# SUPPLEMENTARY INFORMATION

## Interactions of phosphate groups of ATP and aspartyl phosphate with the sarcoplasmic reticulum $Ca^{2+}$-ATPase. An FTIR study.

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**Abbreviations:** AMPPCP, (β,γ-methyl)adenosine 5'-triphosphate; ATP, adenosine 5'-triphosphate; $Ca_2E1$, $Ca^{2+}$ bound form of $Ca^{2+}$-ATPase; $Ca_2E1$AMPPCP, AMPPCP-ATPase complex; $Ca_2E1$ATP, ATP-ATPase complex; $Ca_2E1P$, ADP-sensitive phosphoenzyme; NPE caged ATP, $P^3$-1-(2-nitrophenyl)ethyl ATP; DTT, dithiothreitol; E2P, ADP-insensitive phosphoenzyme; FTIR, Fourier transform infrared; IE, isotope effect; SR, sarcoplasmic reticulum; $\nu_{as}$, asymmetric or antisymmetric stretching vibration; $\nu_s$, symmetric stretching vibration; vu, valence units.

### Introduction

This supplementary information contains a reproducibility test of our spectra and a comprehensive discussion of Fig. 2 of the main text. All references to figures and published data refer to the main text except for Fig. S1.

### Reproducibility test

Fig. S1 shows a reproducibility test that serves to distinguish between isotope related and unrelated effects in our IE spectra. It thus establishes a level of confidence for the spectra shown in the main text. We chose the IE phosphorylation spectrum of γ-labeling for this purpose. It was re-calculated with independent subsets of our data. These control spectra show that the bands in the IE phosphorylation spectrum are significantly larger than bands which are due to isotope unrelated differences between the spectra from different sets of experiments.

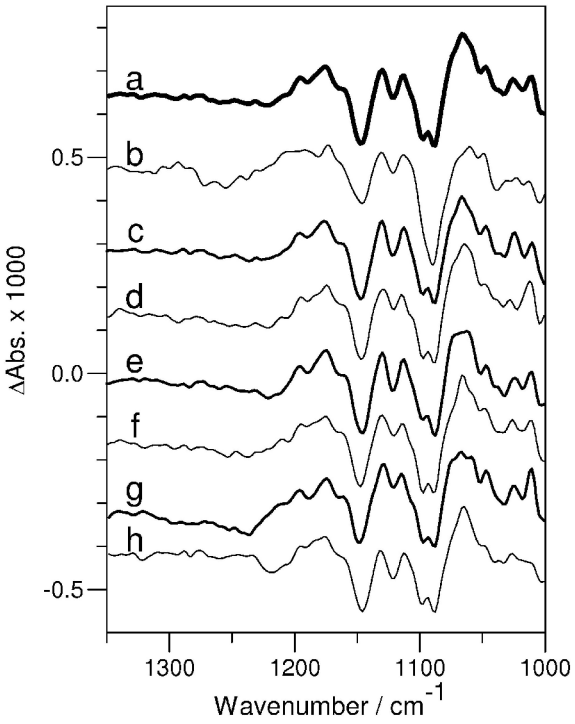


FIGURE S1. Reproducibility test of the IE phosphorylation spectrum obtained with γ-labeled ATP. a, IE phosphorylation spectrum shown in Fig. 2E; b, IE phosphorylation spectrum from previous independent experiments done at slightly different conditions of which phosphorylation spectra were published (Barth & Mäntele, 1998); c - f, IE phosphorylation spectra calculated from different subsets of experiments with γ-labeled ATP and all experiments with unlabeled ATP; g - h, IE phosphorylation spectra calculated from different subsets of experiments with unlabeled ATP and all experiments with γ-labeled ATP

**IE absorption spectra**

In addition to the spectra shown in the main text, we have also obtained IE spectra for the absorption of ATP in aqueous solution that we call IE absorption spectra of free ATP (data not shown). They were calculated from absorbance spectra of [β-$^{18}O_2$, βγ-$^{18}O$]ATP, [γ-$^{18}O_3$] ATP, and unlabeled ATP in aqueous solution by subtracting the spectrum of labeled ATP from that of unlabeled ATP. In this way the sign convention in the IE absorption spectra of free ATP is the same as for free ATP bands in the IE binding spectra. Thus for a downshift of a band of free ATP upon isotopic labeling, ∩∪ band profiles are expected in both the IE absorption spectra and the IE binding spectra.

**β-phosphate bands of ATP**

The IE spectra of binding and phosphorylation that we obtained upon β-labeling are shown in the top panel of Fig. 2E. The expected downshifts of bands upon isotopic labeling will give rise to ∪∩ band profiles in both IE spectra for bound ATP, ∩∪ band profiles for free ATP in the IE binding spectrum and for ADP in the IE phosphorylation spectrum.

*Absorption of $\nu_{as}$(β-$PO_2^-$) of free ATP (1240 - 1180 cm$^{-1}$)* - Published spectra of ATP or GTP in aqueous solution show the $\nu_{as}(PO_2^-)$ band near 1234 cm$^{-1}$ with a shoulder near 1216 cm$^{-1}$ (Takeuchi et al., 1988; Wang et al., 1998). Labeling of the β-phosphate replaces a $P^{16}O_2^-$ group by a $P^{18}O_2^-$ group. As a result, the $\nu_{as}(PO_2^-)$ band loses intensity and becomes a clear double band with component bands at 1220-1226 cm$^{-1}$ and 1178-1190 cm$^{-1}$ (Takeuchi et al., 1988; Wang et al., 1998). In our respective IE absorption spectrum of free ATP (not shown) these spectral changes give rise to a ∩∪ band profile of which the positive band at 1236 cm$^{-1}$ has a shoulder at 1215 cm$^{-1}$ and the negative band is found at 1182 cm$^{-1}$. This indicates that β-phosphate vibrations of unlabeled ATP contribute of both, main band at 1234 cm$^{-1}$ and shoulder at 1216 cm$^{-1}$. Upon labeling, the β-phosphate $\nu_{as}(PO_2^-)$ vibration separates out and becomes a vibration that is likely to be predominantly localized on the β-phosphate.

*Absorption of $\nu_{as}$(β-$PO_2^-$) of free ATP in the presence of ATPase (1250 - 1190 cm$^{-1}$)* - The band profile for free ATP in the IE absorption spectrum is also found in our IE binding spectrum (thin line in the top panel of Fig. 2E): a positive band at 1248 cm$^{-1}$ (for free unlabeled ATP) and a negative band at 1196 cm$^{-1}$ (for free β-labeled ATP). The upshift of the two free ATP bands by ~10 cm$^{-1}$ with respect to the the IE absorption spectrum of free ATP (not shown) can be explained by chelation of a divalent cation (Takeuchi et al., 1988), here $Ca^{2+}$.

*Absorption of $\nu_{as}$(β-$PO_2^-$) of bound ATP in the presence of ATPase (1270 - 1150 cm$^{-1}$)* - Similar bands with the expected opposite sign are observed for bound ATP. The intensity decrease of the main $\nu_{as}(PO_2^-)$ band is observed as a weak minimum at 1260 cm$^{-1}$ for unlabeled bound ATP in the IE spectrum of phosphorylation (bold spectrum in the top panel of Fig. 2E).

The shoulder in the absorption spectrum of ATP and GTP (Takeuchi et al., 1988; Wang et al., 1998) near 1216 cm$^{-1}$ gives rise to the bands between 1210 and 1150 cm$^{-1}$ in our IE spectra (top panel of Fig. 2E). The IE phosphorylation spectrum shows a ∪∩ band profile, attributable to bound ATP, with the negative band at 1207 cm$^{-1}$ (for unlabeled bound ATP) and the positive band at 1174 cm$^{-1}$ (for β-labeled bound ATP). This interpretation is supported by bands of bound ATP in the binding spectra, where they are positive, and in the phosphorylation spectra, where they are negative. In the binding spectrum with unlabeled ATP (top panel in Fig. 2B), a broad positive band is observed at 1200 cm$^{-1}$. The second derivative of the binding spectrum (not shown) locates this band at 1207 cm$^{-1}$. In the phosphorylation spectrum of unlabeled ATP (top panel in Fig. 2D) a small negative band is observed at 1209 cm$^{-1}$. Both bands are absent in the respective spectra with β-labeled ATP (grey lines in Figs. 2B and 2D) in line with their assignment to unlabeled ATP. The band of β-labeled ATP at 1174 cm$^{-1}$ gives rise to higher intensity in this region in the binding spectrum (grey line in Fig. 2B) and is clearly observed in the phosphorylation spectrum with β-labeled ATP (grey line in Fig. 2D) as a negative band at 1178 cm$^{-1}$ .

*Absorption of $\nu_s(PO_2^-)$ of free ATP (1130 - 1060 cm$^{-1}$)* - The ATP or GTP band found near 1121 cm$^{-1}$ in absorption spectra of aqueous solutions is composed of contributions of the in-phase $\nu_s(PO_2^-)$ vibration at 1116 cm$^{-1}$ and the $\nu_{as}(PO_3^{2-})$ vibration (Takeuchi et al., 1988; Wang et al., 1998). Apart from the main peak, a shoulder at 1087 cm$^{-1}$ is clearly visible and has been assigned to the out-of-phase $\nu_s(PO_2^-)$ vibration which couples to the $\nu_{as}(PO_3^{2-})$ vibration (Takeuchi et al., 1988; Wang et al., 1998). This shoulder is found at 1092 cm$^{-1}$

in the photolysis spectrum obtained in the absence of ATPase (grey spectrum in Fig. 1). Upon labeling of the β-phosphate, the main band and the shoulder both downshift by 10-20 $cm^{-1}$ (Takeuchi et al., 1988; Wang et al., 1998). These isotope shifts of main band and shoulder give rise to two overlapping ∩∪ band profiles in the IE absorption spectrum of free ATP (not shown). The first band profile consists of a positive band at 1121 $cm^{-1}$ and a minimum at 1098 $cm^{-1}$ due to a ~11 $cm^{-1}$ isotope shift of the main band and the second produces a positive band at 1083 $cm^{-1}$ and a negative band at 1060 $cm^{-1}$ due to a similar isotope shift of the shoulder. Upshifts of these bands by 5-10 $cm^{-1}$ are expected upon complexation with $Mg^{2+}$ (Takeuchi et al., 1988), smaller upshifts with $Ca^{2+}$ (Brintzinger, 1963).

*Region of in-phase $\nu_s$(β-$PO_2^-$) absorption of bound and free ATP in the presence of ATPase (1150 - 1100 $cm^{-1}$)* - In the IE binding spectrum and the IE phosphorylation spectrum (top panel of Fig. 2E), the isotope shift of the in-phase $\nu_s(PO_2^-)$ band gives rise to the four bands at 1145 (-), 1128 (+), ~1110 (-) and ~1103 (+) $cm^{-1}$ which we attribute to two ∪∩ band profiles of bound ATP and a ∩∪ band profile of free ATP. The first ∪∩ band profile of bound ATP exhibits its negative band at 1145 $cm^{-1}$ (for unlabeled ATP) and its positive band at 1128 $cm^{-1}$, (for β-labeled ATP) the second has its negative band at ~1110 $cm^{-1}$ (for unlabeled ATP) and the positive band at ~1103 $cm^{-1}$ (for β-labeled ATP). Overlapped is the expected ∩∪ band profile of free ATP with the positive band contributing at 1128 $cm^{-1}$. The negative band of this band profile expected at 1098 $cm^{-1}$ is not obvious, but might contribute to the high wavenumber shoulder of the 1085 $cm^{-1}$ band.

That these four bands in the IE spectra correspond to isotope shifts of three bands is probably more obvious in the binding spectra (Fig. 2B) where bands of bound ATP are positive and those of free ATP negative. The binding spectrum for unlabeled ATP (top panel in Fig. 2B) shows a maximum at 1149 $cm^{-1}$ that is reduced in the spectrum with β-labeled ATP (grey line in Fig. 2B). Instead there is a positive shoulder at 1128 $cm^{-1}$ superimposed on a larger negative band and we attribute the positive shoulder to the shifted 1149 $cm^{-1}$ band. Since these bands are positive in the binding spectrum, they can be assigned to bound ATP.

The negative 1128 $cm^{-1}$ band in the binding spectrum with unlabeled ATP (top panel of Fig. 2B) is shifted to ~1117 $cm^{-1}$ or below upon β-labeling. The adjacent positive shoulder at 1106 $cm^{-1}$ corresponds to the ~1110 $cm^{-1}$ negative band observed in the IE spectra (top panel of Fig. 2E) assigned to bound ATP. With β-labeled ATP it is found at 1100 $cm^{-1}$ as a positive shoulder in the binding spectrum (grey line in Fig. 2B). Thus the three shifts inferred from the IE binding spectrum can be clearly identified by a comparison of the binding spectra. In summary, instead of one band of free ATP at 1116 $cm^{-1}$, we observe two bands for bound ATP at 1145 and 1110 $cm^{-1}$ likely because of splitting of the free ATP band into two components.

*Region of out-of-phase $\nu_s$(β-$PO_2^-$) absorption of bound and free ATP in the presence of ATPase (1100 - 1060 $cm^{-1}$)* - The absorption spectrum of free ATP shows a shoulder at 1087 $cm^{-1}$, which is assigned to the out-of-phase $\nu_s(PO_2^-)$ vibration (Takeuchi et al., 1988). The isotope shift of the corresponding band of bound ATP gives rise to a ∪∩ band profile that is observed in both, the IE binding spectrum and the IE phosphorylation spectrum (top panel of Fig. 2E). It consists of the negative band at 1085 $cm^{-1}$ with a shoulder at 1094 $cm^{-1}$ (for unlabeled ATP) and a positive band at ~1070 $cm^{-1}$ (for β-labeled ATP). The corresponding ∩∪ band profile for free ATP is not obvious probably because the bands are broader or less intense than for bound ATP.

In support of the assignments for bound ATP, the bands of unlabeled bound ATP are also present at similar positions in the ATP binding spectrum as a positive band (at 1093 $cm^{-1}$ with a hint of a shoulder near 1085 $cm^{-1}$, see top panel of Fig. 2B) and as negative bands in the phosphorylation spectrum (at 1087 $cm^{-1}$ with a shoulder at 1096 $cm^{-1}$, see top panel of Fig. 2D). Also, the band of β-labeled ATP at ~1070 $cm^{-1}$ is seen as positive band in the respective ATP binding spectrum (at 1069 $cm^{-1}$, see grey line in Fig. 2B) and as negative band in the phosphorylation spectrum (at 1069 $cm^{-1}$, see grey line in Fig. 2D).

**Summary of band assignments for the β-phosphate of bound ATP**

*Region of $\nu_{as}(PO_2^-)$ absorption (1270 - 1170 $cm^{-1}$)* - Summarizing the region of $\nu_{as}(PO_2^-)$ absorption, we have identified two bands for bound ATP that involve the β-phosphate: a weak band at 1260 $cm^{-1}$ and a band at 1207 $cm^{-1}$ for unlabeled ATP, and one band at 1174 $cm^{-1}$ for β-labeled ATP. The IE spectra (top panel of Fig. 2E) indicate that the main component of the $\nu_{as}(PO_2^-)$ band absorbs at higher wavenumber for bound ATP (negative

band in IE phosphorylation spectrum at 1260 $cm^{-1}$) than for free ATP (positive band in IE binding spectrum at 1248 $cm^{-1}$).

The $\nu_{as}(PO_2^-)$ band is difficult to interpret in terms of interaction with the environment since it represents a coupled vibration of α-, β-, and γ- phosphate (see below). Because of coupling, not only the interaction with the environment will contribute to the band position but also the conformation of the phosphate chain.

Labeling of the β-phosphate reduces the coupling between the α- and β-phosphate group, and thus the bands observed for the β-labeled compound better correspond to a $PO_2^-$ vibration localized on the β-phosphate. This makes it more reliable to interpret the vibrations in terms of the strength of interaction with the environment. Bands for β-labeled ATP were found at ~1196 and 1174 $cm^{-1}$ for free and bound [β-$^{18}O_2$, βγ-$^{18}O$] ATP, respectively. The 1174 $cm^{-1}$ band is downshifted from 1207 $cm^{-1}$ upon β-labeling and this -33 $cm^{-1}$ shift is indicative of a vibration that is predominantly localized on the β-phosphate. The lower wavenumber of the band of bound β-labeled ATP compared with that of free β-labeled ATP in our samples indicates a lower P-O bond strength of bound ATP due to stronger interactions with the environment. The band at 1174 $cm^{-1}$ for bound [β-$^{18}O_2$, βγ-$^{18}O$]ATP is close to the band near 1180 $cm^{-1}$ in absorbance spectra of [β-$^{18}O_2$, βγ-$^{18}O$]ATP (our unpublished spectrum) and of [β-$^{18}O_2$, βγ-$^{18}O$]GTP (Wang et al., 1998) and in the photolysis spectrum of caged ATP (Barth et al., 1997) which were all recorded in the absence of divalent cations. The similar band positions may thus indicate that no divalent cation binds to the β-phosphate in the ATP-ATPase complex. This is in line with the structure of $Ca_2$E1AMPPCP (Sørensen et al., 2004; Toyoshima et al., 2004). In contrast, the free ATP in our samples is expected to bind $Ca^{2+}$ as indicated by an upshift of the respective free β-labeled ATP band to 1196 $cm^{-1}$ discussed above.

*Region of $\nu_s(PO_2^-)$ absorption (1150 - 1060 $cm^{-1}$)* - In the spectral region of $\nu_s(PO_2^-)$ vibrations, we have identified three bands for bound ATP that involve the β-phosphate: those at 1145, ~1110, and 1094/1085 $cm^{-1}$ for unlabeled ATP as well as those at 1128, 1103 and ~1070 $cm^{-1}$ for β-labeled ATP. The 1145 $cm^{-1}$ and the 1094/1085 $cm^{-1}$ band of unlabeled ATP both experience ~20 $cm^{-1}$ downshifts upon labeling and correspond therefore to vibrations with a significant contribution of the β-phosphate. However, these shifts are considerably smaller than the 40 $cm^{-1}$ shift that is expected for a $\nu_s$ ($PO_2^-$) vibration completely localized on the β-phosphate (Deng et al., 1998) and thus the corresponding normal modes contain additional significant contributions from the other phosphates. Compared to free ATP, the 1145 $cm^{-1}$ band of unlabeled ATP is at 20 $cm^{-1}$ higher wavenumber, the 1094/1085 $cm^{-1}$ band at similar wavenumber, the 1128 $cm^{-1}$ band of β-labeled ATP at 30 $cm^{-1}$ higher wavenumber and the ~1070 $cm^{-1}$ band at 10 $cm^{-1}$ higher wavenumber (based on our IE absorption spectrum, data not shown).

The ~1110 $cm^{-1}$ band shifts down only by a few wavenumbers and corresponds therefore to a vibration that is predominantly localized on phosphates other than the β-phosphate. Upon γ-labeling this band is unaffected and observed in the binding spectra with γ-labeled and unlabeled ATP at the same position (1106 $cm^{-1}$, see top and bottom panel of Fig. 2B). Therefore it does not correspond to a γ-phosphate vibration either. Thus we assign the ~1110 $cm^{-1}$ band to a vibration of unlabeled bound ATP that is predominantly localized on the α-phosphate. It absorbs approximately 10 $cm^{-1}$ lower than free ATP.

**Bands of ADP**

ADP is expected to contribute to the IE phosphorylation spectrum upon β-labeling in the top panel of Fig. 2E. For GDP in solution a shift upon β-labeling from 1115 to 1089 $cm^{-1}$ has been observed (Wang et al., 1998) and our IE absorption spectrum of free GDP (as a model for ADP) shows a ∩∪ band profile with the positive band at 1139 $cm^{-1}$ (for unlabeled GDP) and the negative at 1081 $cm^{-1}$ (for β-labeled GDP). For GDP bound to Ras, two shifts were observed upon [αβ-$^{18}O$,β-$^{18}O_3$] labeling because the asymmetric environment removes the degeneracy of the $\nu_{as}(PO_3^{2-})$ vibration: from 1136 to 1105 $cm^{-1}$ and from 1101 to 1072 $cm^{-1}$ (Du et al., 2000). The band positions for unlabeled bound GDP are in agreement with studies using singly labeled β-phosphate (Allin & Gerwert, 2001). In our spectra, the absorption of bound unlabeled ADP might contribute between 1160 and 1103 $cm^{-1}$ to the differences between the IE phosphorylation spectrum and the IE binding spectrum to which the latter does not contribute. Likewise, bound β-labeled ADP might contribute between 1100 and 1050 $cm^{-1}$.

**γ-phosphate bands of bound ATP**

IE spectra upon γ-labeling are shown in the bottom panel of Fig. 2E and are discussed in this section. Downshifts of bands because of isotopic substitution will give rise to ∪∩ band profiles for bound ATP in both types of IE spectra, and to ∩∪ band profiles for free ATP in the IE binding spectrum and for the phosphoenzyme phosphate group in the IE phosphorylation spectrum. A very similar IE phosphorylation spectrum (see Fig. S1) was obtained from independent experiments at slightly different conditions of which phosphorylation spectra were published previously (Barth & Mäntele, 1998).

*Influence of γ-phosphate vibrations on the $\nu_{as}(PO_2^-)$ band of free and bound ATP (1270 - 1200 $cm^{-1}$)* - An overlay of spectra of unlabeled and γ-phosphate labeled ATP or GTP in aqueous solution found in the literature (Takeuchi et al., 1988; Wang et al., 1998) indicates a decrease of $\nu_{as}(PO_2^-)$ band intensity upon γ-phosphate labeling, probably due to coupling between γ-$PO_3^{2-}$ and $\nu_{as}(PO_2^-)$ vibrations for the unlabeled molecules. This is confirmed by our absorbance spectra and leads to a positive band at 1230 $cm^{-1}$ in the IE absorption spectrum of free ATP (not shown).

In our IE binding spectrum of γ-phosphate labeling (thin line in bottom panel of Fig. 2E), a positive band at 1249 $cm^{-1}$ is found which corresponds to the expected decrease in free ATP absorption in this spectral region upon labeling the γ-phosphate. Thus this band is assigned to the free ATP $\nu_{as}(PO_2^-)$ vibration, in line with the above assignment based on β-phosphate labeling. The corresponding negative bands of bound ATP are not obvious in the IE binding and phosphorylation spectra (bottom panel of Fig. 2E) indicating that binding reduces the effect of γ-labeling on the $\nu_{as}(PO_2^-)$ vibration. The 1175 $cm^{-1}$ band in the IE phosphorylation spectrum upon γ-labeling (bold line in the bottom panel of Fig. 2E) is assigned to the unlabeled $Ca_2E1P$ phosphate group and will be discussed below in the respective subsection.

*Absorption of $\nu_{as}(\gamma\text{-}PO_3^{2-})$ of free and bound ATP (1150 - 1040 $cm^{-1}$)* - The $\nu_{as}(PO_3^{2-})$ band of free ATP or GTP shifts from ~1120 $cm^{-1}$ to ~1085 $cm^{-1}$ upon γ-phosphate labeling (Takeuchi et al., 1988; Wang et al., 1998). In the IE absorption spectrum (not shown) this leads mainly to a broad positive band at 1126 $cm^{-1}$ and a small negative band at 1063 $cm^{-1}$. The corresponding spectral region shows a complicated band structure in our IE binding and IE phosphorylation spectrum (bottom panel of Fig. 2E). The IE binding spectrum (thin line) is more positive than the IE phosphorylation spectrum (bold line) between 1150 and 1113 $cm^{-1}$. We attribute this to the expected positive band of free unlabeled ATP that only contributes to the binding spectrum.

In both IE spectra in the bottom panel of Fig. 2E, a band near 1130 $cm^{-1}$ is observed. This is indicative of a band of bound ATP which is the only compound contributing to both IE spectra. Its positive sign makes this band assignable to γ-labeled ATP and this interpretation is supported by a shoulder at 1131 $cm^{-1}$ in the binding spectrum with γ-labeled ATP (bold line in Fig. 2B) and in the respective uncorrected spectrum (not shown). The corresponding band for unlabeled bound ATP is expected at higher wavenumber and seems to be located near 1150 $cm^{-1}$ where the binding spectrum with unlabeled ATP (thin line in the bottom panel of Fig. 2B) is more positive in the region around 1150 $cm^{-1}$ than that with γ-labeled ATP (bold line). The band of bound unlabeled ATP near 1150 $cm^{-1}$ leads to the minimum in the IE binding spectrum around 1154 $cm^{-1}$ (thin line in the bottom panel of Fig. 2E). It will also contribute to the clear negative band observed at 1147 $cm^{-1}$ in the IE phosphorylation spectrum (bold line in the bottom panel of Fig. 2E) to which also the phosphate group of $Ca_2E1P$ contributes as discussed in the respective subsection below. In line with the assignment of a band near 1150 $cm^{-1}$ to bound unlabeled ATP, a clear negative band is observed in the phosphorylation spectrum at 1148 $cm^{-1}$ (thin line in Fig. 2D) that is not observed in the phosphorylation spectrum with γ-labeled ATP (bold line in Fig. 2D).

We consider the above assignments above of the 1154 $cm^{-1}$ band and the 1130 $cm^{-1}$ band to bound ATP as being most consistent with our data, in particular because the 1130 $cm^{-1}$ band is clearly observed in both IE spectra (bottom panel of Fig. 2E) and in the binding spectrum with γ-labeled ATP (bold line in Fig. 2B). However, alternative assignments are possible. The 1130 $cm^{-1}$ band in the IE binding spectrum (thin line in the bottom panel of Fig. 2E) could be due to the positive band of free unlabeled ATP and the 1130 $cm^{-1}$ band in the IE phosphorylation spectrum (bold line in the bottom panel of Fig. 2E) to the positive band of the unlabeled phosphoenzyme phosphate group. The identical band position in both IE spectra would then be a coincidence. In summary, we tentatively assign the 1154 and 1130 $cm^{-1}$ bands to unlabeled and γ-labeled bound ATP, respectively, although we are aware that

alternative assignments might also be possible. These assignments are not exclusive. In particular the clearer appearance of the 1130 $cm^{-1}$ band in the IE phosphorylation spectrum as compared to the IE binding spectrum might indicate and additional contribution from the phosphoenzyme phosphate group. The 1154 $cm^{-1}$ band is close to the 1145 $cm^{-1}$ band of bound ATP, sensitive to β-labeling, and might therefore indicate sensitivity towards β- and γ-labeling of the same absorption band.

Another ∪∩ band profile for bound ATP is evident in both, the IE binding and phosphorylation spectrum upon γ-labeling (bottom panel of Fig. 2E), with the negative band between 1098 and 1089 $cm^{-1}$, indicating absorption of unlabeled ATP, and the positive band between 1072 and 1047 $cm^{-1}$, indicating absorption of γ-labeled ATP. These bands are observed at similar positions in the ATP binding spectra for unlabeled and γ-labeled ATP (positive band of the thin line spectrum in Fig. 2B found at 1093 $cm^{-1}$ for unlabeled ATP and more intensity between 1075 and 1040 $cm^{-1}$ in the bold line spectrum for γ-labeled ATP). The band of unlabeled ATP can be also identified as the expected negative band in the phosphorylation spectrum (top panel in Fig. 2D, found at 1087 $cm^{-1}$).

**Summary of band assignments for the γ-phosphate of bound ATP**

In summary, we have identified two bands for bound ATP that involve the γ-phosphate: presumably one near 1154 $cm^{-1}$ shifting to 1130 $cm^{-1}$ upon γ-labeling, and a band between 1098 and 1089 $cm^{-1}$ shifting to 1072 - 1047 $cm^{-1}$. These bands are also affected by β-labeling and represent therefore coupled vibrations between β-, γ-phosphate, and possibly the α-phosphate. The bands at 1154 and 1098 - 1089 $cm^{-1}$ are in the region of $\nu_{as}(PO_3^{2-})$ absorption, where free ATP shows only one band near 1120 $cm^{-1}$. Thus upon binding this band seems to split into two components. The average wavenumber is similar to the wavenumber of the free ATP band indicating a similar bond strength of bound and free ATP.

**Bands of the phosphate group of the $Ca_2E1P$ phosphoenzyme**

In our previous study (Barth & Mäntele, 1998) a band near 1131 $cm^{-1}$ in the phosphorylation spectrum was tentatively assigned to the phosphoenzyme phosphate ($Ca_2E1P$). Our more complete study here does not exclude this assignment, but suggests an alternative or additional assignment of the 1131 $cm^{-1}$ band to γ-labeled bound ATP. In addition, the bands at 1175 and 1113 $cm^{-1}$ are assigned to the phosphoenzyme phosphate as detailed below.

A band at 1175 $cm^{-1}$ appears in the IE phosphorylation spectrum upon γ-labeling (bold spectrum in the bottom panel of Fig. 2E). Because it is not observed in the respective IE binding spectrum (thin line in the bottom panel of Fig. 2E) it is assigned to the $Ca_2E1P$ phosphate group which contributes only to the IE phosphorylation spectrum and for which ∩∪ profiles are expected in the IE phosphorylation spectrum. Thus, the positive sign of this band indicates the absorption of the unlabeled phosphate group. In support of this assignment there is a band at similar position (1170 $cm^{-1}$) in the phosphorylation spectrum with unlabeled ATP that is reduced with γ-labeled ATP (bold line in Fig. 2D). A clear band at this position is observed in the uncorrected spectrum (not shown) of $Ca_2E1P$ formation ($Ca_2E1$ + caged ATP → $Ca_2E1P$ + ADP + byproducts) for unlabeled and β-labeled ATP but not for γ-labeled ATP. The 1175 $cm^{-1}$ band seems to shift to 1148 $cm^{-1}$ where a negative band in the phosphorylation spectrum with unlabeled ATP (thin line in Fig. 2D) is filled up in the spectrum with γ-labeled ATP (bold line in Fig. 2D) and a positive shoulder is observed at 1144 $cm^{-1}$. In the IE phoshorylation spectrum (bold line in the bottom panel of Fig. 2E) this band of γ-labeled $Ca_2E1P$ phosphate contributes to the negative band at 1147 $cm^{-1}$.

In the following we will assign the positive 1113 $cm^{-1}$ band in the IE phosphorylation spectrum (bold line in the bottom panel of Fig. 2E) to the unlabeled $Ca_2E1P$ phosphate group. . The sensitivity of the 1113 $cm^{-1}$ band on isotopic labeling is also evident in the phosphorylation spectra, in which the positive shoulder at 1115 $cm^{-1}$ in the phosphorylation spectrum with unlabeled ATP (thin line in Fig. 2D) is not present in the phosphorylation spectrum with γ-labeled ATP (bold line). Instead, positive bands at 1099 and 1091 $cm^{-1}$ were observed. The assignment of the 1113 $cm^{-1}$ band to the phosphate group of the phosphoenzyme is further strengthened by the observation of a positive band at 1111 $cm^{-1}$ in the unprocessed spectrum of $Ca_2E1P$ formation (not shown) and at 1113 - 1115 $cm^{-1}$ in the phosphorylation spectra with β-labeled ATP (grey line in Fig. 2D), ITP and 2'-dATP (Liu & Barth, 2004). In the latter cases, the likely reason for the clear

observation of the 1113 $cm^{-1}$ phosphoenzyme band is a shift of the overlapping 1110 $cm^{-1}$ negative band of bound ATP due to isotopic labeling or due to a different binding mode of ITP and 2'-dATP. The 1113 $cm^{-1}$ band represents the positive component band of the expected $\cap\cup$ band profile for the $Ca_2E1P$ phosphate. The corresponding negative band seems to overlap with negative bands of $\cup\cap$ band profiles of unlabeled bound ATP in the 1098 to 1089 $cm^{-1}$ region.