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Detection of Ligand Binding to Proteins through Observation of Hydration Water

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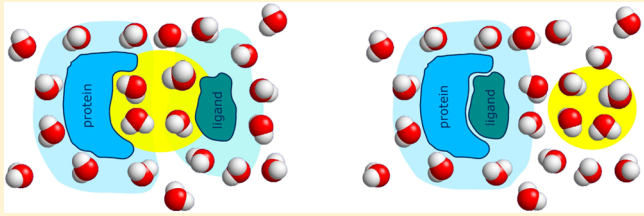
Supporting Information

ABSTRACT: Drug development is impeded by the need to design for each drug target a test that detects the binding of drug candidate molecules to the target protein. Therefore, a general method to detect ligand binding is highly desirable. Here, we present an observation toward developing such a method, which is based on monitoring a change in water absorption by infrared spectroscopy. Infrared spectroscopy has high sensitivity for water, and changes in its hydrogen bond pattern can be observed. We studied absorption changes of water upon the addition of phosphenolpyruvate or Mg^{2+} to pyruvate kinase. In each case, there is a decrease in the absorption of water in the 3000−3100 cm^{-1} region on the low wavenumber side of the OH stretching vibration when a ligand binds to the protein. Our results suggest that the weaker water absorption is due to the release of protein-bound water into bulk water during ligand binding. This observation has high potential for drug development as well as for basic research because it can lead to a general method for detecting molecular association events that (i) is label-free, (ii) works with both binding partners being in aqueous solution, and (iii) is based on a universal process that takes place in all binding events.



INTRODUCTION

The recognition of molecules through proteins is the basis of a vast number of cellular processes. Examples include hormone receptor interaction, binding of antigens to antibodies, recognition of substrates by enzymes and the allosteric regulation of proteins. These processes also guide the action of drugs and are therefore of eminent scientific and economic importance.

New drugs for the therapy of diseases are identified with the help of high throughput screening (HTS) procedures. Current screening techniques for individual drug targets are based on optical spectroscopy, in particular on fluorescence.[1] Common assays are based on fluorescent or absorbing substrates or on fluorescent ligands. They detect binding of drug candidates indirectly via competition with non-native ligands or substrates and often require extensive work for their development. The assay is often specific for a particular drug target or a class of drug targets and cannot easily be transferred to new drug targets. In addition, assay artifacts occur[2] and fluorescence approaches seem to be limited to the presently known fluorophore families.[3] These difficulties and limitations impede the development of new drugs and make developing the binding assays the limiting factor and the most cost-intensive step in the screening of drug candidate molecules. To circumvent the problems, universal, label-free methods are required that directly report on binding.[2] This necessity has recently become even more urgent because the number of potential drug targets has increased dramatically with the knowledge of the human genome. Addressing this need, we study here whether binding events can be detected via the expulsion of water from the hydration shell of proteins to bulk water when a ligand binds.

Hydration of proteins is important for their three-dimensional structure and their activity. The structure of bound water is altered from its structure in bulk water.[4,5] The first hydration shell around proteins is ordered: with high proton transfer rates, well resolved time averaged hydration sites, and coherent hydrogen bond patterns.[6] The hydrogen bonds between these water molecules and the protein are stronger and have longer lifetimes than in bulk water.[7−9]

Ligand binding to biomolecules induces changes in their structure, conformation, and hydration. When a ligand binds to a protein, water molecules must be expelled from the hydration shell of the binding site and transferred to bulk water.[4,5] In addition, protein conformational changes alter the hydration shell. Both effects lead to a change in the properties of the water molecules involved.[5−7,10−14]

Structural and dynamical properties of water can be monitored by infrared spectroscopy. The OH stretching vibration of water leads to a broad absorption band around 3400 cm^{-1} with several shoulders. These have been interpreted in terms of different hydrogen bonding strengths and intramolecular coupling between the two OH oscillators in a water molecule. While these properties influence the spectrum,

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they do not lead to distinct component bands according to recent simulations of the spectrum: hydrogen bonding shifts the absorption to lower wavenumbers for the OH stretching vibration of HOD in D_2O,[15] but different hydrogen bonded species have broad overlapping contributions. The situation is even more complicated in bulk water (H_2O in H_2O or D_2O in D_2O) due to intra- and intermolecular coupling of the OH (OD) stretching vibrations. The spectral effect of intramolecular coupling is less in bulk water than in the gas phase because fluctuations in hydrogen bonding make the intrinsic frequencies of the two OH oscillators different.[16] Intermolecular coupling leads to delocalized vibrations, which extend to the next-nearest neighbors.[17,18] In the low wavenumber region of the water signal, which is of interest here, the intermolecular OH stretching vibrations vibrate in phase.[18]

In summary, infrared spectroscopy has high sensitivity for water structure, and changes in hydrogen bonding are reflected in changes of the absorption of the OH stretching mode. For example, the absorption spectrum of bound water is clearly distinguishable from that of bulk water.[19] Our interest here was whether this absorbance change is detectable when a ligand binds to a protein, and we used the protein pyruvate kinase (PK) as a test case. The concept of monitoring the change in water absorption upon binding could be useful in the selection of lead compounds and in the optimization process during drug development.

EXPERIMENTAL PROCEDURES

Materials. PK (EC 2.7.2.40) from rabbit muscle, monopotassium salt of PEP, and MOPS (3-[*N*-morpholino]-propanesulphonic acid) were purchased from Sigma. Tris-HCl was obtained from Angus. KCl, NaCl, and $MgCl_2$ were obtained from Scharlau. Cellulose dialysis membranes CelluSep F3 of MWCO 12000−14000 were purchased from Orange Scientific, Belgium.

Sample Preparation. For the binding experiments with PEP, 0.8 mM protein was dissolved in buffer (50 mM Tris-HCl + MOPS to adjust the pH to 7.5) containing 100 mM KCl and 4 mM of $MgCl_2$. Fifty millimolar PEP was dissolved in the above buffer and the pH adjusted to 7.5. For experiments with Mg^{2+}, the protein was prepared in a similar manner but without $MgCl_2$. One hundred millimolar Mg^{2+} was dissolved in the above buffer.

Fourier Transform Infrared Spectroscopy. Our ATR-dialysis accessory has been described previously.[20] Six microliters of protein sample were placed between the diamond ATR reflection element of a SensIR ATR unit (9 reflections) and a dialysis membrane separating the sample volume from a reservoir. 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. Protein was equilibrated by continuous diffusion of buffer and salts across the dialysis membrane. 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) or 10 μL of Mg^{2+} (100 mM, pH 7.5) were 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. The resulting spectra are difference spectra (absorbance of protein + ligand sample minus absorbance of protein sample) showing the absorbance change upon the addition of the ligand. The addition of ligands and spectra recording was repeated up to 3 times. The region between 3100 and 2600 cm^{-1} was smoothed over 13 cm^{-1}.

Kinetic plots of water absorption changes were obtained by integrating the spectrum. The integration range was chosen at the low wavenumber side of the OH stretching band between 3050 and 2950 cm^{-1}. The 1214 and 1230 cm^{-1} bands were integrated using integration limits of 1201−1216 and 1230 −1240 cm^{-1}, respectively.

RESULTS AND DISCUSSION

Phosphoenolpyruvate Binding to Pyruvate Kinase. In order to detect a change in the infrared absorption of water upon binding of ligands to proteins, we used a sensitive variant of attenuated total reflection (ATR) infrared spectroscopy. Our setup[20] enables the diffusion of ligands across a dialysis membrane into a protein sample compartment, which makes it possible to detect the very small absorbance changes associated with ligand binding. The protein used was PK, and we studied the binding of phosphoenolpyruvate (PEP) in the presence of Mg^{2+} and K^+ and the binding of Mg^{2+} in the presence of K^+. Since PK needs PEP and ADP for the catalytic reaction, the addition of PEP in our experiments only led to binding of the substrate to PK. Panels A and B of Figure 1 show the effects of two additions of PEP to PK in the mid-infrared spectral range. This range covers the low wavenumber side of the OH stretching band of water (~3000 cm^{-1}), the amide I region (1700−1610 cm^{-1}), which reflects protein conformational changes, and the absorption of PEP (1600−900 cm^{-1}). The region below 1800 cm^{-1} has been studied by us before[21] and will be briefly described first because it reveals the processes that take place after addition of substrate. Further discussion can be found in ref 18.

Figure 1A shows spectra obtained within 240 s after the first addition of PEP. The spectra shown are difference spectra, revealing the absorbance change due to the addition of substrate. Since it took time for PEP to diffuse into the sample compartment, the signals rose gradually. The PEP concentration in the sample compartment at the end of the experiment was estimated to be ~4.6 mM using the 974 cm^{-1} band of PEP by comparison with the absorption of a 100 mM solution recorded with the same ATR setup.[21] This corresponds to ~6 PEP molecules per PK. Since the dissociation constant for PEP in the presence of Mg^{2+} and K^+ is ~70 μM,[22−24] in the presence of Mg^{2+} and K^+ and when the number of binding sites is 4,[24,25] the total concentration of PEP is higher than necessary to saturate the binding sites. The estimated PEP concentration is considerably higher than expected from the calculated concentration in the reservoir (25 μM), probably because PEP binds to dialysis membranes[24] and concentrates close to the membrane. Once the concentration in the sample compartment is above the dissociation constant, PEP binds, and more PEP diffuses into the sample compartment until the binding sites are saturated.

Complex formation between PEP and PK is evident, for example, from the amide I signals (e.g., at 1696, 1660, and 1646 cm^{-1}) revealing conformational changes of the protein backbone and from the band at 1552 cm^{-1}, which was assigned to a carboxylate group due to its characteristic upshift in D_2O and in particular to an Asp or Glu side chain because of its

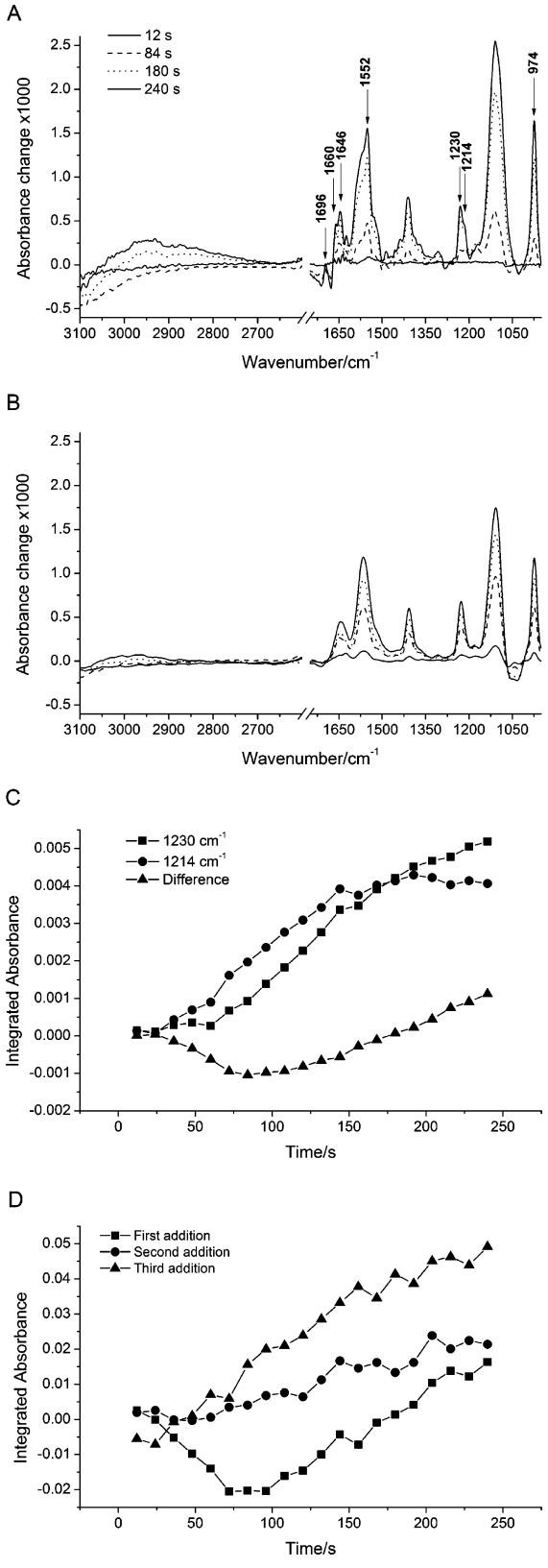

Figure 1. Infrared absorbance changes of water upon the addition of 25 μM PEP to PK in the presence of Mg^{2+} and K^{+}. (A) Spectra obtained upon the first addition. (B) Spectra obtained upon the second addition. (C) Kinetics of the 1214 and 1230 cm^{-1} bands indicating the concentrations of PK:PEP complex and of free PEP, respectively. (D) Kinetics of the water absorbance change near 3000 cm^{-1} of three additions of PEP.

insensitivity to isotope labeling of PEP.[21] After ~180 s, the amide I signals saturated indicating that the conformational change of the protein was complete and that the binding sites were saturated. However, signals due to PEP continued to increase, which was due to an increase in the concentration of free PEP. This transition from PK:PEP complex formation to an increase of free PEP concentration is best seen at the double band in the 1230−1210 cm^{-1} range. Initially the main band is found at 1214 cm^{-1}, which has been assigned to bound PEP.[21] Later the band at 1230 cm^{-1}, which is observed both for the complex and for free PEP in aqueous solution, becomes the dominant band. Partial areas of these bands are plotted in Figure 1C. It is evident that the 1214 cm^{-1} band (circles), that characterizes complex formation, rises earlier and saturates at the end of the experiment, whereas the free PEP band at 1230 cm^{-1} (squares) rises later and continues to rise until the end. The difference between these band areas (triangles) highlights the two dominant processes. The initial drop is due to complex formation and the subsequent rise due to an increase in the free PEP concentration. The change in the dominant process is also revealed in a difference spectrum (not shown) between the last two spectra of Figure 1A, which essentially resembles that of adding PEP to a protein-free sample. After the second (Figure 1B) and third (not shown) addition of PEP, only bands of PEP in aqueous solution,[21] evolved and none of the above characteristics of complex formation were seen. This demonstrates that no further binding took place because the amount of PEP added in the first addition was sufficient to saturate all binding sites.

Turning now to the region of water absorption near 3000 cm^{-1} (Figure 1A) similar phases as observed below 1800 cm^{-1} can be distinguished. After the first addition of PEP, there were initially no changes in the water absorption region (see spectrum recorded at 12 s) because PEP had not reached the detection volume. This indicates the stability of the baseline in these experiments. Later, the absorbance decreased in a broad spectral region around 3000 cm^{-1}, and at the end of the experiment, it increased. As motivated below in detail, the absorbance changes around 3000 cm^{-1} are assigned to a change in water absorption. The kinetics of the absorbance changes is shown in Figure 1D. It mirrors the kinetics of the band area difference between the two bands near 1200 cm^{-1} (triangular data points in Figure 1C), which reflects the different dominant processes after PEP addition as discussed above. Both curves go through a minimum around 80 s after the addition. The subsequent rise of the water signal occurs at a time when the free concentration of PEP increases more than the concentration of the PK:PEP complex as indicated by Figure 1C. By comparison with Figure 1C, we attribute the decrease of the absorption in the water region to the binding of PEP to PK and its increase to the increase in the free concentration of PEP. In line with this interpretation, subsequent additions (second and third) of PEP to PK showed no decrease in the absorption, only a monotonous increase.

The second and third additions served as inherent control experiments since they repeated the first addition under conditions where binding could not take place because the binding sites were already saturated. In addition, the spectral region below 1800 cm^{-1} serves as a control that the signals in the water region are not due to artifacts like, e.g., settling of the protein on the ATR crystal or a change in penetration depth. If these processes were taking place, a change in the amide I and II protein absorption would be observed in the region between

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1700 and 1500 cm^{-1} as two broad positive or negative bands as in Figure 2B. In contrast, the difference spectrum (Figure 1A) in this region contains many narrow bands with different signs. This rules out that the above-mentioned artifacts give rise to the observed changes in the water region.

An increase in water absorption as found for the second and third addition was also observed in control experiments upon the first addition of PEP to a protein-free solution (see Supplementary Figure S1). The amplitude of the water signal is correlated with the amplitude of the PEP bands below 1800 cm^{-1}, i.e., the PEP band amplitudes in the second addition (3.3 mM in the sample compartment) and the integrated water signal (0.021 cm^{-1}) are half as large as those of the third addition (6.3 mM and 0.049 cm^{-1}), which are half of those of the PEP addition to a protein-free solution (10 mM and 0.13 cm^{-1}).

Our published work[21] presented an independent set of three experiments that correspond to those shown in Figure 1. In these experiments, there is less free PEP at the end of the experiment than in those shown in Figure 1 as indicated by the ratio of the 1214 and 1230 cm^{-1} band pair (Figure 1 in this work and Figure 3 in ref 21). We observed a negative water signal like the one shown in Figure 1, which went through a minimum and then increased. However, the increase was less than in Figure 1, and the water signal remained negative throughout the experiment (not shown). This is in line with the interpretation above that the negative signal is associated with complex formation. In the second addition, the PEP band at 1230 cm^{-1} dominated due to the predominance free PEP. A small shoulder at 1214 cm^{-1} was also observed indicating a small extent of complex formation. In line with the minor contribution of complex formation to the spectra, no decrease in absorption at 3000 cm^{-1} was observed, only a slow increase due to free PEP.

For a further investigation into the different kinetic phases of the change in water absorption upon PEP binding to PK, we repeated the experiment with a lower PEP concentration (10 μM final concentration); see Supplementary Figure S2. In the first addition, very little PEP (~0.3 mM) reached the detection volume, 10 times less than required to saturate the binding sites, and there is only a very small decrease in water absorption (Supplementary Figure S2A,D). PK:PEP complex formation was observed after the second addition as indicated by the marker bands of complex formation (Supplementary Figure S2B). The estimated concentration of bound and free PEP in this addition was 3.3 mM, which corresponds to slightly more than 4 PEP molecules per PK, which is sufficient to saturate the binding sites. Accordingly, the absorbance of water decreased. The water signal remains negative throughout the experiment because the concentration of free PEP was too small to lead to a significant increase. Little change of water absorption was observed in the third addition (1 mM PEP in the sample compartment), which correlated with the virtual absence of marker bands for complex formation and only small bands of free PEP in the region below 1800 cm^{-1} (Supplementary Figure S2C).

The amplitude of the PEP bands is much larger after the second addition than in the other additions due to the peculiarities of our experiment. The protein concentration is higher than the concentration of free PEP. When the PEP concentration is sufficient for binding, PK sucks PEP from the reservoir into the sample compartment until the binding sites are saturated. This produces a higher concentration of bound PEP than of free PEP, and therefore, the amplitude of the PEP bands is dominated by the absorption of PEP bound to the protein with little contribution of free PEP. This is indicated also by the position of the symmetric PO_3^{2-} stretching vibration band at 968 cm^{-1} throughout the experiment, which reveals that mainly bound PEP is observed.[21] In line with the low concentration of free PEP at the end of the second addition experiment, the water signal reaches a plateau at 84 s and does not strongly increase thereafter as it did when more PEP was added (Figure 1D). Weak shoulders at 1214 cm^{-1} might indicate that there is also some binding occurring in the first and third additions. The experiment demonstrates again that decrease in water absorbance and conformational changes of the protein due to complex formation are correlated. The persistence of the absorbance drop over several minutes indicates also that the absorbance change is not due to temporary heating of the sample, which is in contact with diamond and metal surfaces.

Control Experiment in the Absence of Added Mg^{2+} and K^+. Apart from the inherent control of adding PEP to PK with saturated binding sites (Figure 1b and Supplementary Figure S2c), we did a further control experiment shown in Supplementary Figure S3 where PEP was added to PK in the absence of Mg^{2+} and K^+. In the absence of these ions, the dissociation constant for PEP increases from ~70 μM[22−24] to 250−300 μM.[26,27] Otherwise, the experiment was performed in the same way as the one shown in Figure 1. In the control experiment, the estimated increase in PEP concentration in the sample compartment is 1.6 and 1.8 mM in the first and second addition, respectively, which together is more than enough to saturate the binding sites. Signals of complex formation were observed near 1690, 1663, 1646, and 1214 cm^{-1} but were strongly reduced by a factor of 3. In the spectra of the second addition, an asymmetry in the 1230 cm^{-1} band also indicates the presence of the band at 1214 cm^{-1} and therefore some complex formation. In contrast to the experiment shown in Figure 1, the band of free PEP at 1230 cm^{-1} was present at all times of the control experiment, indicating a higher proportion of free PEP in line with the lower affinity under these conditions and in line with the smaller signals of complex formation. No negative water signal near 3000 cm^{-1} is observed in the control experiment because the signals of complex formation and of free PEP largely compensate. We note that, after this experiment, the buffer in the reservoir was exchanged several times, Mg^{2+} and K^+ added, and the PEP titration repeated. Under these conditions, large signals of complex formation were observed, and this experiment was one of the three experiments averaged in our previous work[21] discussed above.

Assignment of the 3000 cm^{-1} Signal to a Change in Water Absorption. The observed absorption change occurs in a region where OH and NH groups may absorb. In agreement with this general assignment, the signal is absent in D_2O (data not shown), which deuterates OH and NH groups and shifts their infrared absorption bands. A corresponding absorbance change of D_2O could, however, not be detected due to the strong absorption of the diamond reflecting element in the spectral region of D_2O absorption. An assignment is therefore in principle possible to protein NH and OH groups as well as to water. The latter assignment is in accordance with the available information as the following discussion reveals.

Dried proteins show a band at 3070 cm^{-1},[28,29] which has been assigned to the amide B vibration, the lower wavenumber

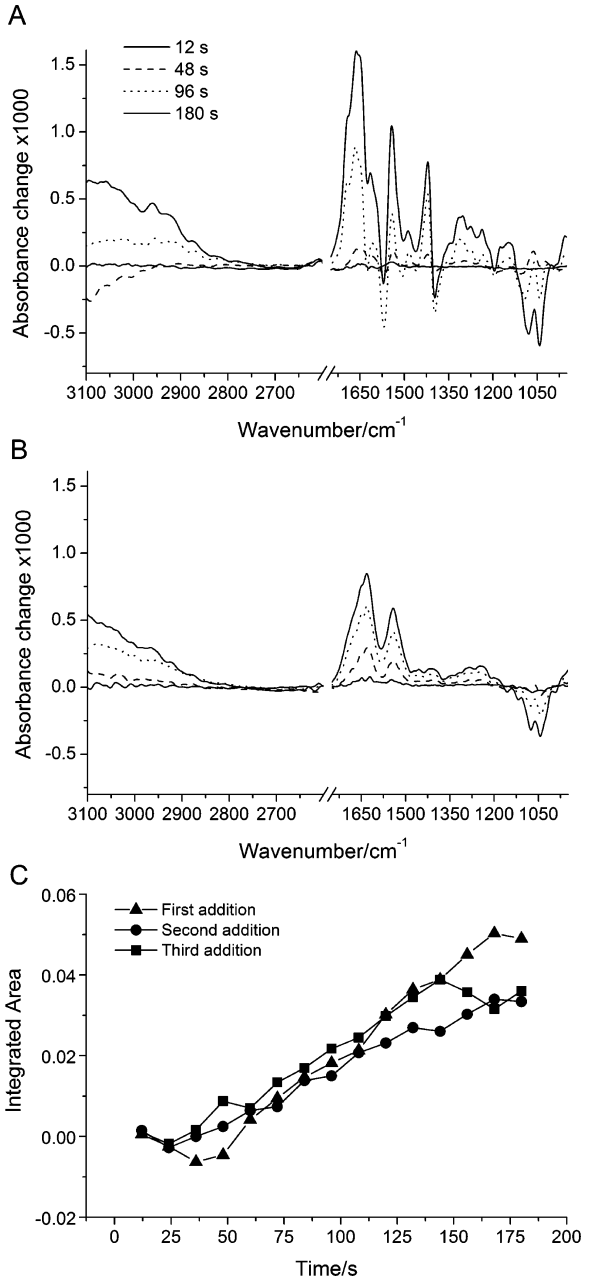

Figure 2. Infrared absorbance changes of water upon the addition of 250 μM Mg^{2+} to PK in the presence of K^+. (A) Spectra of the first addition; (B) spectra of the second addition; (C) kinetics of the water absorbance changes near 3000 cm^{-1}.

component of a Fermi resonance doublet involving NH stretching and amide II vibrations.[30] Both vibrations that contribute to the amide B band are expected to be affected by hydrogen bonding changes, and this affects also the amide I and amide II bands. However, the amide B band has considerably lower intensity than the amide I and amide II bands. Therefore, amide B absorbance changes that are comparable in amplitude to those in the amide I and II regions seem to be inconceivable and an assignment of the observed absorption change around 3000 cm^{-1} to the amide B band unlikely. In addition, the 3070 cm^{-1} amide B band has a spectral width that causes it to have considerably less intensity at 3100 and 3000 cm^{-1}.[31] If our signal were caused by this band, one would therefore expect to observe a clear minimum near 3060 cm^{-1}. In contrast, the band that causes the absorbance change observed here is much broader causing a decrease in absorbance between 2800 and 3100 cm^{-1} without distinct features.

Likewise we consider an assignment of the absorbance change to protein OH groups as unlikely. These groups have much lower abundance in proteins than NH groups and therefore contribute little to the overall absorption. Furthermore, isolated OH oscillators absorb in a much narrower spectral range compared with the coupled OH oscillators in water, as also observed for the isolated OH oscillator of HOD in D_2O or the OD oscillator of HOD in H_2O.[18,32]

For these reasons, we attribute the absorbance change around 3000 cm^{-1} observed upon binding of PEP to PK to a structural change of water. This interpretation is supported by previous reports on the effects of solutes on the water spectrum. For example, salts like NaCl decrease water's absorption in the low wavenumber region around 3000 cm^{-1},[33,34] whereas molecules containing polar and hydrophobic groups increase it.[35] Thus, it is well conceivable that the protein surface alters the absorption spectrum of water in the hydration shell. In line with this, a shoulder in the absorption of water near 3250 cm^{-1} shifts down to 3140 cm^{-1} when a few number of water monolayers are sandwiched by purple membranes, which contain protein and lipid.[36] Therefore, we conclude that the absorption decrease of water around 3000 cm^{-1} arises due to displacement of water from the hydration shell of PK to bulk water when PEP binds. This implies a stronger absorption in the 3000 cm^{-1} region of hydration water as compared to bulk water. Water expulsion from the hydration shell might either be due to the ligand taking up the space of the hydration water molecules or due to a conformational change of the protein. Later in the experiment, the absorption of water in this region increases due to the absorption of water molecules that are transferred from bulk water into the hydration shell around the added, free PEP molecules. Again, this is in line with a stronger absorption of hydration water in this spectral region.

Mg^{2+} Binding to PK. Water absorption detects also the binding of ions to proteins as shown here for the case of Mg^{2+} binding to PK, which is the essential ion for PK activity. Panels A and B in Figure 2 show spectra of the first and second additions of Mg^{2+} to PK in the presence of K^+. The kinetics of the water signal of the three additions is shown in Figure 2C. We detect the same trend as we observed in the case of PEP binding to PK: after the first addition, the water absorption was unchanged initially, this was followed by a decrease and finally an increase of absorbance near 3000 cm^{-1} (Figure 2a). Only a positive water signal was observed upon the second and third additions of Mg^{2+} to PK. The binding of Mg^{2+} to PK is also confirmed by the difference spectrum below 1800 cm^{-1}, which is highly structured with narrow positive and negative bands, which have been tentatively assigned previously.[21] The added Mg^{2+} concentration was 250 μM, which is slightly less than the dissociation constant of Mg^{2+} from PK (~450 μM[37,38]). Nevertheless, the following discussion shows that this concentration saturated the Mg^{2+} binding sites. We note also that the concentration of added Mg^{2+} was varied and optimized in initial experiments. Therefore, we assume that either the affinity of PK for Mg^{2+} is higher under our high protein conditions or that Mg^{2+} is concentrated close to the dialysis membrane as was concluded above for PEP. After the second addition, only broad bands were observed likely due to Mg^{2+} induced settling of the protein on the diamond surface of our ATR setup leading to a general increase in protein

concentration in the detection volume. Similar changes were already observed in the late phase of the first addition, as revealed by a difference spectrum (not shown) between the two last spectra, which shows broad amide I and II bands and an increase in absorption near 3000 cm^{-1}. When this spectrum is multiplied with an appropriate factor and subtracted from the spectra of the second addition, all features largely cancel in the resulting spectrum, including the signal near 3000 cm^{-1}. Therefore, no binding of Mg^{2+} to PK is detected in the second addition.

An increase in protein concentration was not observed in the baseline control done before the first addition and in that before the second addition where spectral noise and fluctuations were typically less than 0.05 mOD. Neither was protein settling observed in our experiments with PEP. This was checked by subtraction of the difference spectrum for the addition of PEP to a protein free sample (Supplementary Figure S1) from the spectra of the second addition of PEP to a PK sample (Figure 1b). Very small signals (0.2 mOD) remain because of nonperfect subtraction, which do not indicate an increase in protein concentration.

Potential of Using Water Absorption for Analytical Purposes. Many techniques are available to investigate the binding of ligands to proteins, such as X-ray crystallography, nuclear magnetic resonance (NMR), circular dichroism (CD), fluorescence, differential static light scattering (DSLS), isothermal titration calorimetry (ITC), surface plasmon resonance (SPR), dual polarization interferometry, analytical gel filtration, dielectric spectroscopy, etc. Those methods that give most molecular information, i.e., X-ray crystallography and NMR, are time-consuming and either require a non-native environment or provide less information for larger proteins. Others require modifications of the biological system like labeling (e.g., fluorescence) or immobilization (e.g., SPR). There are presently only very few techniques (e.g., ITC) that are label-free and where both binding partners are in the aqueous phase.

This study shows that water absorption provides a new label-free and sample-economical monitor of ligand binding; our results were obtained with only 6 nmol of protein. The extent of the water absorption change is expected to depend on the number of water molecules replaced during binding and associated conformational change but also on their absorption index, which depends on the polarity of the OH bonds and therefore on hydrogen bonding. This will make it difficult to quantify the dehydrated area from the amplitude of the water signal. Regarding the spectral shape of the absorbance change, it is too early to speculate whether a decrease of absorption around 3000 cm^{-1} reflects a general feature of protein hydration water, but this assumption is in line with the low wavenumber water band observed for purple membranes.[36] The spectral shape is also expected to depend on the nature of the protein surface that becomes dehydrated, for example, on the balance of charged, polar, and hydrophobic groups, which partly influence water absorption in opposite ways. Thus, the approach has the potential to distinguish between different binding sites on a protein.

Our present setup is not optimal for the detection of water absorbance changes. The light intensity in the spectral region of interest is small, and this precludes recording throughout the entire water region because of the high absorbance in the center of the water band. The current status can be improved by limiting the infrared illumination to the OH stretching region of water. This enables an increase of the light intensity in the relevant spectral region without saturating the detector and can be achieved either by using a filter or a laser source. This improvement of illumination will increase the sensitivity and make it possible to reduce the protein concentration or to detect binding processes with little perturbation of the water spectrum.

In our present implementation, the concentration of the protein is rather high (~millimolar), which has the advantage that also low affinity binders can be detected but dissociation constants of high affinity binders cannot be determined accurately. With the suggested technical improvement regarding the light source, the protein concentration can be reduced, and dissociation constant measurements should be possible.

■ CONCLUDING REMARKS

The strong absorption of water is usually considered a problem by protein infrared spectroscopists. Here, we show that it can be turned to an advantage to study molecular association events by monitoring a change in water absorption. These lead to a release of bound water into the bulk, which can be monitored by infrared spectroscopy, as shown here. This observation outlines a novel concept for detecting protein−ligand binding. Since water is a universal participant in all binding events, the approach has the potential to become a new label-free monitoring technique for drug development as well as for basic research. While we demonstrated the concept for protein−ligand interactions, it is not restricted to these. For example, binding to DNA can also be expected to modify water absorption. In conclusion, the observation presented here has high potential for drug development as well as for basic research because it can lead to a general method for detecting molecular association events that (i) is label-free, (ii) works with both binding partners being in aqueous solution, and (iii) is based on a universal process that takes place in all binding events.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Results obtained with protein free samples (Figure S1) of experiments where less concentrated PEP was added to a PK sample (Figure S2) and where PEP was added to PK in the absence of Mg^{2+} and K^{+} (Figure S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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