

# Lipid-Protein Interactions Mediate the Photochemical Function of Rhodopsin<sup>†</sup>

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**ABSTRACT:** We have investigated the molecular features of recombinant membranes that are necessary for the photochemical function of rhodopsin. The magnitude of the metarhodopsin I to metarhodopsin II phototransient following a  $25\% \pm 3\%$  bleaching flash was used as a criterion of photochemical activity at 28 °C and pH 7.0. Nativelike activity of rhodopsin can be reconstituted with an extract of total lipids from rod outer segment membranes, demonstrating that the protein is minimally perturbed by the reconstitution protocol. Rhodopsin photochemical activity is enhanced by phosphatidylethanolamine head groups and docosahexaenoyl (22:6 $\omega$ 3) acyl chains. An equimolar mixture of phosphatidylethanolamine and phosphatidylcholine containing 50 mol % docosahexaenoyl chains results in optimal photochemical function. These results suggest the importance of both the head-group and acyl chain composition of the rod outer segment lipids in the visual process. The extracted rod lipids and those lipid mixtures favoring the conformational change from metarhodopsin I to II can undergo lamellar ( $L_\alpha$ ) to inverted hexagonal ( $H_{II}$ ) phase transitions near physiological temperature. Interaction of rhodopsin with membrane lipids close to a  $L_\alpha$  to  $H_{II}$  (or cubic) phase boundary may thus lead to properties which influence the energetics of conformational states of the protein linked to visual function.

The process of vision is initiated by *cis* to *trans* photoisomerization of the retinal chromophore of rhodopsin, an integral protein located in the disk membranes of the rod outer segment (ROS)<sup>1</sup> (Wald, 1968). The events that provide a current paradigm for generation of a nerve impulse by the rod can be summarized as follows (Kühn, 1984; Chabre, 1985). Hyperpolarization of the rod plasma membrane in response to absorption of a photon by rhodopsin is mediated by an intracellular second messenger (Miller & Nicol, 1979; Yoshikami et al., 1980). The photoactivated rhodopsin ( $R^*$ ) molecules interact with a signal-transducing G-protein, leading to amplified exchange of GTP for GDP, phosphodiesterase activation, and hydrolysis of cyclic GMP (Fung & Stryer, 1980; Kühn et al., 1981; Lewis et al., 1984; Kühn, 1984; Chabre, 1985; Kohl & Hofmann, 1987). Closure of cyclic GMP dependent plasma membrane channels (Matesic & Liebman, 1987) then results in hyperpolarization. Photoactivation of rhodopsin is generally considered to involve the metarhodopsin II (MII) photointermediate, which is in equilibrium with its precursor metarhodopsin I (MI) (Kühn, 1984; Chabre, 1985; Kohl & Hofmann, 1987). The MI-MII transition comprises a shift in the absorption maximum of rhodopsin from 478 to 380 nm, occurs on the millisecond time scale of visual phototransduction, and is known to involve a

protein conformational change (Applebury et al., 1974; Lamola et al., 1974). Previous studies have suggested that the extent and rate of the MI-MII transition are markedly dependent on the lipid environment of rhodopsin (Applebury et al., 1974; Litman et al., 1981; Beach et al., 1984; Baldwin & Hubbell, 1985) and that unsaturated acyl chains may be important (O'Brien et al., 1977). However, neither the chemical constituents of the rod disk membranes nor their associated properties required for photochemical function have been identified. We have found that the amount of MII produced by photolysis of rhodopsin is dependent on the membrane lipid composition and that both polyunsaturated (22:6 $\omega$ 3) acyl chains and phosphatidylethanolamine head groups may be involved. The results suggest that the retinal disk membrane phospholipids are implicated in control of visual transduction at the molecular level.

## EXPERIMENTAL PROCEDURES

All manipulations were carried out in dim red light (15-W bulb; Kodak Safelight filter 1), and all buffers had been dispersed with argon to minimize oxidation of unsaturated lipids. Native ROS membranes were prepared from frozen bovine retinas (Papermaster & Dreyer, 1974) and were osmotically shocked by suspension in distilled water, followed by centrifugation to remove peripheral proteins [cf. Kühn et al. (1981)]. Rhodopsin was extracted with dodecyltrimethylammonium bromide (DTAB), purified by hydroxylapatite chromatography, and recombined with phospholipids as described (Hong & Hubbell, 1973). All phospholipids were synthesized or purified by using standard techniques (Singleton

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<sup>1</sup> Abbreviations: BHT, butylated hydroxytoluene; DTAB, dodecyltrimethylammonium bromide; EPR, electron paramagnetic resonance; MI, metarhodopsin I; MII, metarhodopsin II; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PMT, photomultiplier; PS, phosphatidylserine; ROS, rod outer segment; symmetric (like-chain) phospholipids are referred to as di(*X:Y*)Z, where *X* is the number of carbon atoms in the chains, *Y* is the number of double bonds, and *Z* denotes the head group (PC or PE).

Table I: Characterization of Rhodopsin-Containing Membranes

	native ROS membranes	ROS phospholipids	di(22:6)PE/ egg PC (1/1)	di(22:6)PC	di(22:6)PC/ egg PC (1/1)	egg PE/egg PC (1/1)	egg PC
$A_{280}/A_{498}^a$	2.6	1.7	2.4	2.8	2.2	1.9	2.0
% bleached	7	0	0	0	10	0	0
% regenerable <sup>b</sup>	100	89	100	98	74	82	84
$\Delta A_{380} \times 10^2 \text{ nmol}^{-1} \text{ c}^{-1}$	3.4	3.4	3.3	3.1	2.5	2.2	1.6
$k \times 10^{-2} \text{ (s}^{-1}\text{)}^d$	2.7	4.2	3.0	4.8	3.0	2.5	3.0
L/P ratio <sup>e</sup>	70	118	92	105	105	88	112
% PE/	42	41	48			44	
% PC	39	40	52	100	100	56	100
% 16:0 <sup>f</sup>	14	19	18		18	26	32
% 18:0	18	24	7		6	18	12
% 18:1	6	7	14		15	24	30
% 18:2	1		5		8	18	15
% 20:4	7	4	1		1	12	4
% 22:4	2						
% 22:5	6				1	2	1
% 22:6	41	40	52	97	50	1	1

<sup>a</sup> Ratio of absorbance at 280 nm to that at 498 nm of unregenerated samples measured in 3% Ammonyx LO, containing 0.1 M hydroxylamine and 20 mM phosphate buffer, pH 6.5. The  $A_{280}/A_{498}$  ratios of the chromatographically purified rhodopsin samples in DTAB at 0 °C were typically 1.8–1.9 before recombination. The higher values for the recombinants with polyunsaturated phospholipids may be due to impurities from minor lipid oxidation. <sup>b</sup> Percentage of bleached opsin regenerated upon addition of 11-*cis* retinal to form rhodopsin (Wald, 1968; Hong & Hubbell, 1973). <sup>c</sup> Change in absorbance at 380 nm per nanomole of rhodopsin bleached following a yellow actinic flash (Sunpak AP-52 with Schott OG 515 filter;  $\lambda > 500$  nm); determined with a scanning spectrophotometer at 28 °C. The values of  $\Delta A_{380} \text{ nmol}^{-1}$  represent an additional measure of the [MII]/[MI] ratio formed upon photolysis of rhodopsin. The photoreversibility of the changes was investigated by exposure of the samples to a second actinic flash of blue-violet light (Schott BG 3 filter;  $\lambda < 500$  nm). For those recombinants exhibiting nativelike behavior, the absorption at 380 nm was largely attributable to MII rather than free retinal (Baldwin & Hubbell, 1985). <sup>d</sup> Estimates of the apparent rate constant for absorbance changes at 480 nm on the millisecond time scale assuming first-order kinetics. The possibility of isochromic or multiple forms of rhodopsin and its photointermediates is a complication which could lead to nonexponential kinetics. Rapid kinetic studies of native bovine ROS disk membranes have shown that the rates of the lumi-MI and MI-MII transitions are within a factor of 3 under conditions similar to those employed here; the decay of MI is nonexponential but can be approximated by a single fast component (Lewis et al., 1981). <sup>e</sup> Mole ratio of phospholipid to rhodopsin determined from phosphate analysis and absorbance at 498 nm assuming an extinction coefficient of  $40\,000 \text{ cm}^{-1} \text{ M}^{-1}$ ; data refer to single sharp bands obtained by isopycnic sucrose density gradient centrifugation at 28 °C. <sup>f</sup> Mole percentage of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) of samples determined by quantitative thin-layer chromatography. The remainder of the ROS membrane phospholipids comprises phosphatidylinositol (PI), phosphatidylserine (PS), and sphingomyelin (Miljanich et al., 1979). <sup>g</sup> Mole percentage of principal fatty acyl chains of samples determined by gas-liquid chromatography of corresponding methyl esters.

et al., 1965; Yang et al., 1967; Mason et al., 1981) and gave single spots with thin-layer chromatography; butylated hydroxytoluene (BHT) was included as an antioxidant (Brown et al., 1982). The results of characterization of the samples are provided in Table I. The various recombinants had  $A_{280}/A_{498}$  absorbance ratios suggesting the absence of denaturation or bleaching of rhodopsin and were largely regenerable (74–100%). All samples were homogeneous with respect to their lipid/rhodopsin ratio (88–118), as shown by isopycnic sucrose density centrifugation (Table I), and were in the lamellar, liquid-crystalline ( $L_\alpha$ ) phase over the temperature range 15–35 °C by <sup>31</sup>P NMR spectroscopy (not shown). Aqueous suspensions of membranes containing 5  $\mu\text{M}$  rhodopsin in 10 mM phosphate buffer, pH 7.0, were clarified by sonication in a container on ice and under argon, using a microtip for 3 min with a 50% duty cycle. Use of different sonication intervals did not significantly alter the experimental results. The membrane suspensions were then diluted 2-fold with the same buffer and transferred to the 5-cm path-length cell of a home-built kinetic spectrophotometer. The flash photolysis instrument was comprised of a monitoring beam,  $480 \pm 5$  nm band-pass interference filter, iris diaphragm, cylindrical chamber with two xenon arc lamps (5  $\mu\text{s}$ , 6 J), monochromator, photomultiplier, and digital oscilloscope interfaced to an IBM CS-9000 computer. A single flash was delivered to the samples which bleached  $25\% \pm 3\%$  of the rhodopsin initially present, as judged from the loss of absorbance at 498 nm in the presence of 0.1 M hydroxylamine [cf. Raubach et al. (1974)].

## RESULTS

The present studies have focused primarily on changes in the transmittance at 480 nm following flash excitation of

bovine rhodopsin in native and recombinant membranes as a criterion of its photochemical activity. It is assumed (i) that only those intermediates formed upon photolysis of rhodopsin in native ROS disk membranes (Lewis et al., 1981) and detergent solutions (Wald, 1968) are present and (ii) that the photochemical changes occurring on the millisecond time scale arise from the lumi-MI and/or the MI-MII transitions (Lewis et al., 1981). A spectroscopic definition of the photointermediates is adopted in which all photolyzed rhodopsin molecules are assumed to reach the MI stage of bleaching. Representative flash photolysis transients are shown in Figure 1 for native ROS membranes and recombinants of rhodopsin with various unsaturated phospholipids at 28 °C and pH 7.0. The increase in photomultiplier (PMT) voltage following the flash (Figure 1) is related to the loss of absorption at 480 nm and indicates the MI to MII transition of rhodopsin (Lewis et al., 1981). Parallel transient increases in absorption at 380 nm were observed (not shown). Flash photolysis studies were also conducted at 15 and 35 °C, and the spectral changes were further investigated with a scanning spectrophotometer (cf. Table I), yielding results consistent with those depicted here. (The possibility of isochromic forms or multiple states of rhodopsin and its various photointermediates does not affect the analysis.)

As can be seen in Figure 1, substantial differences are evident in the magnitudes of the phototransients, whereas the transition rates are similar to that of native ROS membranes (cf. Table I) (Applebury et al., 1974; O'Brien et al., 1977). In general, multiple photon events are a potential cause of differences in photolytic behavior at relatively high stimulus light intensities, such as employed here. For example, bathorhodopsin, lumirhodopsin, or MI formed within the flash by absorption of a single photon can absorb a second photon

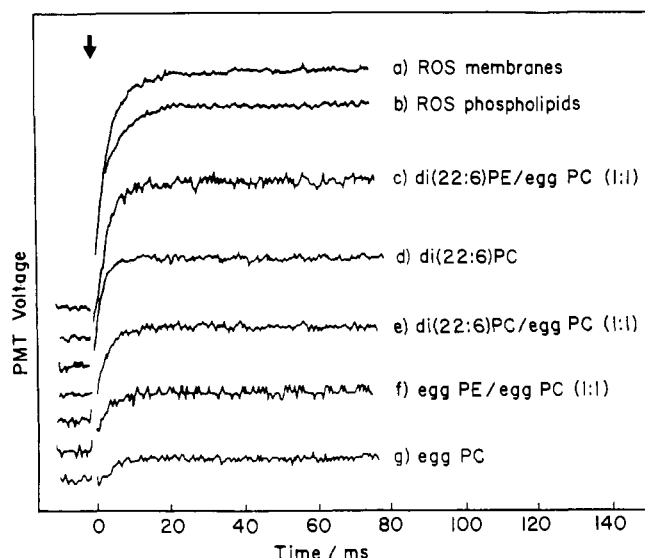


FIGURE 1: Influences of phospholipid environment on photochemistry of rhodopsin in native and recombinant membranes. Flash photolysis transients were measured at a wavelength of 480 nm and employed membrane vesicles containing rhodopsin at 28 °C in 10 mM phosphate buffer, pH 7.0. (a) Native rod outer segment (ROS) disk membranes; (b) ROS phospholipid recombinant; (c) di(22:6)PE/egg PC (1/1) recombinant; (d) di(22:6)PC recombinant; (e) di(22:6)PC/egg PC (1/1) recombinant; (f) egg PE/egg PC (1/1) recombinant; and (g) egg PC recombinant. The increase in photomultiplier (PMT) voltage at 480 nm following the actinic flash (arrow) reflects loss of the lumi and MI intermediates formed upon photolysis, leading to the appearance of MII (see text). The similar flash photolysis transients observed for the native ROS membranes (a) and the total ROS phospholipid control (b) suggest that rhodopsin is largely unperturbed by the recombination procedure. Absorbance measurements conducted with a scanning spectrophotometer before and after the flash indicated that  $25\% \pm 3\%$  of the rhodopsin had been photolyzed in each case.

to yield rhodopsin or isorhodopsin (Wald, 1968). Since all of the bathorhodopsin or lumirhodopsin present after the flash will go on to form MI, the apparent photolysis yield of MII from MI could thus be influenced by secondary photolysis events. However, each of the recombinant membrane samples was bleached to approximately the same extent ( $25\% \pm 3\%$ ), as judged by the loss of absorbance at 498 nm in the presence of hydroxylamine (cf. Experimental Procedures). It is known that the retinal chromophore of rhodopsin becomes susceptible to nucleophilic attack by  $\text{NH}_2\text{OH}$  upon photolysis, such that the various intermediates are trapped in the form of retinal-oxime plus the apoprotein opsin (Van Breugal et al., 1978). Thus, the amount of unphotolyzed rhodopsin plus isorhodopsin remaining after the flash is most likely the same among the series of recombinant membranes. It follows that the observed trends in the phototransient magnitudes (Figure 1) are not a consequence of differences in the yields of secondary photo-reactions. Rather, it is likely that approximately equal amounts of MI are present initially in each case. The absorbance change at 480 nm can be used in conjunction with the amount of rhodopsin bleached to estimate the apparent ratio at pH 7,  $K' = [\text{MII}]/[\text{MI}]$ . The value of  $K'$ , estimated by neglecting spectral overlap and any small absorption differences of the photointermediates, is approximately 4.5 for the native ROS membranes in the absence of peripheral proteins at 28 °C, consistent with earlier results [cf. Figure 5 of Parkes and Liebman (1984)].

We first investigated whether the photochemical activity of rhodopsin is influenced by transbilayer asymmetry of the rod disk membranes (Fung & Hubbell, 1978; Miljanich et al., 1981, 1985). In the native ROS, both rhodopsin (Fung &

Hubbell, 1978) and the lipid constituents (Miljanich et al., 1981) are believed to be asymmetrically disposed with respect to the two monolayers of the disk membrane bilayer. By contrast, in recombinant membranes prepared by detergent dialysis, this asymmetry appears to be lost (Fung & Hubbell, 1978). As can be seen in Figure 1, very similar photochemical behavior is obtained for the native ROS disk membranes (part a) and for rhodopsin recombined with the extracted and purified (Brown et al., 1982) ROS phospholipids (part b). Thus, rhodopsin largely retains its photochemical activity by the reconstitution procedure employed (Hong & Hubbell, 1973), and transmembrane asymmetry is apparently not essential for this aspect of its function. We then corroborated earlier findings (O'Brien et al., 1977; Baldwin & Hubbell, 1985) which demonstrate that the MI to MII transition is essentially blocked—and thus rhodopsin is photochemically disfunctional—in recombinant membranes comprised of phosphatidylcholines (PC) with relatively short, saturated acyl chains, in the liquid-crystalline state, such as di(14:0)PC (not shown). The inability of rhodopsin to undergo the MI–MII transition could be due to a requirement for (i) a minimum phospholipid acyl chain length, (ii) unsaturation or polyunsaturation of the acyl chains, (iii) specific phospholipids other than PC, such as phosphatidylethanolamine (PE), (iv) the presence of some specific mixture of phospholipids, or a combination of (i)–(iv). The roles of the above factors as found in the native system (Stone et al., 1979; Miljanich et al., 1979) were investigated systematically. We studied the influence of the length of the phospholipid acyl chains by recombining rhodopsin with members of a homologous series of monounsaturated 1,2-diacyl-*sn*-glycero-3-phosphocholines. A minimum chain length of about 20 carbon atoms is required to produce significant amounts of MII at 28 °C and pH 7.0, but nativelike photochemical behavior is not obtained (Beach et al., 1984). The influence of acyl chain unsaturation is exemplified by the recombinant of rhodopsin with egg PC in Figure 1; a smaller phototransient (part g) is seen relative to the native ROS disk membrane vesicles (part a), in agreement with previous results (O'Brien et al., 1977). Thus, acyl chain unsaturation is insufficient for full photochemical activity in the case of phosphatidylcholines. We then asked whether polyunsaturation of the acyl chains of phosphatidylcholines can lead to nativelike behavior. The flash photolysis traces for the recombinants of rhodopsin with di(22:6)PC (part d) and with a mixture of di(22:6)PC/egg PC (1/1) (part e), compared to that for the less unsaturated egg PC recombinant (part g), show that the magnitudes of the phototransients may depend on the degree of acyl chain unsaturation. However, polyunsaturation of the phosphatidylcholine acyl chains does not yield full activity, as can be seen by comparison of the result for the di(22:6)PC recombinant (part d) to that for the native ROS disk membranes (part a). The influence of phosphatidylethanolamine head groups is illustrated in Figure 1 by the recombinant of rhodopsin with an equimolar mixture of egg PE and egg PC (part f). A small increase in photochemical activity is found relative to egg PC alone (part g), but again significant differences are evident vis-à-vis the native ROS disk membranes (part a). Finally, we investigated whether equimolar PE and PC together with polyunsaturation of the acyl chains is sufficient for nativelike activity. The flash photolysis trace for the di(22:6)PE/egg PC (1/1) recombinant (part c) shows this may be the case—here, the magnitude of the phototransient approaches that of the native ROS membranes (part a).

Thus, 22:6 $\omega$ 3 acyl chains or PE head groups may promote

the formation of MII, but neither alone appears sufficient for native behavior under the conditions employed here. However, when both are present together with PC, the behavior of the native system is approximated. Other factors, including charged lipid constituents such as phosphatidylinositol (PI) and phosphatidylserine (PS) (De Grip et al., 1983), peripheral proteins (Parkes & Liebman, 1984), and the lipid/rhodopsin ratio (De Grip et al., 1983), may affect the photochemical activity and require further study. The possible influences of minor contamination from free fatty acids, impurities from lipid oxidation, and residual DTAB may also deserve attention.

## DISCUSSION

Here, we have addressed the question of whether the characteristic acyl chain or polar head-group composition of the native ROS disk membranes (Stone et al., 1979; Miljanich et al., 1979) may play a role in the photochemical function of rhodopsin. The glycerophospholipids of bovine ROS disk membranes comprise on a molar basis 39% phosphatidylcholine (PC), 42% phosphatidylethanolamine (PE), 14% phosphatidylserine (PS), and 2% phosphatidylinositol (PI), which are fairly typical of other biological membranes. The acyl chain composition, however, is distinguished by the presence of highly polyunsaturated fatty acids; in particular, docosahexaenoic acid (22:6 $\omega$ 3) constitutes up to 47 mol % of the hydrocarbon chains (Stone et al., 1979). Therefore, we investigated influences of the PC and PE head groups and degree of acyl chain unsaturation on the photochemistry of rhodopsin in recombinant membranes. The present studies (conducted at 28 °C and pH 7) indicate that differences exist in the [MII]/[MI] ratio produced by photolysis of rhodopsin in the various recombinants. A number of other factors are known to influence the MI-MII equilibrium, including temperature and pH [cf. Matthews et al. (1963)]. The effects of temperature have not been investigated in detail and are required for a thermodynamic formulation. Uptake of protons renders the transition from MI to MII pH dependent, such that MII is favored by lower pH values; one explanation of the findings would involve shifts in the  $pK$  of a titratable group of rhodopsin due to the membrane environment. However, it seems unlikely that the differences in phototransient magnitudes are due solely to local pH effects, since the membrane hydrocarbon region appears to be involved (Figure 1).

The implication of phospholipids containing docosahexaenoic acid in the process of visual phototransduction is suggested by several, albeit indirect, lines of evidence. First, docosahexaenoic acid is the predominant fatty acid constituent of the ROS membrane phospholipids (*vide supra*) (Stone et al., 1979). The biosynthesis of long-chain, polyunsaturated fatty acids is metabolically costly, suggesting their importance for visual function. Second, docosahexaenoic acid is relatively labile and susceptible to oxidative damage, which may account for the presence of antioxidants (Stone et al., 1979) and disk renewal mechanisms (Wheeler et al., 1975) in the rod, again suggesting an essential role. Finally, reduction of docosahexaenoic acid levels of the ROS membranes of animals (albino rats and rhesus monkeys) through dietary means, that is, by deprivation of  $\omega$ 3 fatty acid precursors, is known to lead to significant alterations of visual function (Wheeler et al., 1975; Neuringer et al., 1986). In particular, Neuringer et al. (1986) have shown that dietary  $\omega$ 3 fatty acids are required for development of a normal electroretinogram response and visual acuity in rhesus monkeys and that changes in visual function are correlated with reduced levels of rod membrane docosahexaenoic acid.

The findings reported here suggest that all of those features of the ROS membrane lipids investigated, namely, docosahexaenoic acid chains and phosphatidylethanolamine and phosphatidylcholine head groups, may be important with regard to the MI-MII transition, and thus in the process of visual photoexcitation. If similar lipid influences exist under physiological conditions, that is, at lower light intensity and in the presence of nucleotides and peripheral proteins [cf. Kühn et al. (1981), Parkes and Liebman (1984), and Kohl and Hofmann (1987)], then the above could explain at the molecular level earlier observations (Wheeler et al., 1975; Neuringer et al., 1986) that polyunsaturated acyl chains are essential for normal visual function. To further test this hypothesis, it is necessary to conduct studies of the early receptor potentials (Cone, 1967) of animals whose retinal lipids have been modified dietarily (Wheeler et al., 1975); in particular, the R2 phase is believed associated with the MI-MII transition (Cone, 1967). It is also noteworthy that the PE fraction of the retinal ROS phospholipids appears very highly polyunsaturated (Miljanich et al., 1979). Such highly polyunsaturated phosphatidylethanolamines are found in other nerve tissues, including cerebral grey matter (O'Brien & Sampson, 1965); impairment of neural functions can occur as one result of essential  $\omega$ 3 fatty acid deficiency (Lamprey & Walker, 1976; Neuringer et al., 1986).

The present investigations may also be significant in a broader context with regard to the role of lipids in protein-mediated functions of biological membranes. If it is assumed that the photolytic pathway of rhodopsin in the recombinants is similar to that of the native system, then the more extensive prior studies of ROS disk membranes (Lewis et al., 1981) can be utilized as a basis for further interpretation of the results. The differences in the amplitudes of the phototransients on the millisecond time scale would then represent variations in the populations of MI and MII in equilibrium; that is, the membrane lipid environment would influence the Gibbs free energies of rhodopsin conformational states linked to visual function. An alternative is that there exists a population of rhodopsin molecules in the recombinant membranes which is arrested at the MI stage (De Grip et al., 1983) and is unable to undergo the MI-MII transition, for example, as observed for delipidated or aggregated rhodopsin (Applebury et al., 1974). In this case, the phototransients would reflect the fraction of rhodopsin molecules undergoing a relatively nativelylike MI-MII transition and would vary with the lipid composition as depicted in Figure 1. This explanation is disfavored by the ability of rhodopsin in the various recombinants to regenerate (cf. Table I); aggregation or delipidation of rhodopsin apparently leads to a decrease in regenerability (Van Breugal et al., 1978). Although further work is needed, we presently favor a model in which the amplitudes of the flash transients reflect the [MII]/[MI] ratio in equilibrium. A number of studies involving nuclear magnetic resonance (NMR) spectroscopy (Brown et al., 1982), spin-label EPR spectroscopy (Watts et al., 1979), and differential scanning calorimetry (Miljanich et al., 1985) have all suggested that specific interactions of rhodopsin with the docosahexaenoyl acyl chains or polar head groups of the ROS membrane phospholipids are unlikely. Thus, interactions of rhodopsin with membrane lipids important for function may involve properties of a more long-range nature. Recent  $^2\text{H}$  NMR studies have suggested that the saturated acyl chains of asymmetric (mixed chain), polyunsaturated phosphatidylcholines may have somewhat greater configurational freedom than those of disaturated phospholipids (Salmon et al., 1987),

for example, due to an increase in the equilibrium cross-sectional area per chain. This property could be important with regard to the balance of opposing repulsive and attractive forces which govern membrane stability [cf. Gruner et al. (1985) and Salmon et al. (1987)] and could be implicated in the energetics of the MI-MII transition of rhodopsin. The effect, however, that phosphatidylethanolamine head groups would be expected to have on the available area per chain appears opposite to that of polyunsaturation (Gruner et al., 1985). The presence of PE is believed to favor a more condensed bilayer surface, due to the smaller effective size of the head group relative to PC. Hence, the apparent requirements for both docosahexaenoic acid chains and PE head groups seem to suggest that other membrane properties may also be significant with regard to the photochemical activity of rhodopsin.

One possible explanation is that the presence of phosphatidylethanolamine with polyunsaturated acyl chains (Miljanich et al., 1979) leads to a reduction of the lamellar to hexagonal phase transition temperature (Gruner et al., 1985) and that proximity of the system to such a boundary in the phase diagram yields bilayer properties favoring the MI-MII conformational change. The above would be consistent with earlier  $^{31}\text{P}$  NMR studies, which suggest that aqueous dispersions of lipids extracted from the ROS membranes undergo a liquid-crystalline lamellar ( $L_\alpha$ ) to inverted hexagonal ( $H_{II}$ ) phase transition near physiological temperature (Deese et al., 1981; Brown et al., 1982). Similar  $^{31}\text{P}$  NMR studies of the native ROS membranes and unsonicated dispersions of the recombinant membranes employed here (not shown) demonstrate that all are in the lamellar phase under the present conditions. The temperature at which hexagonal-type components begin to appear is lowest for the di(22:6)PE/egg PC (1/1) recombinant ( $\sim 35^\circ\text{C}$ ), which has the greatest photochemical activity (Figure 1). An enhancement of the curvature elasticity, area compressibility, or other properties of the bilayer could occur near such a boundary in the phase diagram and may be important with respect to the energetics of the MI-MII conformational change. It should be noted that pressure dependence studies (Lamola et al., 1974) have shown an increase in the partial molar volume of rhodopsin accompanying the MI-MII transition in native ROS membranes. By contrast, little change is seen for rhodopsin solubilized in detergents, suggesting that lipid-protein interactions may be involved (Lamola et al., 1974). A connection between the MI-MII transition and the acyl chain configurational freedom (Salmon et al., 1987) of polyunsaturated bilayers seen with  $^2\text{H}$  NMR, or proximity to a lamellar to hexagonal or cubic phase boundary (Deese et al., 1981), would be consistent with the present results. In this regard, it is interesting that studies of another integral membrane protein, the  $\text{Ca}^{2+}$ -ATPase from muscle sarcoplasmic reticulum, have also shown that function—here the coupling of  $\text{Ca}^{2+}$  transport to ATP hydrolysis—appears promoted in recombinant membranes containing lipids favoring the hexagonal phase near physiological temperature (Navarro et al., 1984). More extensive investigations are needed in the future to test and refine the above inferences.

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## Purification and Characterization of the Glycoprotein Hormone $\alpha$ -Subunit-like Material Secreted by HeLa Cells<sup>†</sup>

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**ABSTRACT:** The protein secreted by HeLa cells that cross-reacts with antiserum developed against the  $\alpha$ -subunit of human chorionic gonadotropin (hCG) has been purified approximately 30 000-fold from concentrated culture medium by organic solvent fractionation followed by ion exchange, gel filtration, and lectin affinity chromatography. The final preparation had a specific activity (by RIA) of  $6.8 \times 10^5$  ng of  $\alpha$ /mg of protein and appeared homogeneous by electrophoresis on reducing/denaturing polyacrylamide gels (SDS-PAGE). Amino acid analysis indicated that HeLa- $\alpha$  had a composition very similar to that of the urinary hCG  $\alpha$ -subunit. Peptide fingerprints of the HeLa protein and hCG- $\alpha$  revealed that several of the Tyr-, Met-, and Cys-containing tryptic peptides were held in common, thus identifying the tumor protein as a glycoprotein hormone  $\alpha$ -subunit with a primary structure similar to that of hCG- $\alpha$ . However, comparison of hCG- $\alpha$  and HeLa- $\alpha$  demonstrated that the tumor-associated subunit was not identical with its normal counterpart. Only two of the three Tyr-containing tryptic peptides present in hCG- $\alpha$  could be detected in HeLa- $\alpha$  after iodination with <sup>125</sup>I. HeLa- $\alpha$  eluted prior to hCG- $\alpha$  during Sephadex G-75 chromatography, but the subunits coeluted when the tumor protein was first subjected to mild acid hydrolysis. The purified tumor protein had an apparent molecular weight greater than that of the urinary  $\alpha$ -subunit when analyzed by SDS-PAGE (Coomassie blue staining), and this difference was even greater when a partially purified preparation was examined by an immunoblot technique (Western). Isoelectric focusing of the HeLa and hCG subunits demonstrated that the tumor protein had a lower pI (4.7-5.5 compared to 6.5-7.8), and removal of sialic acid by mild acid hydrolysis did not entirely eliminate this difference. Immunoprecipitation and electrophoresis of  $\alpha$ -subunit from HeLa cultures labeled with [<sup>3</sup>H]fucose indicated that the tumor subunit was fucosylated, whereas analysis of hCG- $\alpha$  hydrolysates by HPLC confirmed previous reports that the placental subunit does not contain fucose. HeLa  $\alpha$ -subunit was unable to combine with hCG  $\beta$ -subunit to form holo-hCG under conditions where the hCG  $\alpha$ -subunit was able to do so. The results indicate that, regardless of whether or not a single  $\alpha$ -subunit gene is being expressed in both normal and neoplastic tissues, posttranslational modifications lead to a highly altered subunit in the tumor. The differences observed may be useful in diagnosing neoplastic vs hyperplastic conditions and may lend insight into the mechanism of ectopic hormone production by tumors.

**T**umors often produce proteins not characteristic of the cell type from which the cancer originated (Odell & Wolfson, 1975; Blackman et al., 1978). Such ectopic proteins serve as markers for neoplasia and are used clinically for the diagnosis and monitoring of cancer patients. In general, they are a nonrandom collection of polypeptide hormones, embryonic antigens, and fetal isoenzymes. Not all tumors elaborate ectopic products, though often a particular protein may be more commonly associated with a particular type of cancer.

For example, oat cell carcinomas frequently secrete ACTH,<sup>1</sup> bronchial carcinomas often produce parathormone, and pan-

<sup>1</sup> Abbreviations: ACTH, adrenocorticotrophic hormone; ConA, concanavalin A; DTT, dithiothreitol; Fuc, fucose; FSH, follicle-stimulating hormone; Gal, galactose; GlcNAc, N-acetylglucosamine; hCG, human chorionic gonadotropin; HPLC, high-pressure liquid chromatography; LH, luteinizing hormone; Man, mannose;  $\alpha$ -MG, methyl  $\alpha$ -glucoside (methyl  $\alpha$ -D-glucopyranoside); NeuNAc, N-acetylneuraminic acid (sialic acid); PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; POPOP, 1,4-bis(5-phenyloxazol-2-yl)benzene; PPO, 2,5-diphenyloxazole; RIA, radioimmunoassay; RPMI, Roswell Park Memorial Institute; SDS, sodium dodecyl sulfate; T, tryptic peptide; TPCK, N<sup>α</sup>-tosylphenylalanine chloromethyl ketone; TSH, thyroid-stimulating hormone.

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