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Measurement of Molecular Orientation in a Subcellular Compartment by Synchrotron Infrared Spectromicroscopy

Luca Quaroni,*[a] Theodora Zlateva,[b] Diana Bedolla,[c] Sebastiano Massaro,[c] and Vincent Torre[c]

We demonstrate that synchrotron Fourier-transform infrared (FTIR) spectromicroscopy can be used to obtain detailed orientation parameters of biomolecules within a subcellular compartment. FTIR is an informative technique for the study of biological molecules; it provides insight into both structure and mechanism.[1] To date, most FTIR studies are performed using partially or fully purified biomolecules, often reconstituted under conditions that mimic some features of their native cellular environment. It would be highly desirable to be able to perform these measurements directly in vivo, with biomolecules still in their physiological location. The introduction of optical configurations for FTIR spectromicroscopy has opened this possibility, thus allowing measurements on single intact cells.[2] Nonetheless, the application of FTIR spectromicroscopy to subcellular biochemistry is limited by the low throughput of confocal IR microscopes when operated at high spatial resolution. The use of synchrotron IR light sources for spectromicroscopy has allowed overcoming this limitation and has provided good FTIR spectra of subcellular compartments at diffraction-limited spatial resolution.[3] This success has been used for qualitative analysis of cellular composition, to identify the presence of general classes of biomolecules, such as proteins and lipids, and map their subcellular distribution.[4] We are now aiming to extend the scope of the technique by performing detailed spectroscopic analysis of specific biomolecules in vivo. The usefulness of the technique for the direct study of structure and reactivity in subcellular modules has been recently suggested by Hofmann and co-workers.[5] However, to date, no experimental demonstration of the feasibility of such approach has been obtained. This work provides the first such example by showing that synchrotron FTIR spectromicroscopy can be used to measure vibrational linear dichroism (VLD) within individual vertebrate photoreceptor rod cells and to quantify the orientation of major molecular components.

[a] *Prof. L. Quaroni*
Canadian Light Source, Saskatoon S7N 0X4 (Canada)
and
Department of Chemistry
University of Manitoba, Winnipeg R3T 2N2 (Canada)
Fax: (+1) 306 657353
E-mail: Luca.Quaroni@Lightsource.ca

[b] *Dr. T. Zlateva*
Department of Pathology and Biochemistry
University of Saskatchewan, Saskatoon, S7N OW8 (Canada)

[c] *D. Bedolla, S. Massaro, Prof. V. Torre*
International School for Advanced Studies (SISSA)
Via Beirut 2, 34024 Trieste (Italy)

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Retinal rod cells are responsible for vision under conditions of weak illumination.[6] They are composed by an inner segment (RIS: rod inner segment) portion, which is the site of cellular metabolism, and an outer segment (ROS: rod outer segment), which contains the molecular machinery involved in phototransduction. Amphibian ROS are cylinders (30–50 μm long and 4–7 μm in diameter) in which the membrane encloses a stack of disks.[7] Light is absorbed by rhodopsin molecules embedded in the membranes of the disks, thus initiating an enzymatic cascade that leads to the closure of light-sensitive membrane channels and the generation of a nerve impulse.[8]

The composition of the ROS is relatively simple and its morphology and chemical composition are very suitable for an investigation with FTIR. Although the overall number of molecular species is elevated, only few components are present at sufficiently high concentration to be appreciated by a transmission FTIR measurement.[9] The protein complement is dominated by the photoreceptor protein rhodopsin (≈5 mM). Less abundant proteins include transducin and arrestin. Other proteins are at concentrations below the detection limit of the technique. The disk-membrane composition is also relatively simple, with large concentrations of polyunsaturated fatty-acid chains dominating the infrared absorption spectra.[10]

ROS FTIR spectra recorded with polarized light are shown in Figure 1.

Spectra in the 1500–1800 cm^{-1} region, which represent mostly absorption bands from ester and amide carbonyl groups, show marked dichroism. The band corresponding to the amide-I transition is polarized along the ROS axis, whereas that corresponding to the amide-II transition shows stronger polarization in the orthogonal direction, within the plane of the disks, in agreement with the different orientation of its transition moment.[11] This is expected from a system abundant in proteins with transmembrane α helices oriented roughly perpendicular to the disk membrane, and has already been reported for macroscopic preparations of ROS fragments.[12] Curve-fitting analysis of amide-I-band components is used to obtain polarization ratios for the main elements of the protein secondary structure present in the ROS (see the Supporting Information), as reported in Table 1. Derivation of order parameters from polarization ratios provides a measure of the average angle $\langle\gamma\rangle$ between the axis of α helices and the axis of the ROS.[13] From our results, the orientation of α helices within the ROS can be described by a value of $\langle\gamma\rangle = 30^\circ$. Rhodopsin is a predominantly α-helical protein and its abundance within the ROS makes it the dominant contributor to the amide-I absorption in the α-helical region. Therefore, we propose that $\langle\gamma\rangle$ can be specifically interpreted as a quantitative measure of the orientation of the α helices of rhodopsin. To validate the result,

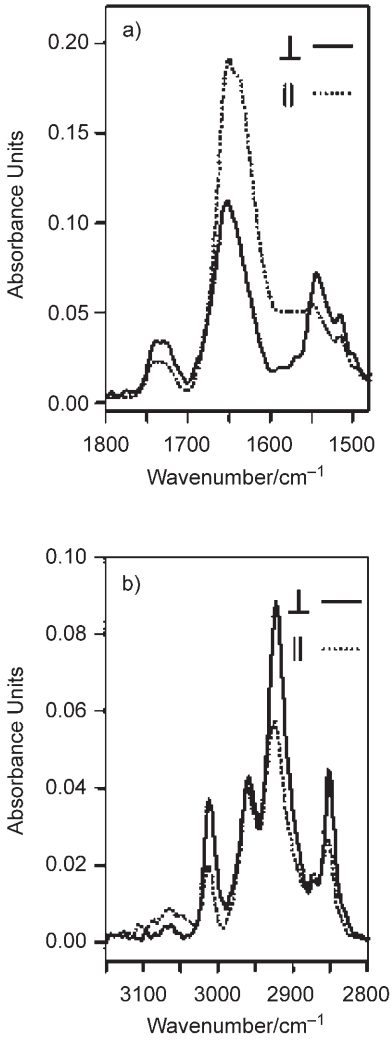

Figure 1. a) ROS spectra recorded in the carbonyl absorption region with light polarized perpendicular (•••••) and parallel (——) to the ROS axis, b) ROS spectra recorded in the methyl absorption region with light polarized perpendicular (•••••) and parallel (——) to the ROS axis.

we derive $\langle\gamma\rangle$ from the crystal structure of rhodopsin[14] [protein data bank (PDB) entry 1F88; see the Supporting Information], thereby obtaining a value of $\langle\gamma\rangle = 24^\circ$, which is in good agreement with our spectroscopic results. The orientation of rhodopsin α helices relative to the normal of the membrane has been previously measured by means of FTIR VLD using reconstituted disks or ensembles of fractured ROS. Values of $\langle\gamma\rangle$ ranging between 38 and 51° were reported in these works.[12,15] To date, our results—obtained in vivo—provide the best agreement between the spectroscopic measurement of the $\langle\gamma\rangle$ value of rhodopsin α helices and the corresponding crystallographic value.

The amide-II band overlaps with bands assigned to tyrosine residues in rhodopsin,[12] which prevents a quantitative assessment of polarization ratios by curve fitting. Nonetheless, the band clearly displays opposite dichroism to that of amide I, as expected based on the orientation of its transition moment. An approximate value of $\langle\gamma\rangle = 33^\circ$ can be calculated for the α helices, in fair agreement with the more accurate value obtained from the analysis of the amide-I absorption.

Dichroism is also observed for the absorption band at 1740–1720 cm^{-1}, which is tentatively assigned to the acylic carbonyl of the phospholipid headgroups. The transition moment for this vibration is taken to be aligned with the C=O group, thus providing an average orientation of $\langle\gamma\rangle = 72^\circ$ and $\langle\gamma\rangle = 67^\circ$, relative to the ROS axis, for non-hydrated and hydrated carbonyl groups, respectively.[16] This result is consistent with measurements on model systems.[17] However, a different orientation for the population of hydrated carbonyl groups has never been reported in the past. The protonated forms of aspartic and glutamic acid residues of rhodopsin absorb in this same region. Although their contribution to the total absorbance is relatively small, it is sufficient to account for the difference in polarization of the two components of the carbonyl band, thus setting a limit to the accuracy of this measurement.

Bands in the 2700–3100 cm^{-1} region also show remarkable polarization, in particular the methylene symmetric and antisymmetric stretching bands at 2852 and 2924 cm^{-1}, respectively, and the stretching vibration of C–H bonds on trigonal carbons at 3012 cm^{-1}. The values of the average angle of the transition moment to the ROS axis, $\langle\theta\rangle$, for these groups are shown in Table 1, together with corresponding values of $\langle\gamma\rangle$. Contributions to the absorption in this spectral region arise predominantly from lipid acyl chains. A contribution from C–H vibrations of amino-acid side chains and other small molecules is also expected. Therefore, evaluation of the orientation of lipid methylene groups is limited in accura-

Table 1. Band frequencies for selected ROS spectral bands measured with polarized light and corresponding polarization ratio (R), average transition moment orientation ($\langle\theta\rangle$), and average structural element orientation ($\langle\gamma\rangle$) relative to the ROS axis.

Frequency of the absorption band [cm^{-1}] $\tilde{\omega}_{\parallel};\tilde{\omega}_{\perp}$	Assignment	Polarization ratio $R = A_{\parallel}/A_{\perp}$	$\langle\theta\rangle$ [°]	$\langle\gamma\rangle$ [°]
1651; 1651	amide-I contribution from α helices	1.94	45	30
≈1545	amide-II contribution from α helices	≈0.5	≈63	≈33
1742; 1741	non-H (or D)-bonded lipid acyl carbonyl groups	0.78	72	72
1724; 1725	H (or D)-bonded lipid acyl carbonyl groups	0.67	67	67
2853; 2852	$\nu_{CH2,S}$	0.57	62	35[a]
2926; 2922	$\nu_{CH2,AS}$	0.72	57	
3013; 3012	$\nu_{=C-H}$	0.61	61	61

[a] Tilt angle of the acyl chain (from the geometry of the acyl chain and assuming that the chain is in the all-*trans* conformation; the transition moment is along the bisector of the CH_2 group). The parameter $\langle\theta\rangle$ is the averaged inclination of the transition moment relative to the ROS axis whereas $\langle\gamma\rangle$ is the averaged inclination of the axis of the structural element or the functional group relative to the ROS axis.

cy. Nonetheless, similar peak frequencies and values of $\langle\theta\rangle$ have been reported for phospholipid bilayers in the liquid crystalline phase.[18] In such systems, acyl chains are mostly in the *trans* conformation, with some disorder due to the formation of gauche rotamers. We are now detecting a comparable organization in the saturated acyl chains of disk phospholipids. Polyunsaturated acyl chains also retain some level of organization, as seen by the preferential average orientation of $\nu_{=C-H}$ transitions, thus indicating that C=C bonds tend to be oriented perpendicular to the disk surface.

These results overall confirm that ROS disk membranes are relatively ordered systems on the molecular scale—exhibiting characteristics of liquid-crystal phases. Concerning the axial orientation of rhodopsin, it appears to be even closer to the crystal structure than previously appreciated from FTIR studies on purified disks and membrane fragments.

From the point of view of methodology, we have shown the feasibility of a VLD study on an intact cell by using synchrotron FTIR spectromicroscopy. The type of spectroscopic study reported herein is traditionally carried out on samples of purified proteins and lipids, or membrane fragments, which involves extraction, purification, and reconstitution of sample components, thus making sample preparation lengthy and often difficult and expensive, in particular for membrane proteins. In addition, the condition and stability of the reconstituted biomolecules is always a major issue. By performing FTIR spectromicroscopy directly on an intact cell we bypass all these problems, thus providing a simpler and cheaper way to probe molecular structure and function in a native environment. The spectra reported in this work show that absorbance values and signal-to-noise levels, at least for this system, are only slightly lower than the ones obtained with purified proteins. This opens the way to the study of dynamic processes by means of differential spectroscopy, where absorbance variations of the order of 10^{-3} absorbance unit must be appreciated.

Application of the technique is limited by the sensitivity of the FTIR measurements, generally requiring the presence of biomolecules at concentrations higher than 0.1 mM, which considerably restricts the number of systems that can be approached. Nonetheless, this limitation can be turned into an advantage in selectivity by considering that the spectral response can be restricted to only the most abundant biomolecules in a particular subcellular system. Several subcellular units satisfy this condition and can be accessed in vivo. We are now inviting the scientific community to further explore the potential of the technique by extending its application to new systems.

Experimental Section

The cross-section of a ROS is close to the diffraction limit for radiation in the mid-IR spectral region, and an IR synchrotron source is necessary to perform spectromicroscopy measurements on individual ROS with high signal-to-noise ratio. We used the MidIR beamline at the Canadian Light Source to perform transmission VLD FTIR measurements of the ROS of intact rod cells from the toad *Bufo americanus* suspended in deuterated Ringer solution (see the Supporting Information).

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