CHAPTER EIGHT

Vibrational spectroscopy of flavoproteins

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Abstract

The flavin cofactor performs many functions in the cell based on the ability of the isoalloxazine ring to undergo one- or two-electron reduction and form covalent adducts with reactants such as amino acids. In addition, the strong visible absorption of the cofactor is also the basis for flavin-dependent photoreceptors. Vibrational spectroscopy is uniquely suited to studying the mechanism of flavoproteins since the frequency of the vibrational modes is very sensitive to the electronic structure and environment of the isoalloxazine ring. This chapter describes the mechanistic information that can be gained using vibrational spectroscopy as well experimental challenges and approaches that are used to obtain and interpret the complex data contained in a vibrational spectrum.

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1. Introduction

1.1 Vibrational spectroscopy: Basic theory and instrumentation

There are many texts that describe the fundamental principles of vibrational spectroscopy (Colthup, Daly, & Wiberley, 1975). Here we give only an outline of the basic concepts in order to provide a foundation for the application of vibrational spectroscopy to the study of flavoproteins. In vibrational spectroscopy, incident electromagnetic radiation exchanges energy with a molecule that results in a change in vibrational energy. Therefore, vibrational spectroscopy is a method for detecting and studying the vibrational frequencies in a molecule. Vibrations arise through motions of the atoms in the molecule and can be broadly categorized into stretching, bending, rocking, twisting, and wagging motions. These motions have different frequencies in the vibrational spectrum and their exact position is very sensitive to both the structure and environment of the molecule. Based on classical theory, in which nuclei are represented as points with mass connected to each other by springs, a molecule has 3N-6 (3N-5 for a linear molecule) normal modes of vibration. In addition, due to anharmonicity, additional bands such as overtones and combinations can also be present, increasing the complexity of the vibrational spectrum. Thus, even a small molecule has many vibrational bands, and the challenge with vibrational spectroscopy is to obtain the vibrational spectrum of only that part of the molecule that is of interest—for example, the flavin chromophore in a flavoprotein.

Vibrational spectra are commonly obtained using two primary methods: infrared spectroscopy (IR) and Raman spectroscopy. IR spectroscopy involves the absorption of infrared light by a molecule and vibrational modes will appear in the IR spectrum provided that there is a change in dipole moment during the vibration. In contrast, Raman spectroscopy utilizes a monochromatic light source such as a laser, which can be of any wavelength but is usually a visible frequency (~400–750 nm). In Raman spectroscopy the incident light is scattered by the sample leading to an exchange of energy if there is a change in polarizability during the bond vibration. The vibrational spectrum is then given by the difference in frequency between the incident light and Raman scattered light. If the excitation frequency is coincident with an electron transition, the vibrational bands associated with the electron transition can be greatly enhanced in intensity (10^4–10^6)

in a process known as resonance Raman spectroscopy. Because IR and Raman spectroscopy operate via different selection rules, bands that are observed using one technique may be weak or absent in the spectrum obtained by the other technique and vice versa. Thus, the two methods are complimentary. Table 1 summarizes some of the advantages and limitations of each technique. Finally, vibrational spectroscopy is a very fast technique so that the vibrational spectrum is a snapshot of all species that are present in solution. Consequently, there is no conformational averaging as can occur in NMR spectroscopy. In addition, the timescale of vibrational spectroscopy also means that very fast processes can be monitored such as activation of a photoreceptor on the picosecond timescale (Wilbrandt, Pagsberg, Hansen, & Weisberg, 1975).

Infrared spectra are normally acquired using a Fourier transform infrared (FTIR) spectrometer (Fig. 1), in which a polychromatic light source is passed through an interferometer (commonly a Michelson interferometer) which separates wavelengths of light in time. The beam is passed through the sample and transmitted light is analyzed using a detector. The signal from the detector is Fourier-transformed to give an absorption spectrum.

Table 1 Techniques for measuring vibrational spectra.

	Advantages	Disadvantages
Infrared	• Information from both chromophore and protein can be obtained • Quantitative: Band intensity is proportional to concentration • Accurate subtractions using fixed pathlength cells	• Strong water absorption • Additional steps are needed to reveal bands from the region of the molecule that are of interest such as isotope editing or light minus dark subtraction • High concentration, ~1 mM required in most cases
Raman	• Observation of chromophore modes without interference from protein modes • The Raman spectrum of water is relatively weak	• Band intensity is dependent on polarizability and cannot be used to determine sample concentration
Resonance Raman	• Strong enhancement of chromophore modes enabling lower concentrations to be used	• Sample fluorescence can obscure the Raman spectrum • Light absorption can lead to the photochemical generation of new species and/or sample damage

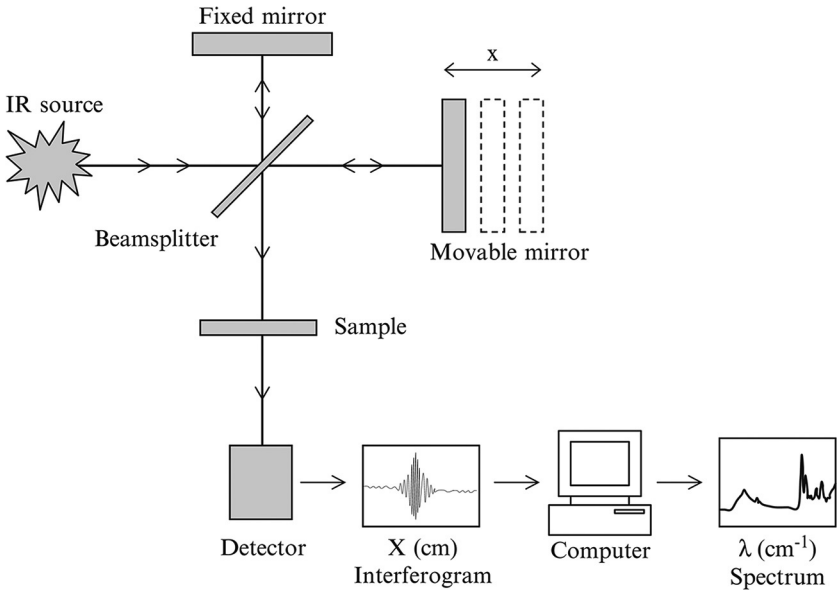

Fig. 1 Schematic Layout of a FTIR Spectrometer. FTIR spectrometers use an interferometer which creates an interferogram from an IR source. After passing through the sample the interferogram is Fourier transformed to give the IR spectrum (Ojeda & Dittrich, 2012).

The sensitivity of commercial FTIR spectrometers can be improved by incorporating a liquid nitrogen cooled HgCdTe (MCT) detector. An advantage of FTIR spectroscopy is that the method is quantitative, so that concentrations can be determined provided that the extinction coefficient and pathlength of the sample is known.

Raman spectroscopy is a scattering technique in which light from a monochromatic source such as a visible laser is focused into the sample (Fig. 2). Scattered light is then collected at 90° to the incident light and focused into a spectrograph in which a grating is used to disperse light onto a CCD detector. A notch filter with near zero transmission at the laser wavelength is used in the collection arm of the optical path to remove scattered light with the same frequency as the excitation source (Rayleigh light). Raman spectroscopy often uses red excitation sources (e.g., 785 nm) to minimize sample fluorescence, an approach that has been aided by the development of high quantum efficiency detectors such as a back-thinned deep-depletion CCD for NIR Raman spectroscopy (Rudik, Bell, Tonge, & Thorpe, 2000).

Each method has its strengths and limitations (Table 1). FTIR spectroscopy is normally performed with high sample concentrations of 1–2 mM. In addition, water has a strong IR spectrum and samples are often prepared

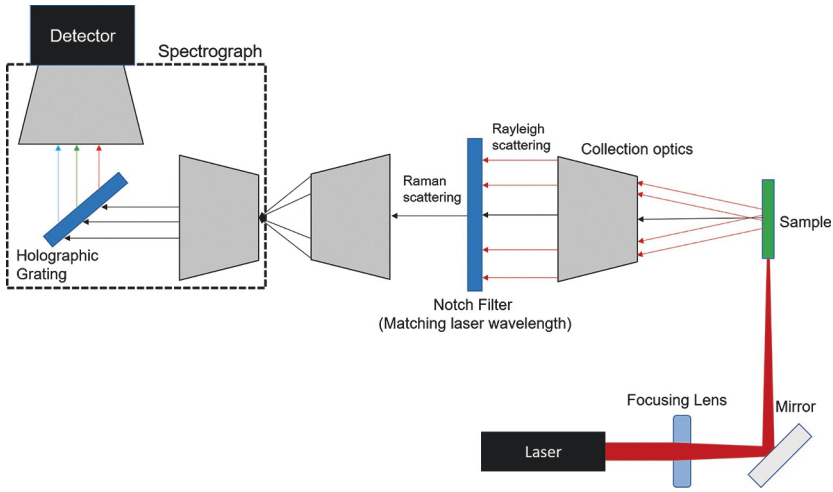

Fig. 2 Schematic Layout of a Raman spectrometer. Scattered light is collected at 90° to the excitation axis and focused onto the entrance slit of a spectrometer equipped with a holographic transmission grating. The grating disperses the spectrum across the exit slit where it is analyzed using a CCD camera. The use of a single grating spectrometer is made possible by a notch filter which efficiently rejects the Rayleigh scattered light.

in D_2O to shift the H—O—H bending vibration from 1650 to 1200 cm^{-1} (D-O-D) thus giving a "window" in the spectrum where important bands from the solute (flavoprotein) can be observed. The vibrational spectrum between 1700 and 1300 cm^{-1} is dominated by vibrational modes associated with the amide backbone of the protein, the amide I, II and III bands, which can be used to estimate the secondary structure content of the protein. However, additional steps are needed to visualize bands from the region of interest such as isotope editing (Deng, Vedad, Desamero, & Callender, 2017; Dong et al., 2001) or, in the case of light-activated proteins, using light minus dark difference spectroscopy. In this regard the use of a fixed pathlength cell enables accurate difference spectra to be obtained.

In contrast to IR spectroscopy, the relationship between Raman band intensity and sample concentration is more complex since the intensity of Raman scattering is dependent on polarizability. Thus, the peak intensity cannot be directly related to concentration. However, the Raman spectrum of water is relatively weak, so that spectra in both H_2O and D_2O can be readily obtained. In addition, Raman spectroscopy has a major advantage if the portion of the structure that is of interest is a chromophore such as the flavin. While resonance Raman spectroscopy can be deployed in this situation, using an excitation frequency that is in resonance with the

electronic transition of the chromophore, as noted above this can lead to unwanted fluorescence and photochemistry (Tonge, Pusztai, White, Wharton, & Carey, 1991). Instead, red excitation can be used (e.g., 785 nm). Although the excitation frequency lies outside the electronic transition, the conjugated π electron system of the chromophore is much more polarizable than the protein and hence vibrational bands associated with the chromophore are much more intense than those associated with protein modes (Carey & Tonge, 1995).

1.2 The vibrational spectrum of the isoalloxazine ring

The chemistry of FMN and FAD is associated with the isoalloxazine ring which can exist in several different ionization/redox states (Fig. 3). Since vibrational band frequencies are very sensitive to structure and environment, vibrational spectroscopy is a powerful method for analyzing the interactions within the flavoprotein binding site that modulate the chemistry of the isoalloxazine ring. The flavin cofactor has several distinct vibrational modes that can be detected in both IR and Raman spectra, and the FTIR spectrum of FMN dissolved in D_2O is shown in Fig. 4. The C2═O (blue) and C4═O (red) carbonyl groups (Fig. 3) have relatively intense IR absorbances at ~1660–1680 and ~1690–1710 cm^{-1}, respectively. The carbonyl frequencies are highly sensitive to hydrogen bonding from solvent or protein amino acids, which is especially useful in determining structural rearrangements that are

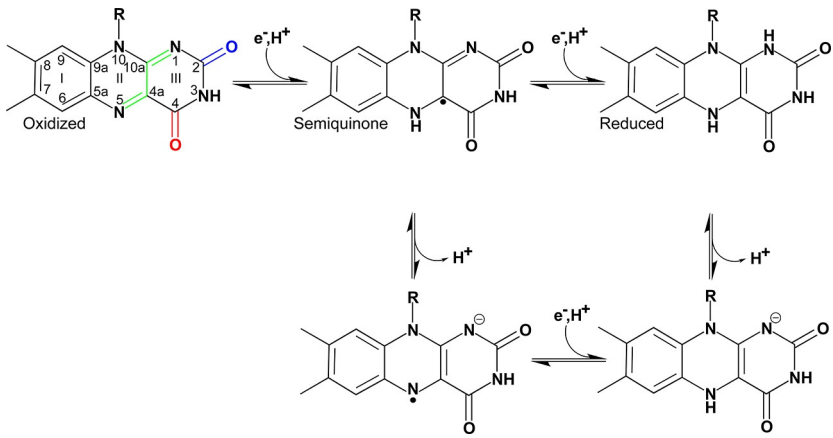

Fig. 3 Ionization and redox states of the isoalloxazine ring. The numbering of the isoalloxazine ring is shown for the oxidized form. *Figure adapted from Conrad, K. S., Manahan, C. C., & Crane, B. R. (2014). Photochemistry of flavoprotein light sensors.* Nature Chemical Biology, *10(10), 801–809.*

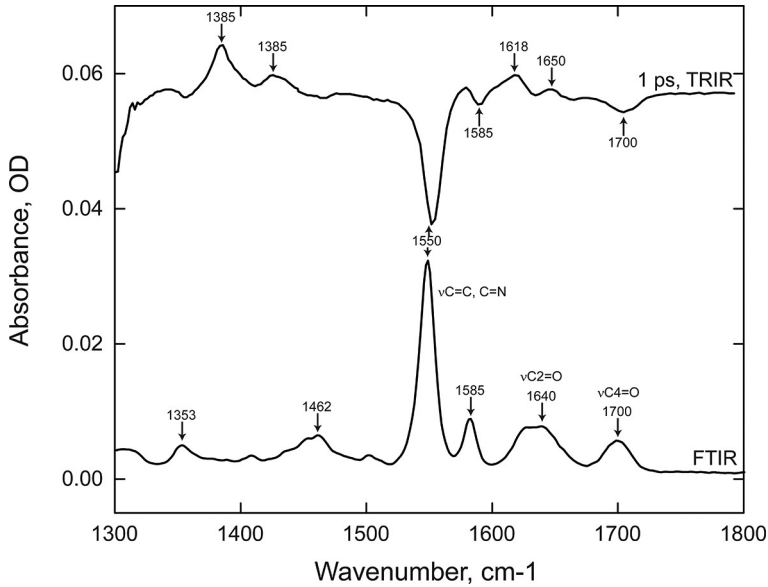

Fig. 4 IR spectra of FMN. The FTIR spectrum of FMN in D_2O (2 mM) after subtraction of the solvent spectrum is shown at the bottom. The light minus dark time-resolved IR (TRIR) spectrum of FMN in D_2O obtained at a delay time of 1 ps after excitation at 450 nm is shown in the upper trace. Negative bands (bleaches) are vibrational modes depopulated following absorption of light and are thus associated with the ground state of the isoalloxazine chromophore. Positive bands are associated with the FMN excited state or species formed by photoexcitation.

associated with biological activity. Another useful vibrational mode arises from motions of N5═C4a—C10a═N1 (green) and is found at ~1550 cm^{-1} in both IR and Raman spectra. This delocalized mode is sensitive to several structural modifications of the isoalloxazine ring including protonation of N5 or N1 upon reduction to the semiquinone and/or covalent adduct formation at C4a during catalysis (Piano, Palfey, & Mattevi, 2017). Normal modes have been assigned for the oxidized, semiquinone and reduced forms of the isoalloxazine ring and a selection of these assignments are given in Table 2.

2. Applications of vibrational spectroscopy to flavoproteins

This section will outline the various methods that have been used to obtain vibrational spectra of flavoproteins. This includes Raman and resonance Raman spectroscopy as well as FTIR spectroscopy and is

Table 2 Selected normal mode assignments of the isoalloxazine ring.

Oxidized (Bowman & Spiro, 1981)		Semiquinone (Sugiyama, Nisimoto, Mason, & Loehr, 1985)		Reduced (Zheng, Carey, & Palfey, 2004)	
1713	C4═O	1532	C4a-N5, C10a-N1	1714	C2═O, N1—H, N3-H
1645	C2═O, C10a═N1	1388	C2-N1	1638	C4═O, N1—H, N5-H
1615	C4═O, C2═O, C10a═N	1268	Ring II and III	1615	C4a═C10a
1511	C4a—N5, N10—C10a, C10a—N1, C4a—C10a	1227	Ring II and III	1560	Ring I, N5-H
1451	C4a—N5, C10a—N1			1539	N1—H, C4a═C10a
1394	N1—C2				
1379	N10—C10a				
1269	N3—H				

followed by a brief introduction to more recent approaches such as time-resolved infrared spectroscopy (TRIR), two-dimensional (2D) IR spectroscopy, and femtosecond stimulated Raman spectroscopy (FSRS). Some examples of flavoproteins that have been studied using vibrational spectroscopy are given in Table 3.

2.1 Raman and resonance Raman spectroscopy of flavoproteins

Many early vibrational spectroscopy experiments on biological systems used resonance Raman (RR) spectroscopy to take advantage of the dramatic increase in Raman band intensity when the laser excitation frequency was in resonance with a chromophore in the sample. Although this was an ideal approach to selectively observe vibrational modes associated with the chromophore, RR spectroscopy was often hampered by strong fluorescence from the chromophore which obscured the Raman spectrum. The flavin chromophore was no exception and initial RR spectra of flavoproteins acquired by excitation into the 450 nm electronic transition were

Table 3 Flavoproteins that have been studied using vibrational spectroscopy.

Protein	Key results
D-Amino Acid Oxidase (DAAO)	• Assignment of reduced flavin spectrum, role of C4═O in catalysis (Nishina, Sato, Miura, Matsui, & Shiga, 1998)
Medium-chain Acyl-CoA Dehydrogenase (MCAD)	• Effect of ligand binding and redox state on C2═O and C4═O vibrations (Nishina et al., 2007)
Glucose Oxidase (GOX)	• TRIR of FAD radicals, assignment of excited state spectra (Lukacs et al., 2012)
Photolyase	• Assignment of the resonance Raman spectra of flavin semiquinone (Schelvis et al., 2003)
Light, oxygen, voltage (LOV)	• Observation of Cys Thiol-FMN C4a adduct formation from a reactive triplet state (Swartz, Wenzel, Corchnoy, Briggs, & Bogomolni, 2002)
Blue-light using flavin (BLUF)	• Hydrogen bond network rearrangement causes perturbations in carbonyl modes upon blue light activation (Masuda, Hasegawa, Ishii, & Ono, 2004)

severely impacted by the intrinsic fluorescence of the isoalloxazine ring (Benecky et al., 1979). Several approaches were taken in an attempt to minimize sample fluorescence including the use of UV excitation (Copeland & Spiro, 1986), and coherent anti-Stokes Raman spectroscopy (CARS) (Dutta & Nestor, 1977). CARS avoids fluorescence since anti-Stokes Raman scattering is at higher frequency than the excitation frequency whereas fluorescence emission occurs at lower frequency than the absorbed light with the caveat that anti-Stokes scattering is much lower in intensity than Stokes scattered light. Dutta et al. used CARS to obtain the RR spectrum of FAD in solution and bound to glucose oxidase (GOX) and were able to assign several vibrational modes from the isoalloxazine ring (Dutta & Nestor, 1977). The authors were also able to determine the frequency shifts associated with binding of free FAD to GOX and to estimate the hydrogen deuterium exchange (HDX) rate in deuterated buffer.

Nishina and coworkers subsequently used RR spectroscopy to measure the vibrational spectra of riboflavin and several derivatives bound to riboflavin

binding protein (RBP) isolated from egg white (Nishina et al., 1978). In this approach, fluorescence was avoided due to the quenching effect of RBP on riboflavin fluorescence. These studies were followed by more sophisticated assignment of spectra using isotope labeling, and it was determined that ring III of the isoalloxazine ring is involved in interactions with RBP (Kitagawa et al., 1979; Nishina, Shiga, et al., 1980). Investigation of the charge-transfer state of old yellow enzyme using RR spectroscopy yielded crucial information about flavoenzyme catalysis and it was suggested, based on resonance enhancement of modes assigned to N5 and C4a, that nucleophilic attack would most likely occur at these two atoms (Nishina, Kitagawa, Shiga, Watari, & Yamano, 1980).

Kim and Carey extended the work on riboflavin and RBP using red excitation at 647.1 nm to obtain the off resonance Raman spectrum and an instrument based on a holographic notch filter, transmission grating spectrometer and a liquid nitrogen cooled red optimized CCD camera (Kim, Owen, & Carey, 1993). Even though the excitation frequency was not in resonance with the chromophore, the Raman spectrum of riboflavin bound to RBP was still dominated by vibrational bands arising from the isoalloxazine ring due to pre-resonance enhancement arising from the polarizability of the chromophore. In addition, off-resonance excitation minimized fluorescence from the sample. Raman spectra of riboflavin in solution and bound to RBP enabled resolution of a carbonyl feature assigned to C4═O in the protein that was previously unresolvable due to low sensitivity (Fig. 5) (Kim & Carey, 1993).

A similar approach using 752 nm excitation was used to study solvent access to the bound flavin in *p*-hydroxybenzoate hydroxylase (PHBH) and, in combination with DFT calculations, led to the assignment of frequency shifts resulting from solvent exposure and an estimate of the relative populations of the "in" and "out" conformations of the flavin (Zheng, Dong, Palfey, & Carey, 1999). These studies ultimately revealed that the flavin carbonyl vibrations, specifically that arising from the C4═O group at 1712 cm^{-1}, are highly sensitive and undergo shifts of >10 cm^{-1} upon binding to the protein.

More recently, Schelvis and coworkers reported RR spectra of the photolyase enzyme from *Escherichia coli*, which binds the neutral semiquinone form of FAD, and GOX, in which FAD can be converted from the oxidized form to $FADH^{\bullet}$ and $FAD^{\bullet -}$ (Schelvis, Pun, Goyal, & Sokolova, 2006; Schelvis et al., 2003). Both studies yielded information on the hydrogen

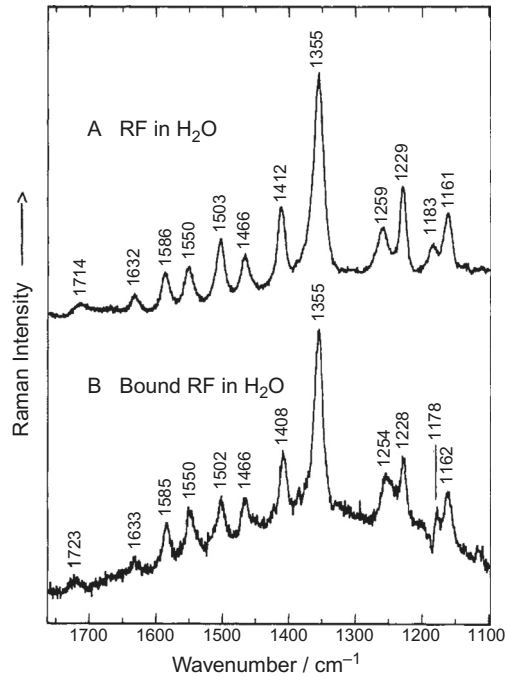

Fig. 5 Raman spectra of riboflavin in solution and bound to RBP. Advances in optics and detector technology enabled the observation of the Raman spectrum of bound riboflavin at 200 μM concentration. *Figure taken from Kim, M., & Carey, P. R. (1993). Observation of a carbonyl feature for riboflavin bound to riboflavin-binding protein in the red-excited raman spectrum.* Journal of the American Chemical Society, 115*(15), 7015–7016.*

bonding environment of the flavin in the protein. The semiquinone form of FAD does not have strong fluorescence enabling subtraction of the weak fluorescence background.

2.2 FTIR spectroscopy of flavoenzymes and light-activated proteins

In contrast to Raman and RR spectroscopy, the IR spectrum of a chromophore bound to a protein cannot be selectively enhanced by resonance or pre-resonance effects. Instead the IR spectrum of the chromophore comprises only a small fraction of the overall IR spectrum of the protein spectrum. Thus, IR spectroscopy of proteins primarily depends on difference methods in which the IR spectrum of the protein is subtracted from

a spectrum of the protein after the structure of the chromophore has been perturbed or after a ligand has bound to the protein. This approach is greatly facilitated by Fourier transform IR spectrometers which can acquire and average IR spectra very rapidly with high sensitivity. In flavoproteins, IR difference spectroscopy has enabled the observation of vibrational modes that have been perturbed due to either redox changes or photoconversion. This approach was first applied to cytochrome *c* oxidase where the IR spectrum of the protein was subtracted from a second spectrum acquired after irradiation with a tungsten lamp to generate a light adapted state (Alben, Moh, Fiamingo, & Altschuld, 1981). The difference spectrum (ΔA) is calculated using the following equation:

$$\Delta A = -\log\left(\frac{T_{light}}{T_{dark}}\right) = A_{light} - A_{dark}$$

where T_{light} is the transmitted IR during or post irradiation and T_{dark} is the transmitted IR pre-irradiation. This is the classic light minus dark (L-D) FTIR spectrum.

FTIR difference spectroscopy has been applied to both light activated flavoproteins and flavoenzymes. Swartz and coworkers used L-D FTIR spectroscopy to study light state formation in the Light Oxygen Voltage (LOV) domain of oat phototropin, and determined that Cys-adduct formation from a reactive triplet state perturbs the N5═C4a vibrational mode (Salomon, Christie, Knieb, Lempert, & Briggs, 2000; Swartz et al., 2002). Additionally, L-D FTIR spectroscopy was also applied to the Blue Light Utilizing FAD (BLUF) photoreceptors, in which photoexcitation leads to a rearrangement in a hydrogen bonding network that surrounds the FAD (Hasegawa, Masuda, & Ono, 2005). From these studies it was determined that the change in hydrogen bonding of the FAD C4═O correlates with the observed red-shift in the visible absorbance spectrum of the FAD transition at 450 nm.

Difference FTIR is not restricted to light-activated proteins and has been applied to studying changes in the redox state of the flavin. A spectro-electrochemical cell, which consisted of a gold electrode attached to CaF_2 windows, was developed for the study of redox sensitive proteins in solution (Moss, Nabedryk, Breton, & Mäntele, 1990). This cell was used to measure the reduced form of FAD in solution and FAD bound to pyruvate oxidase (POX), glucose oxidase (GOX), and D-amino acid oxidase (DAAO) where it was found that POX-bound FAD adopts a bent conformation over the entire isoalloxazine ring in the reduced state (Fig. 6) (Wille et al., 2003).

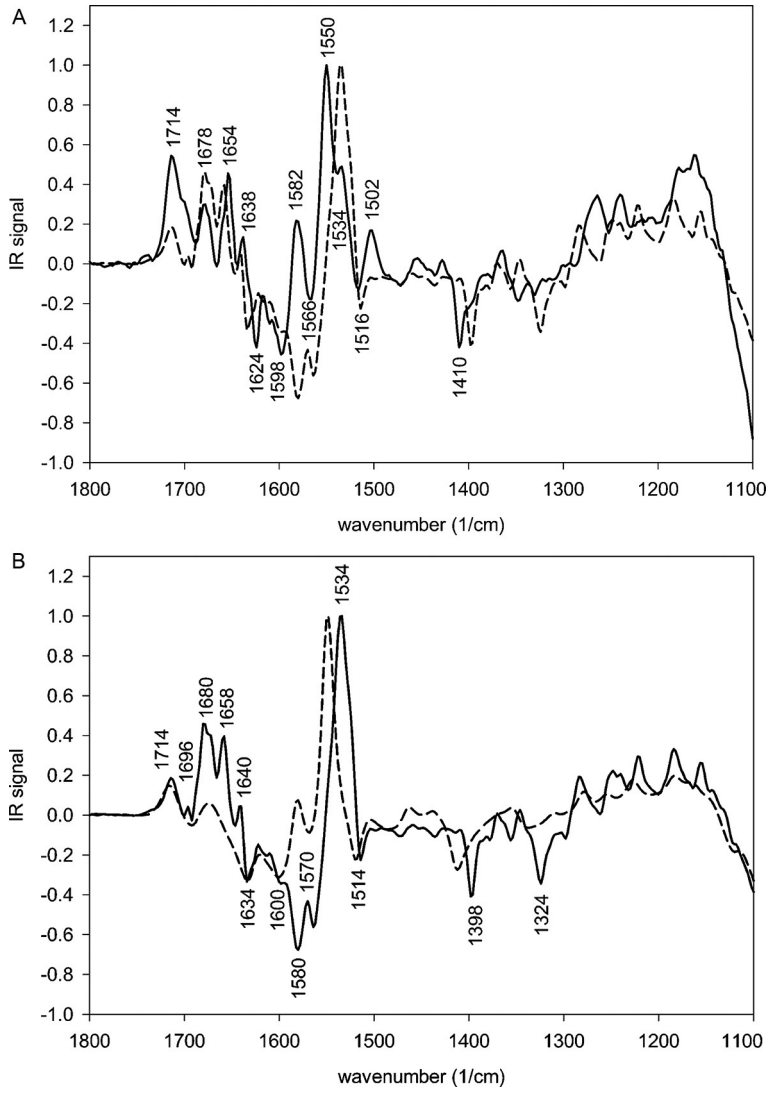

Fig. 6 Redox triggered difference FTIR spectra of (A) GOX and (B) POX. The spectrum of FAD in solution is overlaid on each protein spectrum in dashed lines. The frequency of the strong transient at $\sim$1550 cm^{-1} is sensitive to differences in solvent and protein interactions. *Figure taken from Moss, D., Nabedryk, E., Breton, J., & Mäntele, W. (1990). Redox-linked conformational changes in proteins detected by a combination of infrared spectroscopy and protein electrochemistry. Evaluation of the technique with cytochrome c.* European Journal of Biochemistry, 187*(3), 565–572.*

2.3 Time-resolved infrared (TRIR) spectroscopy of flavoprotein photoreceptors

TRIR combines IR difference spectroscopy with the high time-resolution achievable in vibrational spectroscopy. Most techniques employ a pump-probe approach in which a visible or infrared pump is used to initiate photochemistry or a temperature change in the sample. TRIR spectroscopy was used to study oxidized FAD and the lumiflavin anion in solution by Kondo et al. (2006)), and then extended to the BLUF protein AppA in which the effect of the protein matrix on the FAD vibrational modes was determined and markers for light-state formation were assigned (Stelling, Ronayne, Nappa, Tonge, & Meech, 2007). TRIR also enables the kinetics of excited state decay and ground state recovery as well as the formation and decay of intermediates to be observed, and TRIR datasets are commonly analyzed using global fitting software to extract spectra associated with each component of the fit (Snellenburg, Laptenok, Seger, Mullen, & van Stokkum, 2012). This approach allowed both excited state and ground state vibrational modes to be assigned in combination with DFT calculations. A complete assignment of the TRIR spectrum of AppA was achieved using isotope labeling and reconstituting FAD isotopologues into the protein (Haigney et al., 2011).

TRIR can also be used to characterize the reduced and semi-reduced states and intermediates of flavin in protein and in solution. Reduced FAD was studied by adding sodium dithionite or sodium oxalate to the buffered solution and irradiating the FAD sample for 30 min under anaerobic conditions prior to measurement (Zhao et al., 2011). This work was extended to GOX, in which photoexcitation results in the formation of the anionic radical $FAD^{\bullet -}$ and the corresponding Trp^{+} which decay through internal conversion on the picosecond timescale (Lukacs et al., 2012). In the BLUF protein PixD it was shown that TRIR can separate proton coupled electron transfer (PCET) into distinct steps in which the initial formation of $FAD^{\bullet -}$ is then followed by a proton transfer to yield $FADH^{\bullet}$ (Gil, Laptenok, Iuliano, et al., 2017) (Fig. 7). In the case of the LOV domain protein family, it has been shown that the excited state spectra are sensitive to small changes in hydrogen bonding around the chromophore despite the highly conserved binding pocket (Iuliano et al., 2018).

2.4 Two-dimensional infrared spectroscopy

Multi-dimensional spectroscopy has revolutionized the field of NMR, in which 2D techniques generate high resolution structural information

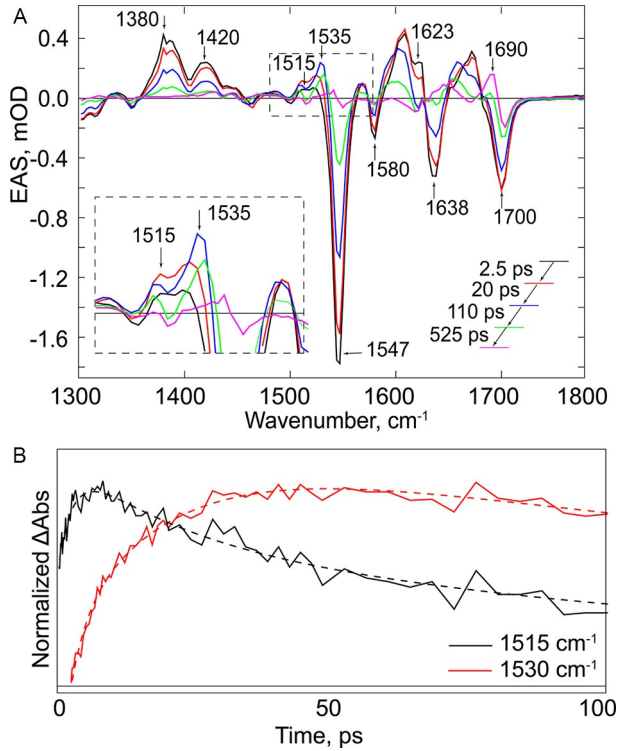

Fig. 7 TRIR of the BLUF protein PixD. (A) EADS derived from global fitting of TRIR data reveals intermediates assigned to $FAD^{\bullet -}$ (1515 cm^{-1}) and $FADH^{\bullet}$ (1535 cm^{-1}). (B) The change in intensity as a function of time is plotted for the two species showing that $FADH^{\bullet}$ (1530 cm^{-1}) forms as $FAD^{\bullet -}$ (1515 cm^{-1}) decays. *Figure adapted from Gil, A. A., Laptenok, S. P., French, J. B., Iuliano, J. N., Lukacs, A., Hall, C. R., et al. (2017). Femtosecond to millisecond dynamics of light induced allostery in the avena sativa LOV domain.* The Journal of Physical Chemistry B, 121*(5), 1010–1019; Gil, A. A., Laptenok, S. P., Iuliano, J. N., Lukacs, A., Verma, A., Hall, C. R., et al. (2017). Photoactivation of the BLUF protein PixD probed by the site-specific incorporation of fluorotyrosine residues.* Journal of the American Chemical Society, 139*(41), 14638–14648.*

in complex protein systems. In two-dimensional infrared spectroscopy (2DIR), coupling between vibrational modes can be observed on any timescale with high time-resolution. This provides information on bond environment, distance, and bond angle through electrostatic and hydrogen bonding interactions which manifest as off diagonal cross peaks in the 2D spectrum (Hamm & Zanni, 2011). 2DIR has already been applied to several enzyme-substrate systems and advances in laser technology and IR detectors

have advanced to a point where lower, more physiological-relevant protein concentrations can be studied (Bagchi, Boxer, & Fayer, 2012; Pagano, Guo, Kohen, & Cheatum, 2016). Although 2DIR has so far not been used to study flavoproteins, El Khoury and coworkers have applied 2DIR to the isolated flavin in solution. In this study a spectroelectrochemical cell was used to measure the 2DIR spectra of oxidized and reduced FAD in solution where it was found that reduced FAD exhibits stronger carbonyl coupling to the isoalloxazine ring modes (El Khoury, Van Wilderen, & Bredenbeck, 2015). These developments in 2DIR and its application to FAD in solution hold promise for the extension of this method to the flavin chromophore in more complex systems such as flavoproteins.

2.5 Femtosecond stimulated Raman spectroscopy

Femtosecond stimulated Raman spectroscopy (FSRS) utilizes the high time and spectral resolution of vibrational spectroscopy to analyze excited state chromophore modes. This is achieved by using several laser pulses: the first is an actinic or visible pulse usually <100 fs, the second is a Raman pulse overlapping with an electronic transition in the chromophore (the excited state absorbance of the flavin is $\sim$700 nm), and a white-light probe pulse to measure the Raman scattering on top of the transient absorption generated by the actinic pulse (Kukura, McCamant, & Mathies, 2007). FSRS was first applied to flavins by Weigel and coworkers where they assigned excited state spectra using DFT calculations and observed vibrational modes sensitive to deuteration (Weigel et al., 2011). Shortly after, Hall and coworkers used FSRS to study the flavin bound to the BLUF domain of AppA in both the dark and photoexcited light states, establishing that FSRS can be used to study ultrafast chromophore dynamics in proteins (Hall et al., 2017).

3. Methods to interpret vibrational spectra

This section summarizes experimental and computational methods that aid in the interpretation of vibrational spectroscopy data. Isotope labeling is the gold standard for the assignment of vibrational bands and can be applied to either the flavin itself or to the protein. In addition, computational methods such as density functional theory (DFT) are used for the isolated chromophore while QM/MM methods are used for the protein-bound flavin.

3.1 Selective isotope labeling

The synthetic method developed by Tishler has been exploited to develop selectively labeled flavins that can be incorporated into proteins, replacing the natural flavin chromophore (Bacher, 1986; Tishler et al., 1947). Recently, Illarionov and coworkers developed a biosynthetic method for converting purine bases to FMN (Illarionov et al., 2015). This approach was exploited in the assignment of the TRIR spectrum of AppA. For example, reconstitution of FAD labeled with carbon-13 at either the C2═O ([2-$^{13}C_1$]-FAD) or C4═O/C10a ([4,10a-$^{13}C_2$]-FAD) into the BLUF domain of AppA allowed unambiguous assignment of a bleach at 1700 cm^{-1} to C4═O and a bleach at 1663 cm^{-1} to C2═O (Haigney et al., 2011). This approach was also used in the study of the BLUF protein BlsA (Brust et al., 2013) and the LOV protein AsLOV2 (Fig. 8) (Gil, Laptenok, French, et al., 2017). In addition to AsLOV2 reconstituted with site-specifically labeled FAD isotopologues, AsLOV2 was also expressed in ^{13}C-glucose enriched media in a bacterial strain lacking the ability to synthesize flavin. The isotope-labeled apo protein was subsequently reconstituted with unlabeled FAD, enabling assignment of modes arising from the protein (Gil, Laptenok, French, et al., 2017).

3.2 Computational methods

In addition to isotope labeling, vibrational spectra calculations play a key role in vibrational band assignments. Density functional theory (DFT) is a common quantum mechanical method implemented in software packages such as Gaussian that can be used to estimate the vibrational spectra of small organic molecules such as the isoalloxazine ring. DFT calculates the electronic structure which is then used to calculate other properties, such as the vibrational frequencies of the molecule. DFT calculations can use several different basis sets, which are groups of Gaussian functions that describe the molecular orbitals of a molecule, to run the calculation and, in addition to ground state spectra, excited state spectra can be also be calculated using time-domain (TD)-DFT (Klaumünzer, Kröner, & Saalfrank, 2010). Choice of the basis set depends on computing power and accuracy required. For example, split-valence basis sets are constructed in increasing complexity at the expense of computing power and time. Zhao and coworkers used DFT to calculate the vibrational spectra of FAD in the gas phase and simulated a solution phase measurement by adding water molecules around the flavin thereby enabling assignment of the spectra of the reduced forms of FAD (Fig. 9) (Zhao et al., 2011). The calculations were performed using the B3LYP/6-31G basis set to calculate shifts due to H/D exchange.

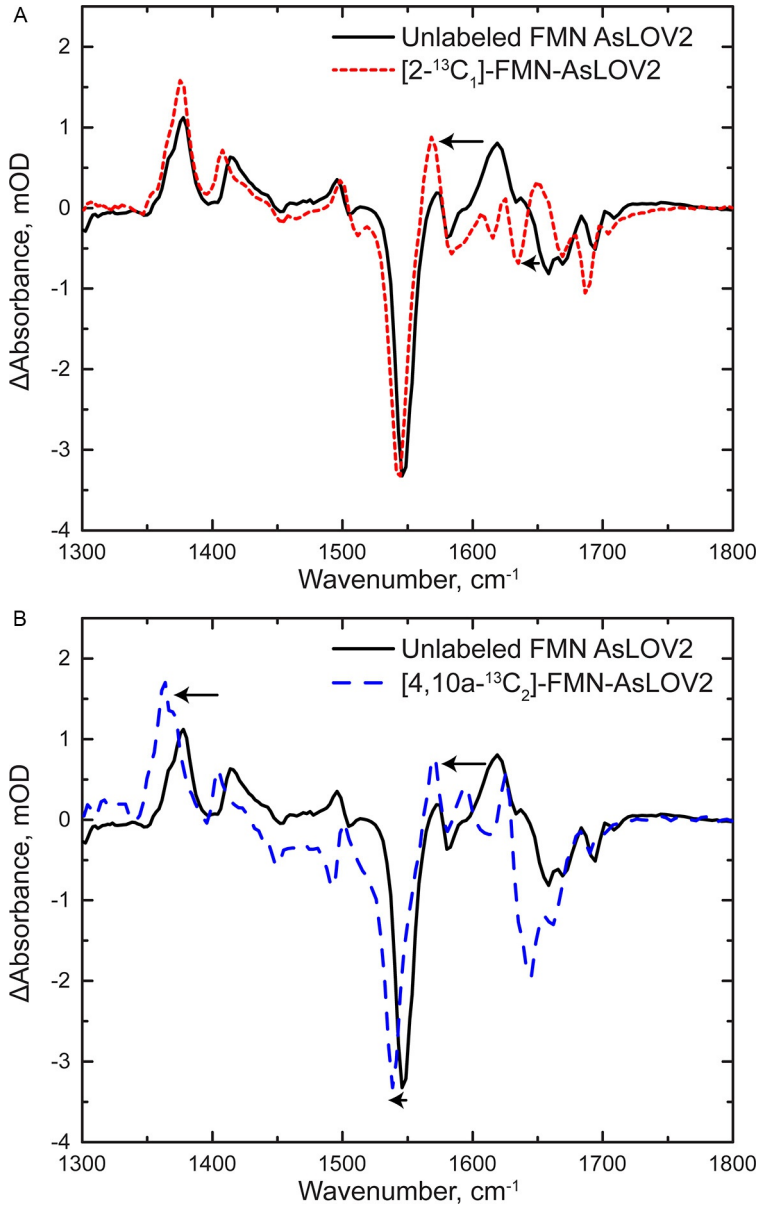

Fig. 8 Effect of isotope labeling on the TRIR spectra of AsLOV2. (A) [2-$^{13}C_1$]-FMN and (B) [4,10a-$^{13}C_2$]-FMN were reconstituted into AsLOV2. The 1 ps TRIR spectrum reveals that bands associated with the C2═O and C4a—C10a modes are red-shifted in the labeled samples. *Figure adapted from Gil, A. A., Laptenok, S. P., French, J. B., Iuliano, J. N., Lukacs, A., Hall, C. R., et al. (2017). Femtosecond to millisecond dynamics of light induced allostery in the avena sativa LOV domain.* The Journal of Physical Chemistry B, 121*(5), 1010–1019; Gil, A. A., Laptenok, S. P., Iuliano, J. N., Lukacs, A., Verma, A., Hall, C. R., et al. (2017). Photoactivation of the BLUF protein PixD probed by the site-specific incorporation of fluorotyrosine residues.* Journal of the American Chemical Society, 139*(41), 14638–14648.*

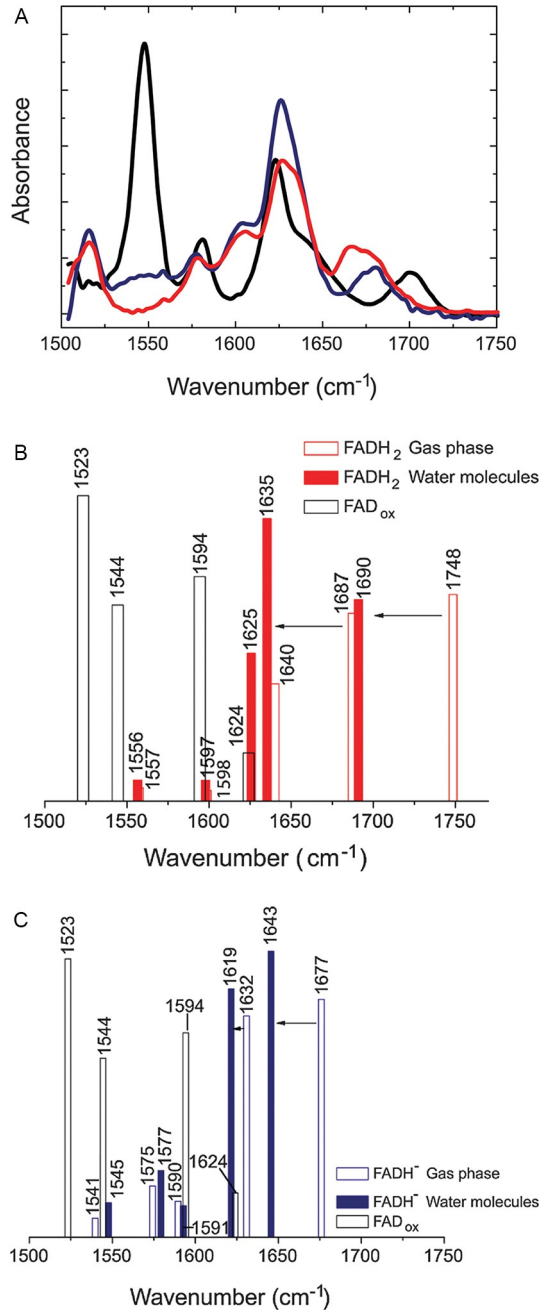

Fig. 9 FTIR and DFT spectra of FAD in solution. (A) FTIR spectra of FAD_{ox} (*black*), $FADH_2$ (*red*), and $FADH^-$ (*blue*) in phosphate buffer. DFT calculations in the gas phase and in water are shown for (B) $FADH_2$ and (C) $FADH^-$. *Figure adapted from Zhao, R.-K., Lukacs, A., Haigney, A., Brust, R., Greetham, G. M., Towrie, M., et al. (2011). Ultrafast transient mid IR to visible spectroscopy of fully reduced flavins.* Physical Chemistry Chemical Physics, 13*(39), 17642.*

QM/MM is especially useful in predicting the magnitude of frequency shifts induced by isotope labeling of the flavin chromophore as well as protein modes hydrogen bonded to the isoalloxazine ring. This approach was exploited by Domratcheva and coworkers in the assignment of vibrational modes associated with a conserved glutamine that are coupled to the flavin in BLUF photoreceptors (Domratcheva, Hartmann, Schlichting, & Kottke, 2016). Overall, the combination of biochemical approaches that enable site specific labeling and computational methods to predict frequencies affords much higher information content from vibrational spectroscopy studies.

4. General experimental considerations

4.1 Concentration determination

While methods such as the BCA assay can be used to determine protein concentration when the extinction coefficient is not known, in most cases the concentration of the flavoprotein can be determined with the visible absorption spectrum of the flavin chromophore and the associated extinction coefficient (Table 4) (Whitby, 1953). In this case it is critical to ensure that there is no unbound FAD or FMN, which can be removed either using a size exclusion or desalting column, or by ultrafiltration using a spin column with a semipermeable membrane which retains the protein but not the free chromophore. In the latter approach, the absorbance of the flow-through can be checked to ensure that no unbound cofactor is present. These methods are generally successful since FAD or FMN has high (nM) affinity for the protein and with mM protein concentrations the equilibrium lies strongly in favor of the complex.

4.2 Protection from ambient light

For flavin photoreceptors it is important to protect the sample from light before and during any measurements. Purification can be conducted in the dark to ensure that the protein is in the dark state. In addition, sufficient

Table 4 Extinction coefficients (Whitby, 1953)

	450 nm	375 nm	260 nm
Riboflavin	12,200 M^{-1} cm^{-1}	10,600 M^{-1} cm^{-1}	27,700 M^{-1} cm^{-1}
FMN	12,200 M^{-1} cm^{-1}	10,400 M^{-1} cm^{-1}	27,100 M^{-1} cm^{-1}
FAD	11,300 M^{-1} cm^{-1}	9300 M^{-1} cm^{-1}	37,000 M^{-1} cm^{-1}

time must be allowed for the photoexcited protein to return to the dark state if multiple measurements are being conducted. The rate of light to dark state recovery can vary widely from minutes to hours. In general, all flavoproteins should be kept in the dark even if they do not have obvious photosensory properties.

4.3 Buffer for protein stabilization

Careful choice of buffer is necessary for vibrational spectroscopy experiments. The buffer salts and solvents used should not have absorbances overlapping with modes from the isoalloxazine ring or protein. Commonly used buffers include Tris, sodium phosphate, or potassium phosphate supplemented with either sodium chloride or potassium chloride. An additional consideration for FTIR spectroscopy is the solvent used for the buffer. As noted above, H_2O strongly absorbs IR light, especially in the 1600–1700 cm^{-1} region which overlaps with important vibrational bands from the chromophore such as the carbonyl modes. Therefore, H_2O is normally replaced by D_2O. Solvent exchange can be accomplished by lyophilizing the protein and redissolving in D_2O, or using a desalting column equilibrated with D_2O buffer or by successive rounds of ultrafiltration in which the sample is repetitively diluted with D_2O buffer and then reconcentrated. Successful exchange can be determined by obtaining an IR spectrum of the ultrafiltration column flow through. While some enzymes require glycerol in buffer for stabilization, high concentrations of glycerol will interfere with acquisition of Raman spectra (Schelvis et al., 2003).

4.4 Sample size

FTIR spectroscopy of proteins in solution is most commonly performed using a demountable transmission cell in which the sample is placed between two BaF_2 or CaF_2 windows with a spacer to set the pathlength. Choice of pathlength depends on solvent: a very small pathlength (6–12 μm) should be used for strongly absorbing solvents such as H_2O while a 50 μm spacer is commonly used for measurements in D_2O. Sample volume is normally 30–50 μL when a 50 μm spacer is used. Solution cells come in two main types in which the cell is assembled around the sample or using a refillable cell that does not have to be disassembled between samples. The latter is recommended if the spectra of two different protein samples are being subtracted since this ensures that the pathlength for the two measurements are identical. However, larger volumes are needed to fill refillable cells and

care must be taken when cleaning and drying these cells so that the protein solution flows evenly into the cell without generating bubbles which can cause serious problems in IR data collection. Flow cells are used for photochemically sensitive samples such as photoreceptors, and in this case sample volume is 1–2 mL so that the protein solution can be recirculated through the cell. Such experiments require large amounts of protein since sample concentration is 1–2 mM. In addition, the cell is rastered in the beam to minimize unwanted photochemistry. In addition to solution cells, attenuated total reflectance (ATR) uses a crystal surface as the sample cell such that only molecules that are in contact with the crystal contribute to the vibrational spectrum: the advantage of this approach is that it is amenable to membrane proteins which can be deposited as films, while the smaller pathlength reduces the absorbance of the solvent such as H_2O.

Raman spectroscopy is more flexible in terms of sampling modality. Samples can be analyzed in capillaries or quartz cuvettes while it is also possible to obtain Raman spectra directly from single crystals mounted on a goniometer (Kalp, Totir, Buynak, & Carey, 2009). Raman crystallography yields valuable information on the ligand in the protein crystal and can also be used to assess photodamage during X-ray data collection and to analyze structural differences between solid and solution phase samples (Røhr, Hersleth, & Kristoffer Andersson, 2010). Raman spectra from small samples and cells can also be acquired using a Raman microscope (Carey, 2006), an approach that can also be used for imaging. The amount of sample necessary for Raman spectroscopy is dependent on the size of the cuvette (50–500 μL) and generally sample concentrations range from 0.2 to 1 mM.

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