Molecular reaction mechanisms of proteins as monitored by time-resolved FTIR spectroscopy

Klaus Gerwert

Institut für Biophysik, Ruhruniversität, Bochum, Germany

Although infrared spectroscopy is a classical method for analytical and structural studies, it can also provide valuable insights into the mechanism of chemical reactions. Time-resolved Fourier-Transform infrared difference spectroscopy has recently successfully proved itself as a powerful new method for studies of molecular reaction mechanisms with the generation of nanosecond time resolution for proteins up to 120 kDa in size.

Introduction

The infrared (IR) spectrum of a protein is dominated by its peptide backbone vibrations, amide I ($C=O$, 1680–1620 cm^{-1}) and amide II (CNH, 1560–1520 cm^{-1}) [1]. In addition, water, which is necessary for protein structure and activity, absorbs strongly in the IR (OH, 1650 cm^{-1}). The major problem in using IR radiation is how to select bands of those groups that undergo changes during the activity of the protein against the background absorbance of the whole protein, water, and buffer. The strategy here is to perform difference spectra between the ground and activated states of the protein. Such measurements demand highly sensitive experimental set-ups to record absorbance (A) changes in the IR of down to $\Delta A/A = 10^{-4}$.

For most applications, researchers choose one of two fundamentally distinct methods: time-resolved IR difference spectroscopy and time-resolved Fourier-Transform IR (FTIR) difference spectroscopy. The former method utilizes dispersive instruments and detects sequentially absorbance changes at selected wavelengths only. For restricted spectral ranges, this allows time resolution up to picoseconds (reviewed in [2]). The latter method monitors in parallel complete IR spectra over the range 700–4000 cm^{-1} with up to nanosecond time resolution (reviewed in [3]). Because of the multiplex and Jaquinot advantages, this high time resolution is accompanied by high amplitude and spectral resolution.

In this review, I focus on time-resolved FTIR spectroscopy. A brief introduction is followed by an account of recent FTIR studies on a variety of proteins reported during the past year.

Fourier-Transform infrared spectroscopy techniques

Scanning modes

Various approaches to time-resolved absorbance data collection by FTIR spectroscopy have been adopted. They are: 'static', rapid-scan, stroboscopic and step-scan FTIR.

'Static'

In this, the simplest and most routinely used technique, recording of interferograms (Fourier transform of the spectrum) takes several seconds. The activated protein state is stabilized for several minutes, e.g. by cooling, pH variation or photostationary accumulation [4].

Rapid-scan

The idea of rapid scanning is simple: after taking a reference spectrum of the ground state, the protein is activated and a series of spectra is taken on a time scale faster than the protein reaction half-life. For technical reasons, the time resolution is presently limited to the millisecond time domain [5].

Stroboscopic

In this approach, interferograms are still monitored in the rapid-scan mode but then interferogram segments are rearranged to generate new interferograms using appropriate software. The time resolution is increased to the microsecond range [6].

Abbreviations

bR—bacteriorhodopsin; FTIR—Fourier-Transform IR; IR—infrared; M—metarhodopsin; PS—photosystem; R—rhodopsin; RC—reaction center; SVD—single-value deconvolution.

Step-scan

In the step-scan mode, the scanner stops at respective sampling positions, protein activity is initiated and the time dependence of the interferograms sampling points is measured. After data rearrangement, nanosecond-resolved spectra are obtained. Because of the sensitivity of this method to external disturbance, the stroboscopic and rapid-scan techniques seems more appropriate to applications where microsecond and millisecond time resolution are sufficient, as for many of the biologically relevant reactions [7••].

Reaction initiation

Reaction initiation must be achieved without changing the sample or moving it out of the apparatus, as any disturbance causes larger absorbance changes than those induced by protein activity.

In photobiological systems, the reaction is triggered using simply a laserflash. For proteins lacking intrinsic chromophore, so-called 'caged' substances can be used, e.g. caged ATP for ATPases. Using an intense UV flash, the caged group is quickly separated and the released molecule of interest triggers the protein reaction (reviewed in [8]). As an alternative to laserflashes, reactions have been initiated by a jump in pH, pressure or the potential difference across an electrochemical cell (reviewed in [9]).

Data analysis

After establishing recording techniques during the 1980s, several groups have concentrated recently on the kinetic analysis of the time-resolved data, mainly with two aims in mind: the reduction of the noise in the difference spectra, and the extraction of pure intermediate difference spectra out of the measured ones. The measured difference spectra represent intermediate mixtures due to temporarily overlapping processes. Three different mathematical procedures have been used. In global multiexponential fit analysis, the simultaneously measured difference spectra are also analyzed simultaneously instead of fitting each wavelength separately [6]. This yields more precise apparent rate constants and more reliable timecourses of absorbance changes even in regions with a lower signal-to-noise ratio. Unfortunately, the corresponding amplitude spectra usually represent intermediate mixtures. Single-value deconvolution (SVD) is a matrix-ranged reduction algorithm which reduces the noise in the spectra [10•]. But the basic difference spectra obtained also do not describe directly pure difference spectra. Factor analysis and deconvolution is in principle an extension of SVD [11••]. The matrix describing the absorbance changes is decomposed into two matrices, one reflecting the time dependence of the intermediate concentrations and the other containing the desired pure intermediate difference spectra. This procedure allows one to assign protein reactions to specific intermediates without reference to kinetic models. Restrictions in the iteration are

made only for few spectral characteristics of the difference spectra.

Band assignments

Crucial to the solution of molecular reaction mechanisms is the assignment of IR bands to specific groups of the protein. This is usually accomplished by marking atoms by isotopic labelling or exchanging molecular groups by site-specific mutation, and then comparing the difference spectra of the modified and unmodified proteins for deviations. Isotopic labelling shifts the frequency of the labelled group's absorbance band, whereas site-directed mutagenesis leads to disappearance of the exchanged group absorbance band. Isotopic labelling has the advantage of being non-invasive, but site-specific labelling is as yet only applicable to exchangeable chromophores [12], although it should soon be applicable to protein side groups [13]. Biosynthetic incorporation of isotopically labelled amino acids is not specific to one site [14•]. Site-directed mutagenesis can disturb protein structure and must be used carefully, as the dicussion below will show. The IR difference spectra can also be used to probe whether mutagenesis has induced gross protein structural changes via large deviations in the mutant difference spectra. For band assignments, broad spectral ranges should be compared with each other and therefore FTIR is more suitable than dispersive IR.

Applications

In the following section, the application of time-resolved FTIR spectroscopy to different proteins will be discussed.

Bacteriorhodopsin

The 27 kDa large membrane protein bacteriorhodopsin (bR) is a light-driven proton pump. In the primary photoreaction to intermediate J, the chromophore all-*trans* retinal isomerizes to 13-*cis* retinal. J decays through the intermediates K, L, M, N and O within a few milliseconds back to the BR ground state. During the pump cycle, the Schiff-base, which links retinal and Lys216, is deprotonated in intermediate M. Most of the developments in time-resolved FTIR and time-resolved IR spectroscopy are tightly coupled to the elucidation of bR's pump mechanism (reviewed in [15] and briefly summarized in [16]). The FTIR studies show Asp85 protonation in the L→M transition serving as a catalytic proton binding site on the release pathway [17,18], and Asp96 deprotonation in the M→N transition serving as a catalytic proton binding site on the uptake pathway [5,18]. These events have now been confirmed in all details [19,20].

Also in general agreement, an amide I difference band ($1670/1650\,cm^{-1}$) indicative of structural movements of a few peptide carbonyl groups is observed in the M→N transition [5,6,11••,15,21,22,23•]. This difference band is consistent with a structural movement that switches the central proton-binding site from the proton-release to the

proton-uptake pathway, thereby determining vectoriality of the pump. Assignment of the $1670/1650\,cm^{-1}$ difference band to specific peptide carbonyls is nevertheless still missing. In the Asp212Asn mutant, Asp96 is deprotonated in spite of the absence of an unprotonated Schiff base but the structural movement appears concomitantly. Therefore, Lanyi, Braiman and coworkers [23•] conclude that this structural transition induces reduction in the pK_a of Asp96.

The controversy about the protonation state of Asp96 in L demonstrates the importance of making the absorbance measurements at room temperature. At low temperature, an Asp96 difference carbonyl band is observed, indicating, as is now generally accepted, a change in its environment [15,18,20]. In contrast, at room temperature, only a disappearing Asp96 carbonyl band is seen, which might indicate its partial deprotonation [15]. Further experiments are needed to clarify protonation state of Asp96 in L.

Protonation of Asp212 in intermediates M and N is also a subject of controversy. Disappearance of a band pattern around $1732\,cm^{-1}$ in Asp212 mutant difference spectra suggested its protonation [17]. But the Asp212 mutation causes gross structural changes and changes in the mutant difference spectra, so a straightforward interpretation does not appear valid. Specific isotopic labelling of Asp212 shows no Asp212 carbonyl bands, leading Siebert and coworkers [14•] to exclude its protonation. It has been shown that the $1732\,cm^{-1}$ band pattern comprises two difference bands originating from Asp96 and Asp115 environmental changes (K Gerwert *et al.*, unpublished data). In conclusion, Asp212 seems not to be involved in proton-transfer reactions.

The O intermediate difference spectra have, for the first time, been characterized in the IR. This intermediate does not accumulate significantly in wild-type BR and equilibrates rapidly with the preceeding intermediate N, rendering it very difficult to measure. Two different approaches have been used. Rothschild and coworkers [24] utilized a Tyr185Phe mutant and demonstrated, in contrast to the wild type, the existence of a long-lived intermediate with a red-shifted absorption maximum, which they assigned to O. Gerwert and coworkers analyzed the time-resolved IR spectra by global fitting [6], factor analysis and decomposition [11••] to extract out of the measured intermediate mixtures the pure BR-O difference spectrum for wild-type. The BR-O difference spectra of wild type and mutant share some general similarities, but do not agree in all details. As in the wild type, in the Tyr185Phe mutant, Asp85 is protonated and Asp96 is already reprotonated in O. But the peptide structural change in the wild type still present in intermediate O is in the mutant already relaxed. The differences may reflect mutation-induced structural changes or the existence of two different O species. Nevertheless, further investigations indicate that light adaptation of the Tyr185Phe mutant produces a wild-type and an O-like species, and some deviations between the mutant and wild-type difference spectra can be explained by photoreactions of intermediate O [25].

Hydrogen-bonded networks play an important role in the proton-transfer reactions of bR. Continuum-band changes are good indicators of such networks, as Zundel and coworkers [26••] have shown. On the basis of FTIR investigations of Thr46 and Thr89 mutants, a H-bonded network between Asp96 and Asp212 has been suggested [27]. As bands arising from their protonation changes were not identified, their involvement in proton-transfer reactions is not resolved.

Further evidence for a hydrogen-bonded network in BR is reported in a series of publications by Maeda and coworkers [20,22,28•,29]. They have extended the investigated spectral region to $3400–3700\,cm^{-1}$ in order to be able to assign the OH and NH stretching vibrations of water and protein side groups. The results indicate changes in the hydrogen bonding of one or a few internal water molecules, Asp96 and the Schiff base in intermediate L.

In summary, the past year has witnessed several important developments in IR studies of bR: an improvement in time-resolved IR techniques enabling investigations under physiological room-temperature conditions; the introduction of kinetic analysis methods allowing the extraction of pure intermediate difference spectra; and development in non-invasive isotopic labeling of specific side groups. These advances should now allow the resolution of ambiguity in some earlier interpretations caused by problems inherent in studying temporarily overlapping processes or in working with invasive site-specific mutants at atomic resolution.

Rhodopsin

The light-induced reaction cascade of rhodopsin has been and continues to be widely studied using FTIR spectroscopy. In order to assign cytosolic loop vibrations, Siebert and coworkers [30] have used specific proteases to modify the loops. The results indicate a transition from an ordered loop structure in rhodopsin (R) to a more random configuration in metarhodopsin (M) II [30].

The bleaching reaction has been monitored, globally fitted and the R/MII, R/MIII and the R/opsin difference spectra have been calculated by placing new constraints on the MIII spectrum [10•]. Current investigations of site-specific rhodopsin mutants promise more specific band assignments in the near future.

Photosynthetic reaction centers

Bacterial reaction centers (RCs) and photosystem (PS) II have been the subject of a large number of FTIR studies. Even though proteins of molecular weight $>100\,kDa$ cause large background absorbances, highly reproducible difference spectra of several charged separated states in the different photosynthetic proteins have been obtained (recently summarized in [9]). Assignments of bands based on comparison with prosthetic groups model compounds were performed [9]. The dif-

ference spectra of bacterial RCs are dominated by chromophore and quinone bands and large-scale structural backbone changes during charge separation can be excluded [31•]. Protein side-group assignments based on site-specific mutation will soon appear in a number of conference proceedings by Breton, Maentele, Nabedryk and coworkers.

$P^+Q_B^-/PQ_B$ difference spectra of *Rhodobacter capsulatus* HisM200Leu and HisL173Leu mutants have been measured [32•]. Their mutation causes changes in the local environment of P_L in HisM200Leu and of P_M in HisL173Leu, and therefore the difference spectra cannot be compared directly with wild-type ones. In PSII, low- and high-potential cyt b_{559} were investigated together with the corresponding model compounds. It was concluded that the mechanism of heme oxidation *in vivo* implies different molecular processes of the two forms [33].

Non-photobiological systems

The reaction mechanism of the sarcosplasmic reticulum Ca^{2+}-ATPase has been studied using caged ATP and caged Ca^{2+} as trigger compounds. The experiments demonstrate nicely the unproblematic use of these caged compounds in IR experiments (reviewed in [9]).

The nicotinic acetylcholine receptor has been investigated following two different approaches. In one experiment, binding of carbamoycholine was monitored using caged carbamoycholine [34•]. In the other, protein films were attached to germanium internal reflection cells, which then allowed bathing of the sample with the agonist [35••]. Redox-induced reactions in myoglobin, hemoglobin and cytochrome *c* have been studied using an electrochemical cell [36,37•].

GTPase activity of h-*ras* p21 was recently studied using caged GTP as the trigger molecule. Complexing p21 with α, β, and γ ^{18}O-labelled caged GTP allow the assignment of the disappearing γ-phosphate band during hydrolysis [38].

Conclusion

Important improvements in FTIR spectroscopy were achieved during the past year: the time resolution was increased, sophisticated kinetic analysis has been introduced and non-invasive specific labelling of side-groups has been performed. The results have generated a detailed picture of bR's pump mechanism, even if some central questions remain to be solved. For rhodopsin and photosynthetic RCs, high-quality difference spectra of their reaction sequences have now been amassed and band assignments should soon enable one to draw detailed conclusions on their molecular mechanism to match those for bR. The use of caged compounds and other trigger elements has opened a door to time-resolved FTIR studies of proteins such as ATPases, acetylcholine and h-*ras* p21. In Fourier-Transform Raman spec-

troscopy, a complementary method stands ready to be developed.

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K Gerwert, Fakultät für Biologie, Institut für Biophysik, Ruhruniversität, 44780 Bochum, Germany.