Identification of transient intermediates in the bisphosphatase reaction of rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase by ^{31}P -NMR spectroscopy

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^{31}P -NMR spectroscopy was used to identify reaction intermediates during catalytic turn-over of the fructose-2,6-bisphosphatase domain (Fru-2,6- P_2 ase) of the bifunctional enzyme, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. When fructose-2,6-bisphosphate (Fru-2,6- P_2) was added to the enzyme, the ^{31}P -NMR spectrum showed three resonances in addition to those of free substrate: the phosphohistidine (His-P) intermediate, the C-6 phosphoryl group of fructose-6-phosphate bound to the phosphoenzyme, and phosphate generated by the hydrolysis of substrate. Direct analysis of the alkali-denatured phosphoenzyme intermediate by ^1H - ^{31}P heteronuclear multiple quantum-filtered coherence spectroscopy confirmed the formation of 3-*N*-phosphohistidine. Binding of fructose 6-phosphate to the bis-

phosphatase was detected by a down-field shift and broadening of the C-6 phosphoryl resonance. The down-field shift was greater in the presence of the phosphoenzyme intermediate. Inhibition of Fru-2,6- P_2 hydrolysis by fructose 6-phosphate and Fru-2,6- P_2 was shown to involve binding of the sugar phosphates to the phosphoenzyme. This study provides new experimental evidence in support of the reaction mechanism of Fru-2,6- P_2 ase and suggests that the steady-state His-P intermediate exists primarily in the E-P·fructose 6-phosphate complex. These results lay a solid foundation for the use of ^{31}P -NMR magnetization transfer studies to provide an in-depth analysis of the bisphosphatase reaction mechanism.

INTRODUCTION

The bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6PF-2-K/Fru-2,6- P_2 ase) was first isolated from rat liver and subsequently from other rat tissues and other vertebrate species [1–6]. This 55 kDa bifunctional enzyme catalyses both the synthesis and degradation of fructose-2,6-bisphosphate (Fru-2,6- P_2) [2–4,6–9]. There are five tissue-specific mammalian isoforms which all have kinase and bisphosphatase active sites that are spatially distinct, with the kinase domain comprising the N-terminal region and the bisphosphatase domain comprising the C-terminal region of the enzyme [1,6,8]. In liver, the bifunctional enzyme, via regulation of gene expression and phosphorylation state, controls the cellular level of Fru-2,6- P_2 and is responsible for directing hepatic metabolic flux toward either glycolysis or gluconeogenesis, while in extrahepatic tissues that do not produce glucose, it contributes to the control of glycolysis [1,2,7,8].

The bisphosphatase domain catalyses the hydrolysis of Fru-2,6- P_2 to fructose 6-phosphate (Fru-6-P) and P_i via a covalent phosphoenzyme intermediate [9–13]. Steady-state kinetic studies indicate that Fru-6-P is a non-competitive inhibitor of Fru-2,6- P_2 hydrolysis [14]. Fru-6-P is postulated to stabilize the E-P intermediate by formation of an E-P·Fru-6-P complex that excludes H_2O from the active site, thereby preventing hydrolysis of the phosphoenzyme intermediate [13,15–17]. Likewise, hydrolysis of E-P is also inhibited by excess substrate, presumably

by binding at the active site in an E-P·Fru-2,6- P_2 complex [11,17]. The other product, P_i , is a competitive inhibitor with respect to Fru-2,6- P_2 and has also been postulated to relieve inhibition by Fru-2,6- P_2 and Fru-6-P by competing for binding to E-P [11,16].

Most of the published steady-state kinetic studies of the Fru-2,6- P_2 ase reaction have been done with the rat liver bifunctional enzyme, yet the bisphosphatase domain is active when expressed separately [18]. Recent reports confirm that the separate bisphosphatase domain and the bisphosphatase of the intact bifunctional enzyme have similar kinetic parameters and identical reaction mechanisms [19]. The phosphoacceptor in the bisphosphatase was identified as His-258 after reaction of the enzyme with [2- ^{32}P]Fru-2,6- P_2 , protease digestion and peptide sequencing of the ^{32}P -labelled protein [10–12]. A comparison of the pH stability of the ^{32}P -labelled protein and phosphohistidine (His-P) suggested that the intermediate was 3-*N*-phosphohistidine [12]. However, it was not possible to unequivocally distinguish between 1-*N*-phosphohistidine and 3-*N*-phosphohistidine because both are acid labile, differing only in the rate of hydrolysis. In contrast, spectroscopic analysis by NMR permits direct observation of the phosphoenzyme intermediate and allows its definitive structural identification. The use of NMR methodologies to elucidate structure/function relationships in the bisphosphatase domain had been precluded because of the large amount of protein required. However, the induction and purification schemes reported herein provide the necessary quantities of protein. This

Abbreviations used: 6PF-2-K/Fru-2,6- P_2 ase, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; Fru-2,6- P_2 ase, fructose-2,6-bisphosphatase domain; Fru-6-P, fructose-6-phosphate; Fru-2,6- P_2 , fructose-2,6-bisphosphate; His-P, phosphohistidine; IPTG, isopropyl β -D-thiogalactoside; HMQC, heteronuclear multiple quantum-filtered coherence spectroscopy; pH*, pH uncorrected for the deuterium isotope effect; E-P, phosphoenzyme intermediate; PMSF, phenylmethanesulphonyl fluoride.

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manuscript reports the use of ^{31}P -NMR spectroscopic methods to identify and monitor the transient intermediates during the turnover of the bisphosphatase domain and thus provides a basis for continued study of the bisphosphatase reaction mechanism by these techniques.

EXPERIMENTAL

Expression and purification of the rat liver Fru-2,6-P₂ase

The wild-type bisphosphatase domain (residues 250–470, 24 kDa) of the rat liver 6PF-2-K/Fru-2,6-P₂ase and the Lys-356-Ala point mutant were expressed in the BL21(DE3) strain of *Escherichia coli* (Novagen) using the pET3a vector system originally developed by Studier and Moffat [20]. The construction of the plasmid vectors, sequencing of the DNA and expression of both the separate bisphosphatase domain and the mutant have been reported previously [19]. Synthesis of the target protein was induced by addition of isopropyl β -D-thiogalactoside (IPTG) (Jersey Glove and Lab) to a final concentration of 400 mM. The cells were harvested after 18–24 h by centrifugation, resuspended in buffer B_p (20 mM Na₂HPO₄/2 mM EDTA/2 mM dithiothreitol/500 μ M phenylmethanesulphonyl fluoride (PMSF)/2.5 mg/l leupeptin, pH 7.5) with 2 mg/ml lysozyme (Sigma), and lysed by three freeze/thaw cycles. The DNA was removed by treatment with DNase I at 50 μ g/ml (Sigma) in the presence of Mg²⁺. The cell extract was cleared by centrifugation and applied to a Q Sepharose Fast Flow (Pharmacia) column equilibrated with buffer B_p. The column was eluted with a linear gradient of 0–500 mM NaCl. Active fractions were detected by the E-P assay of El Maghrabi et al. [21], pooled, and precipitated at 70 % (NH₄)₂SO₄. The precipitate was dissolved in buffer D_p (20 mM Na₂HPO₄/2 mM EDTA/1 mM DTT/500 μ M PMSF/10 % glycerol, pH 7.5), applied to a Sephacryl S-200 HR (Pharmacia) column and eluted isocratically. The protein was detected and pooled as above and applied to a small Q Sepharose Fast Flow column and eluted with a linear gradient from 0 to 500 mM NaCl. The enzyme was then dialysed into the appropriate buffer. The purified bisphosphatase domain and the Lys-356-Ala mutant were homogeneous as judged by SDS/PAGE (results not shown). Typical yields of pure enzyme were 50 mg/litre of culture.

NMR sample preparation

Aqueous NMR samples were prepared by concentration of the stock protein by ultrafiltration (Amicon YM10 or Centricon 10). Deuterium-exchanged samples were prepared by precipitation at 70 % (NH₄)₂SO₄, dissolution in deuterium buffer, dialysis against the same, and concentration by ultrafiltration. Protein concentrations were between 0.9 and 3.6 mM as determined by the bicinchoninic acid assay (Pierce). Low-field experiments used 50 mM Tris/300 mM KCl at pH 8.0. High-field samples were in 99.9 % $^2\text{H}_2\text{O}$ containing 50 mM Tris d₁₁ (Cambridge Isotope Laboratories)/300 mM KCl, pH* 8.0 (* indicates the pH is uncorrected for the deuterium isotope effect).

NMR acquisition and processing parameters

The NMR spectra were obtained using either a Bruker MSL 200 NMR spectrometer operating at 81.01 MHz for ^{31}P or a Bruker AM500 NMR spectrometer operating at 202.46 MHz for ^{31}P and 500.13 MHz for ^1H . Spectra from the MSL 200 were obtained at the indicated temperatures using a 10 mm broadband probe with a 60 ° pulse length of 9.4 μ s, a 4.0 s relaxation delay and a spectral width of 5500 Hz with 4000 complex data points. All 81.01 MHz spectra were processed with exponential apo-

dization and line broadening values are specified in the Figure legends. All ^{31}P chemical shifts are referenced to 85 % H₃PO₄ at 0.00 p.p.m. and most utilized an external standard, methyl phosphonate, which has a well-defined quartet centred at 29.22 p.p.m. relative to 85 % H₃PO₄. For ^1H , the residual water resonance was assigned as reference at 4.78 p.p.m. at 25 °C. The $^2\text{H}_2\text{O}$ lock signal was provided either as $^2\text{H}_2\text{O}$ in the samples or as 99.9 % $^2\text{H}_2\text{O}$ as solvent in the coaxial insert. CYCLOPS phase cycling was employed on the MSL 200 to achieve quadrature detection. All spectra of the native enzyme were acquired without proton decoupling owing to the temperature-sensitive nature of the sample.

The pulse sequence and experimental set-up for the heteronuclear correlation through the indirect detection of X-nuclei followed published procedures [22–26]. The heteronuclear multiple quantum-filtered coherence spectroscopy (HMQC) experiment was carried out on a Bruker AM500 NMR spectrometer at 25 °C using a 5 mm reverse broadband probe that had a 90 ° pulse length of 15 μ s for ^{31}P and of 41 μ sec for ^1H . The in-phase HMQC spectrum was acquired with a refocusing interval equal to the preparation interval, $1/2J = 40$ ms. Quadrature detection was used in both dimensions and 32 t_1 values were acquired with 2048 complex data points in ω_2 , a relaxation delay of 1.2 s and 1744 scans per t_1 point. The time domain signal was multiplied by a sine bell of 45 ° in ω_2 and 66 ° in ω_1 which had been zero-filled to 512.

Other experimental procedures

The Fru-2,6-P₂, Fru-6-P and P_i were added as aliquots from 100 mM stock solutions in water to the NMR tube and mixed by inversion. The 81.01 MHz ^{31}P spectra were obtained in 256–1024 transients depending on the protein and ligand concentrations. The time-course of the hydrolysis reaction was monitored by sequential acquisition of single-pulse spectra. Spectra were integrated by use of a digital scanner (Hewlett-Packard) and suitable software (SigmaScan). For the HMQC experiment the denatured phosphoenzyme was prepared by addition of a 2:1 molar ratio of substrate to enzyme, mixing by inversion for 1–2 min and addition of 40 % NaO²H in $^2\text{H}_2\text{O}$ (Cambridge Isotope Laboratories) to a final concentration of 1 M O²H[−] producing a final pH* of 13.5. All ^{31}P -NMR samples were free of phosphate by the criterion of a blank ^{31}P -NMR spectrum obtained before the experiment.

RESULTS

Resonance assignments of the ^{31}P -NMR spectrum

Figure 1A shows the ^{31}P -NMR spectrum of Fru-2,6-P₂ with two resonances at 3.36 and −1.18 p.p.m. that have been previously assigned respectively to the C-6 and the C-2 phosphoryl groups [27]. When an excess of Fru-2,6-P₂ was added to the bisphosphatase, the ^{31}P -NMR spectrum consisted of five resonances as shown in Figure 1. Substrate resonances were assigned by direct comparison to Figure 1A with the C-6 phosphoryl resonance of free Fru-6-P overlapping this resonance in the substrate [27]. The presence of the C-2 phosphoryl resonance (−1.18) indicates that the reaction is still proceeding and the three new resonances detected at 5.91, 1.88, and −7.11 p.p.m. must represent P_i and reaction intermediates. The resonance at 1.88 p.p.m. in Figure 1B was assigned to P_i by comparison with a standard spectrum of P_i in the same buffer and by spiking of the sample with the addition of a small amount of pH 8.0 phosphate buffer.

Assignment of the low-field (5.91 p.p.m.) resonance was facilitated by study of the interaction of Fru-6-P with the enzyme. The

Figure 1 ^{31}P -NMR spectrum of the bisphosphatase domain and substrate at pH 8.0

Trace A is 10 mM Fru-2,6- P_2 and B is 1.2 mM bisphosphatase with 7.2 mM Fru-2,6- P_2 . All spectra were obtained at 21 °C within 1 h, were the result of 1024 transients and employed 7 Hz line-broadening during processing.

^{31}P -NMR spectrum of Fru-6-P given in Figure 2A showed a single resonance at 3.36 p.p.m. When a substoichiometric amount of Fru-6-P was added to the bisphosphatase at pH 8.0, the resulting spectrum, given in Figure 2B, showed that the resonance was shifted down-field by 2.27 p.p.m. and broadened by approximately 10 Hz as a consequence of Fru-6-P binding to the enzyme. As seen in Figure 2C, when excess Fru-6-P was added, both free and bound resonances were observed.

Additional support for assignment of the 5.91 p.p.m. resonance to enzyme-bound Fru-6-P was obtained with the Lys-356-Ala mutant, that has a greatly diminished affinity for Fru-6-P [17,19]. The ^{31}P -NMR spectrum of the Lys-356-Ala mutant in the presence of substoichiometric Fru-6-P revealed a single resonance at 3.36 p.p.m. as shown in Figure 2D. The absence of the low-field resonance at 5.91 p.p.m. in the spectrum of this mutant, acquired under the same conditions that showed only this resonance with the wild-type enzyme, confirmed the assignment to an enzyme-bound C-6 phosphoryl group.

Comparison of the low-field region of the spectra in Figures 1B and 2C indicated that the bound C-6 resonance detected during enzymic turnover, resonates 0.28 p.p.m. further down-field than that detected when the enzyme was incubated with Fru-6-P. The chemical shift of all other resonances remained constant whether the enzyme was incubated with Fru-6-P or Fru-2,6- P_2 . The difference in the chemical shift of the bound C-6 resonance was related to the presence or absence of the His-P intermediate and provided the basis for distinction between ligand bound to E and bound to E-P.

The high-field resonance (−7.11 p.p.m.) in Figure 1B was assigned as the E-P intermediate on the basis of the spectra shown in Figure 3. The spectrum in Figure 3A was obtained

Figure 2 ^{31}P -NMR spectra of the bisphosphatase domain in the presence of Fru-6-P at pH 8.0

Trace A is 3.4 mM Fru-6-P; B is 1.7 mM wild-type bisphosphatase with 0.85 mM Fru-6-P; C is 1.7 mM wild-type bisphosphatase with 3.4 mM Fru-6-P; and D is 2.3 mM of the Lys-356-Ala mutant with 1.2 mM Fru-6-P. All spectra were obtained at 21 °C and represent 1024 transients, except D which was obtained with 400 transients; all employed 7 Hz line-broadening during processing.

within 45 min after addition of substrate to the bisphosphatase at a 2:1 molar ratio, and it shows the high-field resonance along with P_i , the free and bound C-6 resonances, and a very small amount of remaining substrate C-2 phosphate. The spectrum in Figure 3A shows some heterogeneity of the chemical shift of the free C-6 resonance. Figure 3B shows the spectrum of the same sample 3.5 h after addition of the substrate, when the reaction has gone to completion as judged by the loss of the −7.11 p.p.m. resonance and absence of the C-2 resonance. Although it is well established that Fru-2,6- P_2 ase utilizes a covalent His-P intermediate during catalysis [10–12], these results identify for the first time a transient ^{31}P resonance at −7.11 p.p.m. that is due to the His-P intermediate and can be related to the turnover of the enzyme. The ^{31}P resonance assignments are summarized in Table 1.

Identification of the intermediate as 3-*N*-phosphohistidine

Covalent phosphorylation of the bisphosphatase by Fru-2,6- P_2 was confirmed by the presence of a single resonance (−4.10 p.p.m.) in the ^{31}P -NMR spectrum of phosphoenzyme after addition of 1.0 M NaOH and removal of excess substrate and products by ultrafiltration (results not shown). The ^{31}P chemical shift determined from this alkali-denatured sample corresponds exactly to that reported by Gassner et al. for 3-*N*-phosphohistidine at pH 9.3 [28] and is also consistent with other reports [29–31]. Consistent with His-258 being the phospho-acceptor, no E-P intermediate was detected with a His-258-Ala mutant [13] that did not catalyse Fru-2,6- P_2 hydrolysis (results not shown).

Figure 3 The transient nature of the -7.11 p.p.m. resonance in the ^{31}P -NMR spectrum of the bisphosphatase domain in the presence of substrate at pH 8.0 and 19°C

Trace A shows 1.2 mM bisphosphatase at about 45 min after addition of 2.4 mM Fru-2,6- P_2 (512 transients) and B shows the same sample but after 3.5 h. Trace B was acquired from 1024 transients and both were processed with a line-broadening of 7 Hz. Note that the position of the C-2 resonance designated in trace A was determined from other spectra.

Conclusive evidence for 3-*N*-phosphohistidine was provided by the ^1H - ^{31}P HMQC spectrum acquired immediately after the bisphosphatase reaction was terminated by quickly increasing the solution pH* to 13.5. Cross-peaks in this experiment will occur only at the position of protons that are scalar coupled to ^{31}P . The HMQC spectrum is shown in Figure 4 and it can be seen that in the ^1H dimension between 3.5 and 4.0 p.p.m., cross-peaks were observed from Fru-2,6- P_2 and Fru-6-P. The only other cross-peaks observed fall in the aromatic region of the ^1H spectrum where resonances are observed that are diagnostic of the H-2 and H-4 protons of histidine. The observation of cross-peaks for both side chain histidine protons is consistent only with phosphorylation at N-3. (The intensity of the cross-peaks observed in the HMQC experiment is determined by the magnitude of the heteronuclear coupling constant and the value used for the $1/2J$ preparation period for this experiment. Phosphorylation at N-3 gives rise to three bond PNCH coupling into both H-2 and H-4 protons.

Time-course of Fru-2,6- P_2 hydrolysis

The hydrolysis of Fru-2,6- P_2 by the bisphosphatase domain was monitored by addition of an 8:1 molar excess of substrate to the enzyme and acquisition of a series of ^{31}P -NMR spectra. A plot of the integrated area of the C-2, P_i and His-P resonances versus time is given in Figure 5, which shows a decrease in the C-2 resonance with a concomitant increase of the P_i resonance. The existence of steady-state His-P is evident from the relative constancy of this resonance over the entire course of the reaction. After 10 h the His-P resonance disappeared (results not shown),

Table 1 A summary of the ^{31}P NMR chemical shifts of the substrate, products and reaction intermediates

All chemical shifts were determined in 50 mM Tris/300 mM KCl, pH 8.0, and were relative to 85% H_3PO_4 at 0.00 p.p.m., except that for the His-P in alkali-denatured enzyme which was in 1.0 M NaOH (pH 13.5).

Species	^{31}P Chemical shift (p.p.m.)
Fru-6-P	3.36
C-6 of Fru-2,6- P_2	3.36
C-2 of Fru-2,6- P_2	-1.18
C-6 bound in E·Fru-6-P	5.53
C-6 bound in E-P·Fru-6-P	5.91
C-6 bound in E-P·Fru-2,6- P_2	5.91
His-P in native enzyme	-7.11
His-P in alkali-denatured enzyme	-4.10
P_i	1.88

Figure 4 Identification of 3-*N*-phosphohistidine as the His-P intermediate in the bisphosphatase reaction

The heteronuclear ^1H - ^{31}P multiple quantum coherence spectrum of bisphosphatase acquired in-phase on a Bruker AM500 NMR instrument. The preparation period ($1/2J$) of 40 ms ($J = 12.5$ Hz) was used, and 32 t_1 data-points were acquired at 25°C . The sample contained 3.3 mM bisphosphatase and 6.6 mM Fru-2,6- P_2 . After 1–2 min of reaction, the protein was denatured by addition of sufficient NaOH to bring the final $[\text{OH}^-]$ to 1 M, pH* 13.5.

Figure 5 Time-course of Fru-2,6-P₂ hydrolysis at 21 °C in 50 mM Tris/300 mM KCl, pH 8.0

Inhibition of E-P hydrolysis by Fru-6-P at pH 8.0. The plot was generated by monitoring the hydrolysis of 26.4 mM Fru-2,6-P₂ in the presence of 3.3 mM bisphosphatase domain by a series of spectra acquired at 16 min intervals (256 transients) that were digitally integrated: ●, C-2; ○, P₁; and □, His-P. All spectra were processed with 5 Hz line-broadening. The solid lines drawn through the data are qualitative only.

reflecting substrate depletion and breakdown of the E-P intermediate as shown in Figure 3.

Inhibition of Fru-2,6-P₂ase by Fru-6-P

The effect of excess Fru-6-P on Fru-2,6-P₂ hydrolysis was also investigated by ³¹P-NMR. Spectra were generated by titrating a sample of bisphosphatase domain with substrate at pH 8.0 in the presence of a 6:1 molar excess of Fru-6-P relative to enzyme. After addition of Fru-2,6-P₂, inhibition by Fru-6-P was evident from the very low P₁ resonance in these spectra (results not shown). However, the His-P resonance was present, indicating that excess Fru-6-P did not inhibit E-P formation, but rather hydrolysis of His-P.

Inhibition of Fru-2,6-P₂ase by Fru-2,6-P₂

Inhibition of E-P hydrolysis by excess substrate was also investigated by ³¹P-NMR spectroscopy. The comparison of ³¹P spectra generated by addition of substrate at an 8:1 molar ratio relative to enzyme, and the titration of the bisphosphatase to the same level of substrate, suggests the occurrence of substrate inhibition at pH 8.0. The same total amount of substrate was added to both samples and spectra were acquired 120 min after the initial substrate addition. Inhibition of Fru-2,6-P₂ hydrolysis was apparent from the relatively low amount of P₁ observed in either spectrum, and hydrolysis was estimated to represent less than half of the added substrate, even after 2.0 h (results not shown). The sample to which a high level of substrate was added initially exhibited a greater degree of substrate inhibition, as estimated by the relative amount of P₁ released. Presumably this is because the high concentration of Fru-2,6-P₂ was present over the course of the entire experiment. Earlier reports have suggested that substrate inhibits by preventing hydrolysis of the His-P [18,19], and the His-P resonance was present in both spectra, which is consistent with the formation of an E-P·Fru-2,6-P₂ complex.

DISCUSSION

The experiments described in this report provide for the first time the identification of a His-P enzyme intermediate in the actively turning over Fru-2,6-P₂ase domain. Previous reports only demonstrated labelling of the enzyme upon incubation with [2-³²P]Fru-2,6-P₂ and enzyme denaturation. The ¹H-³¹P HMQC experiment reported here is the first definitive evidence that the phosphoenzyme intermediate in the Fru-2,6-P₂ase reaction is 3-*N*-phosphohistidine. This result is significant because the methods used by Pilkis et al. [12] to identify the His-P intermediate are subject to misassignment of the phosphoacceptor due to migration of the phosphate during hydrolysis of the labelled protein [32] and could not completely distinguish between 1-*N*- and 3-*N*-phosphohistidine. The HMQC experiment allowed direct observation of the His-P and therefore provides a more reliable determination of the identity and structure of the intermediate. The His-P intermediate was observed at -7.11 p.p.m. in the active enzyme. The transient nature of this resonance was demonstrated by the loss of this resonance upon completion of the hydrolysis reaction.

This report also provides the first evidence of specific, non-covalent and stoichiometric binding of Fru-6-P to the bisphosphatase domain. The appearance of two distinct resonances when Fru-6-P is in excess implies that exchange of Fru-6-P between the free and bound states is slow on the NMR timescale. The E·Fru-6-P complex is strongly favoured at pH 8.0, since when ligand and enzyme were present in equimolar amounts, only the bound resonance was detected. The ³¹P-NMR data are consistent with Fru-6-P occupying a specific site in the E·Fru-6-P complex and it is likely that this is the same site that the sugar phosphate occupies in the E-P·Fru-6-P complex. Previous studies have shown that Fru-6-P is a non-competitive inhibitor of the reaction by formation of an E-P·Fru-6-P complex in which Fru-6-P occupies the active site [16]. This view was supported by lack of detectable binding of Fru-6-P to the Lys-356-Ala mutant, which has a *K_m* for Fru-2,6-P₂ and *K_i* for Fru-6-P 1000-fold higher than those of the wild-type enzyme [17,19].

The observation of an E·Fru-6-P complex suggests the possibility of competitive inhibition by Fru-6-P, however no experimental evidence exists for this type of inhibition. It is likely that competition between Fru-6-P and Fru-2,6-P₂ for the active site is not observed because of the high affinity of the enzyme for substrate and the rapid formation of E-P [11,13].

The reaction mechanism shown in Figure 6 was derived from previous steady-state kinetic analyses of the bifunctional enzyme [11,13,15-17] and the results of the ³¹P-NMR experiments reported here. The reaction profile for the hydrolysis of Fru-2,6-P₂ (Figure 5) provides clear evidence for the existence of a steady-state with respect to the transient reaction intermediate, yet according to Figure 6, the His-P can exist in one of four possible states. Which of these states is responsible for the His-P resonance in the ³¹P-NMR spectra was determined from the dependence of the resonant frequency of the bound C-6 group upon the source of the ligand. When Fru-6-P was added to the enzyme the down-field shift of the C-6 resonance can only be due to the E·Fru-6-P species. The greater down-field shift produced when the C-6-bound resonance was derived from addition of Fru-2,6-P₂, may be related to either E·Fru-2,6-P₂, E-P·Fru-6-P or E-P·Fru-2,6-P₂. The presence of a significant level of E·Fru-2,6-P₂ is unlikely because previous work demonstrated that steady-state levels of His-P were attained within 80 ms after addition of substrate [11,13] and hence the lifetime of this species was too short to be detected by NMR. Therefore the chemical shift variation in the C-6-bound resonance was related to the presence of the E-P

Figure 6 Postulated reaction mechanism of the Fru-2,6-P₂ase reaction

intermediate. However, the ³¹P-NMR results do not distinguish between E-P · Fru-6-P and E-P · Fru-2,6-P₂. Comparison of the inhibition constants for Fru-6-P (0.16 μM) and Fru-2,6-P₂ (3.1 μM) reported by Li and co-workers [16,17,19], suggests that Fru-6-P is a more potent inhibitor of the bisphosphatase than Fru-2,6-P₂. These values were determined from experiments using substrate:enzyme ratios of 20–200:1 [16,17,19] as opposed to the 2–8:1 ratios used for the ³¹P-NMR experiments. Therefore, under the conditions used for the NMR experiments, it is likely that the C-6-bound resonance detected during turnover is due primarily to Fru-6-P in the E-P · Fru-6-P complex. The His-P resonance detected must also be due to this complex because the C-6-bound and His-P resonances appear with equal intensities in Figure 1B.

Further support for this interpretation is provided by the observation that the loss of the C-2 resonance from the ³¹P-NMR spectral series of the enzyme during turnover precedes that of the His-P resonance. Therefore, binding of substrate and formation of E-P were fast relative to the hydrolysis of the His-P, thus indicating that the rate limiting step in the bisphosphatase reaction is subsequent to E-P formation. In fact, the view of Stewart et al. [11] that dissociation of E-P · Fru-6-P is the rate limiting step, is supported by the observation that excess Fru-6-P inhibits His-P hydrolysis rather than His-P formation. Fructose-6-phosphate probably inhibits the reaction by competing with H₂O for E-P and clearly an excess of Fru-6-P will be able to compete more effectively (Figure 6). In a similar manner, E-P · Fru-2,6-P₂ acts as a 'dead-end' complex, since Fru-2,6-P₂ must dissociate before the His-P can be hydrolysed [19]. The ³¹P-NMR results imply that the E-P · H₂O complex is unstable and rapidly hydrolyses. The level of E · P_i is predicted to be low as no perturbation of the P_i resonance was detected in the presence of bisphosphatase domain, implying that interaction of P_i with the enzyme is weak, even though P_i has been shown to be a competitive inhibitor with respect to Fru-2,6-P₂ [16,17].

Fructose-2,6-bisphosphatases are members of a larger family of enzymes, including phosphoglycerate mutases and acid phosphatases, that catalyse their reactions via a His-P intermediate [32]. Analysis of their reaction mechanisms by ³¹P-NMR spectroscopy has not been possible due to a requirement for high concentrations of protein and the transient nature of the E-P intermediates. While this is the first reported use of ³¹P-NMR spectroscopy to analyse the reaction of an enzyme of this enzyme family, His-P intermediates have been studied in a number of other enzymes including pyruvate phosphate dikinase [29], the

phosphoenolpyruvate-dependent phosphotransferase involved in sugar transport in bacteria [28,33,34], succinyl Co-A synthetase [35] and ATP citrate lyase [36]. These enzymes catalyse their reactions via a His-P intermediate that exists at equilibrium in the absence of a second phosphoacceptor [8,29,33,34,36]. In contrast, catalysis of the reverse bisphosphatase reaction has not been observed [2], and thus establishment of chemical equilibrium is not possible. Furthermore, since the Fru-2,6-P₂ase reaction has no known inhibitors other than products and substrate, nor is it dependent on metal ions [2,9], it cannot easily be maintained with the E-P and reactants at equilibrium. These aspects of the bisphosphatase reaction complicate the acquisition and analysis of ³¹P-NMR data. However, the very low turnover number of the enzyme and the substrate/product inhibition, permit the use of experimental conditions wherein a steady-state E-P · Fru-6-P complex can be maintained for up to 9 h and provide a basis for the design and interpretation of magnetization transfer studies of the reaction mechanism.

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