Site-directed mutagenesis of rat muscle 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase: role of Asp-130 in the 2-kinase domain

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Asp-130 of the recombinant skeletal-muscle 6-phosphofructo-2-kinase (PFK-2)/fructose-2,6-bisphosphatase was mutated into Ala in order to study its role in catalysis and/or substrate binding. The D130A mutant displayed a 30- to 140-fold decreased 2-kinase $V_{max.}$, depending on the pH, and a 30- and 60-fold increase in K_m for MgATP and Fru-6-P respectively at pH 8.5 compared with the wild-type. Mutagenesis of Asp-130 to Ala had no effect on the 2-phosphatase activity, and fluorescence measurements indicated that the changes in kinetic properties of PFK-2 in the D130A mutant were not due to instability. The role of Asp-130 in the 2-kinase reaction is discussed and compared with that of Asp-103 of 6-phosphofructo-1-kinase from *Escherichia coli*, which binds Mg^{2+}.

INTRODUCTION

Different isozymes of the bifunctional enzyme 6-phosphofructo-2-kinase (PFK-2)/fructose-2,6-bisphosphatase (FBPase-2) have been found in liver, heart and skeletal muscle and termed the L, H and M isozymes [1]. The isozymes differ in molecular mass, kinetic and immunological properties and in their response to phosphorylation by protein kinases. The amino acid sequence of the M isozyme is identical with that of the L isozyme, except at the N-terminus, where a nonapeptide in the M isozyme replaces the first 32 amino acids of the L isozyme [2], which contains a phosphorylation site for the cyclic AMP-dependent protein kinase.

The PFK-2 and FBPase-2 reactions are catalysed at separate sites on each subunit of a homodimeric protein. The FBPase-2 reaction is catalysed in the C-terminal half of the enzyme subunit in a classical Ping-Pong reaction mechanism [3], and a histidine residue (His-258 in the L isozyme) is phosphorylated from Fru-2,6-P_2. The FBPase-2 domain is homologous with the phosphoglycerate mutases [4], and the molecular mechanism of FBPase-2 has been extensively studied by site-directed mutagenesis [5–8].

The PFK-2 reaction is catalysed in the N-terminal half of the subunit and involves a ternary complex mechanism [9,10] with direct in-line transfer of the γ-phosphate of ATP to the 2-OH group of Fru-6-P [11]. However, little is known about which amino acid residues are involved in catalysis and substrate binding, and the three-dimensional structure of the enzyme has not been solved. A consensus sequence for the start of a nucleotide-binding fold (residues 48–54 in the L isozyme) has been recognized [12], and, as expected, mutation of Gly-48 to Ala abolished PFK-2 activity [13]. Bazan and co-workers [12] have modelled the three-dimensional structure of the PFK-2 domain on bacterial PFK-1, suggesting roles for certain amino acids in catalysis and substrate binding. Accordingly, Arg-162 in bacterial PFK-1, which binds Fru-6-P [14], corresponds to Arg-195 in the L isozyme, and mutagenesis of Arg-195 to Ala increased the K_m of PFK-2 for Fru-6-P more than 3000-fold compared with the wild-type [13]. Chemical-modification experiments with the arginine-specific reagent, phenylglyoxal, suggest that another arginine residue, Arg-225 in the L isozyme, is also involved in Fru-6-P binding [15]. However, the analogy between bacterial PFK-1 and the PFK-2 domain cannot be extended to include the active site. In bacterial PFK-1, Asp-127 acts as a general base catalyst [16–18], and an adjacent aspartate residue, Asp-129, is one of the residues that binds Mg^{2+} [18]. These two residues correspond to Cys-160 and Asp-162 in the PFK-2 domain [12], but site-directed mutagenesis experiments indicate that they are not directly involved in catalysis [19].

The PFK-2 domain contains a sequence that presents some similarity to the so-called 'B motif' of *E. coli* PFK-1, recognized by Walker et al. [20] for adenine nucleotide-binding proteins (Figure 1). This motif contains a conserved aspartate residue (encircled), which follows a sequence of hydrophobic residues. The Walker B motif is more difficult to recognize than the GX_4GKS/T 'A motif' [20], which is the classic nucleotide-binding fold signature, the structure of which was first described in adenylate kinase [21]. In fact, in adenylate kinase the conserved aspartate residue (Asp-119) in the B motif does not appear to bind Mg^{2+}, as its mutation had little effect on the kinetic properties, and this is fulfilled by Asp-93 [22]. However, in PFK-1, Asp-103 does bind Mg^{2+}, and mutagenesis of Asp-103 to Ala decreased the $V_{max.}$ about 30-fold [18]. In the PFK-2 domain of the L isozyme, this residue corresponds to Asp-130 and it is conserved in all the PFK-2 domains of the isozymes studied so far. Asp-130 in the L isozyme corresponds to Asp-107 in the M isozyme. In the present paper we have studied the effect of mutation of Asp-130 to alanine (D130A) on the kinetics of PFK-2 and FBPase-2 in the M isozyme. We have used the numbering of amino acids in the L isozyme, as the internal sequences of the L and M isozymes are identical.

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; Glc-6-P, glucose 6-phosphate; PFK-1, 6-phosphofructo-1-kinase (EC 2.7.1.11); PFK-2, 6-phosphofructo-2-kinase (EC 2.7.1.105).

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Protein	Residues	Sequences
Rat liver PFK-2/FBPase-2	116–141	H K Y L S R E - E G - - H V A V F - Ⓓ A T N T T R E R R S L
Adenylate kinase	102–130	G E E F E R K - I G Q P T L L L Y V Ⓓ A G P E T M T K R L L
E. coli PFK-1	85–113	G I E Q L K K - H G I Q G L V V I G G Ⓓ G S Y Q G A K K L T
Bovine ATPase β	241–270	V A E Y F R D Q E G Q D V L L F I Ⓓ N I F R F T Q A G S E V
E. coli ATPase β	227–255	M A E K F R D - E G R D V L L F V Ⓓ N I Y R Y T L A G T E V
E. coli ATPase α	265–293	M G E Y F R D - R G E D A L I I Y Ⓓ D L S K Q A V A Y R Q I
ATP/ADP translocase	275–297	S N V L - R G M G G A F V L V L Y Ⓓ E I K K F V

Figure 1 Alignment of the nucleotide-binding-fold sequences of rat liver PFK-2/FBPase-2 and other adenine nucleotide binding proteins

This Figure is adapted from Walker et al. [20] and includes the sequence of rat liver PFK-2/FBPase-2. The boxes contain conserved residues. The aspartate residue that has been proposed to bind Mg^{2+} [20] is encircled.

MATERIALS AND METHODS

Materials

All materials and reagents were from sources previously cited [19,23]. Radiochemicals were from Amersham International. Phosphocellulose P11 was from Whatman.

Construction of the pET-PFK2M-*sf* expression plasmids

Standard DNA manipulations were performed as described [24]. Mutagenesis [25] was carried out on single-stranded phagemid DNA with a kit purchased from Amersham. The T7 RNA polymerase-based pET3d expression vector of Studier and Moffat [26] was first engineered to produce pET-PFK2M-*sf*, which encodes the wild-type M PFK-2/FBPase-2 isozyme [23]. The oligonucleotides used were:

wild-type 5′-GT GTT GGT GGC **ATC** AAA AAC CGC-3′

D130A 5′-GT GTT GGT GGC **AGC** AAA AAC CGC-3′

They were annealed to the single-stranded DNA of pBlueScript (KS)II + /PFK2M, and the mutagenesis reaction was performed according to the manufacturer's instructions. Positive mutants were identified by sequencing [27]. The selected mutant was then introduced into the expression vector pET/PFK2M-*sf* as described [19].

Purification of recombinant wild-type and D130A mutant PFK-2/FBPase-2

The D130A mutant and wild-type PFK-2/FBPase-2s were expressed in *E. coli* BL21(DE3)-pLysS and BL21(DE3)-pLysE respectively. Cultures (2 litres) were grown and induced as described [19]. Bacteria were harvested and lysed for poly(ethylene glycol) fractionation as described [19]. The poly(ethylene glycol) fraction was applied to a DEAE-Trisacryl column (6 cm × 5 cm) equilibrated with buffer containing 20 mM Hepes, 50 mM KCl, 5 mM EDTA, 1 mM EGTA and 1 mM potassium phosphate, at pH 7.4, and enriched with 0.1 mM Fru-6-P, 0.3 mM Glc-6-P, 15 mM 2-mercaptoethanol, 20% (v/v) glycerol, 2 mM benzamidine hydrochloride and 0.5 mM phenylmethanesulphonyl fluoride (buffer A). PFK-2/FBPase-2 was eluted at about 250 mM KCl with a linear gradient (0–550 mM KCl in 500 ml of buffer A) and applied to a column (1.5 cm × 7 cm) of Blue Sepharose equilibrated in buffer A. The enzyme was eluted with a linear gradient of KCl (0–1 M in 70 ml of buffer A). Fractions containing activity, which were eluted at about 700 mM KCl, were dialysed overnight against 15 vol. of 20 mM Hepes, pH 7.5, containing 5 mM $MgCl_2$, 0.1 mM EDTA, 15 mM 2-mercaptoethanol, 0.2 mM phenylmethanesulphonyl fluoride, 2 mM benzamidine hydrochloride and 20% (v/v) glycerol (buffer B) and loaded on to a column (1.5 cm × 5 cm) of phosphocellulose equilibrated with buffer B. After extensive washing overnight with about 300 ml of buffer B, PFK-2/FBPase-2 was eluted with either 50 ml of buffer B supplemented with 2 mM Fru-6-P or with a linear gradient of potassium phosphate (0–200 mM in buffer B supplemented with 2 mM Fru-6-P). Fractions containing activity were concentrated by ultrafiltration, dialysed against 200 vol. of buffer B for 48 h and stored at −80 °C.

Fluorescence measurements

PFK-2/FBPase-2 (10 μg/ml) was incubated at room temperature for 15 min with the indicated concentrations of guanidinium chloride (GdmCl) in a buffer containing 50 mM Hepes, pH 7.5, 5 mM potassium phosphate and 0.5 mM dithiothreitol. Protein fluorescence was recorded in a Perkin–Elmer LS-5 luminescence spectrometer from 300 to 400 nm with an excitation wavelength of 278 nm (the excitation and emission slit widths were 10 nm and 5 nm respectively).

Other methods

Protein was measured [28] using γ-globulin as a standard. PFK-2 and FBPase-2 were assayed [23] under the conditions described in the legends to the Figures and Table. During the purification procedure, PFK-2/FBPase-2 was detected by assaying either PFK-2 for the wild-type or FBPase-2 for the mutant. SDS/PAGE analysis in 10% acrylamide [29] and Western blotting [23] were performed as described. Kinetic constants were calculated by fitting the data to a hyperbola by non-linear least-squares regression using a computer program [30].

RESULTS AND DISCUSSION

Purification

The recombinant wild-type and D130A mutant of the PFK-2/FBPase-2 M isozyme were purified by the same procedure, which involved poly(ethylene glycol) fractionation, ion-exchange chromatography on DEAE-Trisacryl, affinity chromatography on Blue Sepharose and chromatography on phosphocellulose with elution in the presence of Fru-6-P. The chromatographic

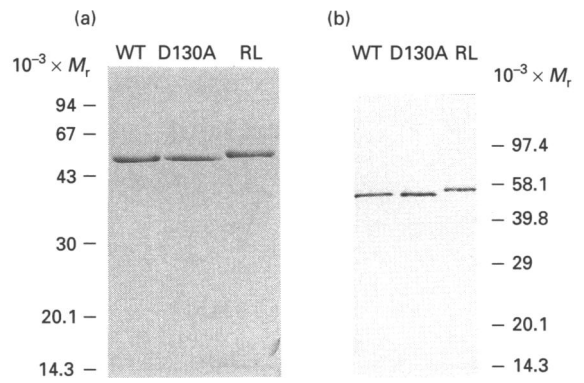

Figure 2 SDS/PAGE and Western blotting of the wild-type and D130A recombinant PFK-2/FBPase-2 preparations

Wild-type recombinant skeletal-muscle, D130A recombinant skeletal-muscle mutant and recombinant rat liver PFK-2/FBPase-2 preparations (5 μg) were subjected to SDS/PAGE in 10% acrylamide followed by Coomassie Blue staining (**a**). Immunodetection of the wild-type recombinant skeletal-muscle (0.2 μg), D130A recombinant skeletal-muscle (0.4 μg) and rat liver PFK-2/FBPase-2 (0.2 μg) was performed by Western blotting after SDS/PAGE (**b**). WT, Wild-type recombinant skeletal-muscle PFK-2/FBPase-2; D130A, D130A recombinant skeletal-muscle PFK-2/FBPase-2 mutant predation; RL, recombinant rat liver PFK-2/FBPase-2.

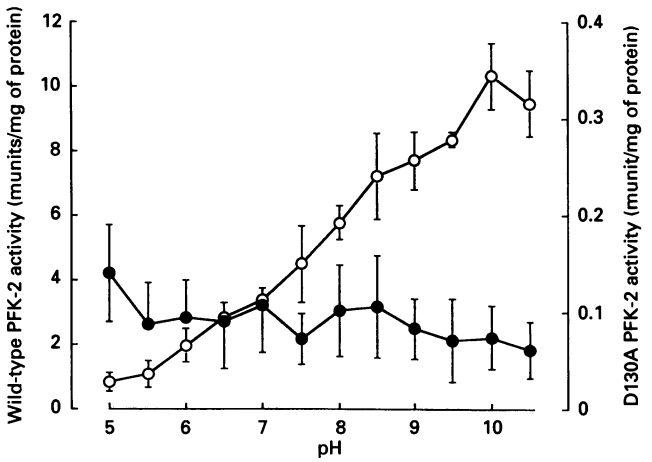

Figure 3 pH profile of PFK-2 in the recombinant wild-type and D130A PFK-2/FBPase-2 preparations

PFK-2 activity in the wild-type (○) and D130A mutant (●) PFK-2/FBPase-2 preparations was measured in buffer containing 25 mM Mes, 25 mM glycylglycine, 25 mM Hepes, 25 mM sodium borate, 50 mM KCl, 20 mM KF, 5 mM potassium phosphate, 1 mM dithiothreitol adjusted to the indicated pH and 5 mM Fru-6-P. The reactions were started with 5 mM MgATP at 30 °C. The results are means ± S.E.M. of four determinations on one wild-type enzyme preparation and two D130A enzyme preparations.

behaviour of the two enzymes was the same. During the course of purification, PFK-2/FBPase-2 was monitored by measuring the activity of PFK-2 for the wild-type enzyme and FBPase-2 for the D130A mutant, as its PFK-2 activity was barely detectable. Two D130A preparations were studied, both of which were homogeneous as judged by SDS/PAGE. The recombinant wild-type and D130A skeletal-muscle PFK-2/FBPase-2 preparations showed a major band migrating with an M_r of 51000 (Figure 2a), which is close to the calculated M_r of 51880 [23]. In the same gel, a preparation of purified recombinant rat liver PFK-2/FBPase-2 displayed an M_r of 55000, as expected. The 51000-M_r band in the wild-type and D130A recombinant muscle PFK-2/FBPase-2 preparations was recognized in a Western blot using an antibody raised against chicken liver PFK-2/FBPase-2 (Figure 2b).

Table 1 Kinetic properties of PFK-2 and FBPase-2 in the recombinant wild-type and D130A mutant PFK-2/FBPase-2 preparations

PFK-2 activity was measured in 50 mM Tris/HCl buffer, pH 8.5, as described previously in the presence of 5 mM potassium phosphate [23]. For the Fru-6-P and MgATP saturation curves the concentrations of MgATP and Fru-6-P were each 5 mM and the concentration of the other substrate was varied up to 1–2 mM (wild-type) or 10–20 mM (D130A). FBPase-2 was measured [23] with 0.25–10 μM [2-^{32}P]Fru-2,6-P_2 in the presence of 1 mM potassium phosphate, 5 mM *sn*-glycerol 3-phosphate and 1.5 mM MgGTP at pH 7. The $I_{0.5}$ of FBPase-2 for Fru-6-P was measured with 1 μM Fru-2,6-P_2 in the presence of 5 mM potassium phosphate. The results are the means ± S.E.M. of the number of determinations shown in parentheses on one wild-type enzyme preparation and two D130A enzyme preparations. *Significant effect of the mutation ($P < 0.05$) compared with the wild-type.

Kinetic parameters	Wild-type	D130A
PFK-2 activity: Fru-6-P saturation curve		
$V_{max.}$ (munits/mg of protein)	17.0 ± 3.6 (4)	0.58 ± 0.06 (8)*
$K_m^{app.}$ Fru-6-P (mM)	0.08 ± 0.02 (4)	5.03 ± 1.34 (8)*
PFK-2 activity: MgATP saturation curve		
$V_{max.}$ (munits/mg of protein)	13.6 ± 5.9 (4)	0.63 ± 0.05 (8)*
$K_m^{app.}$ MgATP (mM)	0.10 ± 0.01 (4)	2.92 ± 0.30 (7)*
FBPase-2 activity		
$V_{max.}$ (munits/mg of protein)	68.0 ± 10.0 (4)	80.7 ± 5.4 (6)
$K_m^{app.}$ Fru-2,6-P_2 (μM)	1.68 ± 0.23 (4)	2.20 ± 0.17 (6)
$I_{0.5}$ for Fru-6-P inhibition (μM)	9.9 ± 1.2 (3)	11.7 ± 2.0 (6)

Kinetic properties

PFK-2 activity in the D130A mutant preparation was decreased by 30-fold at pH 7, and 140-fold at pH 10 compared with the wild-type (Figure 3). The pH profile of PFK-2 activity indicates that, for the wild-type enzyme, the pH optimum measured under $V_{max.}$ conditions was about 10, as described previously [31]. However, for the D130A mutant, the alkaline part of the profile was abrogated. The pH profile of PFK-2 activity for the wild-type M isozyme indicates that a residue with a pK_a of about 8.5, which could be a histidine, lysine or arginine residue, participates in catalysis. Moreover, the striking shift in pH profile of the mutant suggests that Asp-130 interacts with such a residue.

The kinetic properties of the wild-type and mutant enzymes were studied further at pH 8.5. Table 1 shows that, in addition to decreasing the $V_{max.}$, mutation of Asp-130 to Ala increased the K_m of PFK-2 for MgATP by 30-fold and for Fru-6-P by 60-fold. The Mg^{2+}-dependence of PFK-2 activity of the wild-type and D130A mutant was also studied. Figure 4 shows that Mg^{2+} is required for PFK-2 activity and that the half-maximal effect for the wild-type is obtained with about 0.5 mM Mg^{2+} at the concentration of ATP present in the assay. This demonstrates that the MgATP complex is the substrate in the PFK-2 reaction rather than ATP alone. For the D130A mutant, the curves describing Mg^{2+} saturation at 0.5 mM and 3 mM ATP were shifted towards higher Mg^{2+} concentrations. This shift in Mg^{2+} sensitivity reflects the increased K_m of PFK-2 for MgATP of the D130A mutant and possibly a decreased affinity of the D130A mutant for Mg^{2+}.

Mutation of Asp-130 to Ala had no effect on the kinetics of FBPase-2. Table 1 shows that, for the D130A mutant, the $V_{max.}$, K_m for Fru-2,6-P_2 and $I_{0.5}$ for Fru-6-P inhibition of FBPase-2 were all not affected compared with the wild-type. Therefore it is

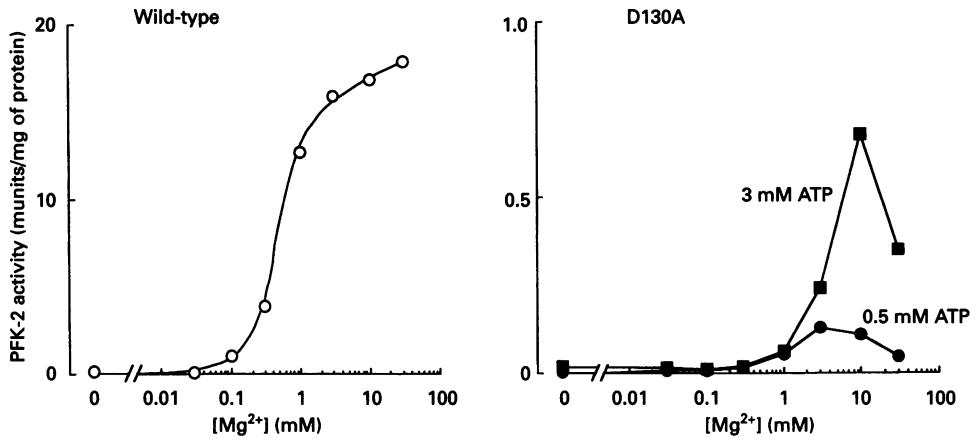

Figure 4 Effect of Mg^{2+} on PFK-2 activity in the recombinant wild-type and D130A PFK-2/FBPase-2 preparations

PFK-2 activity in the wild-type (open symbols) and D130A PFK-2/FBPase-2 preparations (closed symbols) was measured at pH 8.5 [23] in the presence of 5 mM Fru-6-P, the indicated concentrations of Mg^{2+} and 0.5 mM ATP (○, ●) or 3 mM ATP (■). The results are the means of two determinations on one wild-type enzyme preparation and one D130A enzyme preparation.

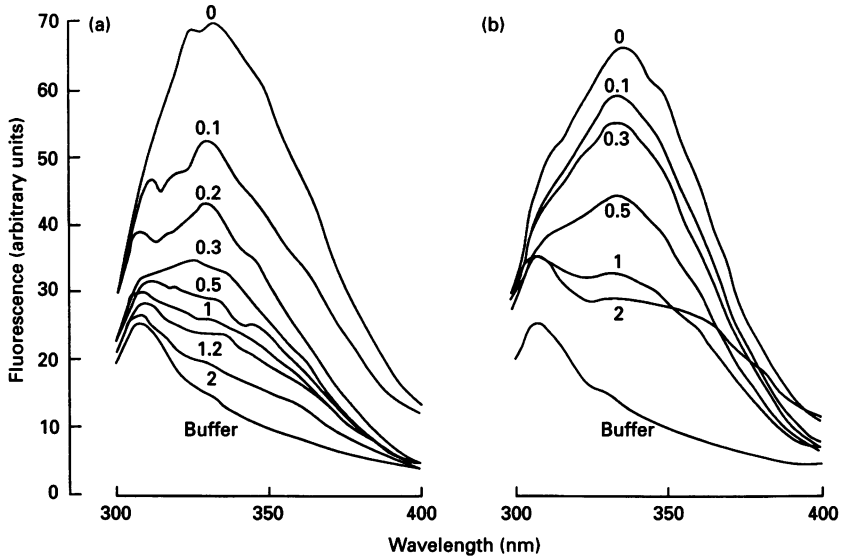

Figure 5 Quenching of the fluorescence emission spectra of wild-type (a) and D130A (b) recombinant skeletal-muscle PFK-2/FBPase-2 by GdmCl

The wild-type and D130A PFK-2/FBPase-2 enzyme preparations were incubated with the indicated concentrations (M) of GdmCl and the fluorescence emission spectra were recorded as described in the Materials and methods section.

unlikely that mutation of Asp-130 to Ala caused structural changes in the enzyme.

Structure

Changes in the structure of the wild-type and mutant enzymes were studied by fluorescence spectroscopy. When measured under identical conditions, the fluorescence emission spectra of the wild-type and D130A mutant enzymes were similar (Figure 5). The emission spectra were of a similar intensity and had a peak at about 335 nm, suggesting that the mutation did not affect the structure of the protein. Incubation with GdmCl caused a quenching of fluorescence, but both the sensitivity and extent of fluorescence quenching for the wild-type and D130A mutant enzymes were different. At 335 nm, incubation with 2 M GdmCl quenched the fluorescence of the wild-type enzyme by 93%, whereas for the D130A mutant the extent of fluorescence quenching was 71%. Furthermore, the fluorescence quenching of the wild-type was more sensitive to GdmCl (Figure 5). These findings suggest that the mutant enzyme was less prone to unfolding by GdmCl and might have a slightly more stable structure. Therefore it is very unlikely that the changes in PFK-2 kinetic properties brought about by mutating Asp-130 to Ala were due to instability of the mutant enzyme.

Conclusions

The results of these findings show that Asp-130 is important for catalysis in the PFK-2 domain. At pH 8.5, mutation of Asp-130 to Ala decreased the $k_{cat.}$ of PFK-2 by 20–30-fold and increased

the K_m values for the substrates (Table 1). Also the affinity of the D130A mutant for Mg^{2+} might be less than for the wild-type (Figure 4). In contrast, the FBPase-2 activity of the D130A mutant was unaffected, and fluorescence measurements suggest that the changes in PFK-2 kinetics were not due to instability of the mutant enzyme.

The sequence alignment in Figure 1 shows that Asp-130 in the PFK-2 domain corresponds to Asp-103 in bacterial PFK-1, which is included in the Walker B motif [20]. In bacterial PFK-1, Mg^{2+} is bound directly to Asp-103 and indirectly through two co-ordinated water molecules to Asp-129 [18]. The role of Mg^{2+} in the PFK-1 reaction is to orientate the β- and γ-phosphates of ATP and to stabilize the transition state [18]. In *E. coli* PFK-1, mutation of Asp-103 to Ala decreased the $k_{cat.}$ 30-fold and increased the K_m for ATP, when measured in the presence of the positive effector, GDP [18]. Mutation of Asp-130 to Ala in the PFK-2 domain decreased the $k_{cat.}$ to a similar extent and increased the K_m for MgATP (Table 1), suggesting that Asp-130 might directly bind Mg^{2+} by analogy with Asp-103 in bacterial PFK-1 and in agreement with the alignment (Figure 1).

Alternatively, Asp-130 could bind Mg^{2+} indirectly in an analogous fashion to Asp-129 in *E. coli* PFK-1. However, in *E. coli* PFK-1, mutagenesis of Asp-129 to serine decreased the $k_{cat.}$ almost 1000-fold [17,18], which is much greater than the decrease in $k_{cat.}$ observed on the mutagenesis of Asp-130 in these studies. Interestingly, in the sequence alignment of Bazan et al. [12], Asp-129 in bacterial PFK-1 corresponds to Asp-162, in the PFK-2 domain, but our previous mutagenesis studies suggest that Asp-162 does not bind Mg^{2+} [19].

It is unlikely that Asp-130 acts as the base catalyst in the PFK-2 reaction, as a much greater decrease in $k_{cat.}$ would be expected in the D130A mutant. For comparison, in *E. coli* PFK-1, mutagenesis of the base catalyst, Asp-127, to serine decreased the $k_{cat.}$ by four orders of magnitude [16]. We cannot exclude the possibility that Asp-130 might participate in a charge-relay system in PFK-2 catalysis, which would be different from the PFK-1 reaction mechanism. This interpretation is, however, difficult to reconcile in view of the changes in K_m of the substrates.

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