Mapping Interactions between the Ca\textsuperscript{2+}-ATPase and Its Substrate ATP with Infrared Spectroscopy*

Received for publication, December 5, 2002, and in revised form, January 13, 2003
Published, JBC Papers in Press, January 21, 2003, DOI 10.1074/jbc.M212403200

Man Liu and Andreas Barth‡

From the Institut für Biophysik, Johann Wolfgang Goethe-Universität, Theodor-Stern-Kai 7, Haus 74, D-603590 Frankfurt am Main, Germany

Infrared spectroscopy has been used to map substrate-protein interactions: the conformational changes of the sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase upon nucleotide binding and ATPase phosphorylation were monitored using the substrate ATP and ATP analogues (2'-deoxy-ATP, 3'-deoxy-ATP, and inosine 5'-triphosphate), which were modified at specific functional groups of the substrate. Modifications to the 2'-OH, the 3'-OH, and the amino group of adenine reduce the extent of binding-induced conformational change of the ATPase, with particularly strong effects observed for the latter two. This demonstrates the structural sensitivity of the nucleotide-ATPase complex to individual interactions between nucleotide and ATPase. All groups studied are important for binding and interactions of a given ligand group with the ATPase depend on interactions of other ligand groups.

Phosphorylation of the ATPase was observed for ITP and 2'-deoxy-ATP, but not for 3'-deoxy-ATP. There is no direct link between the extent of conformational change upon nucleotide binding and the rate of phosphorylation showing that the full extent of the ATP-induced conformational change is not mandatory for phosphorylation. As observed for the nucleotide-ATPase complex, the conformation of the first phosphorylated ATPase intermediate E1P\textsubscript{Ca\textsuperscript{2+}} also depends on the nucleotide, indicating that ATPase states have a less uniform conformation than previously anticipated.

Ligand binding to proteins controls vast numbers of cellular processes and has attracted great scientific and economic interest. Protein and ligand flexibility are important determinants of the interaction and often lead to ligand binding modes that are not anticipated from structures obtained with other ligands. To these “failure(s) of the rigid receptor hypothesis” (1) is added here an impressive example: induced-fit binding of nucleotides to the Ca\textsuperscript{2+}-ATPase. This finding stems from a systematic mapping of substrate-protein interactions with infrared (IR) spectroscopy. New approaches like this are welcomed in the field of ligand-protein recognition, since the most informative techniques, NMR and x-ray crystallography, are laborious and problematic for some systems. Methods like fluorescence and luminescence that require less expenditure also provide less molecular information. We expect that this technology gap will be bridged by IR spectroscopy.

IR spectroscopy, one of the methods of vibrational spectroscopy, provides direct information on the molecular level, is cost-effective, and can be universally applied from small soluble proteins to large membrane proteins under near-physiological conditions. Work summarized in recent reviews (2–5) has shown that the vibrational spectrum changes characteristically when a ligand binds to a protein. This provides a direct observation of ligand binding: no marker compound has to be introduced to report the binding process, as with many other methods. Previous work has mostly focused on individual interactions between a ligand and a protein by monitoring the influence of the protein environment on the vibrational frequency of a particular group of the ligand, the signal of which is identified in a complex vibrational spectrum with the help of isotopically labeled ligands (6–8).

Here we employ a different approach to probe the role of single functional groups of a ligand in the interaction with a protein: using IR spectroscopy we monitored the protein conformational change induced by binding of substrate analogues, which are modified at specific functional groups of the substrate. This identifies those functional groups that are important in the interaction with the protein; structure-interaction relationships are obtained that are similar to structure-activity relationships in drug development that relate the chemical structure of compounds to their pharmacological activity.

This work studies the ATP binding site of the sarcoplasmic reticulum (SR) Ca\textsuperscript{2+}-ATPase (9–12). The SR Ca\textsuperscript{2+}-ATPase, an intrinsic membrane protein of about 110-kDa molecular mass, catalyzes Ca\textsuperscript{2+} transport from the cytoplasm of muscle cells into SR for relaxing a flexed muscle. The energy required for this active transport process is provided by hydrolysis of the substrate ATP, which phosphorylates the ATPase at Asp\textsubscript{351}. The specificity of the SR Ca\textsuperscript{2+}-ATPase for nucleotides is not high and not only ATP, but also some other nucleotides and non-nucleotide substrates enable Ca\textsuperscript{2+} uptake (13–18).

The ATPase structure (19) of the Ca\textsuperscript{2+}-loaded state E1Ca\textsubscript{2+} shows three cytoplasmic domains, the nucleotide binding domain (N-domain), the phosphorylation domain (P-domain), and the actuator domain (A-domain). The structure has been solved with and without 2’,3’-O-(2,4,6-trinitrophenyl)adenosine 5’-monophosphate (TNP-AMP), which binds to the N-domain at

AMPNP, β,γ-imidoadenosine 5’-triphosphate; E1Ca\textsubscript{2+}, the nucleotide-free ATPase; &E1TPC\textsubscript{a\textsubscript{2+}}, the nucleotide-ATPase complex; DTGS, deuterated triglycine sulfate; MSA, maximum signal amplitude; IMPNP, β,γ-iminoinosine 5’-triphosphate.

* This work was supported by Deutsche Forschungsgemeinschaft: Grant Ba1887/2-1. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be advertised solely to indicate this fact.
‡ To whom correspondence should be addressed: Dept. of Biochemistry and Biophysics, Arrhenius Laboratories for Natural Sciences, Stockholm University, S-106 91 Stockholm, Sweden. Tel.: 46-8-16-2452; Fax: 46-8-15-5597; E-mail: Andreas.Barth@dbb.su.se.

The abbreviations used are: IR, infrared; SR, sarcoplasmic reticulum; N-domain, the nucleotide binding domain; P-domain, the phosphorylation domain; A-domain, the actuator domain; TNP-AMP, 2’,3’-O-(2,4,6-trinitrophenyl)adenosine 5’-monophosphate; caged nucleotide, P\textsuperscript{3}-1-(2-nitrophenyl)ethyl nucleotides; A23187, calcium ionophore; A23187, calcium ionophore; D-60590 Frankfurt am Main, Germany

From the Institut für Biophysik, Johann Wolfgang Goethe-Universität, Theodor-Stern-Kai 7, Haus 74, D-603590 Frankfurt am Main, Germany

This paper is available on line at http://www.jbc.org
Mapping Interactions between Nucleotide and the Ca^{2+}·ATPase

EXPERIMENTAL PROCEDURES

Materials

IR samples were prepared as described previously (28). Approximate concentrations of the samples based on 1-µl sample volume were: 1.2 mM Ca^{2+}·ATPase, 0.5 mM Ca^{2+} ionophore (A23187), 150 mM methylimidazole (pH 7.5), 150 mM KCl, 10 mM CaC_{2}O_{4}, 5 mM DTT, and 10 mM caged nucleotide. ITP samples were also prepared with 10 mM DTT replacing 5 mM CaC_{2}O_{4} from the samples for the AMPPNP spectrum. After nucleotide release while slowing down the phosphorylation reaction (29), replacement of Mg^{2+} by Mn^{2+} and MnATP was used to subtract the photolysis spectrum from the raw difference spectra as described (33) using the same time interval for both spectra. This photolysis spectrum is identical to that of other caged nucleotides above 1300 cm^{-1}, i.e. outside the region of phosphate absorption.

Nucleotide Binding Spectra—For the nucleotide binding spectra time windows after the photolysis flash were evaluated in which the nucleotide-ATPase complex (E1NTPC_{2}) accumulates. They were between 0.46 and 0.90 s for ATP or between 0.46 and 3.24 s for ATP analogues. 23 experiments from 12 samples were averaged for the ATP binding spectrum (−3 ms released ATP, one flash), four experiments from four samples for the IP binding spectrum (−6.6 ms released ATP, three flashes), eight experiments from two samples for the 2′-deoxy-ATP binding spectrum (−3 ms released 2′-deoxy-ATP, one flash), and two experiments from three samples for the 3′-deoxy-ATP binding spectrum (−3 ms released 3′-deoxy-ATP, one flash).

Absorption Spectra of Nucleotides—Absorption spectra of 500 mM ATP, 2′- and 3′-deoxy-ATP dissolved in H_{2}O were measured using two BaF_{2} windows (5-µm path length) with a Bruker Vector 22 spectrometer equipped with a deuterated triglycine sulfate (DTGS) detector at 20 °C at different pH values. The population of the C_{3}·endo puckering modes of the three nucleotides were obtained by calculating the ratio of the areas of bands fitted to the spectrum near 830 cm^{-1} for C_{3}·endo puckering and near 814 cm^{-1} for C_{3}·endo puckering (36–38).

RESULTS

Titrations of IR Signals with Nucleotide Binding—We first established the nucleotide concentration needed to obtain saturating IR signals. For that we used the difference in amplitude of the band pair at 1628 and 1641 cm^{-1} in the amide I region of the IR spectrum, which is sensitive to conformational changes. This difference is termed maximum signal amplitude (MSA). Fig. 2 shows the result of titrations with ATP, ITP, 2′-deoxy-ATP, and 3′-deoxy-ATP applying a total of eight flashes, which released ~9.4 mM nucleotide. According to Fig. 2, the binding-induced amplitude difference MSA reached saturating values with the first flash for ATP, 2′- and 3′-deoxy-ATP, and with the third flash for ITP. From the photolysis efficiency of 30% we then calculated the saturating nucleotide concentration.
Assuming 1 µl of sample volume: 3 mM (one flash) for ATP and 2'- and 3'-deoxy-ATP and 6.6 mM (three flashes) for ITP.

For these titration experiments, spectra were evaluated in a time slot where the first phosphoenzyme E1PCa2 accumulates. Strictly speaking, they have therefore determined the nucleotide concentration necessary for saturating E1PCa2, not for saturating E1NTPCa2. However, the reactions of nucleotide binding and of phosphorylation are well separated in time, which ensures that E1NTPCa2 also saturates in the time slot evaluated for the nucleotide binding spectra: time constants for nucleotide binding ($t_n$) and phosphorylation ($t_p$) and the time slot of spectra recording for the nucleotide binding spectra ($t_s$) were: for ATP, $t_n = 0.11$ s, $t_p = 2.0$ s, $t_s = 0.46–0.90$ s; for 2'-deoxy-ATP, $t_n = 0.28$ s, $t_p = 6.7$ s, $t_s = 0.46–3.24$ s; for 3'-deoxy-ATP, $t_n = 0.49$ s, $t_p > 100$ s, $t_s = 0.46–3.24$ s; and for ITP, $t_n = 0.23$ s, $t_p = 8.66$ s, $t_s = 0.46–3.24$ s (see “Methods”). The starting time of 0.46 s for spectra averaging might seem to be early for ITP and 2'- and 3'-deoxy-ATP, since it is close to the time constant for nucleotide binding. However, increasing the starting time to 1.5 s for 3'-deoxy-ATP and 0.8 s for ITP and 2'-deoxy-ATP did not change the maximum signal amplitude MSA by more than 3%. Therefore we kept the time slot for averaging consistent for the three ATP analogues and as large as possible for an optimum signal to noise ratio.

Nucleotide Binding Spectra—Fig. 3A shows IR absorbance changes induced by nucleotide binding to the Ca$^{2+}$-ATPase. The spectra reflect the difference in absorbance between the initial nucleotide-free state E1Ca2 and the nucleotide-ATPase complex E1NTPCa2. Negative bands are characteristic of E1Ca2 and positive bands of E1NTPCa2. Groups or structures not involved in the conformational change do not show up in the difference spectra.

The difference spectra reflect conformational changes of the protein backbone in the amide I ($1700–1610$ cm$^{-1}$) and amide II ($1580–1500$ cm$^{-1}$) region of the spectra. In addition, environmental and structural changes of side chains and nucleotide contribute in the whole mid-IR region shown. We will focus here on the binding-induced absorbance changes in the amide I region.

The spectrum of ATP binding is in close agreement with the AMPPNP binding spectrum as noted before (33). The positive signal near 1653 cm$^{-1}$ is characteristic of an α-helical structure, the signals near 1693, 1641, and 1628 cm$^{-1}$ of β-sheets. Turn structures likely contribute to the signals near 1665 cm$^{-1}$. The spectrum indicates that α-helices, β-sheets, and turns are affected by ATP binding in line with previous findings (33).

The contour of all nucleotide binding spectra is similar; the main difference is the amplitude of the signals indicating various extents of conformational change. For further evaluation we used the MSA (difference between the absorbance change at 1628 cm$^{-1}$ and that at 1641 cm$^{-1}$). As shown in Fig. 3B, the largest binding-induced signals were obtained with ATP and AMPPNP (28) (MSA $\approx 3 \times 10^{-3}$), medium size signals (MSA $\approx 2 \times 10^{-3}$) with ADP (28), and 2'-deoxy-ATP, and the smallest signals (MSA $\approx 1 \times 10^{-3}$) with ITP and 3'-deoxy-ATP. The different amplitudes of the nucleotide binding spectra cannot be explained by incomplete binding to the ATPase, since we have verified that saturating signals have been obtained (see above). MSA values shown in Fig. 3 for nucleotide binding spectra differ slightly from MSA values shown in Fig. 2, for which a time slot was evaluated in which E1PCa2, not E1NTPCa2, accumulated, because of conformational changes accompanying the phosphorylation reaction.

Spectra of Phosphoenzyme Formation—Phosphorylation leads to the appearance of two bands at 1721 and 1549 cm$^{-1}$, which serve here as marker bands for the first phosphorylated intermediate E1P1Ca2 (29, 35). ATP, 2'-deoxy-ATP, and ITP, but not 3'-deoxy-ATP, phosphorylate the ATPase at a rate that is sufficiently high to observe accumulation of the E1P1Ca2 state. Fig. 4 shows spectra of E1P1Ca2 formation from E1Ca2, i.e. the absorbance of E1P1Ca2 minus the absorbance of E1Ca2 (see “Methods”). As found for nucleotide binding, the shape of the E1P1Ca2 formation spectra is similar for the analogues but the amplitude is different. In contrast, the same amplitude is observed for the band at 1721 cm$^{-1}$, which has been tentatively assigned to the C=O group of Asp$^{331}$ formed upon phosphorylation (34, 35). This local probe of the phosphorylation reaction shows that E1PCa2 accumulates to the same extent with ITP.
and 2'-deoxy-ATP as with ATP. The smaller signals obtained with the analogues can therefore not be explained by incomplete phosphorylation. Instead they are due to a smaller extent of conformational change showing that the conformation of E1PCa2 depends on the nucleotide used for phosphorylation of the ATPase.

Phosphorylation Rate—The rate of phosphorylation was measured using the marker bands at 1721 and 1549 cm⁻¹ (29, 35). The kinetics of the two bands is shown in Fig. 5. Phosphorylation of the ATPase by 2'-deoxy-ATP or ITP is slower (0.15 ± 0.01 s⁻¹ and 0.12 ± 0.01 s⁻¹, respectively) than by ATP (0.51 ± 0.03 s⁻¹). Slower phosphorylation of the ATPase with ITP and 2'-deoxy-ATP compared with ATP has been observed (39, 40), but without specifying the rate for ATP. Previous findings revealed a low phosphoenzyme concentration for both 2'- and 3'-deoxy-ATP (40), which we found only for 3'-deoxy-ATP. This is likely due to the different buffers used, since nucleotide binding (41) and the associated conformational change (28) depend on the composition of the medium.

**DISCUSSION**

Interaction between ATPase and ATP—Our results show that modifications to the amino, 2'-OH, 3'-OH, and γ-phosphate groups of ATP affect the binding-induced conformational change of the ATPase. These groups are therefore important for the interaction between ATP and the ATPase. 3'-OH and the region near the amino group have the most significant influence on the induced-fit movement of the ATPase, since modification to either of these groups reduces the extent of backbone conformational change seen by IR spectroscopy to only one-third of that obtained with ATP. They are therefore important groups of ATP that anchor ATP to the ATPase. The importance of the functional groups of ATP investigated here for several partial steps of the ATPase reactions cycle has been shown before (39, 40, 42). The new finding here is that modifications of the ATP molecule have a direct effect on the structure of the nucleotide-ATPase complex. This is valid not only for side chain orientation as often found (1) but also for backbone conformation: with some ATP analogues the binding-induced conformational change of the backbone seen by the IR spectroscopy was found to be only one-third of that for ATP.

The effects of modifying ATP on nucleotide binding might have several causes; (i) a direct interaction of the modified group of ATP with the ATPase, (ii) a direct interaction of the "new" group of the ATP analogue, and (iii) an indirect effect on the interactions between protein and ATP via a change in electron density or conformation of ATP.

A direct interaction is the most likely cause for the reduced extent of conformational change observed for the deoxy-ATPs, since (i) no sterical restraints are expected from the replacement of the hydroxyl groups by the smaller hydrogen atoms, and (ii) effects on the equilibrium between sugar conformations of ATP in solution seem to be less relevant for the sugar conformation of the bound ATP molecule discussed as follows. 2'- or 3'-H substitution influence the sugar conformation in solution; according to the absorption spectra of ATP and 2'- and 3'-deoxy-ATP with deprotonated phosphate groups, the ratios of C₂-endo and C₃-endo puckering of these three nucleotides are 60:40, 80:20, and 70:30, respectively. This shows that free nucleotides prefer C₂-endo puckering, particularly deoxynucleotides. Similar results for ATP and 2'-deoxy-ATP were obtained before (43–45). NMR investigations demonstrate that C₂- and C₃-endo types of conformations are in rapid equilibrium in solution (43), indicating only a small activation barrier between the conformations. Despite the predominant C₂-endo puckering in solution, the ATPase seems to choose the C₃-endo conformation for binding, as determined by NMR (45). Therefore the conformation of the nucleotide-ATPase complex will not depend on the predominant sugar puckering in solution and the effects of ribose OH substitution are best explained by direct interactions of the ribose hydroxyls with the ATPase.

It is less certain whether the reduced extent of conformational change observed for the deoxy-ATPs, since (i) no sterical restraints are expected from the replacement of the hydroxyl groups by the smaller hydrogen atoms, and (ii) effects on the equilibrium between sugar conformations of ATP in solution seem to be less relevant for the sugar conformation of the bound ATP molecule discussed as follows. 2'- or 3'-H substitution influence the sugar conformation in solution; according to the absorption spectra of ATP and 2'- and 3'-deoxy-ATP with deprotonated phosphate groups, the ratios of C₂-endo and C₃-endo puckering of these three nucleotides are 60:40, 80:20, and 70:30, respectively. This shows that free nucleotides prefer C₂-endo puckering, particularly deoxynucleotides. Similar results for ATP and 2'-deoxy-ATP were obtained before (43–45). NMR investigations demonstrate that C₂- and C₃-endo types of conformations are in rapid equilibrium in solution (43), indicating only a small activation barrier between the conformations. Despite the predominant C₂-endo puckering in solution, the ATPase seems to choose the C₃-endo conformation for binding, as determined by NMR (45). Therefore the conformation of the nucleotide-ATPase complex will not depend on the predominant sugar puckering in solution and the effects of ribose OH substitution are best explained by direct interactions of the ribose hydroxyls with the ATPase.

It is less certain whether the reduced extent of conformational change observed for the deoxy-ATPs, since (i) no sterical restraints are expected from the replacement of the hydroxyl groups by the smaller hydrogen atoms, and (ii) effects on the equilibrium between sugar conformations of ATP in solution seem to be less relevant for the sugar conformation of the bound ATP molecule discussed as follows. 2'- or 3'-H substitution influence the sugar conformation in solution; according to the absorption spectra of ATP and 2'- and 3'-deoxy-ATP with deprotonated phosphate groups, the ratios of C₂-endo and C₃-endo puckering of these three nucleotides are 60:40, 80:20, and 70:30, respectively. This shows that free nucleotides prefer C₂-endo puckering, particularly deoxynucleotides. Similar results for ATP and 2'-deoxy-ATP were obtained before (43–45). NMR investigations demonstrate that C₂- and C₃-endo types of conformations are in rapid equilibrium in solution (43), indicating only a small activation barrier between the conformations. Despite the predominant C₂-endo puckering in solution, the ATPase seems to choose the C₃-endo conformation for binding, as determined by NMR (45). Therefore the conformation of the nucleotide-ATPase complex will not depend on the predominant sugar puckering in solution and the effects of ribose OH substitution are best explained by direct interactions of the ribose hydroxyls with the ATPase.

It is less certain whether the reduced extent of conformational change observed for the deoxy-ATPs, since (i) no sterical restraints are expected from the replacement of the hydroxyl groups by the smaller hydrogen atoms, and (ii) effects on the equilibrium between sugar conformations of ATP in solution seem to be less relevant for the sugar conformation of the bound ATP molecule discussed as follows. 2'- or 3'-H substitution influence the sugar conformation in solution; according to the absorption spectra of ATP and 2'- and 3'-deoxy-ATP with deprotonated phosphate groups, the ratios of C₂-endo and C₃-endo puckering of these three nucleotides are 60:40, 80:20, and 70:30, respectively. This shows that free nucleotides prefer C₂-endo puckering, particularly deoxynucleotides. Similar results for ATP and 2'-deoxy-ATP were obtained before (43–45). NMR investigations demonstrate that C₂- and C₃-endo types of conformations are in rapid equilibrium in solution (43), indicating only a small activation barrier between the conformations. Despite the predominant C₂-endo puckering in solution, the ATPase seems to choose the C₃-endo conformation for binding, as determined by NMR (45). Therefore the conformation of the nucleotide-ATPase complex will not depend on the predominant sugar puckering in solution and the effects of ribose OH substitution are best explained by direct interactions of the ribose hydroxyls with the ATPase.

It is less certain whether the reduced extent of conformational change observed for the deoxy-ATPs, since (i) no sterical restraints are expected from the replacement of the hydroxyl groups by the smaller hydrogen atoms, and (ii) effects on the equilibrium between sugar conformations of ATP in solution seem to be less relevant for the sugar conformation of the bound ATP molecule discussed as follows. 2'- or 3'-H substitution influence the sugar conformation in solution; according to the absorption spectra of ATP and 2'- and 3'-deoxy-ATP with deprotonated phosphate groups, the ratios of C₂-endo and C₃-endo puckering of these three nucleotides are 60:40, 80:20, and 70:30, respectively. This shows that free nucleotides prefer C₂-endo puckering, particularly deoxynucleotides. Similar results for ATP and 2'-deoxy-ATP were obtained before (43–45). NMR investigations demonstrate that C₂- and C₃-endo types of conformations are in rapid equilibrium in solution (43), indicating only a small activation barrier between the conformations. Despite the predominant C₂-endo puckering in solution, the ATPase seems to choose the C₃-endo conformation for binding, as determined by NMR (45). Therefore the conformation of the nucleotide-ATPase complex will not depend on the predominant sugar puckering in solution and the effects of ribose OH substitution are best explained by direct interactions of the ribose hydroxyls with the ATPase.
tional change found with ITP can be explained by a localized interaction between the amino group and the ATPase. In ITP the carbonyl group replaces the amino group of ATP, and one of the two endocyclic nitrogen atoms is protonated. These alterations are not localized only on the amino group but will change the electron density distribution in the entire six-membered ring and its hydrogen bonding pattern. Therefore the interaction seems to be located on the six-membered ring of adenine. Since the most drastic alteration is at the amino group, it is likely, but not mandatory, that our results reflect a direct interaction of the ATPase with the amino group.

All functional groups of ATP investigated here are important for inducing the conformation of the ATP-ATPase complex that is competent for phosphoryl transfer. This is shown by the dependence of the phosphorylation rate on the modification of 2'-OH, 3'-OH, and the adenine amino group. Therefore, interactions distant from the phosphate groups contribute to approaching or forming the phosphate binding pocket. Binding of ATP to the ATPase turns out to be an interactive process where the formation of interactions of a given functional group of ATP is reinforced by interactions of other groups, which can be at the opposite end of the ATP molecule.

Distance between γ-Phosphate and Asp$^{352}$—Our data show that phosphorylation does not strictly depend on the full extent of the conformational change achieved by ATP binding. This is in line with the observation that pseudo substrates like acetyl phosphate are able to produce the same kind of phosphoenzyme as ATP (42), although they are not expected to induce the same conformational change as ATP, because their structures are even more different from ATP than those of the nucleotides investigated here.

There is no simple link between the extent of conformational change upon nucleotide binding and the ability to form the phosphoenzyme: (i) the extent is larger for 2'-deoxy-ATP than for ITP but the apparent phosphorylation rates are very close, and (ii) the extent is similar for ITP and 3'-deoxy-ATP, but significant phosphorylation is only observed for ITP. If the conformational change detected in our spectra and the distance between γ-phosphate and phosphorylation site Asp$^{351}$ were correlated, a small conformational change would place the γ-phosphate further away from Asp$^{351}$ than a larger one and result in slower phosphorylation. Therefore the conformational change seen in our spectra seems to be not or only weakly correlated with the distance between γ-phosphate and Asp$^{351}$. This finding is in line with models where the γ-phosphate in the nucleotide-ATPase complex is still some distance away from the phosphorylation site, as proposed by Hua et al. (46) for the Ca$^{2+}$-ATPase and Eittrich et al. (47) for the Na$^+$/K$^+$-ATPase. Then, the γ-phosphate arrives at the catalytic site only after nucleotide binding, which could take place during the conformational change after nucleotide binding that has been identified as the rate-limiting step for phosphorylation (48). A γ-phosphate in some distance to the phosphorylation site provides a possibility of binding a regulatory ATP molecule to the phosphoenzyme at the same site (49).

Concerted Conformational Change—The interactions between nucleotide and protein induce a concerted conformational change upon nucleotide binding: they join forces to induce strain in the protein. If one of the interacting groups is modified to become a less effective binder, the interactions with the respective binding pocket are impaired, the strain is relieved, and a smaller conformational change is produced. A weakened interaction therefore affects the conformational change as a whole instead of producing only local effects. This concept explains that the modifications of ATP studied reduce all bands in the amide I region of the difference spectrum. If an interaction between nucleotide and ATPase had only local effects on the protein structure, a weakened interaction would selectively reduce the amplitude of difference bands associated with that conformational change, but not of all of the bands as observed here. Particularly interesting is that functional groups of ATP, which interact with different domains of the protein, produce the same type of conformational change: the amino function is thought to interact with the N-domain (19, 24, 46) and the γ-phosphate with the P-domain (25, 26, 46). Despite that, the absence of the γ-phosphate in ADP (28) or of the adenine amino group in ITP both reduce the amplitude of the same bands. This shows that the concerted conformational change detected here is caused by interactions of the nucleotide with different protein domains: the N- and the P-domain.

Nucleotide-specific Conformation—Our results suggest that nucleotide binding induces a conformation that is characteristic of the bound nucleotide, as proposed earlier from experiments that did not monitor the conformation of the nucleotide-ATPase complex directly (50). In light of the known flexibility of the N-domain (51, 52), this conformation might represent an average conformation. The (average) conformation adopted in the ATPase-nucleotide complex seems to be very sensitive to individual interactions between ATPase and nucleotide, since the extent of conformational change depends dramatically on the presence of individual functional groups of ATP.

Our finding of a nucleotide specific conformation of the nucleotide-ATPase complexes is supported by previous reports, in which different effects of different nucleotides were found on fluorescence properties (17, 18, 53), partial reaction rates (54–57), protection against proteolysis (21), effects of aromatic compounds (58), nucleotide binding properties of mutants (25), and uncoupling (59).

The structures of the nucleotide-ATPase complexes studied differ in two aspects: (i) the extent of the conformational change induced by nucleotide binding differs as indicated by the different amplitudes of the amide I signals, and (ii) structural details of the nucleotide-ATPase complex differ as shown by the subtle differences of band positions and spectral shape among the nucleotide binding spectra.

A structure characteristic of the nucleotide is inferred not only for the nucleotide-ATPase complex but also for E1Pase, where the conformation of the phosphoenzyme depends on the nucleotide that was used for phosphorylation.

The small conformational change upon ITP binding observed here suggests that soaking E1Pase crystals with β,γ-iminodipropionine 5'-triphosphate (IMPPNP) may not disrupt the crystals as ATP does (19) because of the relatively small conformational change seen here for ITP. This may therefore enable the investigation of nucleotide binding at atomic resolution. The conformational change induced by ATP binding may then be extrapolated from the conformational change seen upon IMPPNP binding.

The Conformational Change Reflected in IR Spectra—Conformational changes in two regions of the protein were proposed to occur upon nucleotide binding (21): (i) movement of the N-domain toward the P-domain and (ii) movement of the A-domain toward the P-domain. The latter does not seem to contribute to a large extent to our spectra for the following reason: Danko et al. (21) studied protection of the ATPase against proteolytic attack by various nucleotides. This effect is thought to reflect a movement of the A-domain. They found no effect for ADP, indicating that ADP does not promote significant movement of the A-domain. Our IR spectra of nucleotide binding, however, show that ADP binding induces a conformational change, the extent of which is two-thirds of that induced by ATP (28) (Fig. 3B). This shows that the conformational change
of the A-domain contributes not or only to a small extent to the IR difference spectra.

Instead, it is likely that the anticipated hinge movement of the N- and P-domain upon ATP binding causes the amide I signals. The hinge movement will, however, not directly reflect in our spectra, because highly mobile structural elements give broad IR bands before and after the conformational change, which largely cancel in the difference spectrum. Therefore IR spectroscopy will largely miss a conformational change in the mobile hinge region itself. In line with this, only small bands in a limited spectral region (1660 to 1680 cm\(^{-1}\)) can be assigned to mobile structures, since they exchange their amide proton upon \(^{2}H_{2}O/^{2}H_{2}O\) exchange (33). Instead the nucleotide binding bands in the amide I region are caused by backbone stretches within well defined and stable structures, since they are hardly affected by \(^{2}H_{2}O/^{2}H_{2}O\) exchange (33). In line with this finding of conformational changes in well structured regions, NMR spectroscopy has detected changes of backbone conformation in the N-domain upon AMP binding (60).

These conformational changes in well structured regions might report the hinge movement indirectly, since a conformational change in the hinge region will also affect the connecting stretches. These stretches become more ordered the more they are incorporated into the domains and therefore give rise to distinct bands in the amide I region; a hinge movement will alter the relative orientation of the connecting amide groups and their hydrogen bonding and therefore affect their amide I signals. From these considerations we think that our spectra detect the hinge movement indirectly because it is reported by structured backbone stretches that link hinge and domains.

**The Hinge Movement between N- and P-domain**—The hinge movement upon nucleotide binding seems, however, to be less pronounced than anticipated in the structural models (61, 62). Fluorescence energy transfer experiments show that distances between fluorescence labels in the N- and the P-domain do not change between E1Ca\(_{2}\) or an E2 conformation, as reviewed in Ref. 63. Of particular interest is the unchanged distance of two pairs of residues for which a change in distance is expected from the two x-ray structures (19, 46). The distance between Cys\(_{8}\) and Lys\(_{8}\) increases from 46 Å in E2 to 50 Å in E1Ca\(_{2}\), and that between Cys344 and Glu439 changes from 38 Å in E2 to 45 Å in E1Ca\(_{2}\). These distance changes should result in decreases in fluorescence energy transfer by 33% and 41%, respectively, which are not observed (64, 65).

The hinge movement can bring the N- and P-domain close together in the E1Ca\(_{2}\) state, since they can be cross-linked with glutaraldehyde (66). The cross-linked cleft of E1Ca\(_{2}\) resembles that of the E2 structure, since the cross-linked residues are only 5 Å apart in the E2 structure but 21 Å in the E1Ca\(_{2}\) structure. In line with these experiments, closure of the hinge could be modeled with the N- and P-domain structures of E1Ca\(_{2}\) without steric clashes, and this brings the two cross-linked residues as close as 4 Å (62). The mobility of the N-domain (51, 52) implies that it is likely to move rather independently from the rest of the protein and that the hinge movement might depend less on the E2 and E1Ca\(_{2}\) state than expected from the crystal structures. The more closed conformation in E2 and the open cleft between the N- and P-domain in E1Ca\(_{2}\) of the crystal structures therefore most likely do not represent the average conformation of these states in solution. They are probably adopted in the crystals because of crystal contacts that are made possible by the mobility of the N-domain in both states (51, 52). In solution the average position of the N-domain will be probably in between those observed in the two crystal structures. Therefore it is plausible to assume that the cleft is less open for E1Ca\(_{2}\) in solution than in the E1Ca\(_{2}\) crystal structure, and that upon nucleotide binding the hinge movement between the N- and P-domain will be smaller than anticipated from the crystal structure.

Our results demonstrate that IR spectroscopy can be used to map ligand-protein interactions and may become an important tool for research as well as for drug and herbicide optimization. In the particular case of ATP binding to the SR Ca\(^{2+}\)-ATPase, modifications to the 2′-OH, 3′-OH, and amino group of ATP reduce the induced-fit movement of the Ca\(^{2+}\)-ATPase, with the six-membered ring of adenine and the 3′-OH of ribose exerting key interactions. Nucleotide binding seems to be a flexible and interactive process: the conformation of the complex is characteristic of the bound nucleotide, and the interactions to a given ligand group depend on interactions of other ligand groups. This finding may also shed new light on the ongoing controversy on the number of nucleotide binding sites. Many of these studies have been conducted with ATP analogues. If binding of an ATP analogue induces a conformation that is characteristic of only that analogue, results with different analogues are not necessarily comparable and do not necessarily reflect the effects of ATP binding. Therefore we propose that some of the conflicting results can be explained by the different conformations of the complexes obtained with different analogues.

**Acknowledgments**—We thank W. Mäntele for continuous support, W. Hasselbach (Max-Planck-Institut, Heidelberg, Germany) for the gift of Ca\(^{2+}\)-ATPase and J. E. T. Corrie (National Institute for Medical Research, London) and F. von Germain for the preparation of caged compounds. We are grateful to C. Toyoshima for sharing unpublished results with us.

**REFERENCES**

1. Davis, A. M., and Teague, S. J. (1999) Annu. Rev. Physiol. 61, 37–55
2. Barth, A., and Zscherp, C. (2000) FEBS Lett. 477, 151–156
3. Wharton, C. W. (2000) Nat. Prod. Rep. 17, 447–453
4. Carey, P. R., and Tonge, P. J. (1985) Acc. Chem. Res. 18, 8–13
5. Deng, H., and Callender, B. (1999) Methods Enzymol. 308, 176–201
6. Belasco, J. G., and Knowles, J. R. (1980) Biochemistry 19, 472–477
7. Cepas, V., Schlegel, A. J., Goody, R. S., and Gerwert, K. (1999) Biochemistry 37, 10263–10271
8. Baenziger, J. E., Miller, K. W., and Rothchild, K. J. (1993) Biochemistry 32, 5448–5454
9. Hasselbach, W., and Makinose, M. (1961) Biochim. Biophys. Acta 28, 518–528
10. Hasselbach, W. (1979) in Membrane Transport (Martonosi, A., ed) 2nd Ed., Vol. 3, pp. 157–191, Plenum Press, New York
11. Andersen, J. P. (1989) Biochim. Biophys. Acta 988, 47–72
12. Lee, A., and East, J. (2001) Biochem. J. 355, 665–683
13. Hasselbach, W. (1979) Top. Curr. Chem. 78, 1–56
14. Hasselbach, W. (1981) in Membrane Transport (Bonting, S. L., and De Pont, J. J. H. M., eds) pp. 183–208, Elsevier Science Publishers B.V., Amsterdam
15. Itzhaki, R., and De Meirleir, L. (1985) in The Enzymes of Biological Membranes (Martonosi, A., ed) 2nd Ed., Vol. 3, pp. 157–191, Plenum Press, New York
16. McIntosh, D. B. (1998) Adv. Mol. Cell Biol. 23A, 33–99
17. Lacapere, J.-J., Bennett, N., Dupont, Y., and Guillain, F. (1995) J. Biol. Chem. 270, 348–353
18. Wakisaka, S., and Shigekawa, M. (1999) Biochemistry 39, 683–688
19. Toyoshima, C., Nakasako, M., Nomura, H., and Ogawa, H. (2000) Nature 405, 647–655
20. McIntosh, D. B. (1998) Biochim. Biophys. Acta 30637–30646
21. Danko, S., Yamasaki, K., Daiho, T., Suzuki, H., and Toyoshima, C. (2001) Acta Physiol. Scand. 171, 129–136
22. Andersen, J. P., and Vilsen, B. (1992) Acta Physiol. Scand. 146, 151–159
23. Mcintosh, D. B., Woolley, D. G., Vilsen, B., and Andersen, J. P. (1996) J. Biol. Chem. 271, 25778–25789
24. Mcintosh, D. B., Woolley, D. G., MacLennan, D. H., Vilsen, B., and Andersen, J. P. (1999) J. Biol. Chem. 274, 25237–25246
25. Clausen, J. D., McIntosh, D. B., Woolley, D. G., and Andersen, J. P. (2001) J. Biol. Chem. 276, 35741–35750
26. Kitazawa, H., Tori, S., and Hoffman, J. F. (1987) Biochemistry 16, 1929–1935
27. Liu, M., and Barth, A. (2002) Biochemistry 41, 267–270
28. Barth, A., von Germain, P., Kreutz, W., and Mantele, W. (1996) J. Biol. Chem. 271, 30637–30646
29. Suzuki, H., Nakamura, S., and Kanazawa, T. (1994) Biochemistry 33, 8240–8246
30. Shigekawa, M., Wakahayashi, S., and Nakamura, H. (1983) J. Biol. Chem. 258, 8698–8707
31. Lacapere, J.-J., and Guillain, F. (1996) J. Biol. Chem. 271, 8583–8589
Mapping Interactions between Nucleotide and the Ca\textsuperscript{2+}-ATPase

33. Von Germar, F., Barth, A., and Mantele, W. (2000) *Biophys. J.* **78**, 1531–1540
34. Barth, A., Kreutz, W., and Mantele, W. (1994) *Biochim. Biophys. Acta* **1194**, 75–91
35. Barth, A., and Mantele, W. (1998) *Biophys. J.* **75**, 538–544
36. Wartell, R. M., and Harrell, J. T. (1986) *Biochemistry* **25**, 2664–2671
37. Thomas, G. A., and Petesch, W. L. (1987) *Biochemistry* **26**, 5361–5368
38. Coan, C., Amaral, J. A., and Verjovski-Almeida, S. (1993) *J. Am. Chem. Soc.* **115**, 4264–4270
39. de Meis, L., and de Mello, M.-C.-F. (1973) *J. Biol. Chem.* **248**, 3691–3701
40. Hua, S., Inesi, G., Nomura, H., and Toyoshima, C. (2002) *Biochemistry* **41**, 11405–11410
41. Hasselbach, W., and The, R. (1975) *Eur. J. Biochem.* **53**, 105–113
42. Bodley, A. L., and Jencks, W. P. (1987) *J. Biol. Chem.* **262**, 13997–14004
43. Clore, G. M., Gronenborn, A. M., Mitchinson, C., and Green, N. M. (1982) *Eur. J. Biochem.* **128**, 113–117
44. Bigelow, D. J., and Inesi, G. (1992) *Biochim. Biophys. Acta* **1113**, 323–338
45. Stefanova, H. I., Mata, A. M., Gore, M. G., East, J. M., and Lee, A. G. (1993) *Biochim. Biophys. Acta* **1147**, 6–12
46. Hobbs, A. S., Albers, R. W., Froehlich, J. P., and Heller, P. F. (1985) *J. Biol. Chem.* **260**, 2035–2047
Mapping Interactions between the Ca$^{2+}$-ATPase and Its Substrate ATP with Infrared Spectroscopy
Man Liu and Andreas Barth

J. Biol. Chem. 2003, 278:10112-10118.
doi: 10.1074/jbc.M212403200 originally published online January 21, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M212403200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 64 references, 18 of which can be accessed free at http://www.jbc.org/content/278/12/10112.full.html#ref-list-1