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The Allosteric Effect of Fructose Bisphosphate on Muscle Pyruvate Kinase Studied by Infrared Spectroscopy

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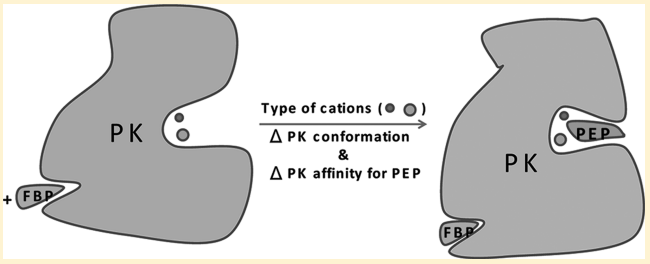
Supporting Information

ABSTRACT: Pyruvate kinase exhibits allosteric properties. The allosteric effect of fructose 1,6-bisphosphate (FBP) on phosphoenolpyruvate (PEP) binding to rabbit muscle pyruvate kinase (PK) in the presence of various ions (Mg^{2+}, Mn^{2+}, K^+, Na^+) was studied by attenuated total reflection infrared spectroscopy in combination with a dialysis accessory. The experiments indicated that FBP binding causes conformational changes of PK that are of the same order of magnitude as those of PEP binding. The conformational change of PEP binding to PK/Mg^{2+}/K^+ in the presence of FBP was about twice as large as in its absence, which is tentatively ascribed to a higher occupancy of the closed state. The affinity for PEP increased in the presence of Mg^{2+} and K^+. No such effects were observed with the other ion combinations Mn^{2+}/K^+ and Mg^{2+}/Na^+ or in D_2O (with Mg^{2+}/K^+), and therefore we did not detect an allosteric effect on PEP binding under these conditions.



INTRODUCTION

Muscle pyruvate kinase (M1-PK) is one of the four isoenzymes of mammalian PK. All of these PK isoenzymes catalyze the conversion of phosphoenolpyruvate (PEP) and magnesium adenosine diphosphate (Mg^{2+} ADP) to pyruvate and magnesium adenosine triphosphate (Mg^{2+} ATP) in the presence of divalent (D^{2+}) and monovalent (M^+) cations.[1,2]

$$\text{PEP} + \text{Mg ADP} + \text{H}^+ \xrightarrow[D^{2+}, M^+]{\text{PK}} \text{pyruvate} + \text{Mg ATP}$$

Rabbit muscle pyruvate kinase is a homo tetramer, and each 530 residue monomer consists of four subunits. Each subunit folds into four domains: A, B, C, and N.[3,4] The active site lies in the pocket between domains A and B of the same subunit. Pyruvate kinase requires divalent cations[5−7] and monovalent cations[8−11] for activity. The active sites of different subunits adopt different conformations that result in different degrees of closure of the cleft that forms the active site.[4,12] One of eight sites is closed, and Mg^{2+} coordinates to L-phospholactate and protein. The other sites are open, and coordination between Mg^{2+} and L-phospholactate is lost. Open and closed conformations have also been observed in magnetic relaxation measurements, indicating an open conformation in the binary complex with Mn^{2+} but closed conformations in the ternary complexes with PEP (PK/Mn^{2+}/PEP) and L-phospholactate (PK/Mn^{2+}/PL).[13]

PK shows allosteric regulation. Fructose 1,6-bisphosphate (FBP) acts as an allosteric activator, whereas phenylalanine, alanine, or ATP acts as allosteric inhibitors for the binding of PEP or ADP or of both substrates.[14−16] FBP binds to domain C of PK away from the active site. In the crystal structure of yeast PK,[17] FBP binds between residues 428−434 (helix) and 512−520 (loop) which are homologous to the residues 475−480 and 512−520 of muscle PK.[18] The possible FBP binding regions are residues 430−436, 454, 480−489, and 510−520.[18] A crystal structure of M1-PK with bound FBP is not available. According to the crystal structure of M2-PK/FBP,[19] we model the interactions between FBP and M1-PK (Figure 1). According to the model, the P1 phosphate group of FBP binds to residues Trp^{481}, Arg^{488}, Gly^{517}, and Lys^{430}. The P2 phosphate group binds to residues Lys^{430}, Gly^{519}, Thr^{431}, Ser^{433}, and Ser^{518}. O3 of FBP binds to Arg^{516} and Gly^{513} and O4 of FBP to Phe^{520}, Gly^{517}, and Thr^{521}. Trp^{481} is rotated toward 512−520 loops.[18,19]

Previous studies have shown that non-M1-PK isoenzymes have allosteric properties.[15,22] The situation is less clear for M1-PK where some studies did not detect an allosteric effect,[20,21] but others showed that M1-PK has allosteric properties.[15,23−26] The discrepancy might be due to an influence of pH and ions on the allostery of PK.[25] No study has been done on the effects of ions on PEP binding to M1-PK in the presence of FBP. In this study, we used *reaction-induced infrared difference spectroscopy* to study the effects of ions on structural changes when PEP binding to PK in the presence of FBP. We used the same approach which was used before by us,[27−29] where we studied the conformational changes of PK upon PEP binding, the interactions between PEP and PK,[27] and the effects of ions on PEP binding.[29] The PEP-induced conformational change observed in the amide I region of

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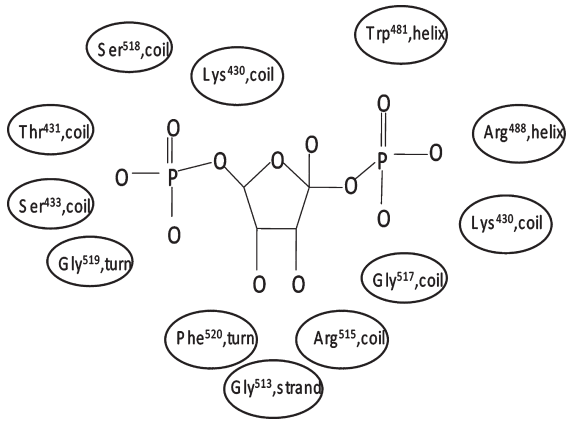
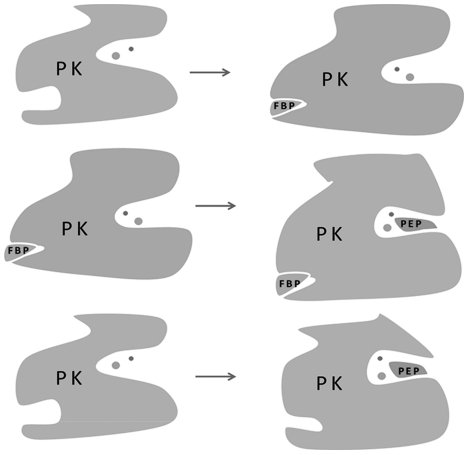


Figure 1. Schematic diagram of the FBP binding site of PK on the basis of crystal structures.[17−19] The indicated amino acids of PK interact via their side chains with FBP.

Scheme 1. Illustration of the Experiments Performed in the Present Study. Top Panel: FBP Binding to PK, Middle Panel: PEP Binding to the Complex of PK and FBP, Bottom Panel: PEP Binding to PK in the Absence of FBP



our spectra is thought to reflect the transition between open and closed forms of PK.[4,13,18,30] Interestingly, this change is significantly larger with Mn^{2+} bound to the catalytic site than with bound Mg^{2+}, which suggests that Mn^{2+} facilitates the adoption of the closed state when PEP binds. The present study focused on conformational changes of PK upon FBP binding as well as on the effect of bound FBP on the conformational changes of PK upon PEP binding in the presence of different ion combinations. Scheme 1 summarizes our experiments.

EXPERIMENTAL PROCEDURES

Materials. PK from rabbit muscle, monopotassium salt of PEP, FBP, MOPS (3-[*N*-morpholino]propanesulfonic acid), magnesium chloride hexahydrate, manganese chloride tetrahydrate, and deuterium oxide (99.9 atom % D) were purchased from Sigma. Tris-HCl was obtained from Angus. Potassium chloride and sodium chloride were obtained from Scharlau. Cellulose dialysis membranes CelluSep F3 of MWCO 12000−14000 were purchased from Orange Scientific, Belgium.

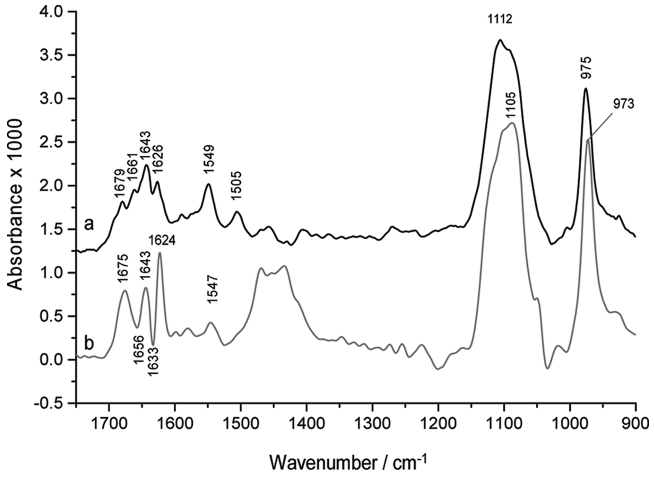


Figure 2. Infrared absorbance changes upon FBP addition to PK in the presence of Mg^{2+} and K^+. Binding of FBP to PK in H_2O (a) and in D_2O (b).

Methods. *PK Sample Preparation.* For difference spectroscopy, 0.8 mM rabbit muscle PK was prepared in 50 mM buffer (Tris-HCl + MOPS, pH, 7.5) containing 100 mM monovalent cations and 4 mM diavalent cations. Deuterated samples of PK were prepared in a similar way and the pD adjusted to 7.6. The pH meter reading was corrected by +0.4 to obtain pD. The PK sample was for 5 h in D_2O before the measurements.

FTIR Studies. Our ATR-dialysis setup has been described previously.[31] The PK sample was placed between the ATR reflection element and the dialysis membrane in the following way: A 2 μL drop of PK sample was placed on the ATR crystal, and another 4 μL were deposited as hanging drop on the dialysis membrane at the bottom of the reservoir. Then the sample compartment was closed by approaching the reservoir toward the ATR crystal. The solution in the reservoir was stirred with a small mechanical stirrer for fast equilibration.

FTIR spectra were recorded at 4 cm^{-1} resolution on a Bruker Vertex 70 FTIR spectrometer equipped with an HgCdTe detector. The experiments were performed at room temperature. PK was equilibrated by continuous diffusion of buffer and salts across the dialysis membrane. The amide II absorption of PK was similar in all experiments, indicating that the same amount of protein is sensed. The absorbance spectrum was recorded in regular intervals. Within 2−3 h, the protein absorption increased because the protein settled on the ATR crystal. After the absorption spectrum of the sample became time-independent, a 500 scan single beam spectrum (background spectrum) was recorded. Then 1 μL of FBP (50 mM, pH 7.5) was added to the 4 mL of solution in the reservoir after which 30 spectra in the absorption mode (150 scans each) were recorded for 6 min. During this time, FBP diffused into the sample compartment which led first to the formation of the PK/FBP complex and later to an increase of the free FBP concentration. The addition of FBP and spectra recording was repeated up to 3 times. Then after 20 min, when the absorption had become stable, a new background spectrum was recorded; PEP (50 mM, pH 7.5) was added to the 4 mL of solution in the reservoir, and spectra were recorded in a similar way. All spectra shown were recorded 84−144 s after the addition of ligand and normalized to the amplitude of the amide I signals in the 360 s spectrum which showed saturated signals of the binding induced conformational change. In this way a spectrum with saturated protein signals was obtained that mainly

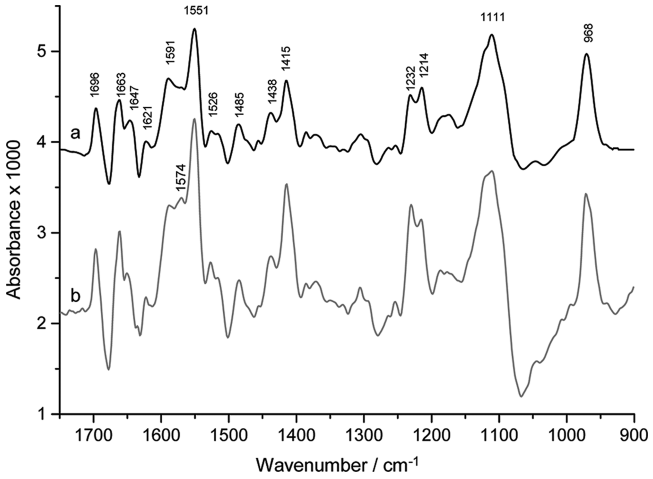


Figure 3. Infrared absorbance changes upon PEP addition to PK in the presence of Mg^{2+} and K^+. Binding of PEP to PK in the absence of FBP (a) and in the presence of FBP (b).

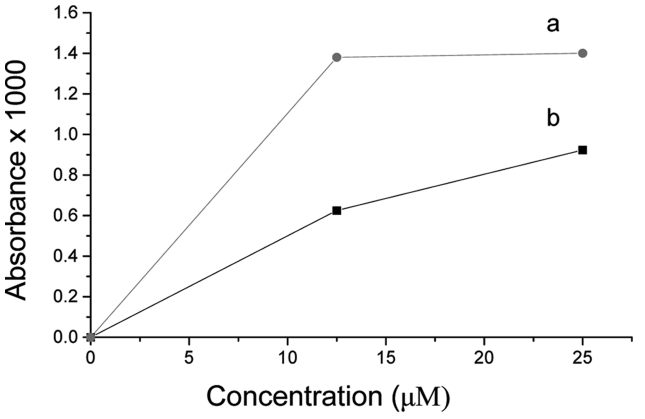


Figure 4. Extent of conformational change of PK as a function of the concentration of PEP in the presence of Mg^{2+} and K^+. The conformational change observed in the first addition of PEP was monitored by integrating the band at 1695 cm^{-1} [27] (a) with FBP and (b) without FBP.

shows bands of bound ligand and minimizes the contribution from the free ligand.

RESULTS

Infrared Difference Spectra of FBP Binding to PK. In this section, we discuss the difference spectra, which reveal the absorbance changes upon FBP binding to PK. Positive bands in the difference spectra are due to bound and free PEP as well as to protein absorption of the complex PK/FBP. Negative bands are due to protein absorption before binding. The absorption of passive groups cancels in the subtraction. Figure 2 shows the difference spectra of FBP binding to PK in H_2O (a) and D_2O (b) in the presence of Mg^{2+} and K^+ at pH 7.5. The amide I region (1700−1610 cm^{-1}) is sensitive to protein backbone structure and used for secondary structure analysis. We tentatively assign the band at 1679 cm^{-1} to coils or turns, the band at 1643 cm^{-1} to coils or β-sheets, and the band at 1626 cm^{-1} to β-sheets. The assignment of the 1679, 1643, and 1626 cm^{-1} bands to amide I vibrations is supported by the spectrum recorded in D_2O (Figure 2b) where these bands either slightly downshift or appear at the same position. The band at 1661 cm^{-1} is not observed in D_2O, which indicates a strong shift upon deuteration, which is in line with the assignment of this band to the side chain of Asn, Gln, or Arg. The assignment to the latter is supported by the appearance of a band at 1600 cm^{-1} in D_2O and reasonable because two Arg residues participate in binding.

The band observed at 1549 cm^{-1} in H_2O is tentatively assigned to the amide II vibration of the protein backbone because its intensity is reduced in D_2O. The 1505 cm^{-1} band not present in D_2O is indicative of a Trp vibration.[32] Because the side chain of Trp^{481} interacts with the phosphate (P1) of FBP,[17,19] we tentatively assigned the band to Trp^{481}. The bands observed at 1112 and 975 cm^{-1} are due to the asymmetric and symmetric PO_3^{2-} vibrations of FBP. We also did experiments of FBP binding to PK in the presence of other ion combinations such as Mg^{2+}/Na^+ and Mn^{2+}/K^+. We observed similar spectral changes with all ion conditions indicating that there is no ion-specific effect on FBP binding.

Binding of PEP to PK in the Presence of FBP and Different Monovalent and Divalent Cations. In this section, we will discuss the effect of the allosteric effector FBP on PEP binding to PK in the presence of different ion combinations. The respective difference spectra are compared to respective spectra in the absence of FBP from our previous work[27,29] where we discussed the secondary structure perturbation and bands of the bound PEP as well as effects of ions on such changes in detail. Figure 3 shows the difference spectra of PEP binding to $PK/Mg^{2+}/K^+$ (a) and $PK/FBP/Mg^{2+}/K^+$ (b) in H_2O. In the amide I region (1700−1610 cm^{-1}), we observed that the magnitude of secondary structure changes of PK upon PEP binding increased significantly in the presence of FBP. In the ATR absorption spectra of the samples before PEP addition, the amide II band had approximately the same intensity with and without FBP, indicating a similar amount of protein sensed by the infrared beam in both experiments and therefore that the increased amide I signals in Figure 3 with FBP must be due to a larger extent of the conformational change. Since PEP binding is associated with the transition between open and closed state of the subunits, it is expected that the infrared signals in the amide I region reflect this transition.[29]

With FBP, a lower concentration of the PEP substrate (12.5 μM) was required to get maximum signals of the conformational change than without FBP. This is demonstrated by the following observations:

(i) Adding 12.5 μM PEP in the absence of FBP, conformational changes of PK are observed in the first and the second PEP addition. This shows that a single addition of 12.5 μM PEP is not sufficient to occupy all binding sites. With FBP, the maximum signals were obtained already in the first addition, and no signals of conformational change were observed in the second addition, showing that 12.5 μM PEP saturates all binding sites in this case.

(ii) Adding 25 μM PEP, all conformational changes occur upon the first addition with and without FBP, showing that 25 μM is sufficient to saturate all binding sites under both conditions.

(iii) The signal of the conformational changes obtained in the first PEP addition is equal for 12.5 and 25 μM PEP in the presence of FBP but is smaller for the lower concentration of PEP in the absence of FBP. This is shown in Figure 4. These observations show that a lower concentration of PEP was needed to get saturating signals in the presence of FBP.

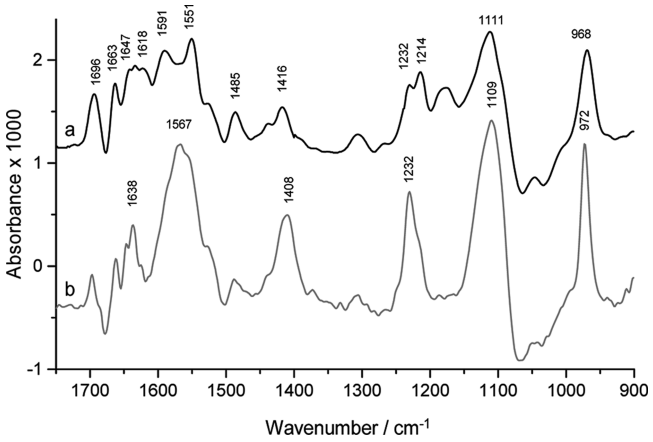


Figure 5. Infrared absorbance changes upon PEP addition to PK in the presence of Mg^{2+} and Na^{+}. Binding of PEP to PK in the absence of FBP (a) and in the presence of FBP (b).

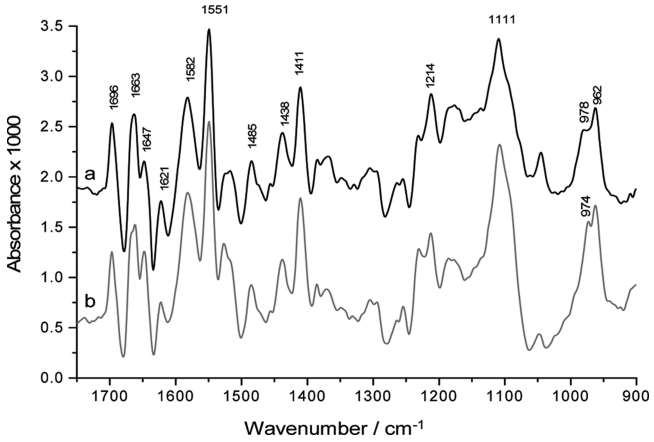


Figure 6. Infrared absorbance changes upon PEP addition to PK in the presence of Mn^{2+} and K^{+}. Binding of PEP to PK in the absence of FBP (a) and in the presence of FBP (b).

Apart from the two effects described above, the spectra with and without FBP were very similar. An exception is the band at 1574 cm^{-1} which is more obvious with FBP. This band is either an amide II band (because absent in D_2O) or a band of the bound carboxylate groups of PEP (because of the effect of isotopic labeling PEP in this region). An assignment to the amide II vibration is less likely, since the band is not observed in the spectrum with Mn^{2+} (see also Figure 6) which shows very similar band patterns and band positions in the amide I region. This gives evidence for a similar character of the PEP-induced conformational change with Mg^{2+} and with Mn^{2+}. An assignment of the 1574 cm^{-1} band to an amide II vibration would therefore indicate a conformational change that is only detectable in the amide II region which seems to be unlikely. Thus we prefer an assignment of the 1574 cm^{-1} band to a band of the antisymmetric carboxylate vibration of bound PEP, which together with a second band from this group at 1591 cm^{-1} indicates several binding modes for PEP in the presence of Mg^{2+}. The different relative amplitude of the 1591 and 1574 cm^{-1} bands with and without FBP therefore seem to indicate a slightly modified population of different binding modes due to allosteric regulation. Otherwise the strength of interactions between PEP and PK seem to be very similar as indicated by the identical band positions of the other carboxylate band (1415 cm^{-1}) and of the phosphate bands (1111 and 968 cm^{-1}).

The same experiments were done also in D_2O (see the Supporting Information); we observed that the band near 1662 cm^{-1} from helices or coils was perturbed slightly differently in the presence of FBP. This band is upshifted by 3 cm^{-1} with FBP which might indicate less H/D exchange and therefore a more rigid structure under these conditions. The spectra in D_2O have a similar amplitude with and without FBP which is as large as for the spectrum with FBP in H_2O. This together with the absence of the band at 1574 cm^{-1} seems to indicate that the conformation of the PK/PEP complex is more closed in D_2O in the absence of allosteric regulation and that there is no further effect of the allosteric regulator. Deuteration is known to alter kinetic constants of chemical reactions and therefore also equilibrium constants. Therefore an isotope effect on the equilibrium between open and closed conformations is a likely explanation for the above observations. In addition, the allosteric regulation of PK is known to be pH-dependent.[14,16,25] Since D_2O affects the pK_a of acidic groups,[33] the protonation state of these groups may be different in D_2O even if pD = pH, and therefore the pH dependency of the allosteric effect is different in H_2O and D_2O.

We did similar experiments with two further ion combinations: PEP binding to PK/FBP/Mg^{2+}/Na^{+} (Figure 5) and to PK/FBP/Mn^{2+}/K^{+} (Figure 6). In the presence of monovalent Na^{+}, we observed the same secondary structure changes with and without FBP; however with FBP the 1696 band is narrower, and the 1638 band is more prominent. With FBP, bands of free PEP are prominent at 1567, 1408, 1232, and 972 cm^{-1} indicating the presence of unbound PEP which shows that the PEP interaction with PK is weak in the presence of FBP and Na^{+}.

In the presence of the divalent ion Mn^{2+}, the PEP induced spectra with and without FBP are very similar. This demonstrates the excellent reproducibility of our spectra and a similar PEP binding mode. One difference between the spectra concerns the sideband of the symmetric PO_3^{2-} vibration which is observed at 974 cm^{-1} in the presence of FBP but at 978 cm^{-1} in the absence of FBP. This indicates that the PEP phosphate binding mode is different with and without FBP.

For both ion combinations, the magnitude of secondary structure change is similar with and without FBP. Therefore the allosteric effector does not have an influence on the adoption of open or closed conformation after PEP binding for these ion combinations.

DISCUSSION

Infrared studies give detailed understanding of conformational changes of proteins upon ligand binding. In our study we observed that the extent of conformational change was similar for FBP and for PEP binding to PK. Our spectra give evidence for an involvement of loops or turns, helical segments, and β-sheets in FBP binding to PK. According to the crystal structure, there are no ions present at the FBP binding site which is also reflected in our infrared study where we observed the same FBP binding spectra with different ions.

In our previous study[27,29] of PEP binding to PK, we concluded that more subunits are in the closed conformation with Mn^{2+} than with Mg^{2+} because of the larger spectral changes in the amide I region. In this work, we observed a similarly large magnitude of spectral changes with Mg^{2+} when FBP was present.

This indicate that more subunits are in the closed conformation when FBP is present than without FBP. Therefore, FBP binding shifts the equilibrium of the PK/PEP/Mg^{2+}/K^+ complex toward the closed conformation. This seems to increase PK's affinity for PEP since a similar magnitude of spectral changes was achieved by half of the PEP concentration needed without FBP. We did not observe an allosteric effect with Na^+ and Mn^{2+} and in D_2O.

Regarding the mechanistic origin of the allosteric effect, there is little information in the spectra apart from the observation that FBP binding changes the structure of the enzyme and that it facilitates the transition to the closed conformation. The binding site of PEP seems to be perturbed only very little since the bands of bound PEP are hardly affected, and the conformational change upon PEP binding is very similar with and without FBP. Only the experiments in D_2O revealed a different band position in the amide I region which could indicate a more rigid structure of a loop or helical region in the presence of FBP.

■ ASSOCIATED CONTENT

Supporting Information. Infrared absorbance changes upon PEP addition to PK in the presence of Mg^{2+} and K^+ in D_2O: spectra without FBP (a) and with FBP (b). This material is available free of charge via the Internet at http://pubs.acs.org.

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