (1987) have located the ATP inhibition site within a heptadecapeptide at the carboxy terminus of rabbit muscle PFK using the method of partial proteolysis. Other than this, little has been elucidated regarding the role of the amino acid residues in the catalytic and regulatory functions of mammalian PFK.

This work presents studies on the structural basis of the allosteric control of RMPFK using DNA technology. We have cloned and expressed the full-length cDNA of this enzyme in E. coli cells and performed site-directed mutagenesis on the glutamine residue at position 200 (Q200) of RMPFK. The rationale for selecting Gln to Asp as the target for mutagenesis is by inference from the highly resolved crystallographic structures of the R (relaxed) and T (tense) forms of BsPFK (Schrinner and Evans, 1990), and the similarity between the primary structures of this bacterial PFK and RMPFK (Poorman et al., 1984). The glutamate 161 of BsPFK, shown to be involved in the allosteric transition, forms a salt bridge to arginine 243 in the T state. In the R state, glutamate 161 rotates away, and a new salt bridge forms between arginine 252 and the substrate, Fru-6-P. Since glutamine 200 occupies the homologous position in the RMPFK sequence, we propose that this residue is similarly important in the allosteric behavior. The results of this series of experiments are presented in this paper.

**EXPERIMENTAL PROCEDURES**

**Cells and Plasmids**—The general methods used for DNA analysis, construction, and purification were those described by Maniatis and co-workers (1982). The cloning and characterization of the full-length RMPFK cDNA has been described previously (Li et al., 1990). In order to insert this cDNA at the correct site and orientation downstream of the P₁ promoter of the expression plasmid pPL2 (Remaut et al., 1987), linkers with unique restriction sites were added to both the cDNA and the plasmid. This plasmid construct (pPL2/RMPFK, Fig. 1) was transformed in E. coli strain M5219 carrying a defective prophage encoding a thermolabile repressor CR57 (Greer, 1975). Minipreparations of plasmid DNA from purified clones were verified as having the RMPFK insert by double-stranded sequencing analysis (Chen and Seeberg, 1985). Plasmids with the correct DNA sequence were prepared in larger quantity and purified through CsCl centrifugation. The purified plasmid DNA was then cotransformed with a second plasmid pC857 (Remaut et al., 1981) encoding the thermolabile C repressor into E. coli strain DF1020 which is deficient in both EcoPφ genes pφA and pφB (Kotlarz and Buc, 1981; Daldal, 1983). The transformed cells were grown on LB agar plates containing ampicillin and kanamycin. Isolated colonies were streaked on M9 (Ausubel et al., 1989) agar plates using mannitol as the carbon source. This vector-host system and the restricted medium provide a visible screening for the bacterial cells which express recombinant PFK. Clones of cells purified through this process were grown in 2 x yeast extract-Tryptone (YT) broth containing ampicillin and kanamycin. The overnight culture was used to inoculate (at 1:100) a large scale culture with the same composition. Incubation at 28°C was carried out until the absorbance at 600 nm reached 0.9 to 1.0. The culture was brought to 42°C rapidly and incubated at this temperature for 3.5 h. The culture was chilled on ice. Cells pelleted by centrifugation were suspended in buffer I containing 0.5 mM ATP (see below) and either lyzed by sonication for enzyme purification, or frozen until used.

**Site-directed Mutagenesis**—The 1-kb 5' fragment of the cDNA coding region for the NH₂-terminal 310 amino acids of RMPFK was excised from plasmid pPL2/RMPFK by digestion with XbaI and BamHI and inserted into the plasmid pBR221 (Biotec Inc., Madison, WI) digested with the same pair of restriction enzymes. The construct was transformed into JM109 E. coli cells. The single-stranded DNA template was prepared by infecting the transformed E. coli cells with helper phage R408 (Dotto and Zinder, 1983). Two mutagenic oligonucleotides, one repairing the UAG triplet in the β-lactamase gene of the phagemid and the other converting the glutamine (G200) codon CAG into alanine GCG, arginine CGG, or glutamate GAG were annealed to the template DNA. The heteroduplex was extended by T4 DNA polymerase and sealed by DNA ligase. The double-stranded phagemid DNA was subsequently transformed into a repair deficient strain of E. coli (BMM 71-18 mutS). The transformed cells were then grown on agar plates containing ampicillin. Plasmid DNA was prepared from colonies randomly picked and selected for correct mutations by sequence analysis using the dideoxynucleotide method (Sanger et al., 1980) and the restriction enzyme EcoRI corresponding to the replacement at the target codon. Wild-type PFK template was included for comparison. The correctly mutagenized RMPFK cDNA fragment was excised from the phagemid DNA by digestion with XbaI and BamHI restriction enzymes, then ligated into pPL2/RMPFK plasmid DNA predigested by the same pair of endonucleases. Procedures for transformation of the plasmid pPL2/RMPFK plasmid DNA into DF1020 E. coli cells, selection of the clones expressing PFK, and DNA sequencing were the same as described in the previous section.

**Purification of Wild-type and Mutated RMPFK**—Cell pellets from a 1-liter culture of E. coli cells expressing wild-type or mutated RMPFK were suspended in 15% (v/v) buffer I (50 mM Tris-HCl, pH 8.0, 25 mM NaF, 0.1 mM EDTA, 1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride) containing 0.5 mM ATP. Cells were lysed by sonication. The supernatant was collected by centrifugation. The pellet was resuspended in 0.15 M NaCl and dialyzed against the same buffer containing 0.1 M NaCl and dialyzed against the same buffer until the absorbance at 280 nm reached 0.5. PFK was eluted with buffer I containing 5 mM each of Fru-6-P and ATP in the absence of magnesium ion. Fractions containing the PFK activity were combined and loaded onto a column (1.5 x 10 mm) of DEAE-cellulose (Whatman DE52) equilibrated with buffer I containing 0.1 mM ATP. The column was eluted stepwise with the same buffer containing increasing concentrations of Tris phosphate. The majority of PFK activity was eluted at 0.2 M phosphate. Fractions containing the PFK activity were combined and concentrated by dialysis against 50% glycerol in buffer I containing 0.1 mM ATP. Aliquots from each step of the purification were analyzed for PFK activity (pH 6.0, 1 mM ATP; and 10 mM Fru-6-P, see below) and protein concentration (Bradford, 1976). Purity of the enzyme was analyzed by SDS-polyacrylamide gel electrophoresis using PFK purified from rabbit muscle (Sigma) as a standard. Gels were stained using Coomassie Brilliant Blue R-250 (Bio-Rad). The enzyme was dialyzed against the phosphate buffer (pH 7.0) and reconstituted to a final concentration of 1 mM DTT, 1 mM EDTA, and 0.1 mM ATP before use.

**Assay of PFK Activity**—Activity was assayed using two different protocols (Foe and Kemp, 1982). For determination of maximum
The enzyme exhibits allosteric behavior: \( n_H \) will be larger than 1.0, and Equation 4 will fit the data much better than Equation 3. The quality of the fits can be compared by calculating where \( n_H \) was followed using a recording spectrophotometer (Gilford Response II) equipped with a cell holder regulated at 30 °C. One unit of PFK activity is the amount of enzyme which catalyzes the conversion of 1 µmol of Fru-6-P to Fru-1,6-P2/min (Remp, 1975). The entire procedure for PFK activity assay was repeated on different preparations of purified enzymes.

**Analysis of Kinetic Data**—The kinetic data were fit to three different equations in order to determine the cooperativity of the enzymes toward Fru-6-P. The cooperative behavior of these enzymes under different conditions was determined by comparing fits to the Hill equation, the Michaelis-Menten kinetics equation, and the Monod-Wyman-Changeux (MWC) model for an allosteric enzyme. The Hill equation (Equation 1, \( a \) is defined in Equation 4) answers all-or-none binding of Fru-6-P molecules. The Hill constant, \( n_H \), is defined by Equation 2 evaluated at half-maximal velocity, \( v = V_m/2 \) (Cantor and Schimmel, 1980; Ferri, 1985). A large Hill constant indicates cooperative interactions, whereas a value of 1.0 indicates no cooperativity.

**Experimental Methods**—The MWC model describes the kinetics of an allosteric enzyme assuming two states of an enzyme, the active \( R \) form and inactive \( T \) form, with an equilibrium constant \( L \) describing the equilibrium between \( R \) and \( T \) forms in the free enzyme (Monod et al., 1965). Assuming Fru-6-P binds only to the \( R \) form, with a dissociation constant \( K_s \), then the rate of reaction as a function of Fru-6-P concentration is given in Equation 4 (Cantor and Schimmel, 1980):

\[
\frac{v}{V_m} = \frac{[\text{Fru-6-P}]}{[\text{Fru-6-P}] + K_s} \quad \text{(Eq. 3)}
\]

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\[
\frac{v}{V_m} = \frac{[\text{Fru-6-P}]}{[\text{Fru-6-P}] + K_s} \quad \text{(Eq. 3)}
\]

\[
\frac{v}{V_m} = \frac{a(1 + a)^2}{(1 + a)^2 + L} \quad \text{(Eq. 4)}
\]

where \( v = [\text{Fru-6-P}]/K_s \). As \( L \) becomes larger, the enzyme exhibits progressively more cooperative behavior.

Several criteria can be used to determine if the kinetics of an enzyme exhibit allosteric behavior: \( n_H \) will be larger than 1.0, \( L \) will be larger than 1.0, and Equation 4 will fit the data much better than Equation 3. The quality of the fits can be compared by calculating

\[
\chi^2 = \frac{\sum (v_{\text{exp}} - v_{\text{calc}})^2}{N - n} \quad \text{(Eq. 5)}
\]

where \( v_{\text{exp}} \) are the experimental values for the rates, \( v_{\text{calc}} \) are the rates calculated by the fit, \( N \) is the number of data points, and \( n \) is the number of parameters in the fit (\( n = 2 \) in Equation 3 and \( n = 3 \) in Equation 4). Since Equation 4 reduces to Equation 3 when \( L \) becomes small, fits to Equation 4 will always result in a lower \( \chi^2 \). In order to determine whether the kinetics exhibit allosteric as opposed to Michaelis-Menten kinetics, we used several criteria: \( L > 50 \), and \( \chi^2 \) for the fit to Equation 4 must be 10 times smaller than the fit to Equation 3.

**Mutagenesis and Purification of Wild-type and Mutants of RMPFK**—Successful expression of recombinant RMPFK was demonstrated by the growth of DF1020 cells transformed with pPL2/RMPFK and pCI857 plasmids on a selective medium using mannitol as carbon source. From 4.5 liters of the culture, 0.71 mg of pure RMPFK can be obtained, a quantity sufficient for studies on the enzymology and regulatory properties of both the wild-type and mutated RMPFK. An alternative procedure was therefore established. The recombinant PFK was purified in three steps: ammonium sulfate fractionation (35%-55% saturation), and chromatography through columns of Cibacron F3GA (Kasten et al., 1983), and DEAE-cellulose. The final purification was 1385-fold with a recovery of 49%. All three mutants of RMPFK, Q200R, Q200E, and Q200A in 10 mM potassium phosphate buffer and 10 mM NaF at pH 7.0. Sample was run in a 1-mm cuvette thermostated at 25 °C. At least four scans were averaged from 270 to 184 nm, and the base-line-corrected spectra were scaled according to their concentration to correspond to the wild-type spectrum. Fig. 7 shows the overlayed spectra.

**Circular Dichroism Spectra**—CD spectra were recorded on an Aviv 62DS circular dichroism spectrometer. Protein concentrations measured by the procedure of Bradford (1976) were 50 µg/ml for wild-type, 68 µg/ml for Q200R, 22 µg/ml for Q200E, and 28 µg/ml for Q200A in 10 mM potassium phosphate buffer and 10 mM NaF at pH 7.0. Sample was run in a 1-mm cuvette thermostated at 25 °C. At least four scans were averaged from 270 to 184 nm, and the base-line-corrected spectra were scaled according to their concentration to correspond to the wild-type spectrum.
Table I shows the results of activity assays at pH 8.0 and 7.0. The properties of recombinant RMPFK, including specific activity and the apparent $K_m$ values for Fru-6-P and ATP, are virtually the same as those of the PFK purified from rabbit muscle. As judged by the results of SDS-polyacrylamide gel electrophoresis, a high degree of purity has been attained for the wild-type and the three mutants of rabbit muscle PFK (Fig. 2). The electrophoretic patterns also indicate that all the recombinant PFK species have the same subunit size as the commercial standard.

Effect of Mutations at Glutamine 200 on the Kinetic Properties of Rabbit Muscle PFK—At pH 8.0, the effect of ATP inhibition is not apparent and RMPFK follows Michaelis-Menten kinetics. The $K_m(Fru-6-P)$ values for Q200R and Q200A are essentially the same as that for the wild-type enzyme, while the value for Q200E is 14-fold higher than that of the native enzyme. Under these conditions, $K_m(ATP)$ values for Q200R and Q200E are 5-fold higher than that of the wild-type enzyme. For Q200A, it is 13-fold higher (Table II).

At pH 7.0, where ATP is both a substrate and an allosteric inhibitor of RMPFK, the effects caused by these mutations are striking. Like the PFK purified from rabbit muscle (Valaïtis et al., 1987), the activity of the wild-type PFK starts to decline when the concentration of ATP is greater than 0.6 mM (Fig. 3). However, the activity of Q200R keeps increasing until 7 mM ATP, after which it starts to decline. The activity of Q200E at pH 7.0 is not detectable except in the presence of high (50 mM) Fru-6-P concentration. Under this condition, the optimum ATP concentration for this mutant is 10 mM. For Q200A, the activity is too low at pH 7.0 to allow proper analysis. Based on these results, the conditions for investigating the effect of Fru-6-P concentration on the activity of native RMPFK and its mutants were adjusted to pH 7.0 in the presence of low (0.6 mM) or high (7 mM) ATP concentration. With the assumption of a concerted mechanism, the native PFK is in its R state at low ATP concentration; thus

![Fig. 2. SDS-polyacrylamide gel electrophoresis of recombinant and mutated rabbit muscle PFK. The gel was stained using Coomassie Brilliant Blue R-250. Lane 1, RMPFK standard; lanes 2-5, lysates of E. coli cells transformed with plasmid pPL2/RMPFK, ammonium sulfate fraction (35-55%), pooled active fractions after Cibacron Blue F3GA column, and after DEAE-cellulose column of native recombinant RMPFK, respectively; lanes 6-8, mutants Q200A, Q200R, and Q200E, respectively. All three mutants were purified through DEAE-cellulose columns. Lane M contains size markers.](image)

**Table I**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity</th>
<th>$K_m$ Fru-6-P</th>
<th>$K_m$ ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 8.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RMPFK (Sigma)</td>
<td>220 units/mg</td>
<td>0.12 ± 0.04</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>RMPFK (cloned)</td>
<td>180 units/mg</td>
<td>0.18 ± 0.04</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>pH 7.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RMPFK (Sigma)</td>
<td>170 units/mg</td>
<td>0.40 ± 0.04</td>
<td>ND</td>
</tr>
<tr>
<td>RMPFK (cloned)</td>
<td>140 units/mg</td>
<td>0.46 ± 0.07</td>
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* ATP concentration was 1 mM.

**Table II**

<table>
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<tr>
<th>Enzyme</th>
<th>Specific activity</th>
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<th>$K_m$ ATP</th>
<th>Hill no.</th>
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<tr>
<td>PFK</td>
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</tr>
<tr>
<td>pH 8.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>180</td>
<td>0.18 ± 0.04</td>
<td>0.10 ± 0.02</td>
<td>NA</td>
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<tr>
<td>Q200R</td>
<td>110</td>
<td>0.22 ± 0.02</td>
<td>0.47 ± 0.03</td>
<td>NA</td>
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<tr>
<td>Q200E</td>
<td>44</td>
<td>2.50 ± 0.53</td>
<td>0.49 ± 0.06</td>
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<td>Q200A</td>
<td>140</td>
<td>0.42 ± 0.04</td>
<td>1.30 ± 0.17</td>
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<tr>
<td>pH 7.0*</td>
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<tr>
<td>Native</td>
<td>150</td>
<td>1.70 ± 0.1</td>
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<tr>
<td>Q200R</td>
<td>85</td>
<td>0.38 ± 0.05</td>
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<tr>
<td>Q200E</td>
<td>16</td>
<td>16.0 ± 1</td>
<td>ND</td>
<td>2.5 ± 0.1</td>
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<tr>
<td>Q200A</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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</tbody>
</table>

* ATP concentration was 7 mM.

**Fig. 3.** Effects of ATP concentration on the activity of the native rabbit muscle PFK and the three mutants. Relative activity is the ratio of $v/V$ where $v$ is the PFK activity at pH 7.0 and Fru-6-P concentration of 1 mM for wild-type RMPFK (●) and Q200R (○) or 50 mM for Q200E (●) and Q200A (○) and ATP concentration as indicated. V is the activity at pH 8.0 in the presence of saturating concentrations of Fru-6-P and ATP.
the rate of reaction versus Fru-6-P concentration exhibits essentially a hyperbolic profile (Fig. 4). Since 0.6 mM ATP is far below the apparent $K_{m(\text{Fru-6-P})}$ for the mutants (Fig. 3), the Fru-6-P saturation profiles cannot be analyzed. On the other hand, under the conditions of 7 mM ATP and pH 7.0, the initial conformation of the native PFK is presumably in the T state, thus the kinetics are cooperative with respect to Fru-6-P concentration with a Hill coefficient of 3.2 (Table II and Fig. 5). Under these conditions, the substrate saturation profile of Q200R (Fig. 5) is shifted to lower Fru-6-P concentration, with an apparent $K_{m(\text{Fru-6-P})}$ which is 1/5 of that for the native enzyme (Table II). By contrast, Q200E exhibits 10-fold higher apparent $K_{m(\text{Fru-6-P})}$ and much reduced activity (Table II and Fig. 5, inset). At pH 7.0, the extremely low activity of Q200A did not allow a significant comparison of its activity and properties with the wild-type enzyme (Fig. 5, inset).

The cooperativity of the enzymes was assessed at pH 7.0 and 7 mM ATP by calculating the Hill constant as shown in Table II. In the absence of allosteric effectors, the wild-type, Q200R, and Q200E enzymes all exhibit sigmoidal kinetics, with Hill constants of 3.2, 3.0, and 2.5, respectively. Fitting the data to the MWC model confirms this, with $L$ values of 1,900 ± 800 for wild-type, 1,100 ± 300 for Q200R, and 100 ± 10 for Q200E (data not shown).

Wild-type RMPFK and its three mutants are activated by inorganic phosphate and Fru-2,6-P$_2$ (Fig. 6, A–D). In the case of Q200R, the effect of inorganic phosphate is not noticeable and only slight activation by Fru-2,6-P$_2$ is observed (Fig. 6B). Mutants Q200E and Q200A require higher concentrations of these activators than the wild-type enzyme and mutant Q200R. Inhibition by citrate is clearly observed for the wild-type PFK and Q200R (Fig. 6, A–D). However, due to the extremely low activities under the assay conditions, the effect of citrate on mutant Q200A is not detectable.

Secondary Structure of Cloned RMPFK and Its Mutants—
In order to assess the effect of mutations at Q200 on the secondary structure of the enzyme, we measured the CD spectra of the native RMPFK and the three mutants. Fig. 7 shows that the CD spectra of these enzyme are essentially identical. These results suggest that mutations at Q200 do not affect the secondary structure of the enzyme.

Quaternary Structure of Cloned RMPFK and Its Mutants—
Rabbit muscle PFK is a tetramer of identical subunits (molecular mass 85 kDa). It is possible that the changes in kinetic properties of the various mutants observed (Table II and Figs. 3, 5, and 6) could be caused by alterations in their quaternary structures. To investigate this possibility, we analyzed the quaternary structure of the different mutants by non-denaturing agarose gel electrophoresis at pH 7.0 as well as pH 8.0 followed by staining for PFK activity and for total protein. As shown in Figs. 8, A and B, the mobilities of the wild-type RMPFK and its three mutants are close to that of the PFK purified from rabbit muscle. Since the minimum enzymatically active form of mammalian PFK is a tetramer (Uyeda, 1979; Dunaway, 1983), the positive staining of these protein bands for PFK activity suggests that the three altered proteins are tetramers. To further support this conclusion, non-denaturing gel electrophoresis was performed in gels containing different concentrations of agarose. The logarithm of $R_p$ (defined under “Experimental Procedures”) of these proteins was plotted versus the concentration of the gel (Ferguson plot). These plots gave parallel lines with the same slope for these PFK proteins at pH 7.0 (Fig. 8C). These results indicate that at pH 7.0, the wild-type rabbit muscle PFK and its three mutants are tetramers of the same size although they may differ from each other in net charge (Hedrick and Smith, 1968).

A slight change in slope of the Ferguson plot was observed for Q200A (0.07 versus 0.08 for other PFK proteins) when the electrophoresis was conducted at pH 8.0 (Fig. 8D) suggesting that, in addition to the difference in net charge, mutant Q200A may also differ subtly in conformation, hence size from the other PFK proteins at this hydrogen ion concentration. On the other hand, Q200A is almost fully active at pH 8.0 (Table II), thus it must have a tetrameric structure. The tetrameric structures of the wild-type PFK and Q200A were also verified by sucrose gradient sedimentation (data not shown).

DISCUSSION
This paper presents the first case of the cloning and expression of a mammalian PFK cDNA in E. coli cells and the first attempt at site-directed mutagenesis on a eukaryotic PFK. Although the level of expression was rather low, the vector-
FIG. 6. Effects of activators and inhibitors on the activities of native and mutated rabbit muscle PFK in response to increasing fructose 6-phosphate concentration. Relative activity is the same as that defined in Fig. 5. Activity of the wild-type enzyme (A), Q200R (B), Q200E (C), or Q200A (D) was measured at pH 7.0, 7 mM ATP in the absence (●) or presence of Fru-2,6-P3 (●), inorganic phosphate (■) or citrate (□). The concentration of Fru-2,6-P3 is 1 μM (wild-type RMPFK or Q200R) or 25 μM (Q200E and Q200A); inorganic phosphate, 1 mM (Q200R), 10 mM (wild-type RMPFK and Q200E), or 20 mM (Q200A); and citrate, 25 μM (Q200E and Q200A), 75 μM (wild-type RMPFK) or 125 μM (Q200R). The program and equations used for computer-fit of the data are described under “Experimental Procedures.”

The major goal of this work has been to study the structural basis for the allosteric control of rabbit muscle PKF. The crystallographic structures of the R and T states of BsPFK (Schirmer and Evans, 1990) showed a 7° rotation between its two pairs of dimers (A:D versus B:C) during the R to T transition. The binding site for the cooperative substrate Fru-6-P is altered by changes involving the 6-P loop. In the R state, the phosphate group of Fru-6-P interacts with Arg162 and Arg243 across the subunit interface (Fig. 9). In the T state, Arg262 swings away from the active site, Glu161 fills the vacated space by interacting with Arg262 through the relay of a water molecule across the subunit interface, and forms a salt bridge with Arg243. This conformational change physically closes the substrate binding cleft. The positive charges of Arg262, Arg143, and Arg252 are therefore no longer available for binding the negative charges of Fru-6-P. Glutamine 200 of rabbit muscle PFK, which corresponds to Glu161 of BsPFK (Poorman et al., 1984; Hellinga and Evans, 1985), was thus chosen for mutagenesis in the current work. Indeed, our results demonstrate the critical role of Gln200 in the activity and allosteric properties of rabbit muscle PKF. Mutations at Gln200 affect its affinities for both substrates, Fru-6-P and ATP.

A positively charged replacement at position 200 (Q200R) results in an enzyme with significantly attenuated ATP inhibition. The mutation might provide an environment for Fru-6-P binding to the “T state,” making it active. Alternatively, the arginine replacement could make the T state structure less stable because its positively charged side chain cannot interact with the intra- and intersubunit arginine residues as shown in the structure of the T state BsPFK (Shirmer and Evans, 1990). The fact that at pH 7.0 and 7 mM ATP the kinetic profile of mutant Q200R is shifted to a lower Fru-6-P concentration region (Fig. 5) argues for both interpretations. The tolerance of Q200R for high ATP concentration could be the consequence of a reduced affinity for ATP at the inhibitor binding site or, more likely, the mutated proteins can no longer effectively transmit the effect of the conformational changes taking place at the inhibitor site to the catalytic site.

A negatively charged residue (Q200E) at this position drastically reduces the affinity of the enzyme for Fru-6-P, most likely by charge repulsion. This is reflected by the 10-fold higher apparent K_mFru-6-P, at pH 8.0 (Table II). At pH 7.0 and 7 mM ATP and at Fru-6-P concentration 10-fold higher than that for wild-type enzyme, the activity of this mutant showed a sigmoidal response. The cooperativity is lower, as shown by
the smaller Hill constant (Table II). The Q200E mutant is activated by Fru-2,6-P₂, but requires 25 μM concentration versus 1 μM for wild-type and Q200R mutant. At pH 7.0, Q200E is inherently less active, and exhibits much weaker allosteric behavior (Fig. 5, inset). Preliminary results have shown that a Q161E mutant of EcPFK (analogous to Q200E of RMPFK) requires a much higher concentration of Fru-6-P to make the T to R transition.²

Finally, mutant Q200A exhibited a relatively high specific activity (78% of the wild-type enzyme) at pH 8.0 with a 13-fold elevation of the apparent $K_m$ for ATP (Table II). Although this mutant was essentially inactive at pH 7.0 over a wide range of Fru-6-P and ATP concentrations (Figs. 3 and 5), it is significantly activated by a high concentration of Fru-2,6-P₂. Both Q200E and Q200A are similar in the difficulty of Fru-6-P to activate the enzymes, while being fully activated by high concentrations of activators. This might suggest that the T state is stabilized in these mutants, perhaps due to a loss of favorable interactions between the active site and residue Q200 in the R state.

The wild-type RMPFK and the mutated enzymes are activated by inorganic phosphate and Fru-2,6-P₂. Citrate may inhibit all forms of RMPFK, although the effect is not discernible for Q200A because of its low activity at pH 7.0. It is interesting to note that the concentrations of the activators and inhibitor for Q200R, wild-type PFK, Q200E, and Q200A are in opposite order (see legend to Fig. 6). Enzymes which are active at lower Fru-6-P concentration in the absence of these effectors require lower concentrations of activators or higher concentrations of inhibitor to reveal their effects.

The changes in kinetic properties of the various mutants could have been caused by alterations in structure at different levels. We investigated this possibility by analyzing the secondary structural content of the wild-type PFK and its three mutants by CD, and their quaternary structures by nondenaturing gel electrophoresis in gels of different agarose concentration followed by Ferguson plots (Ferguson, 1964; Hedrick and Smith, 1968). The results of these experiments showed that there is no significant change in secondary structure, and all three mutants are tetramers (Figs. 7 and 8). Therefore, the changes in kinetic properties observed for these mutants reveal the role of Q200 in the allosteric behavior of RMPFK rather than any changes in the secondary or quaternary structure.

The technique of site-directed mutagenesis has been widely applied to studies on the enzymology of an increasingly larger number of proteins. It is important, when comparing a mutated enzyme to its wild-type counterpart, to know if the altered amino acids would affect the secondary, tertiary, or quaternary structure of the protein. For analysis of the quaternary structures of the altered proteins, methods such as electrophoresis in nondenaturing gels or sedimentation in sucrose gradients would be suitable. However, we have experienced a technical difficulty in that rabbit muscle PFK cannot penetrate nondenaturing polyacrylamide gel when gel concentration is higher than 3.7%. On the other hand, we found that electrophoresis in nondenaturing agarose gels of different concentrations of agarose is much simpler to perform for rabbit muscle PFK than electrophoresis in polyacrylamide gels.

Although only residue Q200 has been investigated by site-directed mutagenesis, this work has demonstrated distinct

² M. Byrnes and S. H. Chang, unpublished observation.
FIG. 9. Schematic view showing the comparison of the T state and R state at the substrates site of B. stearothermophilus PFK. Wavy line represents substrate interface. A, T state, showing Glu241 in the Fru-6-P site; B, R state (with active site ligands Fru-6-P and ADP), showing the phosphate of Fru-6-P bound by Arg252 and Arg256. Reprinted with permission from Nature (Schirmer, T., and Evans, P. R., 1990). Nature 343, 140-145. Copyright (1990) Macmillan Magazines Limited.

differences in kinetic properties among the native rabbit muscle PFK and the three mutants. We speculate that mutations at other residues along the putative 6-F loop (Schirmer and Evans, 1990) of this enzyme may also give useful results which could provide further insights into the structural basis of the allosteric behavior of this enzyme, whether these involve the cooperative binding of Fru-6-P or the many interactions between the Fru-6-P site and several regulatory sites.

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