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SPECTRAL METHODS FOR STUDY OF THE G-PROTEIN- COUPLED RECEPTOR RHODOPSIN. I. VIBRATIONAL AND ELECTRONIC SPECTROSCOPY

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Abstract

Here we review the application of modern spectral methods for the study of G-protein-coupled receptors (GPCRs) using rhodopsin as a prototype. Because X-ray analysis gives us immobile snapshots of protein conformations, it is imperative to apply spectroscopic methods for elucidating their function: vibrational (Raman, FTIR), electronic (UV-visible absorption, fluorescence) spectroscopies, and magnetic resonance (electron paramagnetic resonance, EPR), and nuclear magnetic resonance, NMR). In the first of the two companion articles, we discuss the application of optical spectroscopy for studying rhodopsin in a membrane environment. Information is obtained regarding the time-ordered sequence of events in rhodopsin activation. Isomerization of the chromophore and deprotonation of the retinal Schiff base leads to a structural change of the protein involving the motion of helices H5 and H6 in a pH-dependent process. Information is obtained that is unavailable from X-ray crystallography, which can be combined with spectroscopic studies to achieve a more complete understanding of GPCR function.

Keywords

rhodopsin; G-protein-coupled receptors; optical spectroscopy; electronic spectroscopy; Raman spectroscopy; FTIR spectroscopy; linear dichroism; circular dichroism; fluorescence

INTRODUCTION

Membrane proteins (proteins embedded into the cell membrane or organelle membrane or attached to it) account for about a quarter of all proteins [1]. The most detailed information on protein structure can be obtained by studying their three-dimensional crystals by X-ray diffraction [2–13]. However integral membrane proteins are very difficult to crystallize – being removed from their natural lipid environment, non-polar parts of protein molecules tend to form disordered aggregates unsuitable for X-ray analysis. Recently, significant advances have been achieved in crystallizing G-protein-coupled receptors (GPCRs) for crystallographic studies by modifying their amino acid sequence [8, 14], and/or by adding small molecules [9, 14] that increase their thermo- and conformational stability and facilitate crystallization. However, at this point only few dozens of 3D structures of GPCRs in

different states have been studied out of more than 800 [15]. Thus the application of spectral methods for membrane protein studies is of special interest.

In the present article we review the opportunities provided by modern spectroscopic methods in membrane protein studies using rhodopsin as an example. Rhodopsin (the scotopic vision protein) is a representative of a large family of G-protein-coupled receptors responsible for the transmitting the signal through the cell membrane [16, 17]. We show how different spectral methods (Raman [18], FTIR [17, 18] spectroscopies, ESR [20–22], NMR [23–29]) may provide information on the structure, dynamics, and intramolecular interactions in GPCRs, which is important for understanding their functioning and complementing the results of crystallographic studies.

ELECTRONIC SPECTROSCOPY

Electronic spectroscopy is a very sensitive and convenient method which allows one to measure the absorption, transmission, and reflectance spectra. Usually absorption spectra are diffuse in nature, which limits their use to substances having a chromophoric group (aromatic rings, conjugated systems having multiple bonds, and so on). These spectra allow one to determine the presence of certain groups in the molecule, to study the influence of substituents on the electronic spectra, structure of molecules, tautomerism, and other transformations. In this regard, the rhodopsin has the advantage that its ligand is a covalently linked chromophore (retinal). As a consequence, it has been possible to apply this method for characterization of states of the retinal ligand and receptor as a whole, including the short-lived states with the use of spectroscopy having pico- and femtosecond resolution [30–32].

Figure 1 shows the characteristic absorption spectra of rhodopsin in various states in the UV and visible regions [19]. At high temperatures and in acidic conditions (20 °C, pH 5.0), following the photoisomerization of retinal, rhodopsin is converted to the active metarhodopsin II_bH⁺, while at low temperatures and in an alkaline environment (10 °C, pH 9.5) it is transformed into the inactive metarhodopsin I. In Reference [33] photoreaction of rhodopsin regenerated with three different 9-*cis* retinal analogs (isorhodopsin), modified at or in the vicinity of the β-ionone ring, have been investigated. Results obtained by absorption and FTIR spectroscopy showed the formation of photoproducts such as BSI (blue-shifted intermediate), Lumi and Meta I for all pigments, and also formation of the photointermediates similar to the active Meta II for 5,6-epoxy-ISO and 7,7-diH-ISO pigment with respectively 81 and 65 % of activity in the membrane. However, the diethyl-acyclic-iso-pigment did not formed the active state (18 % of activity in the membrane), which confirms the importance of the β-ionone ring of the retinal to form the active state of the receptor.

RAMAN SPECTROSCOPY AND INFRARED SPECTROSCOPY

These spectral methods can be used to study the secondary structure of proteins. The vibrational spectrum of the polypeptide backbone depends on the type of secondary structure and provides information about the content in a molecule of α- and β-structures

[34]. These methods can be applied to the dried films, aqueous suspensions of membranes and purified proteins.

Information on the structure of the protein from the Raman spectroscopy can be obtained by the simulation of the molecular vibrations whose frequencies depend on the structure of the molecule. The application of modern stimulated Raman spectroscopy with femtosecond resolution allows studying short-lived states (such as photorhodopsin, which is impossible to trap even at very low temperatures). In the paper of Kukura et al. [18], Raman spectra of rhodopsin were recorded in the process of its transformation into bathorhodopsin. The most notable changes were observed in the spectral region corresponding to the hydrogen-out-of-plane wagging motions (HOOP modes) of the retinal polyene chain. The torsion angles for the C8-C14 region of the polyene chain for photo-rhodopsin and bathorhodopsin were estimated using vibrational modeling. The observed results show that while the isomerization of retinal is initiated in the excited state, major structural changes in the ligand takes place already on the ground potential surface of the transition from the photo- to bathorhodopsin [18].

Infrared spectroscopy is very sensitive to conformational changes of rhodopsin in the process of activation [19, 35]. Fourier-transform infrared spectroscopy (FTIR) studies of rhodopsin with modified retinals have been conducted that have revealed the role of the different molecular groups in activation mechanism [33, 35]. Study of retinals with completely removed β -ionone ring (acyclic retinal analogs) and deleted ring methyl groups at positions C1 and C5 showed that any of the above modifications makes the retinal after photo isomerization a partial agonist. The balance between active (Meta II) and preactive (Meta I) states is shifted towards the Meta I state [35]. Typical FTIR difference spectra for the pure Meta II (minus dark) state and Meta I (minus dark) state of rhodopsin are shown in Figure 2. To obtain a pure Meta II or Meta I state, the equilibrium can be shifted, for example, by changing the pH and temperature.

To determine the ratio of Meta II/Meta I in the sample, one can decompose the experimental spectrum in the range of $1600\text{--}1800\text{ cm}^{-1}$, into a linear combination of the Meta I and Meta II reference (basis) spectra, as shown in Fig. 2. Investigations of modified retinals [35] have shown that the acyclic *trans* retinals are weak partial agonists. This reveals the important role of the β -ionic ring for the activation of receptor. The infrared spectra of the retinals with demethylated β -ionone ring indicate interaction of the C18-methyl group with the Glu 122 side chain. Such interaction appears to play an important role in the activation of rhodopsin. It should be noted that in contrast to absorption spectra, which are used to characterize rhodopsin (fig. 1), and which are sensitive to the structure of the chromophore, FTIR spectra as shown in the fig. 2 can identify structural changes in the receptor upon activation. Examples include the rearrangement of the hydrogen bonds around the Asp 83 and Glu 122 amino acid residues [19]. In addition, we showed [19] that by varying the pH and using FTIR and electronic spectroscopy one can investigate the thermodynamic equilibrium between different states of the receptor under various conditions, fig. 3.

Such studies have revealed (fig. 3) that after isomerization of retinal at relatively high temperatures and pH simultaneously, a minimum of four rhodopsin states are present:

inactive Meta I with a protonated Schiff base stabilizing the inactive conformation; an inactive Meta II_a (with deprotonated Schiff base favoring the activation, but still in the inactive conformation); the active Meta II_b state; and the active Meta II_bH⁺ state with protonated Glu 134 residue. Thus, the equilibrium reaction scheme of the rhodopsin activation can be represented as follows:

The above scheme reflects the main activation events (isomerization of 11-*cis* retinal into all *trans* retinal; deprotonation of the Schiff's base; rotation of the cytoplasmic end of H6 helix with the formation of G-protein (transducin) binding site; and protonation of the Glu134, stabilizing the active state). However, it does not explain completely the mechanism of activation. Based on observations of time-resolved absorption spectra, alternative kinetic models have been also suggested, which assume not a strictly linear, but a modified square scheme, where parallel transitions from Lumi to both the protonated (Meta I) as well as earlier deprotonated intermediate substate (Meta I₃₈₀) are observed [30].

LINEAR DICHROISM METHOD

The polarimetry methods [36–45], used for biomedical problems, include kinetic ellipsometry [41, 42] and linear dichroism (LD) [43–45]. For the study of the rhodopsin the LD method, based on a comparison of the absorption coefficients for orthogonal, linearly polarized components of light passing through the sample, allows one to determine angles between the transition dipole moment of the chromophore and the plane of the disk membrane [43]. It can be used as an additional orientation parameter in modeling the structure and orientation of retinal in rhodopsin [44]. In [43] the LD method was used to determine the orientation of the retinal main transition dipole moment in the dark and pre-active (Meta I) states. The orientation of the transition dipole moment in dark state was determined [43], using as a reference the value $\theta_{\text{Rh}} = 16^\circ$ obtained from micro spectrophotometry of the frog rod outer segments [45]. In [46] retinal crystals were investigated by LD, which provided the orientation of the transition dipole moment of retinal relative to the axes of the crystal. It allowed us [44] to determine the orientation of the transition dipole moment of all-*trans* retinal with respect to its own axis for structural studies of the Meta I state.

CIRCULAR DICHROISM METHOD

The method of circular dichroism (CD) is based on a comparison of the absorption spectra for the right and left circular polarized light, which allows one to determine the chirality of molecules. In protein studies, CD spectroscopy is used to analyze the composition of the protein secondary structure. The CD spectrum can be typically decomposed into a linear combination of spectra that represent the different secondary structures of the protein molecule. Once the characteristic spectrum of each of these structures is known, the fraction of each type of secondary structure can be determined [46]. The CD method was originally applied for soluble proteins, but it can also be used for membrane proteins.

Another example of CD application involves studies of the retinal chromophore of rhodopsin. Fujimoto et. al investigated rhodopsin regenerated with synthetic retinoids, where the torsion angle formed by C5=C6–C7=C8 bonds was locked due to additional bonds between carbons C7 and C7 or C16 and C17 [47]. Both retinal analogues had the 6-*s-cis* conformation, but twisting around the C6–C7 bond was the opposite. It turned out that only the enantiomer with a negative torsion angle for the C6–C7 bond forms the pigment, where the CD spectrum is similar to that of native rhodopsin. This finding indicates that the chromophore in rhodopsin has 6-*s-cis* conformation and negative chirality in the C6–C7 region. Moreover, a guanosine triphosphate (GTP) binding assay has shown that the activity of the pigment incorporating retinal with a negative twist of the C6–C7 bond is about 80 % of that of rhodopsin, which suggested that the negative 6-*s-cis* conformation of the retinal cofactor is preserved after activation of the receptor. The CD study of rhodopsin with modified 11-*cis*-locked retinals has also shown a positive torsion angle for the C13–C12 bond of the retinal in the dark state of rhodopsin [48]. Time-resolved circular dichroism measurements [49] have been conducted to investigate the intermediate states of rhodopsin in the activation process. It was found that the CD spectrum of lumirhodopsin was changing in the time interval from 5 to 100 microseconds, which was attributed to the early manifestation of deprotonated state.

FLUORESCENCE

Methods for studying fluorescence of specific substances have a high sensitivity, as well as conveniently short response time, because the emission of fluorescence occurs over a time interval of 10^{-8} s after the absorption of light. It is important that the fluorescence spectroscopy allows one to obtain information about the state of the biosystems without damaging them. However, the fluorescence spectra of complex molecules, as in the case of absorption spectra, are often diffuse and without fine structure. In addition to the values of the fluorescence maxima the ratio of intensities, polarization, and the lifetime of the fluorescence are very informative. In proteins, aromatic amino acids with conjugated systems of double bonds are the fluorophores [50]. The main fluorescent component is tryptophan which is responsible for nearly 90 % of protein fluorescence. Fluorescence of proteins that do not contain tryptophan residues is due to tyrosine residues alone, which have a maximum of fluorescence at 303–304 nm (the intensity is an order of magnitude lower than that of tryptophan).

An insightful review of fluorescence methods to study rhodopsin and other retinal proteins was recently presented by Farrens and Alexiev [51]. In bovine visual rhodopsin, the fluorescence of five tryptophan residues is quenched by very efficient energy transfer to the retinal, because of their proximity to the chromophore. On the one hand, this aspect makes it difficult to use the natural internal fluorophore, yet on the other hand it also allows the use of tryptophan fluorescence for observation of the binding and release of retinal [52–54], folding, and unfolding of rhodopsin [55, 56]. Hoersch et. al observed the transient changes of the tryptophan fluorescence of bovine rhodopsin in the rod outer segment membranes in the interval from 1 μ s to 10 s after the actinic excitation (Fig. 4) [57]. It has been shown that the fluorescence up to 100 μ s does not change, suggesting that tryptophan has similar lifetimes in rhodopsin and in metarhodopsin I. Thereafter, on a millisecond time scale, the

kinetics of the fluorescence decrease fully corresponds to the formation of the active state. The increase in energy transfer is solely due to the overlapping absorption retinal spectral bands (with a maximum of 360 nm at Meta II) and fluorescence of tryptophan, without structural changes (positions of tryptophan residues with respect to the ligands). A significant increase in the transient fluorescence was observed during the transition to the active state (Meta II) for rhodopsin selectively labeled with the fluorophore Alexa594 at cysteine 316 on the H8 helix, Fig. 4. Using transient absorption spectroscopy and the pH indicator dye bromocresol purple, the kinetics of structural changes was compared with the kinetics of proton uptake (Fig. 4). The observations show the following sequence of events in the ROS membranes at pH 6: the deprotonation of the Schiff base, structural changes (at the cytoplasmic surface), and subsequent proton uptake.

In the case of heterotrimeric G-proteins, the tryptophan fluorescence of α -subunit increases when they bind GTP [58]. Thus, the fluorescence can be used to monitor activation of transducin [58]. Other examples of the use of fluorescent spectroscopy to study rhodopsin and other retinal proteins and G-protein-coupled receptors include estimation of distances between the dye and retinal, and between pairs of dyes in selectively labeled protein by fluorescent resonance energy transfer [59]; studies of mobility with time-resolved fluorescence anisotropy experiments [60,61]; investigations of the accessibility of different sites of the fluorophore to the external quencher, which can be either aqueous or hydrophobic [60, 62–64]; studies of the structural changes for helices H6 and H8 in rhodopsin and other G-protein-coupled receptors when activated [62, 65–69]; and investigations of the interaction of rhodopsin with arrestin [53, 65, 70].

CONCLUSION

Optical spectroscopy is a very valuable tool for studies of biomolecular structures due to the high sensitivity to conformational changes. Development of optical methods with extremely high temporal resolution makes them indispensable for the study of the kinetics of molecular transformations and complementary to X-ray diffraction studies. Magnetic resonance (ESR, NMR) studies of rhodopsin will be discussed in the second part of this paper.

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Fig. 1.

UV-visible spectroscopic characterization of the retinal Schiff base for the Meta I/Meta II thermal equilibrium in native disk membranes. (A, B) At 20° C and pH 5.0, the equilibrium is completely shifted to the active state of the Meta II_bH⁺ with a deprotonated Schiff base, while at 10 °C and pH 9.5 the inactive Meta I state with a protonated Schiff base is favored. (C, D) The difference spectra (photoproduct minus the dark state) demonstrate the pH-dependence of the Meta I/Meta II equilibrium at various temperatures (10 °C, 30 °C). One can see that at 30 °C the balance is not fully shifted to Meta I even at very alkaline pH. Figure is adapted from Ref. [19].

Fig. 2.

Example of FTIR difference spectra of rhodopsin in the Meta I or Meta II_bH⁺ minus dark state in native disk membranes at 10 °C, pH 9.5 or 20 °C, pH 5.0, respectively. The spectral range is marked that is sensitive to conformational changes in the protein. Figure is from Ref. [19].

Fig. 3.

Extended reaction scheme for rhodopsin activation in phospholipid membrane predicts a complex equilibrium of photoproducts Meta I, Meta II, and Meta II_b at alkaline pH. By contrast, at low pH the equilibrium is completely shifted to the active Meta II_bH⁺ state. Figure is adapted from Ref. [19].

Fig. 4.

Examples of transient absorption and transient tryptophan fluorescence of the rod outer segment membranes after the photoactivation, showing time order sequence of changes. Note that conformational changes of rhodopsin are delayed with respect to deprotonation of Schiff base, and that subsequent proton uptake is further delayed. Conditions: pH 6, 23 °C (A) Transient absorption at 360 nm and tryptophan fluorescence of rhodopsin. Mirror image of the time-resolved absorption curve is shown by a dotted line to emphasize the similarity of the absorption and fluorescence kinetics. (B) Dependences of the absorption at 360 nm and fluorescence of Alexa594 at cysteine 316 on H8 helix. (C) Time dependences of absorption at 360 nm and 605 nm photoactivated membranes in 100 μ M unbuffered solution of bromocresol purple. Figure is adapted from Ref. [57].