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Effects of Ions on Ligand Binding to Pyruvate Kinase: Mapping the Binding Site with Infrared Spectroscopy

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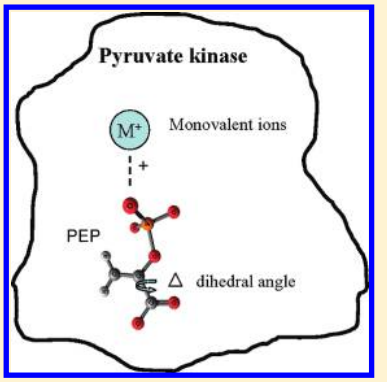
Supporting Information

ABSTRACT: The effects of mono- and divalent ions (Li^+, K^+, Na^+, Cs^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}) on the binding of phosphoenolpyruvate (PEP) to rabbit muscle pyruvate kinase (PK) were studied by attenuated total reflection infrared spectroscopy in combination with a dialysis accessory. The experiments assessed the structural change of the protein as well as the binding mode of PEP. They indicated that a signal at 1638 cm^{-1} assigned to a β sheet was perturbed differently with Na^+ as compared to the other monovalent ions. Otherwise, we obtained similar conformational changes in the presence of different monovalent cations, and therefore, it seems unlikely that the ion effects on activity are due to an ion effect on the structure of the PEP:PK complex. With different divalent cations, a particularly large conformational change was observed with Mn^{2+} and attributed to a more closed conformation of the complex. The absorption of bound PEP was also detected. The antisymmetric stretching vibration of the carboxylate group of bound PEP indicates a more homogeneous binding mode for Mn^{2+} compared to the other divalent ions. The symmetric stretching vibration depends on both monovalent and divalent ions, indicating that the dihedral angle $O-C_1-C_2-O$ is affected by the ions in the catalytic site. Little change in the bond strengths of PEP is observed, indicating that the PEP:PK complex does not adopt a reactive conformation.



INTRODUCTION

Pyruvate kinase (PK) is a key regulatory enzyme of the glycolytic pathway that catalyzes conversion of phosphoenolpyruvate (PEP) and magnesium adenosine diphosphate (Mg^{2+}ADP) to pyruvate and magnesium adenosine triphosphate (Mg^{2+}ATP) in the presence of diavalent (D^{2+}) and monovalent (M^+) cations.[1]

$$\mathrm{PEP} + \mathrm{MgADP} + \mathrm{H^+} \xrightarrow[\mathrm{D^{2+}, M^+}]{\mathrm{PK}} \mathrm{pyruvate} + \mathrm{MgATP}$$

The physiological reaction of pyruvate kinase proceeds in two chemical steps. The first step is phosphoryl transfer from PEP to ADP, which produces ATP and the enolate of pyruvate.[1] The second step is the addition of a proton to the enolate of pyruvate to produce pyruvate.[2]

The pyruvate kinase isozyme found in brain and muscles (M1-PK) is one of four mammalian isozymes of pyruvate kinase.[3] Rabbit muscle pyruvate kinase (EC 2.7.1.40, sequence code AAB61963) consists of four subunits of 530 residues each. Each subunit folds into four domains: A, B, C, and N. Domain N (residues 1−42) is a short helix−turn−helix motif, domain A (residues 43−115 and residues 224−387) is a parallel $(\beta/\alpha)_8$ barrel, domain B (residues 116−223) is a nine stranded β-barrel, and domain C (residues 388−530) is composed of five α-helices and a five stranded β-sheet. The active site lies in the pocket between domains A and B of the same subunit. Structures of the active site with bound ligands indicate that the side chains of Arg^{72} and Lys^{269} bind the phosphate group of PEP or the γ-phosphate of ATP.[4,5]

One subunit of pyruvate kinase has four metal binding sites,[6] it requires divalent cations[6−8] and monovalent cations[9−11] for activity. In the presence of divalent cations, pyruvate kinase is active in a medium containing the univalent cations K^+, Rb^+, or NH_4^+, but only weakly active in the presence of Na^+.[12,13] By use of NMR, Mildvan and Cohn[14] have observed that K^+ enhances the relaxation rate of water protons in the ternary complex PK:Mn^{2+}:PEP. This indicates that K^+ affects the conformation of the enzyme in the presence of PEP and Mn^{2+}. The crystal structure of rabbit muscle pyruvate kinase[15] provides insight into the roles of various protein groups in binding of divalent and monovalent cations. Of the four ion binding sites, two are in the PEP binding pocket; see Figure 1. Mn^{2+} [15] or Mg^{2+} [5] coordinates to the protein through the carboxylate side chains of Glu^{271} and Asp^{295} and K^+ coordinates to four protein ligands: Asn^{74}, Ser^{76}, Asp^{112}, and Thr^{113}. The latter binds only to the phosphate group of PEP whereas the former bridges carboxylate and phosphate groups of PEP.[5,15] In addition to these ions, PK binds also a divalent ion together with ADP.[16] The fourth metal ion binds outside the catalytic site.

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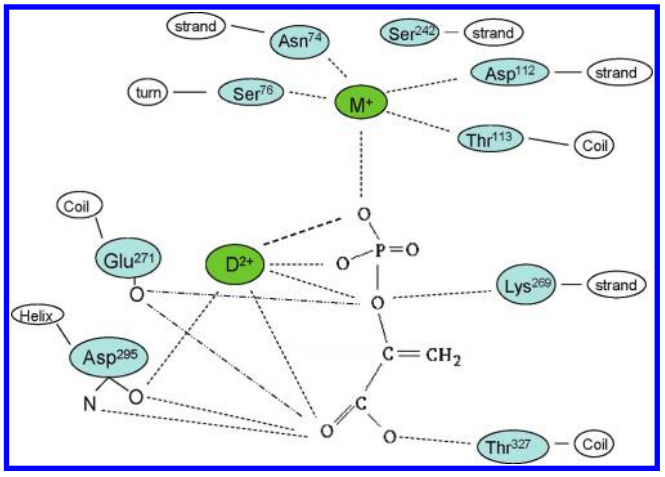

Figure 1. Schematic diagram of the coordination between monovalent (M^+) and divalent (D^{2+}) cations, pyruvate kinase, and phosphoenolpyruvate in the active site on the basis of crystal structures.[4,5,15] The side chain of amino acids of PK interaction with PEP and cations except Asp^{295} where PEP is also interact with nitrogen of main chain.

Crystal structures have been obtained with several ligands[4,5,15] but not with PEP due to the slow hydratase activity of pyruvate kinase. As a close analogue, L-phospholactate (PL) has been used.[5] Interestingly, the active sites of different subunits adopt different conformations that result in different degrees of closure of the cleft that forms the active site.[5] 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 NMR 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).[17]

The potential synergistic effects in the binding of divalent and monovalent cations to the protein[12,18] and the potential of their competitive binding to the enzyme add more layers of complexity.[19] To understand this complexity, we used *reaction-induced infrared difference spectroscopy* to study the effects of cations on structural changes when PEP binds to PK. The method is more sensitive than a comparison of infrared absorption spectra of samples with and without PEP and yields valuable information on ligand induced structural changes.[20−25] We used a variant of the attenuated total reflectance (ATR) technique in which a sample compartment in contact to the ATR crystal is separated by a dialysis membrane from a reservoir.[20,26,27] The absorption of the solution in the sample compartment is probed by the infrared beam. The protein is confined to the sample compartment. The ligands, on the other hand, can exchange freely between the sample compartment and the reservoir via the dialysis membrane. Therefore, the sample composition can be altered by adding a substance to the reservoir. We used the approach before to study PEP binding in presence of Mg^{2+}, K^+, and Na^+.[28] Interpretation focused on the binding mode of PEP and concluded only minor changes in PEP band properties upon binding. Here, we extend the study to include more ions, some of which are used as substitutes for the physiological ions Mg^{2+} and K^+ in several studies. Our results demonstrate that the binding mode of PEP depends on the cations. PEP binding to PK in presence of different cations also helps to map the binding site.

EXPERIMENTAL PROCEDURES

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

Methods. *PK Sample Preparation.* The purity of PK was checked by gel electrophoresis and the activity determined to be 500 units/mg proteins at pH 7.5 and at 37 °C. For difference spectroscopy 0.8 mM rabbit muscle PK was prepared in buffer (Tris−HCl + MOPS, pH, 7.5) containing 100 mM of monovalent cations and 4 mM of 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.[29]

FTIR Studies. Our ATR-dialysis setup has been described previously.[27] 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 was deposited as a 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 2 μL of PEP (50 mM, pH 7.5) was added to the 4 mL solution in the reservoir after which 20 spectra in the absorption mode (150 scans each) were recorded for 4 min. During this time, PEP diffused into the sample compartment, which led first to formation of the PEP:PK complex and later to an increase in the free PEP concentration. Addition of PEP and spectra recording was repeated up to three times. All spectra were recorded 84−144 s after the addition of ligand and normalized to the amplitude of the amide I signals in the 240 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 shows bands of bound PEP and minimizes the contribution from free PEP.

RESULTS AND DISCUSSION

Effects of Monovalent Ions on PEP Binding to PK. The spectra shown in the following are difference spectra, revealing the absorbance change due to binding of PEP. Positive bands in the difference spectra are due to bound and free PEP as well as to protein absorption of the complex. Negative bands are due to protein absorption before binding. Only active groups show in a

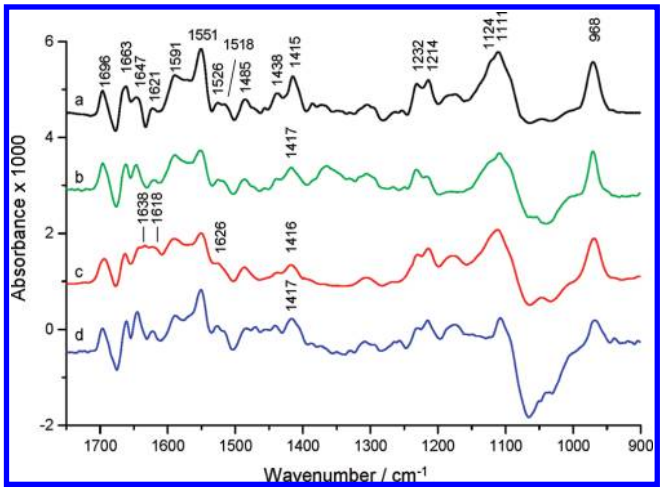

Figure 2. Infrared absorbance changes upon PEP addition to PK in the presence of Mg^{2+} and different monovalent cations in H_2O. The different monovalent cations are (a) K^+, (b) Li^+, (c) Na^+, and (d) Cs^+.

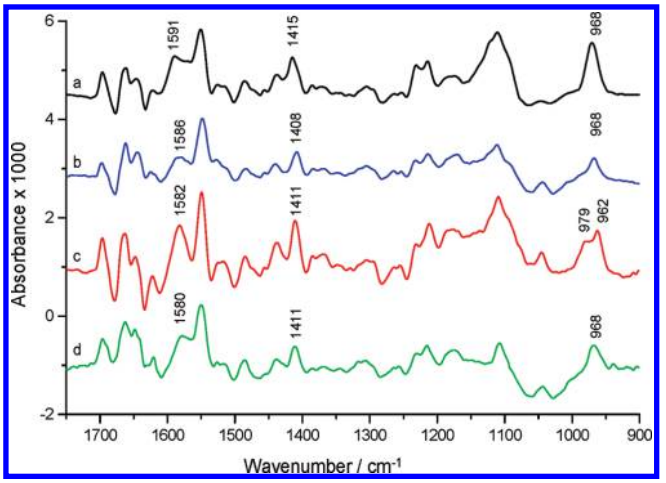

Figure 3. Infrared absorbance changes upon PEP addition to PK in the presence of K^+ and different divalent cations in H_2O. The different divalent cations are (a) Mg^{2+}, (b) Ca^{2+}, (c) Mn^{2+}, and (d) Zn^{2+}.

Table 1. Band of Bound PEP Determined on the Basis of Figures 2 and 3

cations	antisymmetric COO^- vibration (cm^{-1})	symmetric COO^- vibration (cm^{-1})	symmetric P−O vibration (cm^{-1})
Mg^{2+}, K^+	1591	1415	968
Mg^{2+}, Cs^+	1591	1417	968
Mg^{2+}, Na^+	1591	1416	968
Mg^{2+}, Li^+	1591	1417	968
Ca^{2+}, K^+	1586	1408	968
Mn^{2+}, K^+	1582	1411	962
Zn^{2+}, K^+	1580	1411	968

difference spectra. The absorption of passive groups cancels in the subtraction. The effects of different monovalent cations on PEP binding were studied. Figure 2 shows the difference spectra of PEP binding to PK:Mg^{2+} in H_2O in the presence of different monovalent ions K^+, Cs^+, Na^+, and Li^+. Our reference spectra are the spectra with the physiological ions K^+ and Mg^{2+}. The secondary structure perturbation and bands of bound PEP of this spectrum were analyzed in detail in our previous publication.[28] Here we observed that the symmetric carboxylate stretching band near 1415 cm^{-1} of the bound PEP changed spectral position in the presence of different ions. It was observed at 1415, 1417, 1416, and 1417 cm^{-1} in the presence of K^+, Cs^+, Na^+, and Li^+, respectively (Table 1). The bands of the bound PEP at 1591, 1111, and 968 cm^{-1} were not sensitive to the monovalent ion used.

Figure 1 indicates the secondary structure elements involved in the binding of PEP. The shape of the PEP binding spectra above 1650 cm^{-1} is similar in all spectra, which show that the α-helical and turn secondary structure elements are perturbed in a similar way by PEP binding in the presence of different monovalent cations. Differences are observed below 1650 cm^{-1} in the amide I region. The band observed at 1647 cm^{-1} may be tentatively assigned to the loop. This assignment is supported by the rather large shift in D_2O to 1639 cm^{-1} (Figure 1, Supporting Information). The band has different intensities for different monovalent ions. The β-sheet region perturbed differently in the presence of Na^+ than with the other monovalent cations. Two positive bands are observed at 1638 and 1618 cm^{-1} and the negative band at 1632 cm^{-1} is not observed in the presence of Na^+. This indicates that a β-sheet structure that participates in PEP binding is influenced by the monovalent ion and is different with Na^+ compared to the other ions. We observed that PEP induces a larger secondary structural change in the presence of the monovalent cations Cs^+ and K^+ and slightly smaller with Na^+.

The band at 1526 cm^{-1} which shifted to 1170 cm^{-1} in D_2O (see Figure 1, Supporting Information)[28] can be assigned to the symmetric deformation vibration of the NH_3^+ group of lysine.[30] We tentatively assign the band at 1518 cm^{-1} that did not shift in D_2O[28] to a tyrosine.[30] The observed perturbation of lysine and tyrosine residues supports the proposed model[31] of a possible role of Lys^{421} in intersubunit communication via a hydrogen bond with Tyr^{443} of an adjacent subunit. The model says that PEP binding induces a hydrogen bond between these residues. We observed only the 1526 cm^{-1} band with Na^+ whereas two bands at 1526 and 1518 cm^{-1} for the other monovalent cations. The deviating spectrum in the case of monovalent cation Na^+ may be due to the following reasons: (a) no H-bond formation upon PEP binding, (b) the H-bond is weaker, and (c) the H-bond is formed between lysine and another residue. In all cases the intersubunit contact is different with Na^+.

According to a previous study[13] pyruvate kinase is active in a medium containing Cs^+ and K^+ and less active with Na^+ and fails to function with Li^+; however, we observed that the PEP binding to PK is similar in the presence of different monovalent cations. In particular, the spectra obtained with K^+ and Li^+ are very similar with respect to the conformational changes of the protein and the interaction of the bound PEP. Therefore, it seems unlikely that the ion effect on activity is due to an ion effect on the structure of the PEP:PK complex.

Effects of Divalent Ions on PEP Binding to PK. The effects of the activating divalent cations on PEP binding were also studied. We did not observe PEP binding to PK in the presence of Cu^{2+} (spectra not shown) because of protein aggregation upon addition of PEP. Figure 3 shows the difference spectra of PEP binding to PK in the presence of different cations: Mg^{2+}, Ca^{2+}, Mn^{2+}, and Zn^{2+}. The character of the conformational change upon PEP binding is similar for all divalent cations studied, because the

spectral shape and the band positions in the amide I region are preserved, which indicates that the same secondary structural elements are perturbed in a similar way. A significant difference, however, is the larger amplitude of the signals with Mn^{2+}, which indicates a larger extent of conformational change, likely due to more subunits changing from open to closed conformation upon PEP binding.

The bands of the bound PEP in the carboxylate region as well as in the phosphate region changed significantly in the presence of different divalent cations (Table 1). We observed only one sharp band at 1582 cm^{-1} of the antisymmetric carboxylate stretching vibration of bound PEP in the presence of Mn^{2+}. In the presence of other divalent cations (Mg^{2+}, Ca^{2+}, and Zn^{2+}) this band of the antisymmetric carboxylate vibration has less intensity. Some of the absorption of this mode seems to occur around 1574 cm^{-1} where it fills the gap between the ~1591 cm^{-1} band and the adjacent band at 1550 cm^{-1}. This indicates several bands of bound carboxylate group in these cases due to several binding modes and in contrast a more homogeneous binding mode in the presence of Mn^{2+}, probably because more subunits are in the closed conformation. This is in line with the larger spectral changes in the amide I region observed for Mn^{2+} only. Also the band of the symmetric stretching vibration varies between 1408 and 1415 cm^{-1}. The variation is larger than for monovalent ions and its implication will be discussed in more detail below.

In our previous study[28] of PEP binding to PK in the presence of the divalent cation Mg^{2+}, we observed that the $-PO_3^{2-}$ symmetric stretching vibration band is observed 7 cm^{-1} lower for bound PEP than for free PEP. This $-PO_3^{2-}$ stretching vibration shifts more than 12 cm^{-1} from its position in aqueous solution in presence of Mn^{2+} and is observed at 962 cm^{-1}. A second band at 979 cm^{-1} develops later and is assigned to free PEP.[28] Bands of the asymmetric $-PO_3^{2-}$ stretching vibration are similar for Mg^{2+} and Mn^{2+}. Therefore, the additional 6 cm^{-1} shift for Mn^{2+} with respect to Mg^{2+}, indicates a lower average P−O stretching frequency and weaker terminal P−O bonds due to a stronger interaction with protein. In turn, the bridging P−O bond becomes stronger with Mn^{2+}, which makes the bond harder to cleave.[32] However, our quantitative evaluation of the bond strength with Mg^{2+} and K^+ shows that these effects are weak.[28]

Effects of Cations on the Dihedral Angle (O−C_1−C_2−O) of PEP. We observed that the symmetric carboxylate group vibration shifts with both monovalent and divalent cations (Table 1). Monovalent cations coordinate only with the phosphate group of a PEP analogue in the crystal structure[5] whereas a change is observed in the carboxylate region showing that the interaction between PEP and monovalent cations is not restricted to the local interaction but affects the entire binding mode. Divalent cations directly coordinate with the carboxylate group[5] and that may change symmetric carboxylate vibration due to the direct interaction.

In a quantum chemical study[33] we observed that a change of dihedral angle (O−C_1−C_2−O) significantly shifts the wavenumber of the symmetric stretching vibration of the PEP carboxylate group. The dihedral angle has been suggested to be correlated with the chemical reactivity of PEP.[34] In the case of the monovalent cations, we attribute the change in spectral position of the symmetric stretching vibration to a change in dihedral angle because (i) the antisymmetric stretching vibration is not affected and (ii) these ions do not coordinate directly to the carboxylate group. In our calculations,[33] two bands are predicted near 1400 cm^{-1} with nearly equal intensities, whereas only one (main) band is observed in the experiment. Nevertheless, because both predicted bands are similarly affected by the dihedral angle, we are able to estimate the dihedral angle changes. Around the equilibrium position, the spectral position increases by 0.30 cm^{-1} per degree angle change. Therefore, the observed deviations (1414.8−1417.1 cm^{-1}) for the monovalent ions correspond to a change in dihedral angle of 7°. We observed the smallest dihedral angle (O−C_1−C_2−O) with K^+ and the largest for Li^+ and Cs^+. Since PK is active with K^+ and Cs^+ but not with Li^+, the dihedral angle does not correlate with PK activity. There are several possible explanations: (i) the angle is not related to reactivity, (ii) the angle change is too small to be effective, or (iii) the reactive conformation is only adapted when ADP binds. Since the P−O bond strengths are only little affected by PEP binding,[28] option iii is the most straightforward explanation.

In the case of the divalent cations both carboxylate vibrations are affected by the nature of the ion and these ions coordinate directly to the carboxylate group. Therefore, the changes in the symmetric stretching vibration cannot solely be attributed to a change in dihedral angle; they might also be caused by a direct effect of ion coordination on bond properties and geometry of the carboxylate group.

Mn^{2+} Effect on the Conformation of the PK:PEP Complex. It is known that PK fluctuates between expanded and compact forms and binding of PEP favors the compact form[35] and brings the monovalent and divalent cation binding sites closer together.[17] These changes are reflected in our signals in the amide I region, which monitor changes of the protein backbone. The signals arise from distance and orientation changes between amide groups. Thus a rigid body domain movement as such is not visible in the spectrum, neither are flexible loops likely to give a large contribution because they give rise to broad bands before and after the reaction that largely cancel in the subtraction. Rather the signals originate from well-defined structural elements that are affected by the transitions. This interpretation is supported by the assignment of some of the signals in the amide I region to α-helices and β-sheets.[28] At any instant, the infrared spectrum represents a snapshot of the conformer population and thus is a weighted average of the spectra of the open form and of the closed form. If the equilibrium between these forms is shifted by PEP binding, the weight of the closed form increases and the difference spectrum will be due to the percentage of proteins that change conformation. If that percentage is larger under certain conditions, the amplitude of the amide I signals will increase without changing band positions and the shape of the spectrum. This was observed for PEP binding in the presence of Mn^{2+}, and therefore, we conclude that a larger fraction of enzymes shifts from open to closed conformation with Mn^{2+} as compared to Mg^{2+} and, therefore, that Mn^{2+} favors the closed conformation.

Infrared Spectroscopy as a Tool To Map Binding Sites of Proteins. This work highlights the advantages of infrared spectroscopy for the analysis of the binding between a substrate and a protein. First, the physiological substrate PEP could be studied, which has not been possible with slower high-resolution methods because of the slow catalytic cleavage of bound PEP. Second, a large number of structural parameters was obtained, both from the substrate and from the protein. All indicated that the structure of the PEP:PK complex is sensitive to the ions bound to the catalytic site. The signals of the protein backbone provide information on the nature and the extent of the

conformational change. The former demonstrated a particular interaction between protein and ligands in the presence of Na^+ (and Mg^{2+}), and the latter indicated a more closed conformation in the presence of Mn^{2+} (and K^+) than for other ion combinations tested. Several signals from amino acid side chains showed that their environment and interactions change upon PEP binding. One of them was tentatively interpreted in terms of a hydrogen bond that is differently affected by PEP binding in the presence of Na^+. Signals from the bound ligand are particularly valuable for basic research, drug development, and protein design. They assess the distortion of ligand structure upon binding and the strengths of the interactions with the protein and indicated in the present case that both PEP functional groups interact with the protein because they absorb at a spectral position different from that observed in an aqueous environment. A wide ligand absorption band indicates a spread of interaction strengths indicative of a heterogeneous binding mode. This was found here for most conditions, but not in the case of Mn^{2+}, which was unique in showing a well-defined band for the antisymmetric stretching vibration of the carboxylate group. A direct monitor of PEP structure is provided by the band of the symmetric stretching vibration, which is sensitive to the dihedral angle between $O-C_1-C_2-O$. This angle is sensitive to the ions in the catalytic site. However, none of the conditions studied led to a significant change in bond strengths. In summary, the various structural parameters deduced from infrared spectroscopy provide information far beyond the mere detection of binding and allow analyzing the binding mode in detail.

CONCLUSIONS

This work showed that bound mono- and divalent cations influence the binding of the substrate PEP to PK, in particular the binding-induced structural change of the protein and the conformation and interaction of bound PEP. The binding mode of PEP is affected more by different divalent cations than by monovalent cations, which shows that the divalent cation plays a more decisive role for PEP binding to PK than the monovalent ion. These findings highlight that it might be misleading to compare binding studies that were done under different ion conditions.

None of the bonds of PEP seem to be significantly distorted upon binding, in particular, not those of the reacting phosphate group. In addition, there is no obvious correlation between the spectral changes observed for a particular ion combination and the respective activity of the enzyme. This seems to indicate that the enzyme substrate complex does not adopt a reactive conformation upon PEP binding. These reactive ground state conformations have been named near-attack conformers[36] and have structures similar to that of the transition state in an enzymatic reaction. We conclude that near-attack conformers represent at most a minor fraction of the structural ensemble of the PEP:PK complex, undetectable by our study. This makes sense, because near-attack conformers will facilitate phosphate hydrolysis, which would waste the energy contained in PEP. Therefore, we hypothesize that near-attack conformers are formed only when the second substrate ADP is also bound, i.e., that ADP binding influences the bond parameters of the bound PEP.

ASSOCIATED CONTENT

Supporting Information. Infrared absorbance changes of PK upon PEP binding in the presence of K^+ and Mg^{2+} in H_2O (a) and D_2O (b). Two spectra performed under identical conditions, each the average of three experiments, to demonstrate the reproducibility. This material is available free of charge via the Internet at http://pubs.acs.org.

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DEDICATION

We dedicate this work to Maria Krasteva.

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