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Wavelength modulation by molecular environment in visual pigments

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The absorption and regenerability characteristics are compared for rhodopsin contained in rod outer segment membranes and purified in a series of alkyl sucrose esters. It is found that membrane-bound rhodopsin has maximum absorbance from 504 to 500 nm between 1.5 and 40°C. After purification, rhodopsin absorbance can be blue-shifted by up to 6 nm, depending on the detergent species used. Only the longest chain sucrose esters give purified rhodopsin with maximum absorbance comparable to that of the native pigment. In the same manner, detergent-purified rhodopsin will be easily regenerated as long as its native spectral characteristics are maintained. Sucrose esters thus prove to be mild enough to maintain rhodopsin functionality with respect to these two properties and could probably be used successfully to maintain other membrane proteins' integrity.

Introduction

The characterisation of purified membrane proteins always raises the question of the state of 'nativeness' in which the protein is isolated. Membrane proteins do interact more or less with their lipid environment. Destruction of these interactions upon membrane solubilisation and further protein purification in detergents may lead to a final product in a state which is very different from the native membraneous state. For this reason, purification of membrane proteins must be done under the mildest possible conditions. Despite the large variety of detergents tested for membrane protein purification, it appears that the ideal medium has not yet been found.

The visual pigment rhodopsin, an integral membrane protein, is a good system to assess the effects of solubilising agents, since its retinal chromophore enables several aspects of the protein to be easily analysed, under namely, its absorbance, thermal stability, regenerability and bleaching sequence.

Among the new detergents that are available for purification of membrane proteins, alkyl esters of sucrose have been found to be among the mildest and to be able to stabilise membrane proteins which are often unstable in detergents (e.g., rhodopsin from invertebrates [1] or bacteriorhodopsin [2]). Recently, we have shown that bovine rhodopsin purified in a series of sucrose esters retained an intact bleaching sequence and

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an equilibrium between its meta-intermediates I and II which is lacking when the protein is purified in the most commonly used detergents [3]. In addition, the thermal stability of rhodopsin in sucrose ester was found to be very good [4]. In the present paper, we compare the absorption and regeneration characteristics of the rhodopsin contained in its native rod outer segment membranes and purified in sucrose esters and digitonin. We find that only the long-chain esters are mild enough to maintain the native state of the protein with respect to these properties.

Materials and Methods

Unless otherwise stated, all procedures were carried out under dim red light. Rod outer segments were prepared from bovine eyes in the same manner as that recently described [3]. Since the membranes were to be analysed by spectrophotometry, they were clarified by sonication at the end of the preparation procedure. Sonication was achieved by means of a Kubota sonic oscillator or an Ultrasonics (225R) cell disruptor operated at low power for 60 min at 4°C. After sonication, rod outer segments were centrifuged for 1 h at $60\,000 \times g$ and the resulting supernatant was used as the intact rod outer segment membranes.

In some experiments, bleached membranes were needed. For that purpose, we took advantage of the 'in situ' bleaching. Hemisected eyes were bleached under daylight for 1–2 h prior to retina removal. Under these conditions, the visual pigment bleached, the hydrolysed retinal was transported to pigment epithelium and was stored there [5]. Rod outer segments prepared from these retinae contained no retinal (as judged from their absorption spectrum). However, this procedure sometimes failed to give completely bleached membranes. Thus, only those preparations showing no absorption band at 500 nm were retained for experimentation.

Rhodopsin was purified by solubilisation of rod outer segment membranes in 5% (w/v) lauryl sucrose for 1 h at room temperature (final conc.: ca. 5 mg rhodopsin/ml). The solution (10 ml) was clarified by centrifugation at $10\,000 \times g$ for 5 min and applied on a Concanavalin A-Sepharose column (100 × 12 mm) pre-equilibrated with 100 mM Tris-HCl buffer (pH 7.3) containing 1 M NaCl/1 mM $CaCl_2$/1 mM $MnCl_2$. After washing with 100 ml of 50 mM Tris-HCl buffer containing 0.2% (w/v) of the desired detergent, rhodopsin was eluted from the column with the detergent buffer solution in the presence of 0.3 M α-methyl-D-mannoside. The phospholipid content of rhodopsin samples prepared in that way was 1–2 mol phosphate/mol rhodopsin as analysed by the method of Bartlett [6]. Detergent-free rhodopsin was prepared by extensive dialysis of the pigment purified in lauryl sucrose. Typically, 5 ml of a 1 mg/ml sample were diluted ten times with buffer and dialysed for at least 24 h against 2 l of buffer at 4°C. Rhodopsin was then sedimented by centrifugation for 1 h at $60\,000 \times g$. The rhodopsin pellet was resuspended in buffer, sonicated at low power for 10 min and still sedimented at $20\,000 \times g$ for 20 min. The sonication/sedimentation cycle was repeated twice and the rhodopsin was finally pelleted at $15\,000 \times g$ on a quartz slide fitted at the bottom of a centrifuge tube in a swinging bucket rotor.

Regeneration of rhodopsin was measured as follows. A given sample of rhodopsin (in rod outer segments or detergent) was diluted to ca. $1 \cdot 10^{-5}$ M. Its pH was adjusted to a given value between 4 and 10 by use of 50 mM acetate, phosphate, Tris-HCl or carbonate buffer. The spectrum was measured and a 1 ml aliquot, was bleached for 2 min with light from a tungsten lamp filtered through a 550 nm cutoff filter (65-1315, Rolyn, Covina, CA) at an intensity of 530 W/m^2. This was sufficient to completely bleach the samples which were then added with 4 μl of $5 \cdot 10^{-3}$ M 11-*cis*-retinal (a generous gift from Hoffman-La Roche) in ethanol and stored at 37°C in the dark for 12 h. The amount of regeneration was obtained by measuring the 500 nm rhodopsin absorbance which had recovered during that time, relative to the initial rhodopsin content of the sample.

Absorption spectra were measured with a Union SM-401 or Pye Unicam SP8-100 spectrophotometers. The former being equipped with a micro-computer, derivative and difference spectra could be rapidly and accurately obtained. The wavelength scale of both apparatus was calibrated with a Holmium filter and their reproducibility

was better than 0.2 nm. Spectra were recorded with a spectral bandwidth of 0.1 or 0.2 nm. Since maximum absorbance of rhodopsin depends on temperature, thermostated cells and cell holders were used. Their temperature was controlled by water at a constant temperature being circulated from a thermostatic pump and monitored by means of calibrated Cu-Constantan thermocouples located under the surface of the samples. Prior to measurement, samples were equilibrated for 60 min at the desired temperature. Spectra remained stable for at least twice that period of time, even at 40°C, except for those rhodopsin samples dispersed in caprylic (C_8) and capric (C_{10}) sucrose esters which were measured after only 10 min incubation at 40°C.

The sucrose esters used throughout this work were synthesized by Mitsubishi Chemcial Co. (Yokohama, Japan). They all contained less than 0.1% free fatty acids. Lauryl sucrose contained 80% monoesters while caprylyl, capryl, myristyl, palmityl and stearyl sucrose contained 95% monoesters. Digitonin was from Wako Pure Chemicals (Osaka, Japan) or Sigma Chemical Co. (St Louis, MO, U.S.A.); α-methyl-D-mannoside was from Wako Pure Chemicals and Concanavalin A-Sepharose 4B was from Pharmacia (Upsala, Sweden).

Results

An absorption spectrum of bovine rhodopsin is shown in Fig. 1. In that particular case, rhodopsin was dispersed in stearyl sucrose. Measurement of spectra between 1.5 and 40°C clearly indicates a sensitive temperature dependence of the spectrum within that 38.5 Cdeg temperature range. Lowering temperature induces some hyperchromicity and shifts maximum absorbance towards longer wavelengths. From the first derivative of absorbance spectra, maximum absorbance can be located precisely and it is found at 500, 502 and 504 nm at 40, 20 and 1.5°C, respectively, for rhodopsin in stearyl sucrose.

In the sucrose ester series, maximum absorbance of rhodopsin decreases with the length of acyl chain (see Table I). Among the detergents we have used, the lowest maximal absorbance was found for rhodopsin purified in digitonin. In that

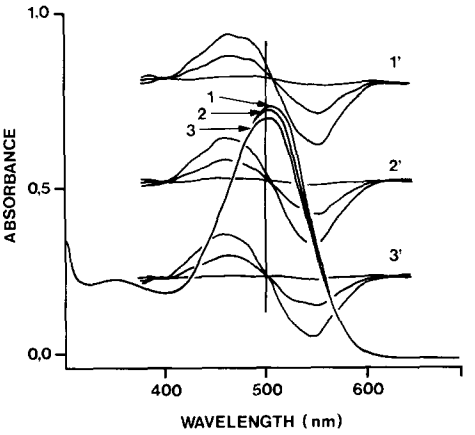

Fig. 1. Absorption spectrum of bovine rhodopsin dispersed in 50 mM Tris-HCl buffer (pH 7.3) containing 0.2% (w/v) stearyl sucrose. Curves 1, 2 and 3 correspond to measurements done at 1.5, 20 and 40°C, respectively. Corresponding derivatives of absorbance spectra (curves 1′, 2′ and 3′) were obtained with Δλ of 1.5, 7 and 15 nm. Maximum absorbance is at 504, 502 and 500 nm at 1.5, 20 and 40°C, respectively. Vertical bar is the 500 nm line.

detergent, rhodopsin has λ_{max} at 498 nm at 20°C. However, it is not necessary to operate a complete purification procedure to observe such an absorbance shift. As a matter of fact, maximum absorbance can be modified by changing the detergent composition after rhodopsin purification. For example, stearyl sucrose-solubilised rhodopsin had its λ_{max} immediately shifted to 499, 498 and 497 nm at 1.5, 20 and 40°C, respectively, when added with digitonin (final conc. 0.5% w/v).

The fact that maximum absorbance depends on

TABLE I

ABSORPTION MAXIMUM OF BOVINE RHODOPSIN DISPERSED IN 0.2% (w/v) FATTY ACID SUCROSE ESTERS AT DIFFERENT TEMPERATURES

Values are given in nm.

Acyl chain length	Temperature		
	2°C	22.5°C	40°C
C_8	503	499	498
C_{10}	503	501	500
C_{12}	503	502	500
C_{14}	503	502	500
C_{16}	504	502	500
C_{18}	504	502	500
Digitonin (0.5%)	499	498	497

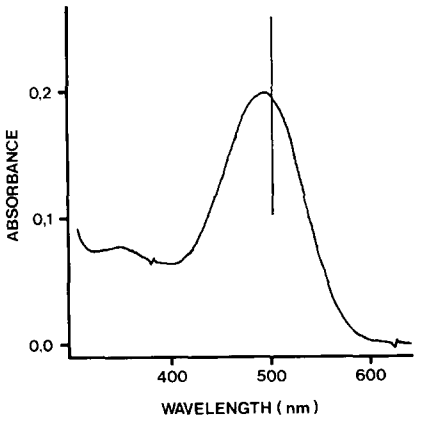

Fig. 2. Absorption spectrum of detergent-free rhodopsin. The sample was deposited on a quartz slide by centrifugation and measured at 22°C.

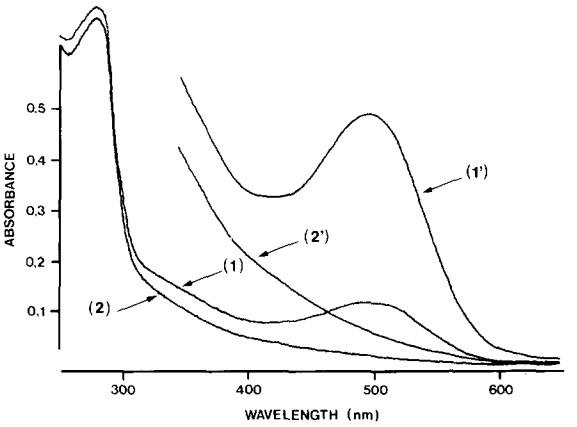

Fig. 3. Absorption spectra of rod outer segment membranes prepared from intact (1) and bleached (2) retinae. Curves 1′ and 2′ are the spectra of the same samples recorded at a 4-times higher sensitivity. Samples were adjusted to the same absolute absorbance at 700 nm and measured against water.

the chemical nature of the detergent used for its dispersion suggests that it might reflect the degree of solvation of the protein by the detergent. In order to get additional information about this problem, detergent-free rhodopsin was prepared. Unfortunately, such preparations showed strong light scattering when dispersed in water and their absorption spectra could not be measured with the desired accuracy. However, when small amounts of detergent-free rhodopsin were deposited on a quartz slide by centrifugation, their spectra could be measured precisely. Fig. 2 shows the spectrum of small aggregates of detergent-free rhodopsin. At room temperature (22°C), they have λ_{max} at 496 nm. Due to the particular physical state and dimensions of these samples, the temperature dependence of their spectra can hardly be measured but it is clear from Fig. 2 that in the absence of solvating amphiphilic molecules, rhodopsin has maximum absorbance at least 2 nm shorter than in detergents.

In order to compare absorption data of purified rhodopsin with those of rhodopsin contained in its native membranes, we have tried to locate precisely the maximum absorbance of rhodopsin in rod outer segments. For this purpose, samples were prepared from intact and bleached retinae. The spectra of both preparations are shown in Fig. 3. The spectrum measured for rod outer segments from bleached retinae shows that they are free from retinal and thus suitable for difference spectroscopy. The spectrum of intact rod outer segments measured against those obtained from bleached retinae is shown in Fig. 4. Spectra were measured at 40, 30, 20, 10 and 1.5°C together with their derivative which show maximum absorbance at 504, 503, 502, 501 and 500 nm, respectively.

Since the absorption properties of rhodopsin depend not only on molecular environment but also strongly on temperature within a rather small temperature range, we finally determined whether this dependence is a property of rhodopsin or of its chromophore. Fig. 5 shows that λ_{max} of 11-*cis*-retinal in ethanolic solution shifts from 374 to 368 nm when temperature is raised from 0 to 40°C. The same result is observed when retinal is dispersed in detergent (e.g., lauryl sucrose). Thus, the temperature dependence of rhodopsin absorbance near physiological temperature reflects the intrinsic properties of the retinal chromophore.

The regenerability of rhodopsin is, to some extent, indicative of its integrity [7]. It was thus interesting to see if the purified rhodopsin samples that had absorption characteristics closer to membrane-bound rhodopsin also had better ability to reform rhodopsin upon addition of 11-*cis*-retinal after bleaching. In a last experiment, rhodopsin purified in different detergents was bleached and the extent of its regeneration in the presence of

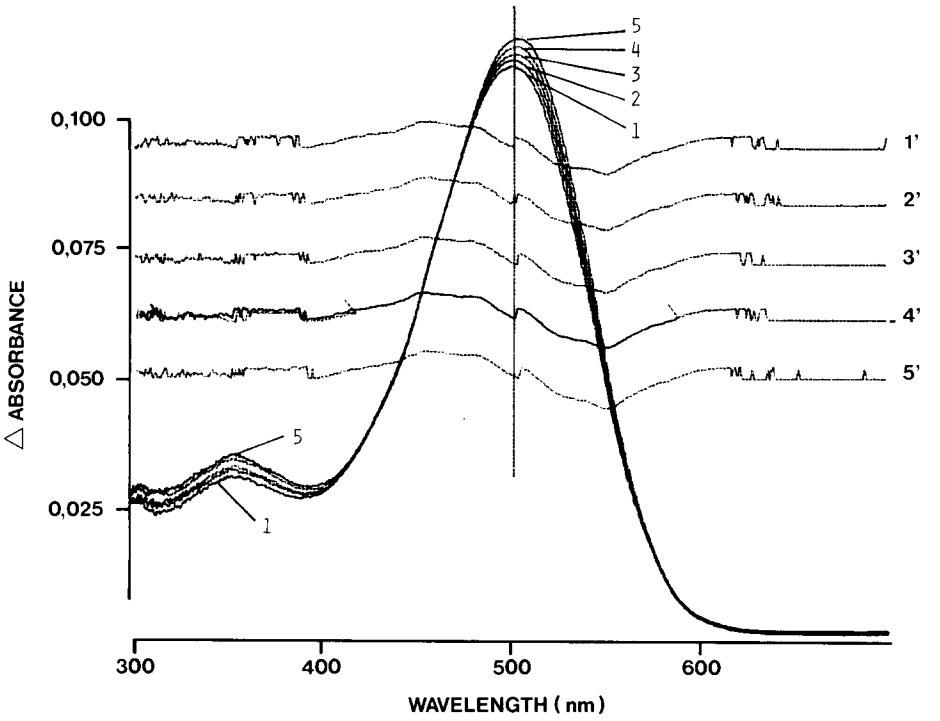

Fig. 4. Difference spectrum between rod outer segments obtained from intact and bleached retinae. Curves 1–5 correspond to measurements done at 40, 30, 20, 10 and 1.5°C and their maxima locate at 500, 501, 502, 503 and 504 nm, respectively.

excess 11-*cis*-retinal was measured. Results of this experiment are depicted in Fig. 6. As expected, only membrane-bound rhodopsin was found to fully regenerate within a large pH range (90–95% regeneration between pH 4 and 10). Purified rhodopsin regenerated maximally at pH = p*I*, i.e.,

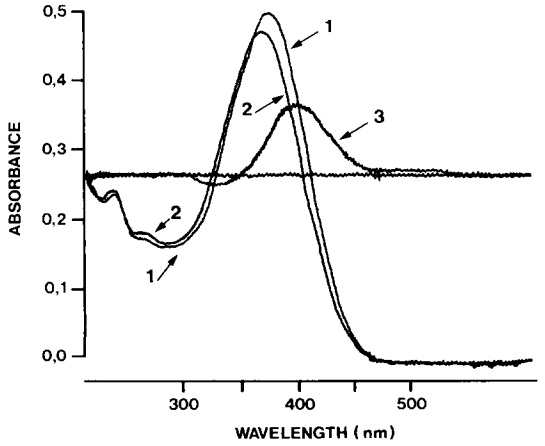

Fig. 5. Absorption spectrum of 11-*cis*-retinal in ethanol, measured at 0 (curve 1) and 40°C (curve 2). Curve 3 is 2.5-times the difference between curves 1 and 2.

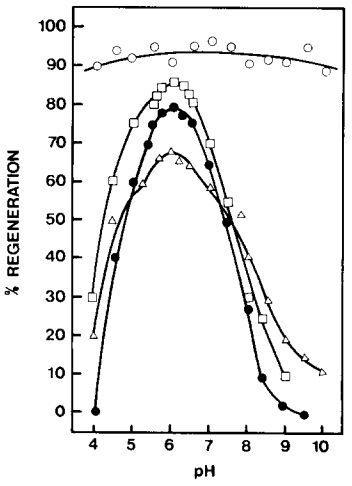

Fig. 6. Effect of pH on the extent of regeneration of rhodopsin. The different curves stand for rhodopsin dispersed in rod outer segment membranes (○), 0.2% palmityl sucrose (□), 0.2% lauryl sucrose (●) and 0.2% digitonin (△).

5.5 [8] and at an extent that depended on the detergent species. The largest regenerability was observed in the longest-chain detergent (90% in palmityl sucrose). Regeneration decreased gradually when chain length decreased (80% in lauryl sucrose) and dropped considerably (ca. 20%) in sucrose esters having a shorter chain length (results not shown). Under the same experimental conditions, rhodopsin dispersed in digitonin showed 70% regeneration.

Discussion

The precise position of the absorption maximum of intact visual pigments in their cells has been a problem for a long time. 30 years ago, Denton and Walker [9] reported that the visual pigment of the conger eel had maximum absorbance shifted by 4 nm towards the blue end of the spectrum when it was extracted with digitonin. A few years later, Dartnall [10] argued that no detectable change could be observed in the absorption properties of visual pigments of frog, eel and carp when extracted with digitonin. From the results presented here, it is clear that λ_{max} of visual pigment may be displaced when its natural surrounding lipids are displaced by other amphiphilic molecules or when its temperature is changed.

The thermal shift of the absorption maximum is an intrinsic property of the retinal chromophore of the visual pigment. Within the temperature range studied here, it is found that free retinal has a larger thermal shift than rhodopsin. However, even if small, the thermal shift of rhodopsin is significant and justifies that the temperature of the measurement should henceforth be given together with maximum absorbance of visual pigments.

On the other hand, the amplitude of the environmental spectral shift depends on the nature of the detergent used for rhodopsin solubilisation. In the sucrose ester series, longer-chain esters give the most red shifted absorbance. In the rod outer segment membranes, we found by comparison of intact and retinal chromophore-free membranes that rhodopsin has maximum absorbance at 502 nm at room temperature. This λ_{max} cannot be distinguished from that of rhodopsin purified in long-chain sucrose esters. At the same temperature, the C_{10} and C_8 esters give pigment with λ_{max} at slightly shorter wavelengths (501 and 500 nm, respectively) while detergent-free rhodopsin has λ_{max} at 496 nm.

This fine tuning of the pigment maximum absorbance surely reflects small changes in the retinal protein interactions that are induced by detergent. In the first approximation, it appears that the intactness of absorption properties depends on the size of the hydrophobic core of the micelles formed by the different detergents and on their ability to operate appropriate solvation of the hydrophobic core of the protein (particularly since detergent-free rhodopsin absorbs at 496 nm at 22°C). However, this argument does not hold if we compare these with detergents other than the sucrose ester series. As a matter of fact, numerous detergents have been used to purify rhodopsin [4,11]. Among them, those in which rhodopsin λ_{max} has been accurately determined all gave pigments absorbing below 500 nm (usually 498 nm). It is also our experience that rhodopsin purified in Triton X-100 or Ammonyx-LO absorbs at 498 nm at 20°C. Thus, there must exist parameters other than micellar size (may be micellar rigidity) that contribute to the maintenance of native absorption properties of rhodopsin.

Micellar rigidity and ordering have been proposed as conditions for regenerability of rhodopsin in detergents [12,13]. In addition to rod outer segments, rhodopsin can be regenerated in digitonin [14], Tween 80 [15], sodium cholate [16] and alkyl glucosides [17]. The results presented here show that rhodopsin can also be regenerated in sucrose esters. The extent of regeneration increases with increasing chain length. Previously, we have observed by means of ESR probes that ordering also increased with chain length in the sucrose ester series [18]. We thus conclude that longest-chain esters provide sufficient rigidity for regeneration. However, it is important to note that the extent of regeneration parallels the red shift of maximum absorbance by rhodopsin. Accordingly, one must be careful when saying that most detergents used for rhodopsin solubilisation leave their absorption characteristics intact, since our results show that only those detergents which do not shift absorption maximum by more than 1 nm allow

the pigment to be fully regenerable.

In conclusion, few detergents can really solubilise rhodopsin without modifying its spectral properties. Accurate measurement of absorption properties of rhodopsin in rod outer segment membranes and sucrose esters reveal that the longest-chain esters give absorption characteristics closer to the native membraneous rhodopsin and allow, at the same time, the best regeneration facility. It is shown thus that they induced little change (if any) in protein functionality. They could become of great importance in the study of membrane proteins in general.

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