Phosphorylation of the sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase from ATP and ATP Analogs Studied by Infrared Spectroscopy*  

Man Liu§§ and Andreas Barth¶  

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§ To whom correspondence should be addressed. Tel.: 46-8-163529; Fax: 46-8-155597; E-mail: liu@dbb.su.se.

¶ To whom correspondence should be addressed. Tel.: 46-8-163529; Fax: 46-8-155597; E-mail: Barth@kth.se.

1 The abbreviations used are: SR, sarcoplasmic reticulum; AMPPCP, adenosine 5’-[β,γ-methylene]triphosphate; AMPPNP, adenosine 5’-[β,γ-imido]triphosphate; caged nucleotide, P\textsuperscript{3-1-(2-nitrophenyl)ethyl} nucleotide; Ca\textsubscript{2}\textsubscript{E1}, the calcium-ATPase complex with high affinity to Ca\textsuperscript{2+}; Ca\textsubscript{2}\textsubscript{E1ATP}, the calcium-nucleotide-ATPase complex; Ca\textsubscript{2}\textsubscript{E1P}, the ADP-sensitive phosphoenzyme; E2P (the ADP-insensitive phosphoenzyme), and E2 (the calcium-free ATPase with low affinity to Ca\textsuperscript{2+}).  

2 Crystal structures of the SR Ca\textsuperscript{2+}-ATPase (7–10) show that the enzyme has two regions, the transmembrane region and the cytoplasmic region. The cytoplasmic region consists of three globular domains: nucleotide domain (N domain), phosphorylation domain (P domain), and actuator or anchor domain (A domain), which are connected by hinge regions. The adenosine part of ATP binds to the N domain (11), and the phosphorylated residue Asp-351 is located in the P domain (7). It has been proposed that the structures of Ca\textsubscript{2}\textsubscript{E1ATP} and of Ca\textsubscript{2}\textsubscript{E1P} are similar, indicated by similar infrared spectra of ATP binding and Ca\textsubscript{2}\textsubscript{E1P} formation (12) and by a proteolysis study (13). Recently crystal structures of the complex Ca\textsubscript{2}\textsubscript{E1AMPPCP} and the Ca\textsubscript{2}\textsubscript{E1P} analogue Ca\textsubscript{2}\textsubscript{E1ADP-2P} were obtained (9, 10). They are almost identical and show a compact arrangement of domains. The aim of this work is to study the conformational difference between the Ca\textsubscript{2}\textsubscript{E1ATP} and Ca\textsubscript{2}\textsubscript{E1P} states with Fourier transform infrared (FTIR) spectroscopy.

3 Infrared spectroscopy (14–18) has several advantages for elucidating the molecular mechanism of proteins, such as high time resolution, universal applicability from small soluble proteins to large membrane proteins, and high molecular information content combined with a sensitivity high enough to detect a change in the environment around a single atom of a large protein. These properties make this method very useful to obtain information on enzyme-substrate recognition (18–21). FTIR spectroscopy (22–27) can provide potent dynamic and structural information, which sheds light on interactions occurring between enzyme and substrate during the catalytic process. In our previous work (21, 28, 29), various nucleotides have been used to probe interactions between nucleotides and the SR Ca\textsuperscript{2+}-ATPase in the nucleotide binding reaction with FTIR spectroscopy, and characteristic binding modes for each nucleotide have been concluded.

Here we investigate enzyme phosphorylation with ATP and ATP analogs inosine 5’-triphosphate (ITP) and 2’- and 3’-dATP to identify important functional groups of ATP for ATPase phosphorylation. The analogs differ from ATP at individual functional groups, which allows us to study the impact of these groups on the conformational change in the phosphorylation reaction (Ca\textsubscript{2}\textsubscript{E1ATP} → Ca\textsubscript{2}\textsubscript{E1P}). Photolabile derivatives, i.e. P\textsuperscript{3-1-(2-nitrophenyl)ethyl} nucleotides (caged nucleotides) (30), were used to trigger the protein reaction directly in the infrared cuvette in order to detect the small infrared absorbance changes associated with phosphorylation of the Ca\textsuperscript{2+}-ATPase.
Phosphorylation of SR Ca\textsuperscript{2+}-ATPase from ATP and ATP Analogs

EXPERIMENTAL PROCEDURES

Materials

SR vesicles were prepared as described (31). After a 60-min dialysis of 50 \mu L of SR vesicles in 100 mL of dialysis buffer (10 mM methylimidazole/HC\textsubscript{1}, pH 7.5, 200 \mu M CaCl\textsubscript{2}, and 10 mM KCl), infrared samples were prepared by drying 15 \mu L of SR suspension on a CaF\textsubscript{2} window with a roughness of 5-\mu m depth and 5-\mu m diameter and immediately rehydrating with ~0.8 \mu L of H\textsubscript{2}O. The sample was sealed with a second flat CaF\textsubscript{2} window. The approximate sample composition based on 1 \mu L of sample volume is 1.2 mM Ca\textsuperscript{2+},-ATPase, 0.5 mg/mL Ca\textsuperscript{2+} ionophore (A23187), 150 mM methylimidazole, pH 7.5, 150 mM KCl, 10 mM CaCl\textsubscript{2}, 10 mM dithiothreitol, 1 mg/mL adenylyl kinase (for ITP samples), and 10 mM caged nucleotide. Adenylate kinase, which regenerates ATP (2ADP + AMP Æ ATP), was added in samples with caged ATP and caged dATPs in order to repeat the kinetic experiments with one sample and ADP: ATP absence of ATPase. Apyrase liberates inorganic phosphate from ATP used in some samples with caged ATP or caged ADP in the presence/absence of adenylate kinase (32–34). The time constants of nucleotide binding (\tau\textsubscript{b}) and ATP hydrolysis were obtained by fitting the absorbance changes of the phosphorylation reaction (12, 36). Its band amplitude is three times smaller than that of the ATP binding spectrum, indicating smaller conformational changes in the phosphorylation step than upon nucleotide binding, in line with studies of FTIR spectroscopy (12), limited proteolysis (13), and crystal structures of complexes Ca\textsubscript{2+}E1AMPPCP and Ca\textsubscript{2+}E1ADP-AlF\textsubscript{4}\textsuperscript{-} (9, 10). Band assignments of the phosphorylation spectrum obtained with ATP have been discussed (36).

The phosphorylation spectrum obtained with ATP in the absence of adenylate kinase (data not shown) is very similar to that in the presence of adenylate kinase except that a larger band at 1241 cm\textsuperscript{-1} was observed compared with that obtained in the presence of adenylate kinase. The 1241 cm\textsuperscript{-1} band is due to a loss of one PO\textsubscript{4}\textsuperscript{3-} group upon ATP splitting.

Adenosine-O-PO\textsubscript{2}-PO\textsubscript{2}-PO\textsubscript{2}\textsuperscript{-} Æ adenosine-O-PO\textsubscript{2}-PO\textsubscript{2}+P,

(or Ca\textsubscript{2+}E1P) (Eq. 1)

This band has been assigned to the antisymmetric stretching mode (v\textsubscript{as}) of the PO\textsubscript{4}\textsuperscript{3-} group of bound nucleotide (36). The smaller amplitude observed in the phosphorylation spectrum with ATP (Fig. 2) is due to the presence of adenylate kinase, which regenerates ATP.

Phosphorylation with 2’d-ATP and ITP—Phosphorylation spectra obtained with 2’d-ATP and with ITP are shown in Fig. 2A and B. Double difference spectra in Fig. 2C show the difference between the phosphorylation spectrum obtained with 2’d-ATP or ITP and that with ATP. We termed them extra conformational change spectra obtained with 2’d-ATP or ITP as explained later.

In Fig. 2A, the phosphorylation spectrum with 2’d-ATP shows signals similar to that with ATP except for the bands at ~1643, 1241, and 1115 cm\textsuperscript{-1}. The difference at ~1643 cm\textsuperscript{-1} in the amide I region is possibly due to different conformational changes of \beta-sheets during phosphorylation. In the presence of adenylate kinase, the 1241 cm\textsuperscript{-1} band obtained with ATP is

integrated band intensities of the marker bands at 1628 and 1641 cm\textsuperscript{-1} for nucleotide binding, at 1718, 1657, and 1549 cm\textsuperscript{-1} (at 1722 and 1707 cm\textsuperscript{-1} for 3’d-ATP) for phosphorylation, and at 1241 cm\textsuperscript{-1} for nucleotide hydrolysis as described (12, 31, 36).

Calculation of Nucleotide Binding, Ca\textsubscript{2+}E1P Formation, and Phosphorylation Spectra—Nucleotide binding spectra show absorbance changes associated with the reaction Ca\textsubscript{2+}E1 Æ Ca\textsubscript{2+}E1P, and Ca\textsubscript{2+}E1P formation spectra show those associated with Ca\textsubscript{2+}E1 Æ Ca\textsubscript{2+}E1P. The time constants of \tau\textsubscript{b} and \tau\textsubscript{h} were used to determine the time intervals used to calculate the nucleotide binding spectra (\Delta t\textsubscript{b}) and Ca\textsubscript{2+}E1P formation spectra (\Delta t\textsubscript{h}) as listed in Table I. We chose the time interval starting at 3\textsubscript{b} and ending at half of \tau\textsubscript{b} for each nucleotide to calculate binding spectra. For obtaining Ca\textsubscript{2+}E1P formation spectra, we chose time intervals starting at 3\textsubscript{h} and ending at 68.1 s for ATP, ITP, and 2’d-ATP, and for 3’d-ATP ending at 224 s.

By subtracting the nucleotide binding spectrum from the Ca\textsubscript{2+}E1P formation spectrum, we obtained the phosphorylation spectrum showing the absorbance changes in the phosphorylation reaction (Ca\textsubscript{2+}E1P Æ Ca\textsubscript{2+}E1P as shown in Fig. 1). Phosphorylation spectra show the spectral differences between the two time intervals, \Delta t\textsubscript{b} and \Delta t\textsubscript{h}. Photolysis signals do not appear in phosphorylation spectra because they are identical in the nucleotide binding and the Ca\textsubscript{2+}E1P formation spectrum and are thus subtracted.

Difference spectra were normalized to a standard protein concentration (amide II absorbance 0.26) before averaging spectra from different samples to avoid the possible predominance of individual samples with high protein content in the averaged difference spectra, as described (29, 36). The number of averaged experiments and the amount of released nucleotides with different flash times are listed in Table I.

RESULTS

Phosphorylation with ATP—The phosphorylation spectrum obtained with ATP is shown in Fig. 2A (see also Table II). It reflects infrared absorbance changes in the reaction Ca\textsubscript{2+}E1ATP Æ Ca\textsubscript{2+}E1P. This spectrum is in agreement with those observed before (12, 36). Its band amplitude is three times smaller than that of the ATP binding spectrum, indicating smaller conformational changes in the phosphorylation step than upon nucleotide binding, in line with studies of FTIR spectroscopy (12), limited proteolysis (13), and crystal structures of complexes Ca\textsubscript{2+}E1AMPPCP and Ca\textsubscript{2+}E1ADP-AlF\textsubscript{4}\textsuperscript{-} (9, 10). Band assignments of the phosphorylation spectrum obtained with ATP have been discussed (36).

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Adenosine-O-PO\textsubscript{2}-PO\textsubscript{2}-PO\textsubscript{2}\textsuperscript{-} Æ adenosine-O-PO\textsubscript{2}-PO\textsubscript{2}+P,

(or Ca\textsubscript{2+}E1P) (Eq. 1)
smaller than that obtained with 2'-dATP as shown in Fig. 2A. However, with ATP in the absence of adenylate kinase, this band shows the same integrated band intensity and kinetic change as with 2'-dATP in the presence of adenylate kinase. Thus, we attribute the difference between ATP and 2'-dATP in the presence of adenylate kinase to faster regeneration of ATP from ADP as compared with that of 2'-dATP from 2'-dADP. Samples with 2'-dATP in the absence of adenylate kinase were made to investigate this further, and we observed no difference (data not shown) compared with 2'-dATP samples in the presence of adenylate kinase. Adenylate kinase was recently found to use ADP more efficiently than 2'-dADP (37). But in an earlier study the efficiency was very similar for ADP and 2'-dADP (38). Here our observation agrees with the most recent result (37). With ITP (no adenylate kinase present), the band amplitude at 1241 cm$^{-1}$ (Fig. 2B) is similar to that in the phosphorylation spectrum obtained with ATP in the absence of adenylate kinase.

As shown in Fig. 2B the phosphorylation spectrum obtained with ITP is significantly different from those obtained with ATP and with 2'-dATP. The differences compared with ATP are particularly pronounced in the amide I region. Larger band amplitudes indicate that larger conformational changes occur when ITP phosphorylates the ATPase. Bands at 1696, 1642, and 1621 cm$^{-1}$ in the amide I region of the phosphorylation spectrum obtained with ITP are different from those with ATP. They indicate different conformational changes of $\beta$-sheets or turns in the phosphorylation reaction with ITP.

In the following we will explore the characteristics of this extra conformational change upon phosphorylation with ITP. A spectrum of the extra conformational change is shown in Fig. 2C. This spectrum was calculated by subtracting the phosphorylation spectrum obtained with ATP from that obtained with ITP. In Fig. 3A this spectrum is compared with the spectra of ATP and ITP binding. As the figure shows, the extra conformational change spectrum with ITP is very similar in shape to the nucleotide binding spectra. A similar shape indicates a similar character of the conformational change, i.e. that secondary structure elements are perturbed in a similar manner. This indicates that conformational changes characteristic of ATP binding take place during phosphorylation with ITP. The band amplitudes in the extra conformational change spectrum are between those of ITP binding and ATP binding. This indicates that the extent of the extra conformational change is larger than that of ITP binding.

### Table I

| Nucleotide       | No. of experiments/no. of samples | Concentration of nucleotide/no. of flash | Time interval of nucleotide binding $\Delta_{\text{ATP}}/\Delta_{\text{E1P}}$ formation $\Delta_{\text{E1P}}$ |
|------------------|----------------------------------|----------------------------------------|--------------------------------------------------|
| ATP              | 23/12                            | 3 mM/1 flash                           | 0.46–0.90/5.84–68.1 s                             |
| 2'-dATP          | 8/4                              | 3 mM/1 flash                           | 0.46–3.24/21.4–68.1 s                             |
| ATP (no ADK)     | 4/4                              | 3 mM/1 flash                           | 0.46–3.24/26.7–68.1 s                             |
| ATP              | 4/4                              | 3 mM/1 flash                           | 0.10–0.33/5.84–68.1 s                             |

### Table II

| Band Positions | Tentative band assignments | Nucleotide |
|----------------|-----------------------------|------------|
| cm$^{-1}$      |                             |            |
| 1696–1690      | The amide I mode of $\beta$-sheet | ITP, 2'-dATP, 3'-dATP |
| 1673           | The amide I mode of turn     | ITP        |
| 1657           | The amide I mode of $\alpha$-helices | 3'-dATP |
| 1645–1610      | The amide I mode of $\beta$-sheet | ITP, 2'-dATP, 3'-dATP |
| 1241           | The $v_{\text{as}}$(PO$\text{2}$) mode of the bound nucleotide | ITP, 2'-dATP, 3'-dATP |
but smaller than that of ATP binding. Fig. 3A also shows that the extent of conformational change upon ITP binding is only about 30% that of ATP binding (21) as judged from the amplitude of the main bands. Because we can expect that the initial state in our samples is the same for all nucleotides, the different amplitudes in the binding spectra indicate a different structure of the nucleotide ATPase complexes as shown previously (21). However, if the extra conformational change spectrum with ITP is added to the ITP binding spectrum, the resulting spectrum has approximately 70% of the amplitude of the ATP binding spectrum (as judged from the band amplitudes of the two largest bands). This comparison is shown in Fig. 3B. The generated spectrum is in fact very similar in shape and amplitude to the 2′-dATP binding spectrum (21), which has about two times larger band amplitudes than the ITP binding spectrum. This indicates that if the extra conformational change upon phosphorylation with ITP had taken place already upon ITP binding, the structure of the ITP ATPase complex would be very similar to that obtained with 2′-dATP and more similar to that with ATP than actually observed.

The consequence of the extra conformational change upon phosphorylation with ITP is that the conformation of Ca₂E₁P obtained with ITP becomes more similar to that obtained with ATP in comparison to the nucleotide ATPase complexes with ITP and ATP. This is evident from the relatively similar band amplitudes of the Ca₂E₁P formation spectra with ITP and ATP (see Fig. 3C and Ref. 21) as compared with the large amplitude differences in the nucleotide binding spectra (see Fig. 3A and Ref. 21). The Ca₂E₁P formation spectrum with ITP is very similar to that obtained with 2′-dATP (21).

Phosphorylation with 3′-dATP—Absorption changes after 3′-dATP binding were also observed as shown in Fig. 4. The spectrum obtained with 3′-dATP differs from the other phosphorylation spectra (Fig. 2) in that it shows different band positions and amplitudes for most of the bands. The spectrum with 3′-dATP does not show the marker band for phosphorylation at 1718 cm⁻¹ observed with ATP and other ATP analogs. Instead, there are two bands at 1722 and 1707 cm⁻¹. The bands at 1674, 1643, and 1533 cm⁻¹ in the amide I region indicate that conformational changes occurred after binding. A band characteristic of ATP splitting is also observed at 1241 cm⁻¹ with 3′-dATP.

Despite the differences to the other phosphorylation spectra, we tentatively attribute this spectrum with 3′-dATP to phosphorylation of the ATPase for the following reasons. (i) It is known that 3′-dATP can phosphorylate the ATPase, although to a lower extent compared with ATP (39); (ii) there are bands at 1722 and 1707 cm⁻¹ close to the marker band of phosphorylation at 1718 cm⁻¹; (iii) the conformational changes after 3′-dATP binding are accompanied by 3′-dATP consumption as indicated by the negative band at 1241 cm⁻¹, which exhibits the same slow kinetic change as the band at 1722 cm⁻¹ (Fig. 5).

The absorbance changes after 3′-dATP binding are very slow; reaction kinetics of the bands at 1722, 1707, and 1241 cm⁻¹ observed with 3′-dATP (τ = 61, and 55 s, respectively) were ~30-fold slower than those with ATP (τ = 2 s). The time course of these bands is shown in Fig. 5. The absorption changes are only partly complete during the measuring time of our experiments. The difference spectrum shown in Fig. 4 was obtained by subtracting the 3′-dATP binding spectrum from the spectrum averaged from 165.4 to 224 s. This spectrum shows ~80% of the absorbance changes associated with the complete reaction (calculated from the fitting of the bands at 1722 and 1707 cm⁻¹).

Kinetics of the Conformational Change—Kinetic evaluation of the bands at 1657 or 1643 cm⁻¹ in the amide I region and at 1547 or 1533 cm⁻¹ in the amide II region (data not shown) gives the time constants of the conformational change of the protein backbone. For all nucleotides the time constants are the same as those of the marker band for enzyme phosphorylation at 1718 cm⁻¹ (at 1722 cm⁻¹ for 3′-dATP). The 1718 cm⁻¹ band has been assigned to the C=O stretching vibration of aspartyl phosphate on the basis of model compound studies (36). This shows that the conformational change of the protein backbone proceeds with the same velocity as phosphorylation at Asp-351.

Effects of ADP on Ca₂E₁P Conformation—To determine the effect of bound ADP on Ca₂E₁P conformation, ADP was removed by a helper enzyme, apyrase. Apyrase experiments were done with caged ADP and caged ATP in the presence/absence of the ATPase. In control experiments without ATPase there were no significant signals observed in the
amide I region with both ATP and ADP, which indicates that ATP or ADP interacting with apyrase does not induce observable absorbance changes at the apyrase concentration used. Therefore, the presence of apyrase will not disturb absorbance changes due to conformational changes of the ATPase. In samples that contain apyrase and ATPase, ATP or ADP binding to the ATPase and ATPase phosphorylation with ATP were observed with the same extent of conformational change and the same reaction rates as without apyrase. However, the largest bands at 1641 and 1628 cm\(^{-1}\) in the amide I region decay to 70% after phosphorylation with ATP or decay to nearly zero after 16 s in experiments of ADP binding, whereas they are constant in the absence of apyrase. For the ATP experiments we evaluated the kinetics of the 1628 cm\(^{-1}\) band by integrating the 1628 cm\(^{-1}\) band with respect to a base line that includes the negative 1641 cm\(^{-1}\) band (see the legend of Fig. 6). The result of integration, therefore, reports on both bands, which were used to quantify the extent of conformational change (21). Phosphorylation of SR Ca\(^{2+}\)-ATPase from ATP and ATP Analogs

**DISCUSSION**

The Conformational Change Observed by Infrared Spectroscopy—Previously we have estimated that the net change in secondary structure involves up to 10 amino acids for all partial reactions of the SR Ca\(^{2+}\)-ATPase (12). The net change upon nucleotide binding was substantial and comparable with those of Ca\(^{2+}\) binding and phosphoenzyme conversion. Changes observed upon phosphorylation were smaller but of the same order of magnitude, in line with very similar structures found for a nucleotide-ATPase complex and a Ca\(_{2}\)E1P analogue (9, 10). With the availability of structures for Ca\(_{2}\)E1 and the ATPase complex with AMPPCP, the value of 10 residues for a net change of secondary structure can now be scrutinized. We analyzed the structures with a modified Kabsch and Sander algorithm (40) as implemented in the program ICMLite and considered the secondary structure types, helical structures, \(\beta\)-strand, and others. The number of residues involved in a net change of secondary structure are 6, 15, and 8, respectively, which is similar to the difference between the two Ca\(_{2}\)E1 structures 1EUL and 1SU4. In fact, the number of residues involved in a net change of secondary structure is 46, about as large as that between the Ca\(_{2}\)E1 1EUL structure and the Ca\(_{2}\)E1AMPPCP 1T5S structure. Because of its higher resolution we chose the 1SU4 structure for Ca\(_{2}\)E1 to explore differences between the two Ca\(_{2}\)E1 structures 1EUL and 1SU4 and molecules A and B of Protein Data Bank entry 1VFP (10). The number of residues involved in a net change of secondary structure are 6, 15, and 8, respectively, which is similar to the differ-

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![Graph A](image1.png)

**FIG. 5.** A, kinetics of bands at 1722, 1707, and at 1241 cm\(^{-1}\) in the experiments with 3\(\text{-}d\)ATP. B, comparison of kinetics at the 1718 or 1722 cm\(^{-1}\) band for phosphorylation with ATP, ITP, 2\(\text{-}d\)ATP, and 3\(\text{-}d\)ATP.

![Graph B](image2.png)

**FIG. 6.** Kinetics of the 1628 cm\(^{-1}\) band in the experiments with ATP in the presence/absence of apyrase. The integration was done with respect to a base line drawn between two points that were the average of data points between 1643 and 1638 as well as 1611 and 1607 cm\(^{-1}\). This gives a similar measure of the extent of conformational change as the one we have used before (21).
ences observed between the 3 Ca$_2$E1AMPcP structures where the number is 3–10. Because infrared spectroscopy can detect only a net change in a structure, many individual compensating changes will not be revealed. In fact, the actual number of residues undergoing a change between the secondary structure types considered is larger; that is, 54–77 residues for the Ca$_2$E1 (1SU4) to Ca$_2$E1AMPcP transition. This discussion indicates that (i) the net change in secondary structure between Ca$_2$E1 and Ca$_2$E1AMPcP is very small and is correctly estimated by infrared spectroscopy (12) and that (ii) caution should be applied when structures are chosen to calibrate infrared spectra for secondary structure analysis. Secondary structure algorithms will give different secondary structure contents depending on resolution and crystallization conditions.

In our previous work (21) we have tentatively attributed most of the amide I signals (1700–1610 cm$^{-1}$) to the N-P hinge region. There we discussed that our infrared spectra will not reflect the N-P hinge movement directly because we assumed the hinge region to be flexible. Instead we anticipated that hinge movements will reflect indirectly in infrared spectra due to conformational changes in adjacent well structured regions. However, a recent NMR study (41) on the isolated N domain of Na$^+$, K$^+$-ATPase showed that the N hinge region has a defined conformation. Therefore, movements in the N-P hinge region might well reflect directly in infrared spectra.

The prominent band near 1628 cm$^{-1}$ in our nucleotide binding spectra (Fig. 3) (21) and in the extra conformational change spectrum obtained with ITP (Fig. 2C) will not be caused by the N-P hinge region. Such low wavenumber bands are due to $\beta$-sheets with a considerable number of parallel or antiparallel strands. The spectral position is sensitive to the number of aligned strands and the planarity of the sheets (for antiparallel $\beta$-sheets) but is nearly independent to the number of residues per strand (42–44). There are three extended $\beta$-sheets in the Ca$_2$E1-ATPase, one in each of the N, A, and P domains. In the following we will tentatively attribute the 1628 cm$^{-1}$ and the adjacent 1641 cm$^{-1}$ band to a structural change of the $\beta$-sheet in the P domain. The N domain moves as a rigid body in the transition from Ca$_2$E1 to Ca$_2$E1AMPcP (10); thus, its $\beta$-sheet is not expected to contribute significantly to the band at 1628 cm$^{-1}$. The $\beta$-sheet in the A domain gains six residues in the transition as revealed by a secondary structure analysis of Ca$_2$E1 (1SU4) and all available Ca$_2$E1AMPcP structures. This, however, will not lead to a change in absorbance near 1628 cm$^{-1}$ since the additional residues are in $\beta$-sheet sections with only 2 antiparallel strands.

Most interesting is the observation that the $\beta$-sheet of P domain alters its structure and acts as a secondary hinge (10), in line with the view that the P domain should not be regarded as a rigid body (45). These changes are expected to reflect in infrared spectra as discussed in the following. In Ca$_2$E1AMPcP a short section of this sheet has 8 parallel strands comprising 2 residues per strand and a total number of 16 residues. The strands are connected by two lines of hydrogen bonding along residues 653–675/676–623/624–350/351–701/702–718/719–733/734–332 and 651–675/674–623/622–350/349–701/700–718/717–733/732–334. In Ca$_2$E1 the first line of hydrogen bonding is disrupted between the fourth and fifth strand (between residues 351 and 701; N-O distance increases from 2.7 to 5.7 Å), which effectively splits the $\beta$-sheet here into two parts. The second line of hydrogen bonding is disrupted in Ca$_2$E1 between the seventh and eighth strand (between residues 732 and 334; N-O distance increases from 2.8 to 3.9 Å), which narrows the $\beta$-sheet by one strand and weakens between the fourth and fifth strand (between residues 349 and 701; N-O distance increases from 2.8 to 3.2 Å). Disruption of hydrogen bonding will also disrupt the transition dipole coupling between these residues, which is the main determinant of the spectral position in the infrared spectrum.

The main consequence for infrared absorption is that transition dipole coupling between the two parts of the $\beta$-sheet is weaker in Ca$_2$E1, which will make the infrared absorption more like that of two $\beta$-sheets with four parallel strands. Thus, the P domain $\beta$-sheet is expected to absorb at higher wavenumber in Ca$_2$E1 than in Ca$_2$E1AMPcP. In line with this we observe a negative band at 1641 cm$^{-1}$ and a positive band at 1628 cm$^{-1}$ upon nucleotide binding (21), and we tentatively attribute a considerable portion of these bands to the change in $\beta$-sheet structure of the P domain.

Effects of the Adenine Ring of ATP on Phosphorylation—Differences between phosphorylation spectra obtained with ATP and with ITP are larger than those between phosphorylation spectra obtained with ATP and 2'-dATP (Fig. 2). However, a similar phosphorylation reaction rate and the same extent of Ca$_2$E1P accumulation were obtained with ITP and 2'-dATP (21), which indicates that the extent of conformational change is not an important factor for these properties.

Interestingly, Scofano et al. (46) observed an overshoot of phosphorylation with 1 mM ITP but not with 5 $\mu$M ATP (pH 7.4, room temperature, 0.1 mM CaCl$_2$, 1.0 mg/ml leaky vesicles). However, a similar steady state level of phosphoenzyme was obtained with 5 $\mu$M ATP and 1 mM ITP, and the same amount of phosphoenzyme formation with ITP or GTP as with ATP was observed at high Ca$^{2+}$ concentration (47, 48). Our results are partly consistent with these observations. In our study the accumulation of the Ca$_2$E1P state with 6.6 mM ITP and 3 mM ATP is similar. However, an overshoot of phosphorylation is not observed with ITP because the amplitude of the marker band at 1718 cm$^{-1}$ in the phosphorylation spectrum obtained with ITP is the same as that with ATP.

In the phosphorylation spectrum obtained with ITP, additional signals in the amide I region (Fig. 2C) were observed, which are similar to nucleotide binding signals as shown in Fig. 3A. Several explanations are in principle possible for the larger bands observed in the phosphorylation spectrum obtained with ITP as follows.

Nucleotide binding might be slow with ITP so that there is a convolution of binding and phosphorylation spectra. This can be excluded from the kinetic evaluation discussed before (21). The time constants for binding and phosphorylation with ITP are 0.23 and 8.66 s, respectively. The nucleotide-ATPase complex and the phosphoenzyme with ITP are even better separated in time than with ATP. The slow phase of the bands at 1628 and 1641 cm$^{-1}$ (where the main differences with respect to ATP are observed) has the same rate as the phosphorylation marker band at 1718 cm$^{-1}$ (data not shown).

In ITP samples, ~6.6 mM ITP was released, ~3.6 mM more than in ATP samples. The additional bands in the amide I region of the phosphorylation spectrum of ITP could be due to the extra amount of ITP interacting with the ATPase in the phosphorylation reaction. However, in samples with 3 mM released ITP, a phosphorylation spectrum with a similar shape was observed (data not shown), showing that the extra amount of ITP has no influence on the appearance of the additional bands in the ITP phosphorylation spectrum. In addition, ITP was found not to bind to the regulatory site of the ATPase (49).

A fraction of ATPase could exist in dimers or higher oligomers that allow ITP to bind only to one of the ATPase molecules. Phosphorylation of this molecule then allows ITP to bind to the second ATPase molecule followed by rapid phosphorylation. One would then have to assume that ATP binding is not governed by this allosteric effect, although the ATP binds.
ing-induced conformational changes are similar in character to those induced by ITP.

The most likely explanation is that larger conformational changes occur upon phosphorylation with ITP. In the proceeding step of the reaction cycle, the conformational change induced by ITP binding is smaller than that induced by ATP binding (21). As discussed above the extra conformational change with ITP during phosphorylation is similar to that upon nucleotide binding. If the extra conformational changes occurring during phosphorylation with ITP occurred in the ITP binding reaction, the conformational changes would be very similar to those occurring upon 2'-dATP binding (Fig. 3B). The 1628 cm\(^{-1}\) band was tentatively attributed above to the secondary hinge movement of the \(\beta\)-sheet in the P domain. Therefore, our phosphorylation spectrum with ITP indicates that this \(\beta\)-sheet hinge movement is promoted upon phosphorylation by ITP but not by the other nucleotides used.

In line with our result, a fluorescence study (50) showed also that a fluorescence change observed for ATP binding was not observed for ITP binding but later for phosphorylation by ITP. Transient interactions during phosphorylation might be responsible for the extra conformational change with the result that the enzyme snaps into the closed conformation. For 2'-dATP and ATP this conformational change is largely induced upon binding. Thus, it seems that upon phosphorylation with ITP the enzyme catches up on a conformational change that cannot be achieved by ITP binding because the interactions between protein and base moiety are impaired. Analysis of crystal structures of Ca\(_2\)E1AMP1PPC (9, 10) shows that Glu-442 is close to the adenine amino group. This residue will repulse the negative partial charge on the inosine oxygen. This may explain the weaker binding of ITP and the smaller extent of conformational change upon ITP binding (21). After ATPase phosphorylation with ITP, IDP may dissociate from the phosphoenzyme under our conditions, which will relieve the repulsion, resulting in extra conformational change upon phosphorylation with ITP.

Effects of the Ribose OH Groups of ATP on Phosphorylation—
The crystal structure of Ca\(_2\)E1AMP1PPC (10) shows that the ribose 2'-OH interacts by van der Waals contacts with the N domain but not with the P domain. Arg-678 and Arg-560 may interact electrostatically with the negative partial charge on the oxygen of 2'-OH. This may explain the effect of 2'-OH modification on binding and phosphorylation rate (21). The phosphorylation spectra of ATP and 2'-dATP are very similar. This demonstrates that 2'-OH has little impact on the conformational change upon phosphorylation. Small differences are evident in the extra conformational change spectrum shown in Fig. 2C. The extra conformational change spectrum of 2'-dATP is similar to that of ITP in shape, but the band amplitudes are considerably smaller. This indicates that there are also extra conformational changes occurring with 2'-dATP upon enzyme phosphorylation, although their extent is much smaller than for ITP.

In our previous study (21) we inferred that Ca\(_2\)E1P could not be formed from 3'-dATP because there was no clear marker band at 1718 cm\(^{-1}\) for Ca\(_2\)E1P formation. In this work, however, the kinetic analysis reveals slow absorbance changes that we attribute to the phosphorylation reaction as discussed under “Results.”

The ribose 3'-OH is important for enzyme phosphorylation since 3'-dATP has unique properties, it phosphorylates the ATPase very slowly (\(t_\text{p} = 54\) s), its phosphorylation spectrum is very different from the other nucleotides, and its Ca\(_2\)E1P formation spectrum (data not shown) exhibits only very small absorbance changes in the amide I region. This implies that the Ca\(_2\)E1P conformation obtained with 3'-dATP is more similar to that of Ca\(_2\)E1 than to Ca\(_2\)E1P obtained with ATP. Thus, the hinge between the N and P domain will be as open after phosphorylation with 3'-dATP as before, i.e. the phosphoenzyme would not adopt the closed conformation, as obtained with ATP. Phosphorylation might not require the closed conformation since non-nucleotide substrates like acetyl phosphate can also phosphorylate the ATPase but may not be able to link N and P domain upon binding.

It was found (39) that 3'-NH\(_2\)-ATP was utilized in a manner equivalent to ATP. The 3'-NH\(_2\) group is similar to the 3'-OH group as a donor of hydrogen bonds, whereas the 3'-H group is an acceptor of a hydrogen bond. The similar results obtained with 3'-NH-ATP and ATP and the differences observed with 3'-dATP and ATP can, therefore, be explained by the 3'-OH group forming a hydrogen bond (39), which was found to be with the \(\beta\)-phosphate in the crystal structure of Ca\(_2\)E1AMPPCP (9). This hydrogen bond stabilizes a bend conformation of the triphosphate chain, helping stabilize the AMP-PCP-ATPase complex for the subsequent enzyme phosphorylation (9). Crystal structures of Ca\(_2\)E1AMPPCP (9, 10) also show that the 3'-OH interacts with Arg-678 of the P domain, which appears to form hydrogen bonds with residues both in the N and P domains. The position of Arg-678 is such that the 3'-OH hydrogen is directed toward the \(\beta\)-phosphate. Our infrared results agree with these crystal structures and point out the importance of the 3'-OH on enzyme phosphorylation. Its existence and interaction with other groups seem to be prerequisites for the closed conformation upon nucleotide binding (21) and for fast phosphorylation.

Previously, the rate of phosphorylation of the Ca\(_2\)E1-ATPase was found to decrease by more than one order of magnitude with 2'-dATP and 3'-dATP (39). We also observed slower phosphorylation with the two dATPs, but the extent of decreased phosphorylation rate was less (~4-fold) with 2'-dATP.

**Effect of ADP on Phosphoenzyme Conformation**—The results obtained in our apyrase experiments in the presence of ATP and the ATPase elucidate the ADP effect on the conformation of Ca\(_2\)E1P. With the helper enzyme apyrase, ADP dissociation from the phosphoenzyme Ca\(_2\)E1P is irreversible because apyrase removes ADP. ADP dissociation reverses some of the conformational changes of nucleotide binding with the effect that Ca\(_2\)E1P conformation is more similar to the Ca\(_2\)E1 conformation without ADP than with bound ADP (Fig. 6). We, therefore, conclude that ADP binds to Ca\(_2\)E1P, which promotes the closed conformation of the phosphoenzyme. After phosphorylation with ITP or 2'-dATP, IDP or 2'-dADP seems not to have such an effect on Ca\(_2\)E1P conformation as ADP. Ca\(_2\)E1P without bound ADP may adopt a defined more open conformation of the cytoplasmic domains, or the equilibrium between closed and open conformation is more on the open side than with bound ADP.

**Conclusions**—Our results demonstrate that infrared spectroscopy can be used to probe ligand-protein interactions. In the particular case of phosphorylation of the SR Ca\(_2\)E1-ATPase, modifications at the 2'-OH, 3'-OH, and amino group of ATP affect phosphorylation of the Ca\(_2\)E1-ATPase by affecting the conformational change of the protein backbone and by slowing down the reaction velocity. Conformational changes upon phosphorylation are characteristic of the nucleotide used as shown by the different phosphorylation spectra obtained with different nucleotides. This indicates that interactions of the ATP molecule distant from the phosphatase groups contribute to approaching the \(\gamma\)-phosphate to the phosphorylation site Asp-351 and/or to forming the phosphate binding pocket; the functional groups 2'-OH and 3'-OH and the adenine ring of ATP are
important for inducing the conformational change of the ATP-ATPase complex that is competent for phosphoryl transfer. Therefore, phosphorylation is an interactive process in which the formation of interactions of the γ-phosphate is reinforced by interactions of other ATP groups, which can be at the opposite end of the ATP molecule.

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REFERENCES

1. Hasselbach, W., and Makino, M. (1961) Biochem. Z. 333, 518–528
2. Hasselbach, W. (1974) in The Enzymes (Boyer, P. D., ed) 3rd Ed., pp. 431–467, Academic Press, Inc., New York
3. Makino, M., and The, R. (1965) Biochem. Z. 343, 383–393
4. Toyoshima, C., and Inesi, G. (2004) Annu. Rev. Biochem. 73, 269–292
5. McIntosh, D. B. (2000) Biochemistry 39, 129–135
6. Kendrew, J., and Lawrence, E., eds (1994) The Encyclopedia of Molecular Biology, 1st Ed., p. 624, Blackwell Science, Oxford
7. Toyoshima, C., Nakasako, M., Nomura, H., and Ogawa, H. (2000) Nature 405, 647–655
8. Toyoshima, C., and Nomura, H. (2002) Nature 418, 605–611
9. Sorensen, T. L. M., Moller, J. V., and Nissen, P. (2004) Science 304, 1672–1675
10. Toyoshima, C., and Mizutani, T. (2004) Nature 430, 529–535
11. Hua, S., Nomura, H., and Toyoshima, C. (2002) Biochemistry 41, 11405–11410
12. Barth, A., von Germar, F., Kreutz, W., and Mäntele, W. (1996) J. Biol. Chem. 271, 30637–30646
13. Danko, S., Yamasaki, K., Daiho, T., Suzuki, H., and Toyoshima, C. (2001) FEBS Lett. 505, 129–135
14. Colthup, N. B., Daly, L. H., and Wiberley, S. E. (1990) Introduction to Infrared and Raman Spectroscopy, 3rd Ed., Academic Press, Inc., New York
15. Siebert, F. (1995) Methods Enzymol. 246, 501–526
16. Cantor, C. R., and Schimmel, P. R. (1980) Biophysical Chemistry. Part II: Techniques for the Study of Biological Structure and Function, pp. 466–480, W. H. Freeman and Co., New York
17. Goormaghtigh, E., Cabiaux, V., and Rayschaert, J. M. (1994) Subcell. Biochem. 25, 329–362
18. Barth, A., and Zscherp, C. (2002) Q. Rev. Biophys. 35, 369–430
19. Cepus, V., Scheidig, A. J., Goody, R. S., and Gerwert, K. (1998) Biochemistry 37, 10263–10271
20. Granjon, T., Vacheron, M.-J., Vial, C., and Buchet, R. (2001) Biochemistry 40, 2988–2994
21. Liu, M., and Barth, A. (2003) J. Biol. Chem. 278, 10112–10118
22. Griffiths, P. R., and de Haseth, J. A. (1986) Fourier Transform Infrared Spectrometry, John Wiley & Sons, New York
23. Wharton, C. W. (2000) Nat. Prod. Rep. 17, 447–453
24. Arrondo, J. L. R., Muga, A., Castresana, J., and Guí, F. M. (1993) Prog. Biophys. Mol. Biol. 59, 23–56
25. Jackson, M., and Mantsch, H. H. (1995) Crit. Rev. Biochem. Mol. Biol. 30, 95–120
26. Braiman, M. S., and Rothschild, K. J. (1988) Annu. Rev. Biophys. Chem. 17, 541–570
27. Jun, C. (2000) J. Mol. Recognit. 13, 325–351
28. Liu, M., and Barth, A. (2002) Biospectroscopy 67, 267–270
29. Liu, M., and Barth, A. (2003) Biochem. J. 384, 3262–3270
30. Kaplan, J. H., Forbush, B., and Hoffman, J. F. (1978) Biochemistry 17, 1929–1935
31. De Meis, L., and Hasselbach, W. (1971) J. Biol. Chem. 246, 4759–4763
32. Suzuki, H., Nakamura, S., and Kanazawa, T. (1994) Biochemistry 33, 8240–8246
33. Shigekawa, M., Wakabayashi, S., and Nakamura, H. (1983) J. Biol. Chem. 258, 8698–8707
34. Lapacarpe, J.-J., and Guillain, F. (1990) J. Biol. Chem. 265, 8585–8589
35. Barth, A., Mantele, W., and Kreutz, W. (1991) Biochim. Biophys. Acta 1057, 115–123
36. Barth, A., and Mantele, W. (1998) Biochim. J. 375, 538–544
37. Resnick, S. M., and Zehnder, A. J. B. (2000) Appl. Environ. Microbiol. 66, 2045–2051
38. Ladner, W. E., and Whitesides, G. M. (1985) J. Org. Chem. 50, 1076–1079
39. Coan, C., Amaral, J. A., and Verjovski-Almeida, S. (1993) J. Biol. Chem. 268, 6917–6924
40. Kabsch, W., and Sander, C. (1983) Biopolymers 22, 2577–2637
41. Hilge, M., Siegal, G., Vuister, G. W., Guntert, P., Bloor, S. M., and Abrahams, J. P. (2003) Nat. Struct. Biol. 10, 468–474
42. Chiragdez, V. N., and Nevskaya, N. A. (1976) Biopolymers 15, 627–636
43. Chiragdez, V. N., and Nevskaya, N. A. (1976) Biopolymers 15, 607–625
44. Kuhelka, J., and Keiderling, T. A. (2001) J. Am. Chem. Soc. 123, 12048–12058
45. Reuter, N., Hissen, K., and Lacapere, J. J. (2003) Biophys. J. 85, 2186–2197
46. Scafano, H. M., Vieyra, A., and De Meis, L. (1979) J. Biol. Chem. 254, 10227–10231
47. de Meis, L., and de Mello, M. C. F. (1973) J. Biol. Chem. 248, 3691–3701
48. Souza, D. O. G., and de Meis, L. (1976) Biopolymers 15, 3262–3270
49. Reuter, N., Hissen, K., and Lacapere, J. J. (2003) Biophys. J. 85, 2186–2197
50. Scofano, H. M., Vieyra, A., and De Meis, L. (1979) J. Biol. Chem. 254, 10227–10231
51. Holmgren, A., and de Mello, M. C. F. (1973) J. Biol. Chem. 248, 3691–3701
52. Souza, D. O. G., and de Meis, L. (1976) Biophys. J. 26, 6355–6359
53. Petretski, J. H., Wolusker, H., and De Meis, L. (1989) J. Biol. Chem. 264, 20339–20343
54. Kubo, K., Suzuki, H., and Kanazawa, T. (1990) Biochim. Biophys. Acta 1040, 251–259