Vibrational Spectroscopic Approaches in Phytochrome Research

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Phytochromes are biological photoswitches that translate light into physiological functions. Spectroscopic techniques are essential tools for molecular research into these photoreceptors.

Keywords: Raman ; IR ; spectroscopy ; phytochrome

1. Introduction

Phytochromes constitute a class of sensory photoreceptors that utilize light as a source of information to trigger a physiological response [1][2][3][4]. They harbor a linear methine-bridged tetrapyrrole as the chromophoric unit, which, upon light absorption, undergoes a double bond isomerization around the methine bridge between rings *C* and *D* [2][3]. The primary photoprocess is followed by thermal relaxations that eventually lead to functionally relevant structural changes in the protein for conversion between the parent states. These states are the red-absorbing Pr and the far-red absorbing Pfr state, which represent the physiologically inactive and active forms.

While phytochromes were initially thought to exist exclusively in plants, representatives of this photoreceptor family were later also found in bacteria and fungi [3]. Regardless of the origin, all phytochromes display the same general photo-induced reaction pattern but differ with respect to the domain composition and the type of tetrapyrrole and its binding site. Phytochromes include a tetrapyrrole-binding photosensor module composed of PAS (period/ARNT/single-minded), GAF (cGMP phosphodiesterase/adenylate cyclase/FhlA), and PHY (phytochrome-specific) domains, as well as an output module with an enzymatic domain, which is frequently a histidine kinase. Plant and cyanobacterial phytochromes carry a phytochromobilin (PΦB) or phycocyanobilin (PCB), respectively, both with the Cys binding site in the GAF domain. Bacterial and fungal phytochromes harbor biliverdin (BV) attached to a Cys in the PAS domain [3]. In most of the phytochromes (prototypical phytochromes), Pr is the thermodynamically stable parent state, which is also thermally recovered (dark reversion) after photo-conversion to Pfr. Only in some bacterial phytochromes are the relative thermodynamic stabilities of the parent states reversed, and Pfr is the stable dark state (bathy phytochromes) [5].

Related to phytochromes are a class of cyanobacterial photoreceptors lacking the PHY domain [6][7]. These cyanobacteriochromes (CBCR) carry a PCB chromophore, covalently attached to a Cys in the GAF domain, and also represent photoswitches between two parent states. However, these states can show absorption maxima in the entire visible spectral range from the red to the violet region [8].

The first vibrational spectroscopic experiments on phytochromes were published in the late 1980s, although dedicated Raman and IR spectroscopic approaches were successfully applied to other photoreceptors much earlier [9][10][11][12]. One of the obstacles that made the vibrational spectroscopy of phytochromes an enormous challenge was the tedious isolation and purification of the protein from plants, which was, until the late 1990s, the only source for the relatively large amounts of sample needed for the experiments. Furthermore, the purified phytochrome was by far not as stable as the classical benchmark photoreceptor bacteriorhodopsin (BR) [13], and thus specifically adapted vibrational spectroscopic approaches were required.

2. IR Spectroscopic Techniques

IR absorption spectroscopy provides information about the secondary structure of proteins [14], which, in view of its large size, was not a particularly instructive method for phytochromes [15]. Thus, IR spectroscopy is mainly carried out as reaction-induced difference spectroscopy, comparing the spectra measured after irradiation with the reference spectra obtained in the dark [16][17][18]. These difference spectra exclusively display those vibrational bands of both the chromophore and the protein that undergo changes upon the reaction. Although the difference spectra between the parent

states are obtained at ambient temperature, the spectral changes associated with intermediate states are measured at temperatures at which the desired state is trapped [18].

Time-resolved IR spectroscopy, like rapid-scan and step-scan spectroscopy, requires long signal accumulation times and large amounts of sample [19][20][21]. These techniques were developed on the basis of experiments with BR, which undergoes a photocycle within less than 100 ms [19][20]. Thus, within less than a second, in BR, the original photoreceptor state is recovered as a prerequisite for repetitive probing (fresh sample condition). In phytochromes, thermal recovery (dark reversion) takes much longer [3], which leads to unacceptably long measuring times. Hence, the only solution is the photo-induced recovery of the initial state, implying a demanding three-beam pump-probe-pump setup [22]. Such experiments have been successfully carried out with phytochromes covering a time range from microseconds to seconds [22][23]. Probing faster events requires a different approach based on transient absorption techniques, which allow monitoring the evolution of the IR-active vibrational bands over femtoseconds and above and can cover a wide dynamic range [24][25][26][27][28][29][30]. The experiments are based on narrow IR probe pulses, and their wavelength may be tuned over a spectral region of several hundreds of wavenumbers. In the case of monitoring localized modes, such as the C=O stretching of the chromophore, polarization-dependent measurements may even provide information about the time-dependent orientational changes of the group [29][31]. For all time-resolved IR spectroscopic techniques, a good signal-to-noise ratio is a prerequisite for a reliable spectra interpretation. In this respect, noise-reduction approaches are of particular importance, as shown, for instance, by Kübel et al. [32], who developed a generally applicable method on the basis of the time-resolved spectra of phytochromes.

Recently, two-dimensional IR spectroscopy was applied to phytochromes [33]. This technique is capable of identifying the coupling between different spectral changes and, thus, adds an additional type of information to the analysis of the structural and reaction dynamics of the photoreceptor.

3. Raman Spectroscopic Techniques

Raman spectroscopy is an important tool to selectively probe the vibrational spectrum of a chromophore upon excitation in resonance with its electronic transition (RR spectroscopy) [34][35]. In the case of phytochromes, RR spectroscopy faces the problem of fluorescence, which can obscure the RR signals, as well as uncontrolled photo-conversions. Fodor et al. were the first to present a solution by using excitation lines in the near-infrared (NIR) at 792 or 752 nm, which is shifted from the fluorescence maximum but yet sufficiently close to the first electronic transition of the tetrapyrrole chromophore (670–750 nm) to achieve good resonance enhancement [36][37]. Using a classical Raman spectrometer, these experiments suffered a bit from the low signal detection sensitivity in this spectral region. An alternative, which meanwhile has been widely applied to phytochromes, is the Fourier transform (FT) Raman spectroscopic technique, which offers the advantages of a high optical throughput and excellent frequency stability [38][39][40][41]. It is restricted to 1064-nm excitation, however, this still provides sufficient resonance enhancement of the chromophore bands such that the protein Raman bands are efficiently discriminated [39].

An interesting approach to eliminate fluorescence is shifted-excitation Raman difference spectroscopy (SERDS), in which two spectra are measured with slightly different excitation lines that are in resonance with the electronic transition of the chromophore [42]. The resultant difference spectrum cancels the fluorescence but yields a rather noisy spectrum with positive and negative Raman difference bands. These signals were simulated assuming Lorentzian band profiles and, thus, allow for calculating the absolute RR spectrum. A comparison with the spectra obtained under pre-resonance conditions demonstrated not only the reliability of SERDS but also the close similarity of the RR spectra with rigorous and pre-resonance enhancement [39][42].

The various RR spectroscopic approaches described above exploited the resonance enhancement associated with the lowest electronic transition in the red spectral region. Only a few studies have used excitation lines that were in resonance with the second transition at ca. 360–400 nm, although the fluorescence quantum yield is distinctly lower [43][44][45]. However, the high excitation energy might favor undesired side reactions.

Further techniques that circumvent fluorescence include coherent anti-Stokes (resonance) Raman spectroscopy (CARS) and surface-enhanced resonance Raman spectroscopy (SERRS). CARS probes the vibrational spectrum at the higher energy (anti-Stokes) side of the excitation line and, thus, does not interfere with fluorescence, but it is technically quite demanding [46]. SERRS can be applied when the target molecules are immobilized on nanostructured plasmonic metals like Ag or Au [47][48]. Thus, the RR scattering is enhanced by several orders of magnitude due to the coupling of the surface plasmons with the radiation field. In addition, due to a manifold of decay channels for the excitation energy in the metal, fluorescence is efficiently quenched. Serious drawbacks, however, include adsorption-induced and photo-induced

degradation of the immobilized proteins. In fact, the first results by CARS and SERRS were not very promising [49][50][51], such that both approaches were not employed anymore in further studies.

In contrast, femtosecond (fs)-stimulated Raman scattering (FSRS), which, like CARS, requires a highly complex experimental setup, is a technique with strong future potential [52][53][54]. In FSRS, a picosecond (ps) probe pulse that controls the spectral resolution is combined with a spectrally broad fs pump pulse governing the time resolution to generate a stimulated Raman spectrum. When studying a protein-bound chromophore, the probe pulse is tuned in resonance with its electronic transition. Then, FSRS selectively probes the chromophore, and due to the coherent character of the stimulated Raman scattering, fluorescence interference can easily be avoided. As the most intriguing advantage, however, the transform limit can be overcome. Thus, it is possible to probe ultrafast processes upon the temporal correlation of the FSRS pulses with an additional fs photolysis pulse that initiates the reaction of interest [52].

The RR techniques described so far were designed to establish resonance conditions with the electronic transition of the tetrapyrrole chromophore. Qualitatively different information can be obtained when the excitation line is shifted to the UV spectral region in resonance with the electronic transitions of the aromatic amino acid residues of the protein [55]. UV-RR spectroscopy probes the intramolecular interactions of Trp and Tyr, but its application of phytochrome suffers from the large number of these amino acids, which makes the interpretation of possible changes very difficult [56][57].

In contrast to IR spectroscopy, which is restricted to the (frozen) solutions of phytochromes, RR spectroscopy can also be applied to phytochrome crystals. Such experiments were carried out with low-energy NIR excitation (1064 nm) to avoid photodestruction [58][59][60]. Thus, it was possible to obtain vibrational spectra from the state of the phytochrome sample for which a crystallographic analysis yielded a three-dimensional (3D) structural model. This is particularly advantageous when the Raman spectra of the chromophore are evaluated by quantum-mechanical/molecular mechanics (QM/MM) calculations that are based on the crystal structure (vide infra) [59].

4. Spectra Interpretation

In addition to experimental obstacles, the interpretation of the vibrational spectra represents an enormous challenge. IR difference spectra are typically dominated by signals of the amide I and II modes of the protein and the localized C=O stretching modes of the tetrapyrrole substituents. In some cases, the distinction between protein and chromophore bands was not unambiguous a priori but could be clarified by isotopic labeling [61][62][63]. ^{13}C-labeling of the apoprotein of the bathy phytochrome Agp2 from *Agrobacterium fabrum* and its reconstitution with unlabeled BV chromophore allowed for the identification of unusual protonated propionic C=O stretching by ruling out the alternative assignment to a carboxyl amino acid side chain [63]. Agp2—like other bathy phytochromes—also allowed for the sequential H/D exchange of the pyrrole N-H groups [63]. Thus, the C=O stretching modes of rings *A* and *D* could be distinguished due to coupling with the N-H co-ordinates of the same rings [63], thereby confirming earlier assignments based on the selective ^{18}O-labelling of the ring *A* C=O group [61].

The greatest problem, however, was initially the vibrational assignment of the RR spectra. Until the first crystal structure determination of a phytochrome [64], information about the structure of chromophore was rather vague. Extraction experiments suggested a *ZZZ* and *ZZE* configuration for chromophore in Pr and Pfr, respectively [65]. No direct evidence for the conformation of a methine bridge was available, such that the starting point of a normal mode of analysis, i.e., the molecular geometry, was poorly defined. Thus, one could not build upon the successful empirical normal mode analyses of cyclic tetrapyrroles, i.e., porphyrins [66][67][68].

Instead, emphasis was laid on a spectral comparison using model compounds (e.g., biliverdin dimethyl ester and related tetrapyrrole derivatives) or tetrapyrrole-binding proteins of a known structure and isotopically labeled tetrapyrroles (PCB and derivatives) assembled with apo-phytochromes [39][58][66][67][68][69][70][71][72][73][74][75][76][77][78][79][80][81][82]. However, due to the high conformational flexibility of linear methine-bridged tetrapyrroles, the model compounds could hardly mimic the specific structures of the chromophores in phytochromes stabilized by interactions with surrounding amino acids. Thus, the main benefit of studying the model compound was to use it for testing and training molecules for developing theoretical approaches to calculate vibrational spectra, from semi-empirical to quantum chemical methods [37][39][70][72][73][74][75][81][83][84][85][86][87][88].

Among them, density functional theory (DFT) calculations were the best compromise between computational costs and accuracy. When applying the appropriate scaling procedures, the frequency error could be reduced to less than ±15 cm^{-1} [85][89]. Nevertheless, the inevitable drawbacks were the specific van der Waals and electrostatic interactions with the protein, which affect the structural and electronic properties of the chromophore in a way that could not be mimicked by

calculation of the molecule in vacuo. This problem was eventually solved with the development of QM/MM techniques, specifically when combined with molecular dynamics (MDs) simulations [90][91][92]. Here, the segment of interest, i.e., the chromophore and possibly nearby amino acids, are treated quantum mechanically, whereas, for the remainder of the protein, an empirical force field is used. QM/MM requires a reliable 3D structure as the starting point and was successfully applied to tetrapyrrole-binding proteins and eventually to phytochromes first by the Mroginski group [59][93][94][95].

A good reproduction of the RR spectra by the QM/MM calculations can serve as a criterion for the quality of the 3D structure model, not only as far as the chromophore itself is concerned but also with respect to the protonation pattern of the amino acids adjacent to the chromophore [96], which is not accessible by protein crystallography. The spectroscopic-theoretical characterization of a reference state, typically one of the parent states, may then constitute the starting point for the analyses of those states for which, due to the lack of experimentally determined structures, only tentative 3D models are suggested. Here, the comparison between the calculated and experimental RR spectra then guides the refinement of the model. In addition, with an increasing set of data, for instance, derived from snapshots of an MD simulation, structure–spectra relationships can be obtained that allow for estimating the geometrical parameters of tetrapyrrole, even without extensive calculations [95][97].

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