The ATP-binding Site in the 2-Kinase Domain of Liver 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase

STUDY OF THE ROLE OF Lys-54 AND Thr-55 BY SITE-DIRECTED MUTAGENESIS*

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Didier Vertommen§§, Luc Bertrand§§, Bruno Sontag, Attilio Di Pietro†, Marc P. Louckx‡, Hubert Vidal†, Louis Huet, and Mark H. Rider**

From the Hormone and Metabolic Research Unit, International Institute of Cellular and Molecular Pathology and the University of Louvain Medical School, Avenue Hippocrate 75, B-1200 Brussels, Belgium, the 1Institut de Biologie et Chimie des Protéines, Centre National de la Recherche Scientifique, Passage du Vercors, F-69367 Lyon cedex 07, France, and the 2Unité INSERM 197, Faculté de Médecine Alexis Carrel, Rue Guillaume Parradin, F-69372 Lyon cedex 08, France

All known 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase isozymes contain a sequence (GX₄GK(S/T)) in the 6-phosphofructo-2-kinase domain corresponding to the so-called nucleotide binding fold signature or Walker A motif. Mutagenesis and crystal structure data from several nucleotide binding proteins, which also contain this sequence, showed the importance of the lysine and serine/threonine residues in nucleotide binding. We have studied the role of Lys-54 and Thr-55 in MgATP binding in the 6-phosphofructo-2-kinase domain of rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase by site-directed mutagenesis. Lys-54 was mutated to methionine, whereas Thr-55 was mutated to valine, serine, and cysteine. Three mutants, Lys-54 to Met and Thr-55 to Cys or Val, displayed more than a 5000-fold decrease in 6-phosphofructo-2-kinase activity compared with the wild type. The mutations had no effect on fructose-2,6-bisphosphatase activity and did not affect the activation of fructose-2,6-bisphosphatase after phosphorylation by cyclic 3',5'-AMP-dependent protein kinase. Binding experiments with ATP, ADP, and their analogs (3'-N-methylantraniloyl derivatives) showed that these two residues do not play the same role. Lys-54 is involved in ATP binding, whereas Thr-55 is important for catalysis.

Several isozymes of the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) have been identified in mammalian tissues. They differ in kinetic properties, immunological reactivity, molecular mass, and response to phosphorylation by protein kinases. They are called the L (liver), H (heart), M (skeletal muscle), and T (testis) isozymes according to their tissue distribution. Their amino acid sequences have been determined from the corresponding cDNAs (1–5). The amino acid sequence of the rat M isozyme has been proposed to bind fructose 6-phosphate (Fr6-P), whereas Arg-230 and Arg-238 might be involved in ATP binding (6). The PFK-2 domain is homologous with the so-called histidine phosphatases (7), and residues involved in catalysis and substrate binding in the FBPase-2 domain have been studied by site-directed mutagenesis (8).

The PFK-2 reaction is catalyzed in the NH₂-terminal half of the subunit and involves ternary complex formation (9, 10). Site-directed mutagenesis studies have identified some amino acid residues that are important in substrate binding. For example, Arg-104 (11), Arg-195 (12), and Arg-225 (11) in the L isozyme have been proposed to bind fructose 6-phosphate (Fr6-P), whereas Arg-230 and Arg-238 might be involved in ATP binding (12). The PFK-2 domain contains a sequence (residues Arg-121–Asp-130 in the L isozyme), which shares sequence similarity with the so-called B motif defined by Walker et al. (13) as being important for ATP binding. This motif contains a conserved aspartate residue, which has been proposed to bind ATP via Mg²⁺. Indeed mutation of Asp-130 to Ala in PFK-2 greatly decreased the Kₘ for MgATP (3). Site-directed mutagenesis studies have identified some amino acid residues that are important in substrate binding. For example, Arg-104 (11), Arg-195 (12), and Arg-225 (11) in the L isozyme have been proposed to bind fructose 6-phosphate (Fr6-P), whereas Arg-230 and Arg-238 might be involved in ATP binding (12). The PFK-2 domain contains a sequence (residues Arg-121–Asp-130 in the L isozyme), which shares sequence similarity with the so-called B motif defined by Walker et al. (13) as being important for ATP binding. This motif consists of a conserved aspartate residue, which has been proposed to bind ATP via Mg²⁺. Indeed mutation of Asp-130 to Ala in PFK-2 greatly decreased the Kₘ for MgATP (3).

The PFK-2 domain also contains a sequence (residues Gly-48–Thr-55 in the L isozyme) similar to the A motif defined by Walker et al. (13) for nucleotide-binding proteins. This motif is conserved in the PFK-2 domain of all known PFK-2/FBPase-2 sequences. Interestingly, a second A motif is present in the FBPase-2 domain (residues Gly-274–Lys-280 in the L isozyme) and could play a role in the stimulation of FBPase-2 activity by GTP (15). The A motif consensus sequence (GX₄GK(S/T)) was first described in adenylyl kinase (16). It has the following structure: (β-strand)-GX₄GK(S/T)(α-helix) with the glycine-containing loop forming a tight turn between the β-strand and the α-helix. Mutagenesis of Gly-48 in the L isozyme decreased PFK-2 activity at least 100-fold without affecting FBPase-2 (12). Whether this loss of PFK-2 activity resulted from a change in structure or the affinity for MgATP is not known. The three-dimensional structures of adenylylate kinases from various organisms revealed the importance of the “invariant” Lys
in ATP binding (17). Site-directed mutagenesis of Lys-21 to Met in adenylyl kinase decreased the Kcat 1000-fold (18). The Ser/Thr residue adjacent to the conserved Lys has also been implicated in nucleotide binding. From the crystal structures of p21^ras (19), EF-Tu (20), and RecA (21), Mg^2+ has been proposed to bind the \( \beta \) and \( \gamma \) phosphates of ATP and is itself bound by the OH group of the Ser/Thr residue in the A motif.

We have studied the effect of site-directed mutagenesis of Lys-54 and Thr-55 in the rat recombinant L (rL) isozyme on the kinetic properties of PFK-2 and FB Pase-2 and particularly on nucleotide binding in the 2-kinase domain. Lys-54 was mutated to Met (K54M mutant), because the sizes of the side chains are similar and because the same mutation in adenylyl kinase drastically decreased Kcat (18). Thr-55 was first mutated to Val (T55V mutant), because a similar mutation (Thr to Ile) in F1-ATPase (23) was performed by site-directed mutagenesis. The mutant oligonucleotides were: wild type rL, 5'-GGACACTGTACCTGCCCATTAC--------TGAGCCCTTTTCAAGTGATC-3', 5'-GG-GAGCAACTGTACCTGCCCATTAC(CAT) 6TGAGCCCTTTTCAAGTGATC-3', T55V, 5'-CGGAGCACTGTACCTGCCCATTAC-3', TSSS, 5'-CGGAGCACTGTACCTGCCCATTAC-3', and TSSC, 5'-CGGAGCACTGTACCTGCCCATTAC-3'. The mutagenesis was checked (28), and the mutants were introduced into the pPFK-2/FB Pase-2 expression vector pET/PFK2L (11).

The introduction of a polynucleotide encoding six histidine residues (H6) in front of the termination codon of the rL PFK-2/FB Pase-2 cDNA was performed by site-directed mutagenesis. The mutant polynucleotides were synthesized and purified as described (24). The catalytic subunit of the cyclic 3',5'-AMP-dependent protein kinase was purified (25) and assayed with histone IIA as a substrate (26).

Circular dichroism—Circular dichroism spectra were recorded in a J-600 UV/Vis spectrophotometer of the recombinant L isozyme (+ 6 His residues) of 311,171 Da.

Measurement of Ligand Binding by Fluorescence—The binding of ATP, ADP, and their Mann derivatives were measured by fluorescence. Spectra were recorded at 25 °C with continuous magnetic stirring in a SLM 8000C spectrofluorimeter (SLM Aminco) with spectral bandwidths of 2 and 4 nm for excitation and emission, respectively. The nucleotides were added stepwise in small volumes (1–2 \( \mu \)l) to 7–15 \( \mu \)l of recombinant (H6)-tagged wild type or mutant proteins in 1 ml of buffer containing 50 mM Hepes, pH 7.5, 100 mM KC1, 0.5 mM phosphate, 1 mM dithiothreitol in the presence of the absence of 5 mM MgCl2. Upon excitation at 295 nm, eight scans were scanned at 350 to 500 nm. Corrections were made for buffer fluorescence and for the inner filter effect of the nucleotides from the quenching of N-acetyltryptophanamide (4 \( \mu \)M) under the same conditions (30).

The high affinity binding of 5'-AMP accelerated by a 0.5–1 mg/ml solution were introduced with a long-tipped micropipette into the quartz cell (path length, 0.2 mm), which had been cleaned with acetonitrile and dried under a stream of N2. Spectra were recorded (0.2 mm/s) between 195 and 250 nm with a bandwidth of 2 nm. For each preparation, five to six scans were recorded and averaged using the computer software. Mean residue ellipticities were calculated using the computer software taking the protein concentration, measured by the modified Lowry method described below and taking the protein purity. Molecular masses were determined in triplicate for each preparation. The mean value was used. The correction for the circular dichroism spectra was calculated using the computer software. The mean residue ellipticities were calculated using the computer software.
nase doubled FBPase-2 activity as expected (Table III). Therefore, the phosphorylation site was still able to interact with the FBPase-2 domain in all the four mutants. This suggests that the conformation of the phosphorylation domain, which contains Ser-32 and which is N-adjacent to the GXXGKT motif, is unaffected in all the mutants.

PFK-2 activity was undetectable in the K54M, T55V, and T55C preparations (Table I). We calculated that PFK-2 activity was less than 0.01 milliunits/mg of protein when measured at pH 7.1 with 5 mM MgATP and 2 mM Fru-6-P, which are optimal conditions for the wild type-(H)₆. This represents a more than 5000-fold decrease in PFK-2 activity compared with the wild type-(H)₆ measured under identical conditions.

By contrast with the three inactive mutants, the T55S mutant showed a slight increase (1.7-fold) in PFK-2 Vₘₐₓ and a higher Kₘ for MgATP (4-fold) without change in the Kₘ for Fru-6-P. Moreover, the T55S mutant was 10-fold less sensitive to the inhibition by MgADP when IC₅₀ was measured with MgATP concentrations equivalent to the Kₘ for Fru-6-P. However, the T55S mutant was 10-fold less sensitive to the inhibition by MgADP when IC₅₀ was measured with MgATP concentrations equivalent to the Kₘ for Fru-6-P. This would indicate that in this mutant, the increase in Vₘₐₓ results from an increased rate of ADP release. This is consistent with a compulsory order ternary complex mechanism (9).

We also tested whether Mant-ATP was a substrate for PFK-2. Although the Vₘₐₓ was 10 times lower with Mant-ATP than with authentic ATP, the low Kₘ of 260 mM was comparable with the Kₘ measured by fluorescence quenching (Fig. 1 and see below). This indicates that for wild-type-(H)₆ PFK-2, Mant-ATP binds to the active site of the 2-kinase domain and is a substrate in the PFK-2 reaction.

Substrate Binding—The fact that PFK-2 activity was undetectable in the K54M, T55V, and T55C preparations meant that kinetic measurements could not be used to investigate the effects of the mutations on the Kₘ for the MgATP. Therefore, substrate binding was measured by quenching of the enzyme intrinsic fluorescence, taking advantage of the fact that the rL isozyme contains four tryptophane residues, one being located in the NH₂-terminal domain (Trp-16), one in the PFK-2 domain (Trp-67), and two in the FBPase-2 domain (Trp-301 and Trp-322). Upon excitation at 295 nm, the fluorescence emission spectrum of the wild-type-(H)₆ enzyme was maximal at 325 nm as illustrated in Fig. 2A. All the mutants displayed a similar spectrum (not shown).

The addition of micromolar concentrations of ATP or ADP, with or without magnesium, to the wild type-(H)₆, T55S, and T55C mutants caused a maximum quenching of fluorescence...
Kinetic properties of PFK-2 and FBPase-2 in the phosphorylated recombinant rL PFK-2/FBPase-2 with and without the polyhistidine tag. To obtain phosphorylated forms (phospho-), aliquots (50 μg) of recombinant PFK-2/FBPase-2 preparations were incubated with 20 micromolar of the cyclic 3',5'-AMP-dependent protein kinase for 30 min at 30°C as described (39). Measurements of kinetic parameters were then performed as described in Tables I and II. Individual values are shown.

| Enzymes               | PFK-2 Activity (milliunits/mg) | FBPase-2 Activity (milliunits/mg) | K_m for MgATP (μM) | K_m for Fru-6-P (μM) |
|-----------------------|--------------------------------|-----------------------------------|---------------------|----------------------|
| phosho-rL             | 4                              | ND*                               | 410                 | 21.25                |
| phosho-rL-(H)_6       | 5                              | ND                                | 320                 | 23.27                |
| phosho-K54M-(H)_6     | NM*                            | ND                                | NM                  | 22.26                |
| phosho-T55V-(H)_6     | NM                             | NM                                | NM                  | 24.28                |
| phosho-T55C-(H)_6     | NM                             | NM                                | NM                  | 19.21                |
| phosho-T55S-(H)_6     | 13, 14                         | ND                                | 400, 446            | 20.22                |

*NM, not measurable.

**ND, not determined.

Figure 1. Saturation curves of PFK-2 activity of wild type PFK-2/FBPase-2-(H)_6 for MgMant-ATP and MgATP. PFK-2 was assayed with 2 mM Fru-6-P as described in Table I. The V_max was 6.3 milliunits/mg protein with MgMant-ATP and 65 milliunits/mg protein with MgATP.

Figure 2. MgMant-ATP binding to the (H)_6-tagged wild type PFK-2/FBPase-2 monitored by intrinsic and extrinsic fluorescence. A. Fluorescence resonance energy transfer between protein tryptophan residue(s) and bound MgMant-ATP. Wild type PFK-2/FBPase-2-(H)_6 was excited at 295 nm in the absence (a) or the presence (b) of 200 nm MgMant-ATP, and the emission fluorescence was scanned between 310 and 480 nm. B. High affinity binding of MgMant-ATP to the (H)_6-tagged wild type enzyme as monitored by spectral enhancement of the extrinsic fluorescence of the analog. MgMant-ATP binding was measured upon excitation at 350 nm. The extrinsic fluorescence of 100 nm MgMant-ATP was measured in the absence of protein (c) or in the presence of 72 nm PFK-2/FBPase-2-(H)_6 (a). The differential spectrum corresponds to bound MgMant-ATP (b).

binding was observed whether the Mg^2+ complex was present or not. This could indicate that the partial charge compensation is not present because of the different nature of the side chain in serine and cysteine. The addition of Mant-ATP lowered the intrinsic fluorescence on excitation at 295 nm, whereas a new peak appeared at 432 nm (Fig. 2A). The fact that the K54M and T55V mutations abolished the high affinity binding component for Mant-ATP and Mant-ADP suggests that it is associated with PFK-2. The low affinity component probably reflects nucleotide binding to the FBPase-2 domain.

The high affinity binding of Mant-ATP and Mant-ADP to the wild type-(H)_6 enzyme was also monitored by the enhancement of extrinsic fluorescence. Upon excitation at 350 nm, Mant-ATP in solution exhibited a characteristic fluorescence emission with a maximum at 445 nm (Fig. 2B). The addition of the wild type-(H)_6 enzyme markedly increased the probe fluorescence and blue-shifted the maximum to 438 nm. The differential spectrum corresponding to bound Mant-ATP was maximal at 432 nm, indicating that a 13 nm blue shift was produced on binding (Fig. 2B). The saturation curve for Mant-ATP was determined by enhancement of fluorescence at 432 nm (not...
enzymes. Kd function of MgMnGTP or MgMnGADP concentration. PFK-2/FBPase-2-(H)6 (72 nM dimer) was mixed with increasing concentrations of either MgMnGTP (20 nM to 80 μM, panel A) or MgMnGADP (10 nM to 24 μM, panel B). Intrinsic fluorescence was measured upon excitation at 295 nm for wild type (○), K54M (●), and T55V (□) mutant enzymes.

![Graph A](image)

![Graph B](image)

FIG. 3. Quenching of PFK-2/FBPase-2 intrinsic fluorescence as a function of MgMnGTP or MgMnGADP concentration. PFK-2/FBPase-2-(H)6 (72 nM dimer) was mixed with increasing concentrations of either MgMnGTP (20 nM to 80 μM, panel A) or MgMnGADP (10 nM to 24 μM, panel B). Intrinsic fluorescence was measured upon excitation at 295 nm for wild type (○), K54M (●), and T55V (□) mutant enzymes.

The calculated apparent Kd was 60 nM. The same spectral change was produced by Mant-ADP with a comparable maximal increase at 432 nm, whereas the affinity was 2-fold greater (Kd = 30 nM).

The Kd for Mant-ATP binding to the high affinity site (50 nM) was about 3 orders of magnitude lower than the Kd for ATP (180 μM) of PFK-2 activity in the wild type-(H)6 enzyme. A large increase in affinity for Mant analogs compared with unmodified nucleotides has also been observed for a number of proteins (30, 31, 35–37).

In conclusion, the results of fluorescence quenching suggest that Lys-54 is involved in ATP binding in the 2-kinase domain. The fact that the high affinity site for Mant-ADP was also abolished in the K54M mutant suggests that Lys-54 binds at least the β-phosphate of ATP. Binding results obtained with the T55V mutant should be regarded with caution, because the structure of the enzyme was locally disrupted (see below). From binding experiments made with the T55S and T55C mutants, one can conclude that these mutations did not affect nucleotide binding, because both mutants have the same Kd values for the nucleotides.

Structure—Because the Lys and Thr residues of the Walker A motif form the start of an α-helix after the Gly-containing loop (17, 19, 20), mutation of Lys-54 and Thr-55 could disrupt the structure. We therefore studied the secondary structures of the K54M, T55V, T55S, and T55C mutants by circular dichroism. The spectrum of the wild type-(H)6 protein was similar to that of the K54M, T55S, and T55C mutants, whereas that of the T55V mutant was notably different (not shown). The spectra were analyzed, and the ratio (R) of the mean residue ellipticities at 208 and 222 nm were calculated (Table V). This ratio has often been taken as an index of helical structures and as a comparative tool for estimating the α-helical content (38). Mutations of Lys-54 to Met and of Thr-55 to Ser and Cys had no effect on the R values compared with the wild type-(H)6. By contrast, the T55V mutant had a different R value, representing a loss of α-helical content. Therefore, the abolition of the high affinity binding site for ATP in the T55V mutant could be due to a structural change in the ATP binding site. Nevertheless, mutation of Thr-55 into Ser or Cys did not affect the structure, and the binding affinity for nucleotides was likewise unaffected.

Conclusions—The site-directed mutagenesis studies reported in this paper show that Lys-54 and Thr-55 do not play the same role in the PFK-2 domain. The fact that nucleotide binding was abolished in the K54M mutant suggests that Lys-54 binds the β- or γ-phosphate of the nucleotide. Lys-54 in PFK-2 might play a similar role to Lys-21 in adenylate kinase, which wraps around the glycine-containing loop and hydrogen bonds to the β- and γ-phosphates of ATP. In adenylate kinase, it also participates in catalysis by stabilizing the penta-coordinate transition state during in-line transfer of the γ-phosphate

### TABLE IV

| Enzyme          | MgMnGTP-ATP | Mant-ATP | MgMnGTP-ADP | Mant-ADP | MnGTP-ATP | ATP | MnGTP-ADP | ADP |
|-----------------|-------------|----------|-------------|----------|-----------|-----|-----------|-----|
| rl-(H)6         | 53          | 297      | 23          | 20       | 44        | 296 | 76        | 60  |
| K54M-(H)6       | NM*         | NDb      | NM          | ND       | ND        | ND  | ND        | ND  |
| T55S-(H)6       | 38          | 82       | 15          | 15       | 38        | 47  | 39        | 46  |
| T55C-(H)6       | 43          | 77       | 26          | 31       | 51        | 96  | 51        | 73  |
| T55V-(H)6       | NM          | ND       | NM          | ND       | ND        | ND  | ND        | ND  |

* NM, not measurable.

** ND, not determined.

### TABLE V

| Enzymes          | Number of preparations | R (222/208) |
|------------------|------------------------|-------------|
| rl-(H)6          | 4                      | 0.92 ± 0.02 (4) |
| K54M-(H)6        | 2                      | 0.91 ± 0.05 (4) |
| T55S-(H)6        | 3                      | 1.70 ± 0.25 (3) |
| T55C-(H)6        | 1                      | 0.91–0.93    |
| T55V-(H)6        | 1                      | 0.88–0.90    |

Circular dichroism parameters for the wild type-(H)6 enzyme and mutant preparations.
of ATP (17). By analogy, Lys-54 in PFK-2 would thus be involved in ATP binding and in catalysis, as indicated by the drastic change in substrate binding and $V_{\text{max}}$.

Thr-55 is more likely to be involved in catalysis because mutation of Thr-55 to Cys abolished PFK-2 activity without affecting the binding affinity of the enzyme for nucleotides. The role of Thr-55 in catalysis is also illustrated by the consequences of the mutation of Thr into Ser on the kinetic properties. This residue might participate in ADP release as suggested for the F$_1$-ATPase, where the same mutation also increased the $V_{\text{max}}$ by modifying the rate of ADP dissociation (23), or it could play a role in catalysis by stabilizing the transition state via hydrogen bonding to the $\gamma$-phosphate of ATP or by co-ordinating Mg$^{2+}$.

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