Site-directed mutagenesis of Lys-174, Asp-179 and Asp-191 in the 2-kinase domain of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase

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In a structural model of the 2-kinase domain of the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase based on the analogy with adenylate kinase, Lys-174, Asp-179 and Asp-191 residues are located in the putative active site. Asp-179 and Asp-191 are conserved in all known 6-phosphofructo-2-kinase sequences. In contrast, Lys-174 is conserved except in a yeast isoenzyme, fbp26, where it is replaced by glycine. Yeast fbp26 possesses fructose-2,6-bisphosphatase activity, but is devoid of 6-phosphofructo-2-kinase activity. Mutation of Asp-179 and Asp-191 of the rat liver isoenzyme to alanine increased the K_m of 6-phosphofructo-2-kinase for fructose 6-phosphate 2000- and 1000-fold respectively, whereas mutation of Lys-174 to glycine decreased the V_{max} of 6-phosphofructo-2-kinase more than 4000-fold. In contrast, none of the mutations affected the kinetic parameters of fructose-2,6-bisphosphatase. CD and fluorescence measurements indicated that the mutations had no effect on the structure and stability of the recombinant proteins. The results show that Asp-179 and Asp-191 participate in fructose 6-phosphate binding, whereas Lys-174 is important for catalysis. Therefore the natural mutation of Lys-174 to glycine in the fbp26 yeast isoenzyme could explain the lack of 6-phosphofructo-2-kinase activity. These results support a novel 6-phosphofructo-2-kinase structure model based on adenylate kinase.

INTRODUCTION

Distinct isoenzymes of the bifunctional enzyme 6-phosphofructo-2-kinase (PFK-2)/fructose-2,6-bisphosphatase (FBPase-2) are present in eukaryotic cells from yeast to mammalian tissues. They differ in molecular mass, kinetic and immunological properties and phosphorylation by protein kinases. They are termed the L (liver), M (muscle), H (heart), T (testis), B (brain), fbp26 and pfk26 (yeast) isoenzymes (see [1] for a review). PFK-2/FBPase-2 isoenzyme sequences are available from several species [2–12]. The three-dimensional structure of the FBPase-2 domain has been solved recently [13], but the structure of the PFK-2 domain is not known (see, however, Note added in proof in the preceding paper [20]). The PFK-2 and FBPase-2 reactions are catalysed at separate sites on each subunit of a homodimeric protein. The PFK-2 reaction is catalysed in the N-terminal half of the subunit and involves ternary complex-formation between the enzyme and the two substrates, fructose 6-phosphate (Fru-6-P) and MgATP [14–16]. However, the catalytic mechanism is not understood, despite the fact that some residues involved in substrate binding have been identified by site-directed mutagenesis [17–19]. Theoretical studies suggest that the nucleotide-binding site in the 2-kinase domain is similar to that of adenylate kinase (AK) (see preceding paper [20]). On the basis of this similarity, we have made a structural model of the PFK-2 domain. The model predicts that two conserved negatively charged residues, Asp-179 and Asp-191, are located within the active site. In this paper we have studied the role of these two residues by site-directed mutagenesis to alanine.

Multiple alignment of the available PFK-2/FBPase-2 sequences highlighted seven residues that are conserved in all isoenzymes except the yeast fbp26 [20], which is devoid of PFK-2 activity. These residues are Thr-62 (Ile), Asn-66 (Ser), Glu-137 (Lys), Lys-152 (Gln), Cys-160 (Ser), Lys-174 (Gly) and Leu-214 (Met). Two of the residues that are naturally mutated in fbp26, Cys-160 and Glu 137, have already been shown to be not essential for catalysis [21,22]. In the present work, we also studied the role of one of the five other residues, Lys-174, by site-directed mutagenesis. This residue is near Asp-179 and Asp-191 in the sequence and would be situated close to the active site according to our structural model. The other four residues would not be located in the putative active site in the model. Lys-174 was mutated to glycine to mimic the natural change in the yeast fbp26 isoenzyme.

The mutants, termed D179A, D191A and K174G, were produced in the rat L polyhistidine-tagged isoenzyme [PFK-2/FBPase-2-$(H)_6$] [23].

EXPERIMENTAL

All materials and reagents were from previously cited sources [23]. The double-stranded mutagenesis kit was from Stratagene.

Construction of the expression plasmids

The L-type PFK-2/FBPase-2 cDNA was originally cloned in pBlueScript (KS)II$^+$ phagemid for cloning procedures and then in the T7 RNA polymerase-based pET3a vector of Studier and Moffat for expression. The introduction of a polynucleotide encoding six histidine residues immediately before the termin-

Abbreviations used: AK, adenylate kinase (EC 2.7.4.3); 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; Mant, 3′-*N*-methylanthraniloyl; NTA, nitrilotriacetic acid; PFK-2, 6-phosphofructo-2-kinase (EC 2.7.1.105).

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ation codon of the L-type PFK-2/FBPase-2 cDNA was performed by site-directed mutagenesis in the pBlueScript (KS)II$^+$/PFK2L phagemid [23]. This phagemid, called pBlueScript (KS)II$^+$/PFK2L-$(H)_6$, was used to create the mutations. The mutant oligonucleotides are indicated below:

Wild-type L (5′ → 3′): C AAG CAA GTG **AAA** CTT GGT AGT CC
K174G (5′ → 3′): C AAG CAA GTG **GGA** CTT GGT AGT CC
Wild-type L (5′ → 3′):
GGT AGT CCT **GAT** TAC ATA GAC TGT GAC CAA
D179A (5′ → 3′):
GGT AGT CCT **GCT** TAC ATC GAT TGT GAC CAA
Wild-type L (5′ → 3′): G GTT TTG GAA **GAC** TTT CTA AAG AG
D191A (5′ → 3′): G GTT TTG GAA **GCT** TTT CTA AAG AG

They were used with the double-stranded DNA of the phagemid for double-stranded mutagenesis according to the manufacturer's specifications. The mutations were verified by sequencing [24]. The selected mutants were then introduced into the expression vector as described [23].

Expression and purification

The recombinant liver wild-type-$(H)_6$ and the three mutant enzymes were expressed in *E. coli* BL21(DE3) pLysE. They all contained a C-terminal tail of six histidines allowing their purification on Ni^{2+}–nitrilotriacetic acid (NTA)–agarose. Cultures (2 litres) were grown at room temperature and induced as described [21]. Bacteria were harvested and lysed for poly(ethylene glycol) fractionation (5–20 %, w/v) [23]. The purification of recombinant wild-type L-$(H)_6$ PFK-2/FBPase-2 and mutants was performed by a two-step procedure including anion-exchange chromatography on Q-Sepharose and affinity chromatography on Ni^{2+}–NTA–agarose [23]. The preparations were concentrated by ultrafiltration and dialysed as described [23]. The enzymes were stored frozen at −80 °C.

Other methods

The ATP analogue, 3′-*N*-methylanthraniloyl-ATP (MantATP), was used to study nucleotide binding by quenching of intrinsic fluorescence as described [23]. Stability measurements were made by incubating 10 μg of protein with increasing concentrations of guanidinium chloride (GdmCl) as described [17]. CD spectra were recorded as detailed [23]. Protein was measured after trichloroacetic acid precipitation in the presence of deoxycholate [25] by the method of Lowry et al. [26] using BSA as standard. PFK-2 and FBPase-2 were assayed under the conditions described in the legends to the Tables. SDS/PAGE analysis in 10 % polyacrylamide was performed as described [27]. Kinetic constants were calculated by fitting the data to a hyperbola by non-linear least-squares regression using the computer package Ultrafit.

RESULTS

Purification of the recombinant enzymes

Engineering of a C-terminal polyhistidine hexapeptide tail in PFK-2/FBPase-2 did not modify the kinetic properties of PFK-2 and FBPase-2 and allowed rapid purification of the protein by a two-step procedure [23]. After ion-exchange chromatography, the recombinant $(H)_6$-enzyme was loaded on Ni^{2+}–NTA–agarose and eluted with an imidazole gradient. The chromatographic behaviour was the same for wild-type and mutant enzymes. PFK-2 activity was measured to monitor the purification of the wild-type and D179A and D191A mutants, whereas FBPase-2 was measured in the K174G mutant, since its PFK-2 activity was barely detectable. A Coomassie Blue-stained SDS/polyacrylamide gel of the purified $(H)_6$-preparations from wild-type and D179A, D191A and K174G mutants revealed a single band with an M_r of 56600 (not shown).

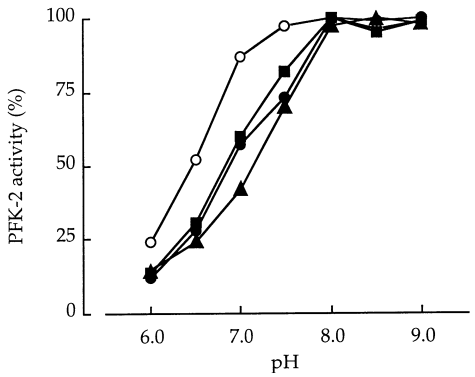

Figure 1 pH-dependence of PFK-2 activity in the wild-type and K174G, D179A and D191A mutant preparations of recombinant PFK-2/FBPase-2-$(H)_6$

PFK-2 activity of the wild-type (○) and K174G mutant (●) was measured at the indicated pH under optimal substrate concentrations. PFK-2 activity of the D179A (▲) and D191A (■) mutants was measured in the presence of 5 mM MgATP and 20 mM Fru-6-P. Activity was measured in a mixed buffer system 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, adjusted to the indicated pH as described [19]. The values are the means of two determinations. The 100% values of PFK-2 activity correspond to 70 (wild-type), 0.014 (K174G), 4 (D179A) and 15 (D191A) m-units/mg of protein.

Kinetic properties

PFK-2 activity

The pH profile of PFK-2 activity of recombinant wild-type and mutant PFK-2/FBPase-2 preparations showed a slight alkaline shift for the three mutants as compared with the wild-type (Figure 1).

The kinetic properties were compared at pH 8.5, i.e. under conditions of optimal activity (Table 1). PFK-2 activity of the K174G mutant was almost undetectable, since the V_{max} was decreased 4000-fold. The mutation also decreased the K_m of PFK-2 for MgATP 10-fold and increased the K_m for Fru-6-P 100-fold. Mutations of Asp-179 and Asp-191 to Ala also induced marked changes: (i) the V_{max} was decreased 10-fold and 3-fold respectively; (ii) more dramatically, the D179A and D191A mutations increased the K_m for Fru-6-P 2000- and 1000-fold without changing the K_m for MgATP. The fact that all the mutations increased the K_m of PFK-2 for Fru-6-P and the IC_{50} for citrate agrees with the proposal that these two molecules bind to the same site [19]. Other minor changes in kinetic properties of PFK-2 were also observed. Indeed, increasing the pH from 7.1 to 8.5 decreased the K_m of wild-type PFK-2 for Fru-6-P (8-fold) without affecting the V_{max}. In contrast, the K_m of the D179A and D191A mutants for Fru-6-P was increased (2.5- to 4-fold). In the case of the K174G mutant, the K_m for Fru-6-P was independent of pH.

Table 1 Kinetic properties of PFK-2 in the recombinant wild-type and mutant 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, 1 mg/ml BSA, at either pH 8.5 or 7.1 [21]. For Fru-6-P-saturation curves, the concentration of MgATP was 5 mM for the wild-type and mutants. For MgATP-saturation curves of wild-type and K174G, D179A and D191A mutants, the concentration of Fru-6-P was 1, 10, 25 and 20 mM respectively. For the IC_{50} for magnesium citrate, Fru-6-P and MgATP concentrations were equal to the K_m and to ten times the K_m respectively. The values are the means ± S.E.M. for three determinations, otherwise individual values are given. NM, not measured. $^*P < 0.01$ compared with the wild-type (unpaired t test).

	PFK-2 activity			
	V_{max} (m-units/mg of protein)	K_m Fru-6-P (μM)	K_m MgATP (μM)	IC_{50} magnesium citrate (μM)
pH 7.1				
Wild-type	63 ± 6	45 ± 4	190 ± 12	22 ± 4
K174G	0.010 ± 0.002*	603 ± 10*	50 ± 10*	NM
D179A	3 ± 1*	3890, 4060	96, 88	NM
D191A	10 ± 2*	1440, 1280	196, 188	NM
pH 8.5				
Wild-type	68 ± 5	6 ± 1	318 ± 32	126 ± 17
K174G	0.018 ± 0.001*	729 ± 55*	28 ± 4*	387 ± 15*
D179A	6 ± 1*	10900 ± 900*	217 ± 15*	1300 ± 100*
D191A	21 ± 2*	5400 ± 600*	370 ± 20	4200 ± 100*

Table 2 Kinetic properties of FBPase-2 in the recombinant wild-type and mutant PFK-2/FBPase-2 preparations

FBPase-2 was measured as described [21] at pH 7.1, in the presence of 1 mM potassium phosphate with concentrations of [2-^{32}P]Fru-2,6-P_2 up to ten times the K_m. The IC_{50} of FBPase-2 for Fru-6-P was measured in the presence of 5 μM Fru-2,6-P_2. The values are the means ± S.E.M. for three determinations.

	FBPase-2 activity		
	V_{max} (m-units/mg of protein)	K_m Fru-2,6-P_2 (μM)	IC_{50} Fru-6-P (μM)
Wild-type	9 ± 3	0.41 ± 0.06	3.0 ± 1.0
K174G	10 ± 2	0.43 ± 0.10	2.3 ± 0.4
D179A	8 ± 1	0.52 ± 0.07	2.6 ± 0.9
D191A	8 ± 1	0.43 ± 0.04	2.1 ± 0.6

FBPase-2 activity

The pH profile of FBPase-2 activity of wild-type and mutants was not different (not shown). Moreover, the K174G, D179A and D191A mutations had no significant effect on the kinetic properties of FBPase-2 (Table 2).

Nucleotide binding

Since mutation of Lys-174 to glycine was shown above to drastically decrease the V_{max} of PFK-2 and to lower the K_m for MgATP, nucleotide binding was measured by quenching of intrinsic fluorescence. On excitation at 295 nm, the fluorescence emission spectra of wild-type and mutant enzymes were similar with a maximum at 325 nm as described [23] (not shown). Increasing concentrations of MantATP were added and produced a quenching of fluorescence that reached a maximum of 40 % for all the preparations. K_d values for MgMantATP were 3–5-fold lower at pH 7.1 than at pH 8.5, but were not significantly changed by mutation (Table 3).

Table 3 Binding constants for MgMantATP to the wild-type and mutant PFK-2/FBPase-2 preparations

Fluorescence measurements were performed as indicated in the Experimental section. The K_d values were determined by quenching of intrinsic fluorescence as described in the Experimental section. Individual values are shown.

	K_d MgMantATP (nM)	
	pH 7.1	pH 8.5
Wild-type	40, 50	150, 180
K174G	30, 40	160, 170
D179A	30, 40	140, 150
D191A	40, 50	150, 170

Table 4 Sensitivity of wild-type and mutant PFK-2/FBPase-2 preparations to GdmCl-induced denaturation assessed by quenching of fluorescence

PFK-2/FBPase-2 preparations were incubated in the presence of increasing GdmCl concentrations as described [17]. The values represent the concentration of GdmCl required to obtain 50% of maximal quenching of fluorescence ($[GdmCl]_{50}$). Individual values are shown.

	Quenching of fluorescence $[GdmCl]_{50}$ (M)
Wild-type	0.40, 0.49
K174G	0.38, 0.41
D179A	0.41, 0.46
D191A	0.42, 0.48

GdmCl-induced denaturation

Fluorescence spectroscopy was used to study the stability of mutant and wild-type preparations by monitoring the quenching of intrinsic fluorescence induced by denaturation with GdmCl. On excitation at 295 nm, the maximum of quenching reached 60 % for all the preparations, suggesting the same conformation of the initial state. Table 4 shows that a similar concentration of GdmCl was required to obtain half-maximal quenching for both the wild-type and mutant preparations.

Structural study by CD

Possible effects of the mutations on the structure of each enzyme preparation were studied by CD. The spectra obtained for all mutant preparations were similar to those observed with the wild type and displayed two characteristic minima of mean residue ellipticity at 222 and 208 nm [23]. The spectra were analysed by calculating the ratio (R) of the mean residue ellipticity at these two wavelengths (Table 5). R is an index of α-helical structure and has previously been used to estimate the average helical structure of wild-type and mutant PFK-2/FBPase-2 preparations and other enzymes [23,28]. Comparison of R values indicates that there was no significant difference in α-helical content between wild-type and mutant preparations.

Table 5 Ratio (*R*) of the mean residue ellipticity at 222 and 208 nm of the wild-type and mutant PFK-2/FBPase-2 preparations

CD spectra were recorded, and *R* was calculated as described [23]. The results are the means ± S.E.M. for the number of determinations shown in parentheses, otherwise individual values are given.

	R
Wild-type	0.94 ± 0.03 (4)
K174G	0.93 ± 0.05 (3)
D179A	0.94, 0.99
D191A	0.95, 0.97

DISCUSSION

None of the mutations studied in this work affected the kinetic properties of FBPase-2, suggesting that the effects on the kinetic properties of PFK-2 were not due to a change in structure. Indeed, no structural changes in the mutant enzyme preparations were detected by either CD measurement or progressive denaturation with GdmCl.

The most striking effect induced by the K174G mutation was the dramatic decrease in V_{max} of PFK-2. This single mutation could explain the lack of PFK-2 activity in the fbp26 yeast isoenzyme. The critical role played by Lys-174 in catalysis might be to stabilize the transition state. In *Escherichia coli* AK, mutation of two positively charged residues (Arg-156 and Arg-167) decreased the V_{max} by 71000- and 9200-fold respectively [29]. In our PFK-2 model based on the structure of AK, Lys-174 is located in the same region as the two arginines. This domain in AK undergoes movement on substrate binding, which is necessary for activity. Similarly, in the PFK-2 domain, Lys-174 could be involved in both the stabilization of the transition state and a putative induced-fit mechanism (Figure 2). Indeed, ATP has been shown to induce changes in PFK-2 structure as monitored by CD [30].

Mutation of Asp-179 and Asp-191 resulted in a large increase in K_m for Fru-6-P. These observations suggest that both residues are involved in Fru-6-P binding. These residues might bind directly to the ribose ring of Fru-6-P by hydrogen-bonding. However, we speculate that Asp-179 and Asp-191 are more likely to interact indirectly with Fru-6-P, by making electrostatic bonds with positively charged residue(s) directly involved in Fru-6-P binding. Arg-195, previously shown to be involved in Fru-6-P binding [1], is a good candidate for such an interaction (Figure 2). Indeed, Arg-195 and Asp-191 residues are predicted to be located side by side in a putative α-helix located near the active site [20]. Moreover, Asp-179 would stabilize a second arginine involved in Fru-6-P binding, Arg-104 [19], situated near the loop including the aspartate residue (Figure 2). The fact that the mutations of the two aspartate residues in PFK-2 substantially decreased the V_{max} could be due to the involvement of these residues in an induced-fit movement.

All these results validate the model of the PFK-2 domain based on the structure of AK. Moreover, they confirm that the PFK-2 and PFK-1 domains are unrelated. Indeed, in the PFK-1-based model [1], at least two of the three residues of PFK-2 (Lys-174 and Asp-179) studied here would not be located near the active site.

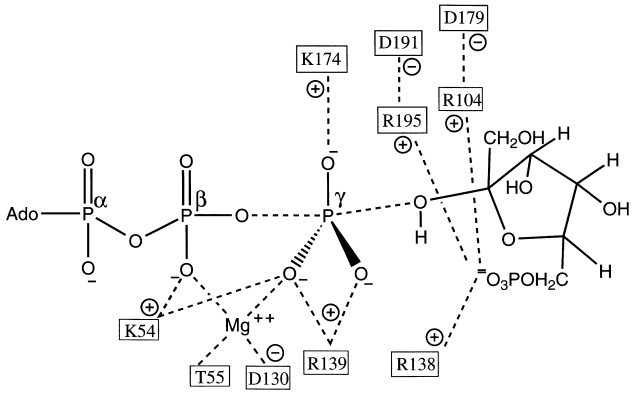

Figure 2 Schematic representation of the transition state in the PFK-2 reaction

This is a hypothetical model based on the results of site-directed mutagenesis and theoretical studies [20].

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