MASTER

BNL 28104

"Methods in Enzymology"

DISCLAIMER

This book was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

"Isolation and Purification of Retinals
from Purple Membranes for Mass
Spectral Analysis"¹

by Stanley Seltzer and Mow Lin

Recent studies concerned with the action of the purple membrane proton pump of Halobacteria have led to proposals concerning the mechanism of this process.² One mechanism suggests that upon photon absorption the excited retinal leads to a charge separated species which can induce proton hopping along the polyene chain to provide part of the route for the translocated proton. Other mechanisms suggest reversible proton or hydrogen atom transfer from the cyclohexenyl ring or from the 5-methyl of the cyclohexenyl ring of the bound retinal to a nearby acceptor amino acid residue of the protein. Similar proposals have been made for the rhodopsin system.³

Were reactions of this sort occurring one might expect some ultimate exchange of the hydrogen atoms of retinal with the migrating proton (i.e. those originally from the solvent) or exchange with the labile protons of the nearby acceptor amino acid side chains of the protein. Such processes can be tested by light-pumping the membrane in a deuterated environment, detaching and isolating the chromophore

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency Thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

from the pigment, purifying it by HPLC, and determining the deuterium content of the chromophore by mass spectrometry.⁴ Other experiments such as those designed to examine kinetic and equilibrium isotope effects might also be carried out in similar fashion.

Removal of the chromophore can be accomplished by one of two general methods: a) irradiation of a suspension of purple membrane with orange light in a neutral hydroxylamine solution,⁵ or b) extraction of the chromophore from the membrane with chloroform-cetyltrimethylammonium bromide (CTAB)⁶ or with methylene chloride.⁷ While the first method leads to intact apomembrane which can be used to regenerate purple membrane or an analogue of the native membrane, residual absorption at 360 nm, even after washing, suggests that some retinal oxime remains in the membrane. In principle it should be possible to recover retinal by this method but some thermal or photocatalyzed cis-trans isomerization of the free retinal or retinal oxime may occur unless precaution is taken to carry out reaction at low temperature⁷ and with long wavelength light. This method will not be discussed further since it is treated more fully in another section.

Extraction of retinals from the purple membrane by methylene chloride or chloroform is preferred since it can be accomplished in a shorter period of time in the absence of light so that there is little chance of post isomerization. Several extractions from a water suspension of the purple membrane have to be carried out to remove the

major amount of the retinal present.

Since methylene chloride at the same time extracts some lipids, lipids must be removed from the retinal before subjecting it to mass spectral analysis. Purification and separation of retinal isomers is ultimately achieved by HPLC on a microparticulate silica gel column with the retinals eluted by a solvent of less than one per cent ether in cyclohexane.⁸ Silica gel, however, binds lipids much more strongly than retinals and the solvent system used to separate retinal isomers does not effectively elute lipids from the column. Thus repeated application of lipid-retinal mixtures would gradually inactivate the HPLC column. This is avoided by simply performing a gross separation of lipids and retinals on a coarser silica gel and eluting retinals and lipids sequentially by a more polar solvent. Alternatively, a microparticulate silica gel-alkyl nitrile column has been used to separate retinal and lipid components which have been extracted from rhodopsin by methylene chloride. Simultaneous purification of the retinals and then elution of the lipids is achieved with two separate solvent systems.⁹ This method is discussed in another section and will not be discussed further here.

The retinal is introduced into the mass spectrometer as a solid. It is important to protect retinal samples against oxidation and therefore to carry out mass spectral analysis as soon after HPLC purification as possible. If samples have to be stored it should be as a solid under a nitrogen atmosphere in the freezer.

The parent ion of retinal is 284. A smaller peak at 285, about 20% the height of the 284 peak, is mainly due to the approximately one per cent natural abundance ^{13}C randomly distributed throughout the molecule. A 285/284 peak intensity ratio in excess of that measured for a control sample of retinal, is taken as a measure of the degree of replacement of a light isotope with one, one unit higher in mass, e.g. due to the incorporation of an atom of deuterium. A similar increase in the 286/284 peak height ratio over the natural abundance ratio found in a control sample of retinal (i.e. ~ 0.03) can be used to detect the incorporation of two atoms of deuterium.

The presence of retinoic acid presents a problem. Retinoic acid exhibits a parent ion at mass 300. A relatively intense peak is generated at mass 285 ($285/300 \sim 0.3$; $286/285 \sim 0.22$)¹⁰ presumably due to the loss of a methyl group. Thus contamination by retinoic acid will lead to an incorrectly determined degree of isotopic enrichment. Depending on the degree of precision desired, it may be reasonable to correct the 285 peak intensity for the presence of a small amount of retinoic acid (i.e., $\leq 5\%$) by measuring the 285/300 for retinoic acid under the same conditions and correcting the 285 peak as determined from the size of the peak at 300.

Methods

Material

Reagent grade methylene chloride, hexanes, and ether

Spectrograde cyclohexane (Eastman Kodak)

Potassium phosphate buffer, 0.01M, pH 7.4

Silica gel for column chromatography (60-200 mesh)

Silica gel HPLC column (60 x 0.2 cm, particle size, 10 μ);

Partisil 10 (Whatman) or equivalent is used.

Procedure

If retinal isomer integrity is to be maintained all operations are to be carried out under dim red light at 0°.

An aliquot of purple membrane containing the equivalent of about 50 μ g of retinal is generally sufficient. Smaller quantities might be used if a more sensitive mass spectrometer, than the one used here, is available. Membranes which are initially in a basal salt solution are transferred to a 0.01M phosphate buffer, pH 7.4, by centrifuging the basal salt suspension at 45,000 x g for 20 min and then resuspending the pelleted material in about 10 ml of 0.01M phosphate buffer, pH 7.4, as a homogeneous suspension. An equal volume of methylene chloride is added and an emulsion is formed by repeatedly sucking up and ejecting the mixture through a syringe needle (16-20 gage).⁷ The emulsion is then centrifuged at 35000 x g for 30 min at 0° and the layers separated. Methylene chloride extractions of the residual

aqueous phase and of the protein zone, located at the interface, are repeated four to five more times. The combined methylene chloride extracts should contain at this point about 80% of the total available retinal. The methylene chloride is removed with a rotary evaporator at ambient temperature.

Contaminating lipids are removed by column chromatography on silica gel. A column (23 x 0.9 cm) is slurry packed with silica gel (60-200 mesh) in hexane-ether (4:1, v/v). The effluent of the column is passed through a uv flow detector operating at 254 or 340 nm (Altex Model 153). The retinal sample is applied and eluted with the hexane-ether solvent mixture. The fraction between 16 and 32 ml generally contains all of the retinal. The solution is concentrated in a stream of nitrogen to a small volume (~20 μ l) for subsequent purification by HPLC.

HPLC is carried out on a column (60 x 0.2 cm) packed with microparticulate silica gel (10 μ). The eluate is passed through a uv flow detector operating at 254 or 340 nm. Ether (0.3 to 0.7%, v/v) in cyclohexane is used as the eluting solvent at a flow rate of 1-3 ml/min. The conditions of flow rate, and ether content of the solvent will vary with the column but are adjusted to give efficient separation with a standard sample of retinals. Typical elution volumes for retinal isomers using 0.5% ether are: 11 cis, 61.5 ml; 13-cis, 72.6 ml; 9-cis, 96.6 ml; all-trans, 112.5 ml. The desired isomers are collected separately and the solutions are concentrated

with a rotary evaporator and then transferred to small conical tubes. Further concentration to a smaller volume ($\sim 50\mu\text{l}$) is achieved with a stream of nitrogen. At this point an aliquot is transferred to the device used to introduce solid samples into the direct inlet of a mass spectrometer. If the sample holder has been previously cleaned in acid it is imperative that it be washed with considerable distilled water, ammonia, and then distilled water before drying. Traces of residual acid in contact with retinal at elevated temperature lead to substantial decomposition and a considerably altered 285/284 ratio. After transfer, the solvent is evaporated off in a stream of nitrogen. The process is repeated until the whole solution is transferred to the sample holder and all of the solvent is removed. The sample is introduced into the direct inlet system and the sample is analyzed. Typical conditions for the Hitachi-Perkin Elmer RMU-7 mass spectrometer are: sample source chamber temperature, 60° ; ionization chamber, 250° ; photomultiplier voltage, 1.25 Kv; ionizing voltage, 80 eV. The source pressure is generally $1 \times 10^{-6}\text{mm}$ during measurement.

Prior to mass spectral analysis of the unknown, pure control samples of all-trans-retinal and/or 13-cis-retinal are analyzed to verify that the system is operational and to measure the 285/284 ratio in the absence of isotopic enrichment.

¹Research carried out at Brookhaven National Laboratory under contract with the U. S. Department of Energy and supported by its Office of Basic Energy Sciences.

²For recent reviews see a) S. R. Caplan and M. Ginzburg, ed.

"Energetics and Structure of Halophilic Microorganisms," Elsevier/

North-Holland Biomedical Press, Amsterdam, 1978; b) W. Stoeckenius,

R. H. Lozier, and R. A. Bogomolni, Biochim. Biophys. Acta. 505, 215

(1978); c) M. Eisenbach and S. R. Caplan in "Current Topics in

Membranes and Transport," F. Bramer, A. Kleinzeller, eds, Vol. 12,

pp. 165-248 (1979).

³K. van der Meer, J. J. C. Mulder, and J. Lugtenburg, Photochem.

Photobiol., 24, 363 (1976).

⁴M. Lin and S. Seltzer, FEBS Lett. 106, 135 (1979).

⁵D. Oesterhelt and L. Schulmann, FEBS Lett. 44, 262 (1974).

⁶L. Y. Jan, Vision Res. 15, 1081 (1975).

⁷M. J. Pettei, A. P. Yudd, K. Nakanishi, R. Henselman, and W.

Stoeckenius, Biochemistry, 16, 1955 (1977).

⁸R. Sack and S. Seltzer, Vision Res., 18, 423 (1978).

⁹K. Nakanishi, Pure and Appl. Chem., 49, 333 (1977).

¹⁰R. L. Lin, G. R. Waller, E. D. Mitchell, K. S. Yang, and E. C.

Nelson, Anal. Biochem., 35, 435 (1970).