

Study of the roles of Arg-104 and Arg-225 in the 2-kinase domain of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase by site-directed mutagenesis

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The roles of Arg-104 and Arg-225 located in the 2-kinase domain of the bifunctional enzyme 6-phosphofructo-2-kinase (PFK-2)/fructose-2,6-bisphosphatase (FBPase-2) have been studied by site-directed mutagenesis. In recombinant rat liver PFK-2/FBPase-2, mutation of Arg-225 to Ser increased the K_m of PFK-2 for fructose-6-phosphate (Fru-6-P) 7-fold at pH 6 and decreased PFK-2 activity at suboptimal substrate concentrations between pH 6 and 9.5. The mutation had no effect on the V_{max} of PFK-2 or on the K_m of PFK-2 for MgATP. The mutation also increased the V_{max} of FBPase-2 4-fold without changing the K_m for Fru-2,6- P_2 or IC_{50} of Fru-6-P. These findings are in agreement with a previous study [Rider and Hue (1992) *Eur. J. Biochem.* **207**, 967–972] on the protection by Fru-6-P of the labelling of Arg-225 by phenylglyoxal, and suggest that Arg-225 participates

in Fru-6-P binding. In recombinant rat muscle PFK-2/FBPase-2, mutation of Arg-104 to Ser increased the K_m for Fru-6-P 60-fold, increased the IC_{50} of citrate, increased the V_{max} 1.5–3-fold at pH 8.5 and altered the pH profile of PFK-2 activity. It did not affect the K_m of PFK-2 for MgATP. The mutation also decreased the V_{max} of FBPase-2 3-fold, increased the K_m for Fru-2,6- P_2 70-fold and increased the IC_{50} of Fru-6-P at least 300-fold. Although the dimeric structure was maintained in the mutant, its PFK-2 activity was more sensitive towards inactivation by guanidinium chloride than the wild-type enzyme activity. The findings indicate that Arg-104 is involved in Fru-6-P binding in the PFK-2 domain and that it might also bind citrate. Structural changes resulting from the mutation might be responsible for the changes in kinetic properties of FBPase-2.

INTRODUCTION

Fructose 2,6-bisphosphate (Fru-2,6- P_2) is a potent stimulator of 6-phosphofructo-1-kinase (PFK-1) and hence of glycolysis [1,2]. The synthesis and degradation of Fru-2,6- P_2 are catalysed by 6-phosphofructo-2-kinase (PFK-2) and fructose-2,6-bisphosphatase (FBPase-2) respectively. In mammalian tissues these two reactions are catalysed at separate sites on each subunit of a homodimeric bifunctional enzyme, PFK-2/FBPase-2. Distinct isoenzymes of PFK-2/FBPase-2 exist in mammalian tissues. They differ in M_r , kinetic and immunological properties and phosphorylation by protein kinases, and are encoded by at least two different genes [3]. The liver (L) and muscle (M) isoenzymes are identical, except for the first 32 amino acids of the L-type, which are replaced by a unique nonapeptide in the M isoenzyme. As the two isoenzymes display very similar K_m values of PFK-2 activity for the two substrates, we believe that the N-terminal sequence has very little impact on these values and we have used the numbering of amino acids in the L isoenzyme for the identical sequence of the L and M isoenzyme.

The FBPase-2 reaction is catalysed in the C-terminal half of each subunit. The catalytic mechanism of FBPase-2 is well understood and involves a classical Ping-Pong reaction in which a histidine residue (His-258 in the L-type) is phosphorylated from Fru-2,6- P_2 [4].

The reaction mechanism of PFK-2 is not well understood and

the amino acids directly involved in catalysis have not been identified yet. In this work we have investigated the role of two arginine residues in the 2-kinase domain by site-directed mutagenesis. Because of their positively charged side chain, arginine residues in proteins are known to bind the negatively charged phosphate groups of substrates and ligands. Arginine residues are also involved in making subunit–subunit contacts in proteins by forming salt bridges with neighbouring aspartate or glutamate residues.

One arginine residue in the PFK-2 domain, Arg-195, has already been studied on the basis of a sequence alignment of PFK-2 on PFK-1 from *Bacillus stearothermophilus* and *Escherichia coli* [5]. The three-dimensional structure of bacterial PFK-1 has been solved and the geometry of residues involved in substrate binding and catalysis is known [6]. Arg-162 in bacterial PFK-1, which corresponds to Arg-195 in the L isoenzyme of PFK-2/FBPase-2 according to the alignment [5], binds the 6-phosphate group of fructose 6-phosphate (Fru-6-P) [7]. Mutagenesis of Arg-195 to Ala in the L [8] and M [9] PFK-2/FBPase-2 isoenzymes increases the K_m for Fru-6-P by two to three orders of magnitude.

The rationale for mutating two other arginine residues, Arg-104 and Arg-225, is the following. We showed in a previous study that pretreatment of purified preparations of rat liver PFK-2/FBPase-2 with the arginine-specific reagent, phenylglyoxal, irreversibly inactivated both PFK-2 and FBPase-2 [10]. Chymo-

Abbreviations used: FBPase-2, fructose-2,6-bisphosphatase (EC 3.1.3.46); Fru-6-P, fructose 6-phosphate; Fru-2,6- P_2 , fructose 2,6-bisphosphate; GdmCl, guanidinium chloride; PFK-1, 6-phosphofructo-1-kinase (EC 2.7.1.11); PFK-2, 6-phosphofructo-2-kinase (EC 2.7.1.105); [R104S]M, Arg-104 to Ser mutant (muscle isoenzyme); [R225S]L, Arg-225 to Ser mutant (liver isoenzyme).

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tryptic cleavage of the modified protein and microsequencing showed that Arg-225 was one of the residues labelled by radioactive phenylglyoxal. Moreover, the inactivation of PFK-2 and the labelling of Arg-225 by phenylglyoxal were completely prevented by Fru-6-P, thus suggesting that Arg-225 is involved in Fru-6-P binding in the PFK-2 domain [10]. Therefore we mutated Arg-225 to Ser in the recombinant rat L isoenzyme ([R225S]L mutant) to investigate its potential role in Fru-6-P binding.

Arg-104 was studied for two reasons. First, it is absolutely conserved in the sequences of all the PFK-2/FBPase-2 isoenzymes studied so far. Secondly, according to the sequence alignment of the PFK-2 domain on bacterial PFK-1 [5], it corresponds to Arg-72 in *B. stearothermophilus* PFK-1, which binds the γ -phosphate of ATP and stabilizes the transition state [7]. Mutagenesis of Arg-72 to Ser in *E. coli* PFK-1 decreased the k_{cat} 33-fold and increased the K_m for Fru-6-P 3-fold [7]. Therefore we mutated Arg-104 to Ser in the recombinant M mutant ([R104S]M mutant) and compared the effects of mutation on activity with the results obtained with the mutated bacterial PFK-1 [7].

In both cases Arg-225 and Arg-104 were mutated to Ser, as the same mutation of several arginine residues in bacterial PFK-1 profoundly changed the kinetic properties of the enzyme [7].

MATERIALS AND METHODS

Restriction endonucleases and isopropyl 1-thio- β -D-galactopyranoside were obtained from Boehringer. T4 DNA ligase, T7 DNA polymerase, oligonucleotide-directed mutagenesis kit, [[α -³⁵S]thio]dATP (600 Ci/mmol) and [γ -³²P]ATP (3000 Ci/mmol) were from Amersham International. [2-³²P]Fru-2,6-P₂ was prepared enzymically from [γ -³²P]ATP and Fru-6-P as described [11]. Protein standards for gel filtration were from Sigma.

Construction of the expression plasmids

The rat M-type PFK-2/FBPase-2 cDNA was originally cloned in pBlueScript (KS)II+ phagemid for construction procedures [12] and then in the T7 RNA polymerase-based pET3d vector of Studier and Moffat [13] for expression to create pET/PFK2M-sf. The mutant oligonucleotide is indicated below:

Wild-type M 5'-CCAGCTTATCAGGAAGCAGTGTGC-3'

[R104S]M 5'-CCAGCTTATCAGCAAGCAGTGTGC-3'

It was annealed to the single-stranded DNA of pBlueScript (KS)II+ /PFK2M and the mutagenesis reaction was performed as described [14]. Mutations in the positive mutants were verified by sequencing [15]. The selected mutant was then introduced into the expression vector pET/PFK2M-sf.

The rat L-type PFK-2/FBPase-2 cDNA was engineered following the same procedure as described for the M-type cDNA [12]. The two *Eco*RI fragments resulting from the cloning procedure, 22C2 and 22C1 [16], were linked together in the pBluescript (KS)II+ phagemid to create pBluescript (KS)II+ /PFK2L (see Figure 2 of ref. [12], replacing 5C2 by 22C2 and 5C1 by 22C1). A single-stranded form of this phagemid was then used as a template for mutagenesis [14] to create an *Nde*I restriction site compatible with the *Nde*I site located within the translation-initiation site of the pET3a expression vector. The mutagenic oligonucleotide used had the following sense sequence:

Wild-type L 5'-CTGCAACGCCTAAGATGTCTCGAG-3'

*Nde*I site 5'-CTGCAACGCCTCATATGACTCGAG-3'

The *Nde*I site is underlined.

The mutants were screened by *Nde*I digestion and their sequences confirmed [15]. The *Nde*I double mutant was digested with a combination of *Nde*I–*Bam*HI to release the complete L-type PFK-2/FBPase-2 cDNA sequence beginning at the ATG and inserted into the compatible ends of the pET3a expression vector to create pET-PFK2-L.

For the mutagenesis reaction, the oligonucleotide was:

Wild-type L 5'-GTGGGCACACGCTACATGGTAAATC-3'

[R225S] L 5'-GTGGGCACATCCTACATGGTAAATC-3'

It was annealed to the single-stranded DNA of pBlueScript (KS)II+ /PFK2L and the mutagenesis reaction was performed as described above. After sequence verification [15] the selected mutant was introduced into the L-type PFK-2/FBPase-2 expression vector pET/PFK2L [R225S]. This was achieved by replacing the entire *Nde*I–*Bam*HI fragment in the wild-type expression vector with that containing the relevant mutation.

Expression and purification

The *E. coli* host for hyperexpression, BL21(DE3) [17], contains an integrated chromosomal T7 RNA polymerase gene with the *lacUV5* promoter. This promoter is inducible by isopropyl 1-thio- β -D-galactopyranoside. The recombinant liver wild-type and [R225S]L PFK-2/FBPase-2s were expressed in *E. coli* BL21(DE3)-pLysE and BL21(DE3)-pLysS respectively, and the recombinant muscle wild-type and [R104S]M PFK-2/FBPase-2s were expressed in *E. coli* BL21(DE3)-pLysE and BL21(DE3)-pLysS respectively. Cultures (4 litres) were grown at room temperature in Luria–Bertani medium until an A_{620} of 0.6 was reached. T7 RNA polymerase was then induced with 0.4 mM isopropyl 1-thio- β -D-galactopyranoside and the incubation was continued for 16 h with vigorous shaking.

The recombinant wild-type L isoenzyme and the [R225S]L mutant were purified by poly(ethylene glycol) fractionation, ion-exchange chromatography on DEAE-Sepharose and specific elution from Blue Sepharose as described [12]. The recombinant wild-type M isoenzyme and the [R104S]M mutant were purified by a similar procedure [18], with modifications for the mutant as described in the text.

Estimation of M_r by gel filtration

The M_r of the recombinant wild-type M isoenzyme and the [R104S]M mutant was measured by gel filtration on a Biosil SEC 125 size-exclusion column (7.8 mm \times 300 mm; Bio-Rad) equilibrated in 50 mM potassium phosphate, pH 7.5, containing 100 mM KCl and 0.1 mM dithiothreitol. The column was first calibrated with 100 μ l of a protein mixture consisting of 0.3 mg/ml β -amylase (approx. M_r 200000), 0.25 mg/ml alcohol dehydrogenase (approx. M_r 150000), 0.25 mg/ml BSA (approx. M_r 66000), 0.15 mg/ml carbonic anhydrase (approx. M_r 29000) and 0.2 mg/ml cytochrome *c* (approx. M_r 12400). The recombinant wild-type M isoenzyme or [R104S]M mutant preparation (50–100 μ g) was injected on to the column. Protein eluted from the column was monitored by measuring A_{214} at a flow rate of 0.4 ml/min.

Incubation with guanidinium chloride (GdmCl) for kinetic studies

The recombinant wild-type M isoenzyme or the [R104S]M mutant (0.1 mg/ml) was incubated in a final volume of 10 μ l in buffer containing 50 mM Hepes, pH 7.5, 100 mM KCl, 0.2 mM EDTA, 2 mM potassium phosphate, 1 mM dithiothreitol and

various concentrations of GdmCl. After incubation for 10 min at 25 °C, PFK-2 and FBPase-2 were assayed as described in the Figure legend.

Other methods

Protein concentration was determined [19] using γ -globulin as standard. PFK-2 and FBPase-2 were assayed [12] as described in the legends to the Figures and Tables. Kinetic constants were calculated using a computer program [20]. One unit of enzyme activity corresponds to the formation of 1 μ mol of product/min under the assay conditions. SDS/PAGE analysis in 10% acrylamide [21] and Western blotting [12] were performed as described.

RESULTS

Purification of the wild-type and mutant enzyme preparations

During the purification procedure, the behaviour of the wild-type L isoenzyme and the [R225S]L mutant was the same. However, the wild-type M isoenzyme was eluted from the phosphocellulose column with buffer containing 2 mM Fru-6-P [18], whereas the [R104S]M mutant could not be eluted under the same conditions. It was eluted with a linear gradient of potassium phosphate (0–0.4 M) in buffer containing 2 mM Fru-6-P and came through at about 0.2 M. Each of the wild-type and mutant enzyme preparations displayed a major band, which migrated with the expected molecular mass, after SDS/PAGE and Coomassie Blue staining or Western Blotting (not shown).

Kinetic properties of the [R225S]L mutant

Mutagenesis of Arg-225 to Ser had no effect on the V_{max} of PFK-2 (Table 1). However, it increased the K_m of PFK-2 for Fru-6-P 7-fold at pH 6, whereas the increase at pH 7 was not significant. The K_m for MgATP was almost doubled at pH 7, but not at pH 6 (Table 1). These changes in kinetic properties were reflected in PFK-2 activity measured under suboptimal conditions: 85% and 50% decreases in activity were observed at pH 6 and pH 8.5 respectively (Figure 1). These results indicate that Arg-225 could interact with the substrates.

Mutation of Arg-225 to Ser increased FBPase-2 activity 4-fold

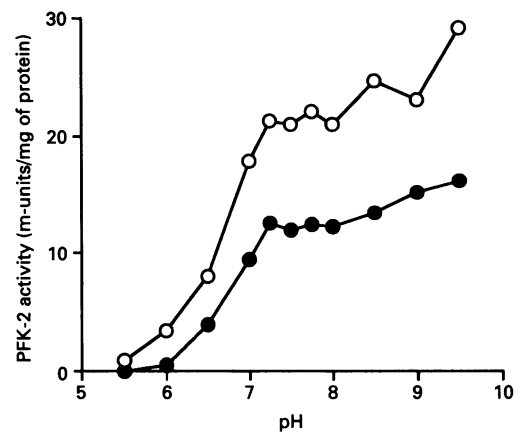


Figure 1 pH profile of PFK-2 in the recombinant wild-type L isoenzyme and recombinant [R225S]L mutant PFK-2/FBPase-2 preparations

PFK-2 activity in the wild-type (○) and [R225S]L mutant (●) PFK-2/FBPase-2 preparations was measured in buffer containing 20 mM Hepes, 20 mM Tris/HCl, 20 mM Mes, 100 mM KCl, 20 mM KF, 5 mM potassium phosphate and 1 mM dithiothreitol at 30 °C, adjusted to the indicated pH, and to which 1 mg/ml BSA was added. The reactions were started with 0.5 mM MgATP and the concentration of Fru-6-P was 0.1 mM.

without affecting the K_m for Fru-2,6- P_2 , the IC_{50} of Fru-6-P (Table 1) or the pH profile, measured under optimal assay conditions (not shown).

Kinetic properties of the [R104S]M mutant

Mutagenesis of Arg-104 to Ser in the M isoenzyme did not affect the K_m for MgATP (Table 2). This is at variance with the prediction of Bazan et al. [5]. However, the mutation increased the K_m for Fru-6-P 60-fold, and increased the IC_{50} of citrate 800-fold (Table 2). These large changes in affinity suggest that Arg-104 is important for binding Fru-6-P and citrate.

Table 1 Kinetic properties of PFK-2 and FBPase-2 in the recombinant wild-type L isoenzyme and [R225S]L mutant PFK-2/FBPase-2 preparations

PFK-2 activity was measured in buffer containing 50 mM Hepes, 100 mM KCl, 20 mM KF, 1 mM dithiothreitol, 5 mM potassium phosphate, at the indicated pH, to which 1 mg/ml BSA was added [12]. For the Fru-6-P and MgATP-saturation curves, the concentrations of MgATP and Fru-6-P were 5 and 10 mM respectively, and the concentration of the other substrate was varied up to 10 mM. FBPase-2 was measured [12] with concentrations of [32 P]Fru-2,6- P_2 5–10 \times the K_m in the presence of 5 mM potassium phosphate, 5 mM *sn*-glycerol-3-phosphate and 1.5 mM MgGTP at pH 7. For the IC_{50} of FBPase-2 for Fru-6-P, 5 mM potassium phosphate and 5 μ M [32 P]Fru-2,6- P_2 were included in the assays and the concentration of Fru-6-P was varied between 0.001 and 1 mM. The results are means \pm S.E.M. of the number of determinations shown in parentheses. *Significant ($P < 0.05$) effect of the mutation compared with the wild-type. PKF-2 and FBPase-2 activities are expressed per mg of protein.

Enzymes	PFK-2 activity (Fru-6-P-saturation curve at pH 7)		PFK-2 activity (MgATP-saturation curve at pH 7)		PFK-2 activity (Fru-6-P-saturation curve at pH 6)		PFK-2 activity (MgATP-saturation curve at pH 6)		FBPase-2 activity at pH 7		
	V_{max} (m-unit/mg)	K_m for Fru-6-P (μ M)	V_{max} (m-unit/mg)	K_m for MgATP (mM)	V_{max} (m-unit/mg)	K_m for Fru-6-P (mM)	V_{max} (m-unit/mg)	K_m for MgATP (mM)	V_{max} (m-unit/mg)	K_m for Fru-2,6- P_2 (μ M)	IC_{50} of Fru-6-P (μ M)
Wild-type L	39 \pm 3 (3)	35 \pm 8 (3)	41 \pm 1 (3)	0.24 \pm 0.02 (3)	40 \pm 6 (4)	0.07 \pm 0.01 (4)	46 \pm 7 (3)	0.52 \pm 0.08 (3)	16 \pm 1 (3)	1.5 \pm 0.1 (3)	3.4 \pm 0.3 (3)
[R225S]L	38 \pm 2 (3)	57 \pm 8 (3)	58 \pm 5 (3)*	0.40 \pm 0.05 (3)*	36 \pm 3 (4)	0.55 \pm 0.05 (4)*	36 \pm 5 (3)	0.53 \pm 0.08 (3)	58 \pm 3 (3)*	1.5 \pm 0.1 (3)	3.2 \pm 0.2 (3)

Table 2 Kinetic properties of PFK-2 and FBPase-2 in the recombinant-wild-type M isoenzyme and [R104S]M mutant PFK-2/FBPase-2 preparations

PFK-2 activity was measured in buffer containing 50 mM Hepes, 100 mM KCl, 20 mM KF, 1 mM dithiothreitol and 5 mM potassium phosphate, pH 8.5, to which 1 mg/ml BSA was added [12]. For the Fru-6-*P* and MgATP-saturation curves, the concentrations of MgATP and Fru-6-*P* were 5 and 10 mM respectively, and the concentration of the other substrate was varied up to $5-10 \times K_m$. For citrate inhibition of PFK-2, the concentrations of Fru-6-*P* and MgATP were 10 and 5 mM respectively and citrate concentration was varied up to 30 mM. FBPase-2 was measured [12] with concentrations of $[2-^{32}\text{P}]\text{Fru-2,6-}P_2$ $5-10 \times K_m$ in the presence of 5 mM potassium phosphate, at pH 7. For the IC_{50} of FBPase-2 for Fru-6-*P*, 5 mM potassium phosphate and $5-10 \mu\text{M}$ $[2-^{32}\text{P}]\text{Fru-2,6-}P_2$ were included in the assays and the concentration of Fru-6-*P* was varied between 0.001 and 1 mM. The results are means \pm S.E.M. of the number of determinations shown in parentheses, otherwise individual values are given. *Significant ($P < 0.05$) effect of the mutation compared with the wild-type. PFK-2 and FBPase-2 activities are expressed per mg of protein.

Enzymes	PFK-2 activity (Fru-6- <i>P</i> -saturation curve at pH 8.5)		PFK-2 activity (MgATP-saturation curve at pH 8.5)			FBPase-2 activity at pH 7		
	V_{\max} (m-units/mg)	K_m for Fru-6- <i>P</i> (mM)	V_{\max} (m-units/mg)	K_m for MgATP (mM)	IC_{50} of citrate (mM)	V_{\max} (m-units/mg)	K_m for Fru-2,6- <i>P</i> ₂ (μM)	IC_{50} of Fru-6- <i>P</i> (mM)
Wild-type M	31 \pm 2 (4)	0.10 \pm 0.01 (4)	30 \pm 1 (3)	0.24 \pm 0.02 (3)	0.027, 0.033	33 \pm 1 (3)	0.7 \pm 0.1 (3)	0.01
[R104S]M	91 \pm 9 (5)*	5.7 \pm 0.6 (5)*	45 \pm 1 (3)*	0.21 \pm 0.02 (3)	25	12 \pm 1 (5)*	51 \pm 7 (5)*	> 1

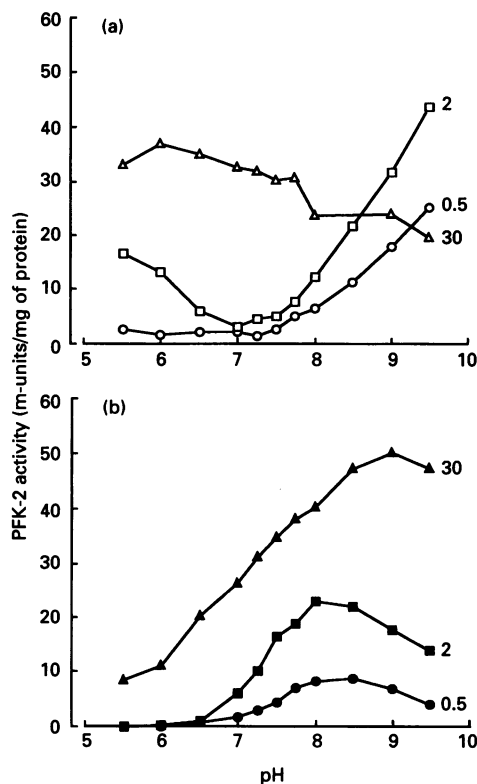


Figure 2 pH profile of PFK-2 in recombinant wild-type M isoenzyme (a) and recombinant [R104S]M mutant PFK-2/FBPase-2 preparations (b)

PFK-2 activity in the wild-type (a) and [R104S]M mutant (b) PFK-2/FBPase-2 preparations was measured in buffer containing 20 mM Hepes, 20 mM Tris/HCl, 20 mM Mes, 100 mM KCl, 20 mM KF, 5 mM potassium phosphate and 1 mM dithiothreitol at 30 °C, adjusted to the indicated pH, and to which 1 mg/ml BSA was added. The reactions were started with 2 mM MgATP. PFK-2 activity of the wild-type and [R104S]M mutant preparations was measured with 0.5 mM (○, ●), 2 mM (□, ■) and 30 mM (△, ▲) Fru-6-*P*. The results are the means of two to three determinations.

The pH curves of PFK-2 activity of the wild-type M isoenzyme and the [R104S]M mutant measured with different concentrations of Fru-6-*P* are shown in Figure 2. For the recombinant wild-type

M isoenzyme (Figure 2a), PFK-2 activity increased at alkaline pH with Fru-6-*P* concentrations of 0.5 and 2 mM. This suggests that a basic residue is responsible for the alkaline part of the pH profile. However, when measured in the presence of 30 mM Fru-6-*P*, which represents a substrate concentration $300 \times K_m$ at pH 8.5 (Table 2), PFK-2 activity was relatively unaffected by pH change, but, when compared with PFK-2 activity measured at low substrate concentration, it was greater at pH 5.5 and decreased with increasing pH (Figure 2a). For the [R104S]M mutant, the pH profile of PFK-2 activity was dramatically altered (Figure 2b). With suboptimal concentrations of Fru-6-*P*, the alkaline part of the pH profile was abolished and the pH optimum of PFK-2 was between 8 and 8.5. These findings indicate that Arg-104 might contribute to the alkaline part of the pH profile of PFK-2 activity in the wild-type enzyme. In addition to modifying the alkaline part of the pH profile, mutagenesis of Arg-104 to Ser decreased PFK-2 activity below pH 7 at the three Fru-6-*P* concentrations tested, compared with the wild-type. Interestingly, a similar effect on the pH profile of PFK-2 activity was observed when Cys-160, a residue that has been proposed to maintain the conformation of the Fru-6-*P*-binding site, was mutated to Asp or Ser [22].

Mutagenesis of Arg-104 to Ser also affected FBPase-2 activity. The [R104S]M mutant displayed a 3-fold decrease in V_{\max} of FBPase-2 and a 70-fold increase in K_m for Fru-2,6-*P*₂ (Table 2). Furthermore, with concentrations of Fru-6-*P* up to 1 mM, the FBPase-2 activity of the [R104S]M mutant was not inhibited, whereas for the wild-type, the IC_{50} of Fru-6-*P* was about 10 μM (Table 2). The pH profile of FBPase-2 activity was unaffected by the mutation (not shown).

Structural studies on the [R104S]M mutant

The fact that so many kinetic properties of both PFK-2 and FBPase-2 activity of the [R104S]M mutant were different compared with the wild-type M isoenzyme suggested that Arg-104 might not only bind Fru-6-*P* and citrate in the PFK-2 domain, but could also play a structural role. To check whether the mutation affected the dimerization state of the enzyme, the M_r of the wild-type and [R104S]M mutant preparations was measured by gel-filtration HPLC. In two separate determinations, the M_r of the wild-type was 96200, whereas for the [R104S]M mutant, values of 112600 and 100400 were obtained, thus

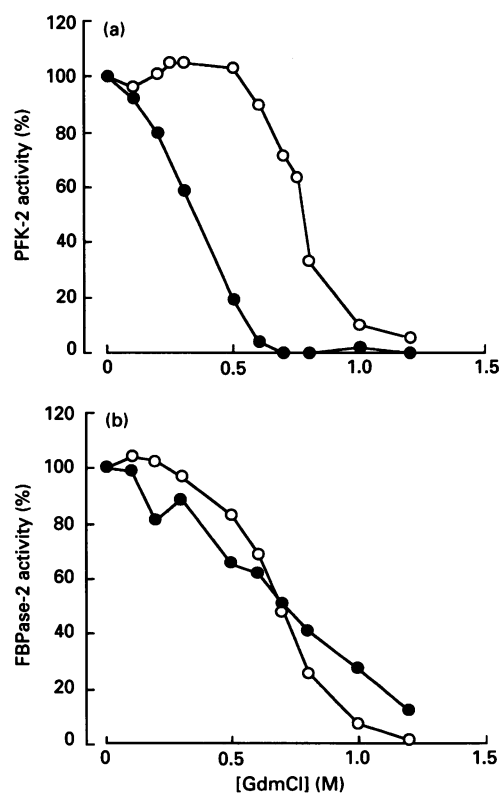


Figure 3 Sensitivity of wild-type M Isoenzyme and [R104S]M mutant PFK-2 (a) and FB Pase-2 (b) activities towards inactivation by GdmCl

The recombinant wild-type (○) and [R104S]M mutant (●) PFK-2/FBPase-2 enzyme preparations were diluted in buffer containing 20 mM HEPES, pH 7.5, 50 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol and 20% (v/v) glycerol and incubated with the indicated concentrations of GdmCl, as described in the Materials and methods section. After 10 min of incubation at room temperature, PFK-2 was assayed [12] at pH 8.5 and 30 °C with 2 mM Fru-6-P and 2 mM MgATP. Full (100%) PFK-2 activity was 17.7 ± 0.9 m-units/mg of protein (mean \pm S.E.M., $n = 4$) for the wild-type and 9.6 ± 1.8 m-units/mg of protein (mean \pm S.E.M., $n = 3$) for the [R104S]M mutant. Aliquots were also assayed for FB Pase-2 at pH 7.1 [19] in the presence of 10 μ M Fru-2,6- P_2 (wild-type) or 30 μ M Fru-2,6- P_2 ([R104S]M mutant). Full (100%) FB Pase-2 activity was 19.8 ± 0.9 (mean \pm S.E.M., $n = 3$) for the wild-type and 5.4 ± 2.2 (mean \pm range, $n = 2$) for the [R104S]M mutant. Each point is the mean of two to four separate determinations.

excluding dissociation of the dimers into monomers. However, the small increase in M_r of the [R104S]M mutant preparation together with the changes in kinetic properties of both PFK-2 and FB Pase-2 suggest that its structure might have been modified. Therefore incubations with GdmCl were carried out to examine the sensitivity of PFK-2 and FB Pase-2 activities of the wild-type and [R104S]M mutant preparations towards subunit dissociation and protein unfolding. Incubation with GdmCl decreased both PFK-2 and FB Pase-2 activity of the wild-type and the IC_{50} was about 0.7 M (Figure 3). For the [R104S]M mutant, the sensitivity of FB Pase-2 towards GdmCl-induced inactivation was unchanged with respect to the wild-type (Figure 3b). However, PFK-2 activity of the [R104S]M mutant was more sensitive to inactivation by GdmCl with IC_{50} about 0.25 M (Figure 3a). The difference in sensitivity of PFK-2 activity towards GdmCl was not due to the fact that the enzyme activity of the [R104S]M mutant was measured under suboptimal conditions, as identical results were obtained under optimal assay conditions (not

shown). The effect of GdmCl was not simply a salt effect, as incubation with KCl up to 1 M had no effect on PFK-2 activity (not shown).

DISCUSSION

Mutagenesis of Arg-255 to Ser in the L isoenzyme increased the K_m of PFK-2 for Fru-6-P at pH below 7 and decreased PFK-2 activity measured with suboptimal concentrations of substrates between 6 and 9.5. This observation is in agreement with our previous studies on the protection by Fru-6-P of the labelling of Arg-225 and PFK-2 inactivation by phenylglyoxal. It suggests that Arg-225 is located in or near the Fru-6-P-binding pocket in the three-dimensional structure of the PFK-2 domain. To explain why the K_m for Fru-6-P was increased below pH 7, but not above, one can speculate that the hydroxy group of Ser-225 might be hydrogen-bonded to the phosphate group of Fru-6-P and that this interaction would be weakened at pH 6 because of protonation of the phosphate group (pK_a 6.1). This implies that the phosphate group of Fru-6-P could be bound in two different ways: one, in the mutant, by hydrogen-bonding with serine and another, in the wild-type, by electrostatic interaction with arginine.

Arg-225 is conserved in all the mammalian PFK-2/FBPase-2 isoenzymes, except in testis where it is replaced by serine [23]. The K_m for Fru-6-P of the testis enzyme is similar to that of other mammalian PFK-2s at pH 7.5 [23]; however, it has not been measured at lower pH values. The two yeast isoforms of PFK-2/FBPase-2, which contain the two domains (PFK-2 and FB Pase-2), differ in their catalytic properties. One isoform, called *pfk26*, is devoid of FB Pase-2 activity [24], whereas the other, called *fbp26*, lacks PFK-2 activity [25]. The primary structures of the PFK-2 domain of the yeast isoforms differ from their mammalian counterparts at several locations, including Arg-225. One may indeed wonder whether the Lys substitution for Arg-225 in *pfk26* might explain its higher K_m of PFK-2 for Fru-6-P (0.5–1.3 mM [24,26]), and whether the replacement of Arg-225 by Gln in *fbp26* is responsible for its lack of PFK-2 activity. Obviously, understanding the peculiar properties of the two yeast isoforms would require complete analysis of the various substitutions.

Mutagenesis of Arg-104 to Ser did not have the expected effect on the affinity for MgATP, as the K_m of PFK-2 for the nucleotide was not affected. In contrast, it increased the V_{max} of PFK-2 3–10-fold, depending on the pH, and increased the K_m for Fru-6-P 60-fold (Table 2). This is in sharp contrast with Arg-72 in bacterial PFK-1, which corresponds to Arg-104 in the PFK-2 domain according to Bazan et al. [5]. In bacterial PFK-1, Arg-72 has been proposed to stabilize the transition state, and mutagenesis of Arg-72 to Ser decreased the k_{cat} 33-fold and increased the K_m for Fru-6-P 3-fold [7]. Clearly, Arg-104 in the PFK-2 domain does not play an analogous role to that of Arg-72 in bacterial PFK-1. This lends further support to our previous mutagenesis studies [22], which suggested that the sequence alignment of Bazan et al. [5] is not valid.

Mutagenesis of Arg-104 to Ser increased the K_m of PFK-2 60-fold and increased the IC_{50} of citrate 800-fold. Therefore Arg-104 might be involved in binding the negative charge of the substrate and inhibitory ligand in the same binding site. The altered pH profile of PFK-2 activity suggests that Arg-104 contributes to the alkaline part of the pH profile. However, the fact that mutagenesis of Arg-104 to Ser in the PFK-2 domain affected both PFK-2 and FB Pase-2 suggests that Arg-104 might also play a structural role or that the mutation induced a conformational

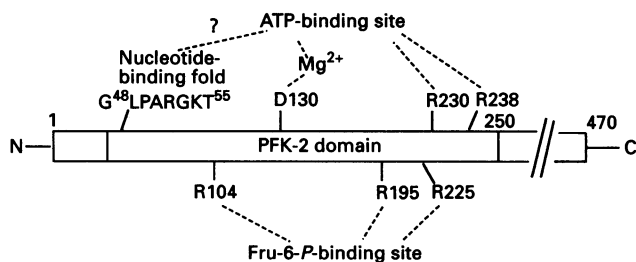


Figure 4 Schematic representation of the PFK-2 domain of the rat L isoenzyme showing the locations and roles of the residues studied by site-directed mutagenesis

For the effects of mutagenesis of Gly-48, Arg-195, Arg-230 and Arg-238, see refs. [8,9]. For mutagenesis of Asp-130, see ref. [18].

change. Indeed, the mutation changed the affinity of FBPase-2 for Fru-2,6- P_2 and Fru-6- P , both of which bind to a separate site in the FBPase-2 domain [27]. On gel filtration the [R104S]M mutant behaved as a dimer, but with a different apparent M_r . Moreover, incubations with GdmCl showed that the PFK-2 activity of the [R104S]M mutant was less stable towards denaturation. It is noteworthy that mutagenesis of an essential histidine residue in the FBPase-2 domain, His-392, to phenylalanine or lysine abolished PFK-2 activity [28], which is again indicative of interactions between the two catalytic domains.

A schematic representation of the residues involved in substrate binding in the PFK-2 domain is shown in Figure 4.

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