Minireview

Substrate binding and enzyme function investigated by infrared spectroscopy

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Abstract Protein conformational changes triggered by molecule binding are increasingly investigated by infrared spectroscopy often using caged compounds. Several examples of molecule-protein recognition studies are given, which focus on nucleotide binding to proteins. The investigation of enzyme mechanisms is illustrated in detail using the Ca^{2+}-ATPase of the sarcoplasmic reticulum membrane as an example. It is shown that infrared spectroscopy provides valuable information on general aspects of enzyme function as well as on molecular details of molecule–protein interactions and the mechanism of catalysis.

Key words: ATPase; Kinase; H-*Ras* P21; GroEL; Fourier transform infrared spectroscopy; Nucleotide

1. Introduction

Elucidating the functions of proteins is a major challenge for the life science community. With developments in recent years, infrared spectroscopy [1–7] joined those biophysical methods that investigate enzyme function [8–11] and molecule-protein recognition on the molecular level. It is a non-invasive technique that can be applied to small soluble proteins as well as to large membrane proteins. The infrared spectrum of a protein encodes information about protein backbone conformation, side chain structure and environment. The sensitivity has been increased to the point where a change in the environment around a single atom can be detected.

The absorbance changes usually observed for protein reactions are very small, in the order of 0.1% of the maximum absorbance. In consequence, simply comparing the spectrum of a sample where the protein is in state A with a spectrum where it is in state B does not usually allow the sensitive detection of the small absorbance changes between the two protein states. Instead, the protein reaction of interest has to be initiated directly in the cuvette. To further complicate the experiment, the application of infrared spectroscopy to substrate-driven enzyme reactions is hindered by the strong water absorbance in the mid-infrared spectral range that requires an optical pathlength of 5–10 μm for H_2O solutions (30–50 μm in D_2O). Thus, adding the substrate to a protein sample is difficult. To overcome this problem, three approaches to generate a concentration jump in an infrared sample have been reported: (i) the use of an infrared stopped flow apparatus [12], (ii) the use of the ATR (attenuated total reflection) technique [13] and (iii) the photolytic release of effector molecules from biologically 'silent' precursors (termed 'caged compounds') as shown in Fig. 1 [14] for the archetype of caged compounds, caged ATP [15,16].

Given the limitations of space we will focus here on the third approach. A typical infrared experiment is performed as described in Fig. 2. At the beginning of the measurement the sample contains the protein of interest and the caged effector molecule. In its caged form, the effector molecule is modified such that it does not react with the protein of interest (see Fig. 1 for caged ATP). Upon a UV flash (300–350 nm) the caged molecule photolyses which leads to a sudden concentration jump (<10 ms) of free effector molecule. This triggers the enzymatic reaction which can be followed with a maximum time resolution of 10 ms using the rapid scan mode of Fourier transform infrared spectrometers. Time resolution in the sub-μs range can be achieved in single wavelength measurements [17] using a UV laser for photolysis and caged compounds with fast effector molecule release times [18]. Recently, an interesting new approach to obtain faster time resolution has been introduced using the step scan mode in combination with an infrared microscope [19].

From the spectrum recorded before effector molecule release and the spectra recorded after effector molecule release, difference spectra are calculated that originate only from those protein residues that are affected by effector molecule binding and/or consecutive reactions (Fig. 3). All 'passive' residues are invisible in the difference spectrum which, therefore, exhibit details of the reaction mechanism on the molecular level despite a large background absorption. Negative bands in difference spectra are characteristic of the initial state, while positive bands reflect the state(s) after effector molecule release. In addition to protein and effector molecule bands, the photolysis reaction is reflected in the difference spectra [20–22] (red, horizontal bars in Fig. 3).

2. Molecule-protein recognition studies

The characterisation of molecule binding sites of proteins

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Abbreviations: AMP-PNP, β,γ-iminoadenosine 5′-triphosphate; caged ATP, P^3-(1-(2-nitrophenyl)ethyl) adenosine 5′-triphosphate; Nitr 5, 1,2-amino-5-[1-hydroxy-1-(2-nitro-4,5-methylene dioxyphenyl) methyl]-phenoxyl-2-(2′-amino-5′-methylphenoxy) ethane-*N,N,N′,N′*-tetraacetic acid tetrasodium salt

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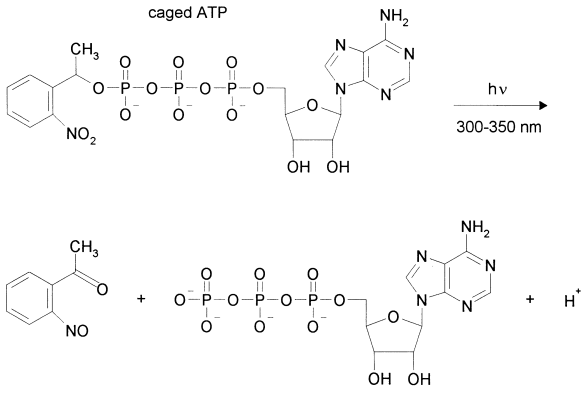

Fig. 1. Photolysis of caged ATP.

by infrared spectroscopy is a very promising approach to molecule-protein recognition studies. Infrared spectroscopy has the advantage that binding of virtually any molecule can be detected, since conformational changes of only a few peptide groups are readily observed in a difference spectrum.

Due to the availability of caged nucleotides, molecule binding studies have focussed on nucleotide binding to several proteins. Mapping of the nucleotide binding site of the sarcoplasmic reticulum Ca^{2+}-ATPase (a Ca^{2+} pump fuelled by ATP hydrolysis) has begun using ADP [23], ATP and β,γ-iminoadenosine 5′-triphosphate (AMP-PNP) [24,25]. Interactions between γ-phosphate and ATPase are highlighted by the different conformational changes upon ADP binding on the one hand and ATP and AMP-PNP binding on the other [25]. Subtle differences between the ATP and the AMP-PNP binding spectra seem to be caused by their different protonation state, due to the higher pK_a value of AMP-PNP. A signal at 1716 cm^{-1} (H_2O) of protonated aspartic and glutamic acid residues is only observed for ATP and may indicate protonation of carboxyl groups as a mechanism to compensate for the negative charges of the phosphate groups. A protonated tyrosine residue is also affected by nucleotide binding.

Creatine phosphate and arginine phosphate serve as reservoirs of high-energy phosphate compounds in muscles of vertebrates and invertebrates, respectively. In periods of muscle action with high energy consumption, both molecules can promptly transfer their phosphoryl group to ADP to re-gen-

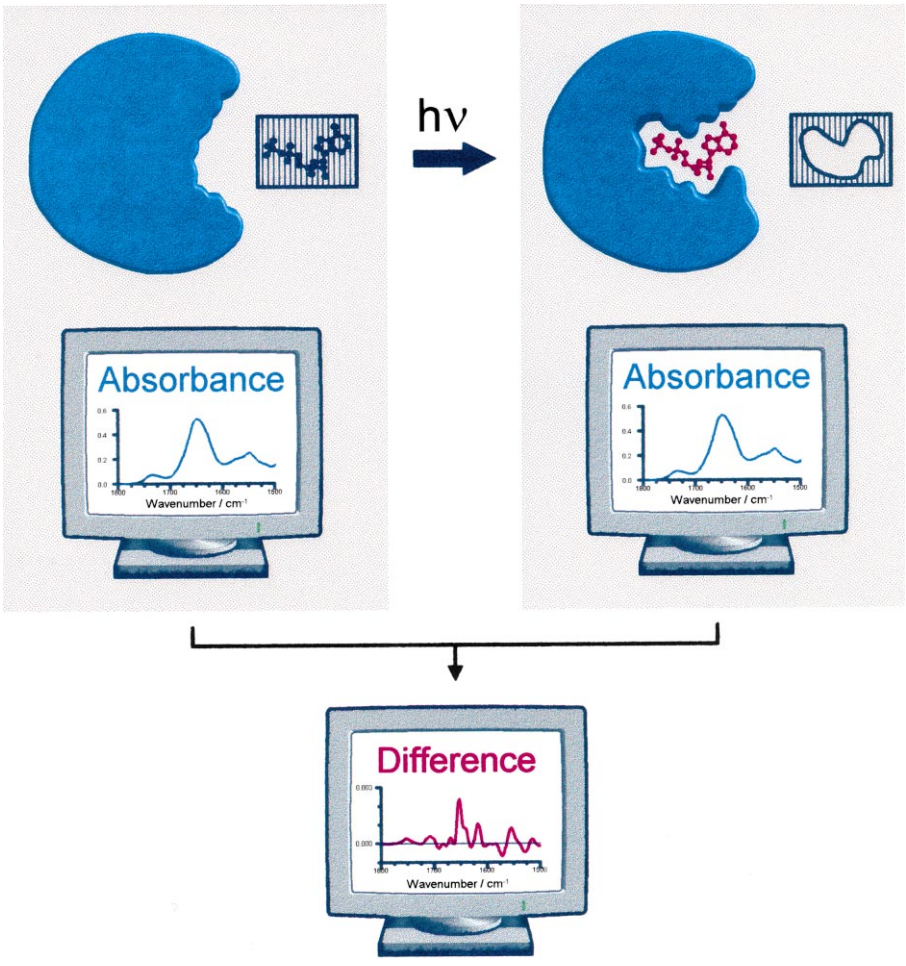

Fig. 2. Molecule binding and enzyme function investigated with the photolytic variant of concentration jump induced infrared difference spectroscopy. Left: the enzyme (blue) and the caged effector molecule (black) are shown. The effector molecule in its caged form does not react with the enzyme and in the case shown here does not bind to the protein. Right: Photolysis of the caged effector molecule releases the free molecule (red) which binds to the protein and triggers the enzymatic reaction. Infrared absorbance spectra are recorded of the free enzyme and of the enzyme–molecule complex. The small absorbance changes upon complex formation are not obvious in the absorbance spectra. They are identified only when the difference between the two absorbance spectra is calculated and the scale is enlarged 100-fold (bottom). The colour of the difference spectrum indicates that it originates from the interaction of the effector molecule shown in red with the protein shown in blue.

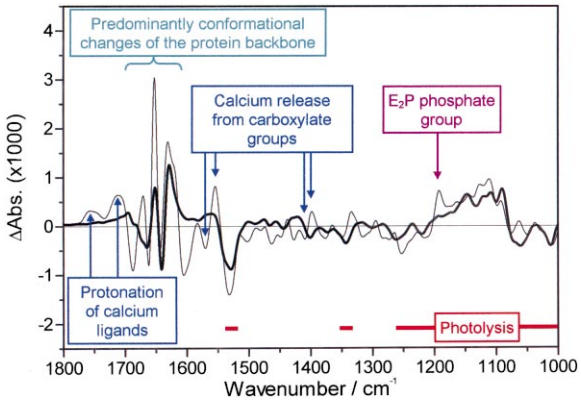

Fig. 3. Changes of infrared absorbance of Ca^{2+}-ATPase samples induced by ATP release (1°C, pH 7) [24]. Bold line: spectrum of ATP binding ($Ca_2E_1 \rightarrow Ca_2E_1ATP$) recorded 71–385 ms after ATP release. Thin line: spectrum of E_2-P formation ($Ca_2E_1 \rightarrow E_2$-P) recorded 1–2 min after ATP release with tentative assignments [39]. Photolysis bands are indicated with red, horizontal bars.

erate ATP. The enzymes catalysing the corresponding reversible reactions are called creatine kinase and arginine kinase. Binding of ADP and ATP to arginine kinase [26] induced similar but not identical conformational changes. This was also observed for creatine kinase [27]. Again, there is evidence of a protonation of an aspartic or glutamic acid residue of arginine kinase when ATP binds to the protein [26]. The conformational coupling between the ADP and arginine binding sites of arginine kinase [26] and of the ADP and creatine binding sites of creatine kinase [27] was found to be only weak, although the interactions between creatine and creatine kinase are altered upon ADP binding. Partial digestion of creatine kinase by proteinase K preserves the overall active site [28] in contrast to the point mutation W227Y where the nucleotide binding site is partially impaired [29].

Two studies that illustrate very well the power of isotopic labeling have dealt with GTP and GDP binding to H-*Ras* P21 [30,31]. H-*Ras* P21 is a regulatory protein that stimulates cell growth and differentiation in mammals when GTP is bound, thereby initiating a kinase cascade resulting in a transcriptional response. Slow hydrolysis of GTP inactivates the protein. This off-switch is important since a permanently activated signal transduction pathway leads to cancer. Using ^{18}O labeling of individual nucleotide phosphate groups, it was possible from the isotopic shifts in the infrared difference spectra to nearly completely assign the phosphate vibrations. As a result, interactions between protein and individual phosphate groups could be characterised, which have a profound impact on the GTP and GDP phosphate vibrations. In particular, the phosphate vibrations of GTP bound to H-*Ras* P21 are largely decoupled [30] and the degeneration of the terminal phosphate vibrations of GDP and possibly GTP is removed [31]. The interaction with the GTP β-phosphate seems to be particularly strong, which in turn weakens the bonds between β- and γ-phosphate and thus facilitates GTP hydrolysis [30]. However, there seems to be no correlation between the GTP hydrolysis rate of mutants and their binding strength to the GTP β-phosphate. In contrast, the hydrolysis rate correlates with the binding strength to the terminal β-phosphate of the product GDP, suggesting that the transition state is GDP-like [31].

Proper protein folding in vivo and in vitro is assisted by heat shock proteins, also known as chaperones. Their affinity for substrate proteins is controlled by nucleotide binding and hydrolysis. ATP binding to the chaperonin GroEL [32] first leads to relatively small conformational changes that are similar to those induced by ADP binding. Following the initial binding, a more pronounced conformational change into the active, ATP-hydrolysing state is observed on a time scale of seconds which relaxes within minutes to the state with bound ADP. The latter is very similar to the initial state with bound ATP.

So far, the difference spectra of nucleotide binding to different proteins show unique patterns in the amide I region of protein backbone absorbance, which is a monitor for conformational changes (see below). This seems to indicate that the conformational changes are unique for each protein studied. However, some common features are observed. The strongest difference bands usually appear at similar band positions in the region of 1655–1615 cm^{-1} where α-helices and β-sheets absorb [25–27,32,33]. Two bands near 1650 and 1640 cm^{-1} with opposite signs are found for four of the five proteins discussed above and a band shoulder structure around 1625/1615 cm^{-1} for all five proteins. The sign of the latter spectral feature is unrelated to the former two bands. It is tempting to speculate that the 1650/1640 band pair and the 1625/1615 band shoulder structure represent two conformational changes that may occur independently of each other. Bands at 1650 cm^{-1} are usually assigned to α-helical structures, while bands below 1630 cm^{-1} are assigned to β-sheets. Evidence has been found in two cases [25,27] that γ-phosphate binding affects the 1625/1615 cm^{-1} bands and it is interesting to see that hydrolysis of GTP at H-*Ras* P21 and release of the cleaved phosphate group causes a band at 1627 cm^{--1} to disappear [30].

3. The transport mechanism of the Ca^{2+}-ATPase

The potential of infrared spectroscopy in the investigation of enzyme function is illustrated here by the example of the sarcoplasmic reticulum Ca^{2+}-ATPase. This enzyme was the first to be studied by the photolytically induced concentration jump technique [14]. The Ca^{2+}-ATPase belongs to the P-type ATPase family and mediates muscle relaxation by the removal of cytosolic Ca^{2+}. Two Ca^{2+} ions are actively transported at the expense of hydrolysis of one ATP molecule [34–37] in a

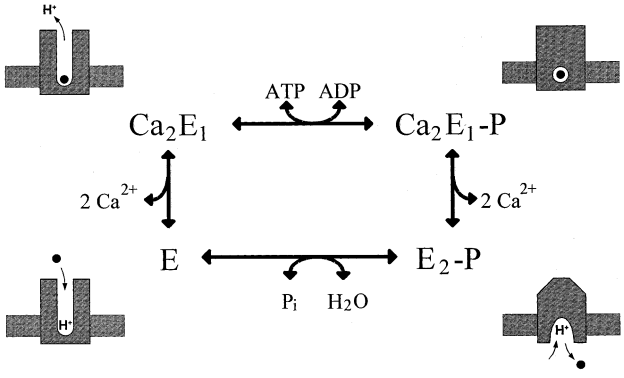

Fig. 4. Reaction scheme of the Ca^{2+}-ATPase, simplified from [44]. The Ca^{2+} free state E without subscript indicates an equilibrium between an E_1 and an E_2 state. Of the transported Ca^{2+} ions (filled circles) and the countertransported protons only one is shown for clarity.

reaction cycle shown in Fig. 4. Binding of cytosolic Ca^{2+} enables the ATPase to react with ATP to form a phosphoenzyme intermediate ($Ca_2E_1 \rightarrow Ca_2E_1$-P), which then converts from an ADP-sensitive form (Ca_2E_1-P) to an ADP-insensitive form (E_2-P) that is more rapidly hydrolysed. This phosphoenzyme conversion is associated with Ca^{2+} release towards the sarcoplasmic reticulum lumen against the concentration gradient. E_2-P picks up protons from the lumenal side of the membrane that are countertransported and released to the cytoplasmic side upon Ca^{2+} binding. Although mutagenesis studies have identified crucial residues for Ca^{2+} transport [36] and a structure at atomic resolution for Ca_2E_1 has appeared recently [38], more work is required to understand the molecular mechanism of active ion pumping.

3.1. The infrared difference spectrum seen as a fingerprint of the conformational change

To investigate the molecular mechanism of Ca^{2+} transport, infrared difference spectra of several partial reactions have been recorded [25,39–43]. Examples of these spectra are illustrated in Fig. 3. A first approach to the interpretation of these spectra is to regard them as a fingerprint of the underlying conformational change. In this way the amplitude of the signals, their spectral shape and their kinetics can be analysed. Similar approaches have a long tradition in fluorescence spectroscopy, where 'high' and 'low' fluorescence have been used to define enzyme conformational states. Infrared spectroscopy has the advantage of providing a wealth of information and of directly monitoring all peptide groups of a protein, therefore making a conformational change hard to miss. Of special interest is the absorbance of the amide I mode of the polypeptide backbone (predominantly a C=O vibration) in the region from 1700 to 1610 cm^{-1} which is sensitive to secondary structure.

This 'fingerprint approach' has been applied to the sarcoplasmic reticulum Ca^{2+}-ATPase. The extent of net secondary structure change of several partial reactions has been quantified using the *COBSI* (change of backbone structure and interaction) index [24]. This index relates the absorbance change in the amide I spectral region to the total absorbance of the polypeptide backbone in the same spectral region. From the similar *COBSI* values of the partial reactions investigated, it does not seem possible to distinguish between minor and major secondary structure changes in the catalytic cycle of the Ca^{2+}-ATPase. This is in contrast to what would be expected from the classical model of the ATPase reaction cycle by de Meis and Vianna [44] that is based on only two main protein conformations, E_1 and E_2. The model, therefore, seems to be a strong simplification of reality. Using a simple model, the net change of secondary structure was estimated to involve only 0.3–1% of the ATPase residues, i.e. 3–10 amino acids. (Similar numbers of amino acids involved in a conformational change were found in infrared studies of nucleotide binding to arginine kinase [26], creatine kinase [27] and annexin VI [45].) These secondary structure changes seem to be surprisingly small given that the events at the Ca^{2+} binding sites in the membrane region of the protein and at the phosphorylation site in the cytoplasmic domain are coupled over a distance of more than 50 Å [38]. As discussed in more detail in [24], the overall picture that emerges from combining the results of several methods is that conformational changes in small flexible regions of the protein lead to significant movements of rigid protein domains relative to each other. Such movements are able to reconcile the different shapes of the protein in the Ca^{2+} free and the Ca^{2+} loaded form [38].

The model of de Meis and Vianna postulates a Ca_2E_2-P intermediate, i.e. an ADP-insensitive phosphoenzyme that binds Ca^{2+}. However, analysing the time course of the infrared difference spectra, such an intermediate could not be identified [24] and thus is short-lived or non-existent in accordance with the view of Myung and Jencks [46].

From the very similar spectra of Ca^{2+} release from the unphosphorylated and from the phosphorylated enzyme ($Ca_2E_1 \rightarrow E$ and Ca_2E_1-P $\rightarrow E_2$-P, respectively), it was concluded that similar conformational changes take place in these partial reactions [47]. This led to a working model of the ATPase reaction cycle that represents the simplest explanation of the observations while other models would require additional assumptions. The model proposes only one pair of binding sites for the two Ca^{2+} ions that are accessible from different sides of the membrane depending on the enzyme state. It is in contrast to the model of Jencks [48] that proposes two different pairs of sites for cytoplasmic high affinity and lumenal low affinity binding sites, respectively. However, it is in agreement with mutagenesis studies [36] that have identified only one pair of Ca^{2+} binding sites and have associated E-309, E-771 and N-796 with high affinity Ca^{2+} binding as well as with low affinity Ca^{2+} binding.

3.2. Ca^{2+} binding to the unphosphorylated ATPase

Ca^{2+} binding ($E \rightarrow Ca_2E_1$) has been investigated by the use of the caged Ca^{2+} compounds 1,2-amino-5-[1-hydroxy-1-(2-nitro-4,5-methylene dioxyphenyl) methyl]phenoxyl-2-(2′-amino-5′-methylphenoxy) ethane-*N,N,N′,N′*-tetraacetic acid tetrasodium salt (Nitr 5) [42] and 1-(2-nitro-4,5-dimethoxyphenyl)-*N,N,N′,N′*-tetrakis[(oxycarbonyl)methyl]-1,2-ethanediamine [41,43]. (Caged Ca^{2+} Nitr 5 has also been used to investigate the effects of Ca^{2+} binding to calmodulin [49].) In spite of the quite different experimental conditions that range from −9°C, pH 6.5, 30% glycerol [43] to 22 or 25°C, pH 7.0 in aqueous solution [41,42], the difference spectra obtained are remarkably similar. This is of interest since the proposed equilibrium between the E_1 and the E_2 forms of the unphosphorylated ATPase E depends on pH and temperature [50]. Small differences between the spectra have been noted [43] and may be due to the $E_2 \rightarrow E_1$ transition.

Especially interesting is the observation of two signals of protonated carboxyl groups [41–43] in the Ca^{2+} free state E near 1760 and 1710 cm^{-1} in H_2O. The earlier suggestion that they are sensitive to the addition of dithiothreitol [42] could not be reproduced [41,43]. Instead these bands were associated with the protonation of residues most likely of the Ca^{2+} binding sites which are involved in proton countertransport [47]. Bands at the same positions have been found in the Ca^{2+} release reaction from the phosphorylated enzyme (Ca_2E_1-P $\rightarrow E_2$-P) which has led to the proposal that the same two amino acid residues become protonated in E and E_2-P and has strengthened the argument for only one pair of binding sites discussed above.

3.3. ATPase phosphorylation

Phosphorylation of D-351 by ATP ($Ca_2E_1ATP \rightarrow Ca_2E_1$-P) occludes the two calcium ions in the protein. Most of the infrared absorbance changes induced by Ca^{2+} binding

($E \rightarrow Ca_2E_1$) are preserved in the phosphorylation reaction [40] in line with the above interpretation that the Ca^{2+} sites of Ca_2E_1 and Ca_2E_1-P are the same. Using the model compounds acetyl phosphate for phosphorylated D-351 and acetate for unphosphorylated D-351, several difference bands of the phosphorylation spectrum could be assigned to the molecular groups involved in the reaction. The C=O group of the aspartyl phosphate seems to undergo special interactions with its environment with a strength comparable to hydrogen bonding in water. This was concluded from the position at 1719 cm^{-1} of the respective difference band and its narrower line width when compared to acetyl phosphate in water. A band at 1131 cm^{-1} was assigned to the phosphate group of the phosphoenzyme on the basis of (i) the model spectra and (ii) isotope labeled phosphoenzyme using [γ-$^{18}O_3$]ATP. From the frequency it can be concluded that the phosphoenzyme phosphate group is deprotonated when bound to the protein.

3.4. Phosphoenzyme conversion and Ca^{2+} release from the phosphorylated ATPase

Phosphoenzyme conversion (Ca_2E_1-P $\rightarrow$ E_2-P) opens the Ca^{2+} binding sites towards the sarcoplasmic reticulum lumen and releases the Ca^{2+} ions from low affinity binding sites. The reactivity of the phosphate group is also altered, it dephosphorylates with ADP in Ca_2E_1-P and with water in E_2-P. Backbone conformational changes of the preceding step of ATPase phosphorylation are preserved. This indicates that the enzyme in the phosphoenzyme conversion step goes even further away from the pre-phosphorylation conformation [39].

Two pairs of bands in the difference spectrum of phosphoenzyme conversion at 1570/1554 and 1411/1399 cm^{-1} were assigned to Ca^{2+} release from carboxylate groups. Their low sensitivity towards $^1H_2O/^2H_2O$ exchange indicates a partial protection from the aqueous environment, which is in line with the proposed location of the Ca^{2+} binding sites in the membranous part of the enzyme. An E_2-P band at 1638 cm^{-1} was also assigned to a carboxylate group on the basis of its higher band position in ^2H_2O. This unusual position may be explained for example by a strong interaction with a positive charge. Such an interaction with one of the Ca^{2+} chelating groups may well explain the low Ca^{2+} affinity of E_2-P.

Especially interesting is a positive band at 1192 cm^{-1} that is sensitive to ^{18}O labeling of the phosphate group. It can therefore be assigned to the E_2-P phosphate. The band shows that there is a conformational change that directly affects the geometry and/or the electron density of the phosphate group and indicates very different interactions between phosphate group and protein in Ca_2E_1-P and E_2-P. The band position is unusual for phosphate groups in water and may be explained by a very hydrophobic environment in line with other findings [51–53].

4. Conclusions

The examples discussed above demonstrate that infrared spectroscopy provides a highly detailed examination of working enzymes. Conformational changes of the polypeptide backbone and alterations of side chains can be followed at the level of individual groups. The enzymes investigated so far represent only a small fraction of the proteins that can be studied with the methods reviewed here. Two methodical aspects seem to be especially interesting for further applications. The first is the enzyme catalysed transfer of a labeled group to a protein as described here for the phosphate group of the Ca^{2+}-ATPase phosphoenzyme intermediate E_2-P. Phosphate groups control enzyme activity and the method sheds light on how this is achieved at 'atomic resolution'. The second aspect is the characterisation of molecule binding pockets of proteins by infrared spectroscopy which will have interesting medical and pharmaceutical applications. With developments under way that will ease binding investigations considerably, the future will see a growing number of molecule-protein recognition studies using infrared spectroscopy. The level of sophistication can be adjusted to the problem, ranging from the simple screening of substances on their ability to bind to a protein of interest to the demanding characterisation of molecule protein interactions.

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