Rhodopsin–Phospholipid Interactions: Dependence of Rate of the Meta I to Meta II Transition on the Level of Associated Disk Phospholipid†

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ABSTRACT: Solubilization of retinal rod outer segment disk membranes in octyl glucoside was employed to prepare rhodopsin samples with varying amounts of associated disk phospholipid. Flash photolysis studies were carried out on these samples to determine the dependence of the meta I to meta II transition kinetics on the level of associated phospholipid. The rate constant for the formation of meta II increased from 6.9×10^3 to 19.5×10^3 s^{-1} as the molar ratio of phospholipid per rhodopsin fell from 35 to 5. The activation free energy for this process had a linear dependence on the level of phospholipid, with a slope of 24 cal/mol of rhodopsin-associated phospholipid. A variety of evidence suggests that rhodopsin undergoes a reversible conformation change during the meta I to meta II transition. No evidence was found for an enhanced effect on the activation free energy for this conformation change at the level of associated phospholipid which corresponds to the formation of a phospholipid boundary layer around rhodopsin.

Integral membrane proteins derive a high degree of stabilization through their interaction with lipids in biological membranes. This is evidenced by both the loss of activity of many membrane-bound enzymes when solubilized and the sensitivity of these enzymes to perturbations of the physical properties of the bilayer phospholipids (Sandermann, 1978). The visual pigment rhodopsin constitutes about 90% of the rod outer segment disk membrane protein (Papermaster & Dreyer, 1974) and is thought to have the major portion of its mass within the phospholipid bilayer of the disk membrane (Saibil et al., 1976). The bovine rod outer segment (ROS)[1] disk membrane has a 75:1 ratio of phospholipid per rhodopsin (Stubbs & Litman, 1978a). About 40% to 50% of the acyl side chains of the disk phospholipid are docosahexaenoic acid (Anderson et al., 1975; Stone et al., 1979). One might anticipate, therefore, that there will be an intimate relationship between rhodopsin function and the microenvironment provided by the lipid matrix of the disk membrane.

The photolytic cycle of rhodopsin is characterized by the sequential appearance of several spectrally characterized intermediates (Ostroy, 1977). The last transition in this cycle occurring on a time scale consistent with that of the light-evoked neural response is the meta I to meta II transition. Previous evidence indicates that opsin, the protein moiety of rhodopsin, undergoes a conformation change at this step (Liebman et al., 1974; Saibil et al., 1976; Ostroy, 1977; Downer & Englander, 1977). The reversibility of this step is implied by both the ability to generate a pigment with spectral properties identical with that of native rhodopsin by addition of 11-*cis*-retinal to opsin and the apparent reversible equilibrium between the meta I and meta II forms of rhodopsin. The dependence of the meta I to meta II transition kinetics on the microenvironment was first demonstrated by Applebury et al. (1974) and extended to rhodopsin reconstituted with a variety of phospholipids by O'Brien et al. (1977). Shichi et al. (1977) have recently demonstrated a similar effect of microenvironment on the lumi to meta I transition. A quantitative interpretation of the dependence of the meta I to meta II transition kinetics, in terms of the level of rhodopsin-associated phospholipid, has not been previously reported, nor has the effect of a boundary layer of phospholipid on the conformational change undergone by opsin during this transition been determined.

Previous studies of opsin–lipid interaction employed the solubilization of bleached disk membranes in octyl glucoside as a means of altering the level of opsin-associated phospholipid (Stubbs & Litman, 1978a,b). The present study utilizes a similar approach, with the goal of determining the mode of interaction of rhodopsin with disk phospholipids. To accomplish this aim, we have examined the effect of varying the level of rhodopsin-associated phospholipid on the activation parameters for the meta I to meta II transition kinetics.

Material and Methods

Disk Membrane Preparation. Disk membranes were prepared from frozen bovine retinas (Hormel) by the Ficoll floatation method of Smith et al. (1975). The disk membranes were washed twice and resuspended in buffer consisting of 0.05 M Tris, 0.05 M sodium acetate, and 0.2 M potassium chloride, adjusted to pH 7 by addition of HCl. When solubilized in octyl glucoside, the resulting disk membranes typically had an A_{280}/A_{500} ratio of 2.3. The phospholipid was assayed by the method of Bartlett (1959). The rhodopsin concentration was measured by recording the difference in A_{500} produced by bleaching a sample solubilized in octyl glucoside in the presence of 0.09 M hydroxylamine. The molar extinction coefficient of rhodopsin at 500 nm employed was 40 000 (Shichi, 1970). All procedures were performed under dim red light unless otherwise stated.

Disk Membrane Solubilization. Octyl glucoside was prepared from acetobromo-α-D-glucose (Sigma) and 1-octanol (Fisher Scientific) by a modification of the method of Noller & Rockwell (1938). The amount of octyl glucoside required to solubilize a specific disk membrane sample was determined by

$$[\text{octyl glucoside}] = 0.0185 + 270[\text{rhodopsin}] \quad (1)$$

where the brackets refer to total concentrations expressed in

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[1] Abbreviation used: ROS, rod outer segment.

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mol/L (Stubbs & Litman, 1978a). The amount of phospholipid associated with rhodopsin, as a function of the varying level of total octyl glucoside concentration, was calculated from the previously determined binding equation:

$$\frac{1}{\bar{\nu}} = \frac{1}{K}\frac{[\text{micellar octyl glucoside}]_f}{[\text{phospholipid}]_{total}} + \frac{[\text{rhodopsin}]}{[\text{phospholipid}]_{total}} \quad (2)$$

where $\bar{\nu}$ is the average number of associated phospholipid molecules per solubilized rhodopsin molecule, K is the distribution coefficient for the partitioning of phospholipid molecules between octyl glucoside micelles containing rhodopsin and those free of rhodopsin, $[\text{phospholipid}]_{total}$ is the total phospholipid associated with the membrane sample, and $[\text{micellar octyl glucoside}]_f$ is that concentration of octyl glucoside present as either pure octyl glucoside or phospholipid–octyl glucoside micelles. The value of $[\text{micellar octyl glucoside}]_f$ was estimated by subtracting the octyl glucoside concentration needed to solubilize all of the rhodopsin in the sample (calculated by eq 1) from the total octyl glucoside concentration. This calculation assumes that the monomer detergent concentration and the amount of detergent bound to the solubilized rhodopsin remains unchanged as the total detergent concentration is increased (Stubbs & Litman, 1978a).

Flash Photolysis Measurements. Samples were prepared by suspending an aliquot of disks in 50 mM Tris-acetate buffer, pH 7, and adding an appropriate volume of a 200 mM octyl glucoside stock solution so as to obtain the desired detergent concentration. Samples were prepared fresh each day for kinetic studies. Solutions exposed to pulsed N_2 laser excitation (331 nm, 0.5 mJ, 8 ns) showed an increase in absorbance at 380 nm, which is ascribed to meta II formation. Photolysis of the rhodopsin samples by the monitoring light was minimized by using short (10^{-2} s) exposures (obtained with a synchronized camera shutter) and appropriate combinations of Corning glass filters (Rosenfeld et al., 1972; Goldschmidt et al., 1976). First-order rate constants were obtained from plots of log $(A_\infty - A_t)$ vs. time, where A_t and A_∞ are the absorbance at 380 nm at times t and infinity, respectively. Activation parameters were determined from the temperature dependence of the meta I to meta II transition kinetics; these activation parameters were used to calculate $\Delta G^\ddagger$ at 23 °C.

Results and Discussion

A major question in the understanding of protein–phospholipid interactions is the mode of interaction of integral membrane proteins with the fraction of bilayer phospholipid surrounding them, i.e., the boundary lipid. By definition, a boundary layer of phospholipid must exist in a protein–phospholipid membrane. Due to the anisotropic nature of the forces acting on the phospholipid molecules at the protein–phospholipid interface, one would expect that the motional properties of this group of phospholipid molecules would be different from those in the bulk phospholipid phase. Information of major interest with respect to the boundary layer population of phospholipid molecules is the residency time of a given phospholipid molecule in the boundary layer, the binding specificity of the protein for a particular phospholipid class, and the nature of the interaction of the boundary layer phospholipid with the protein. The present study is designed to address this latter point for the visual pigment rhodopsin. Upon initial solubilization of disk membranes in octyl glucoside, rhodopsin was shown to have approximately a boundary layer of phospholipid associated with it (Stubbs & Litman, 1978a). As the micellar detergent to disk phospholipid ratio is increased, the level of associated phospholipid drops to yield

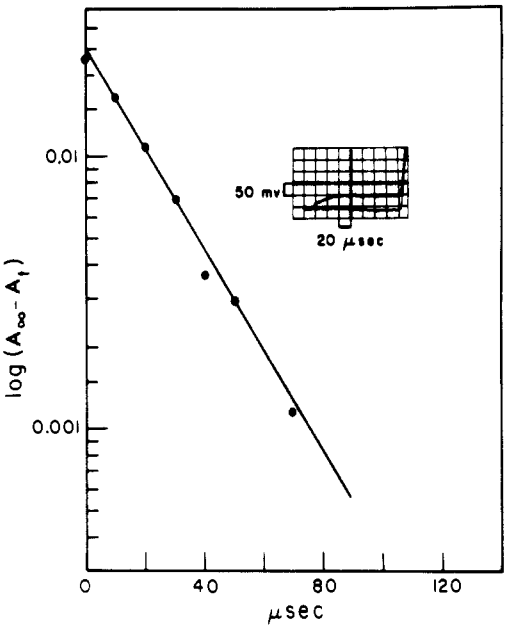

FIGURE 1: Characteristic oscillogram (insert) and first-order rate plot of the meta II generation kinetics following excitation of an octyl glucoside solubilized disk sample. The light (monitoring beam on, lower trace in the oscillogram) to dark (monitoring beam off, not shown) difference is 50 mV. The monitoring wavelength is 380 nm. A_t is the absorbance at time t, in the growing-in region, while A_∞ is the final value in the plateau region. The data given are for 1.37×10^{-5} M rhodopsin dissolved in 35.7 mM octyl glucoside in the standard Tris–acetate buffer, pH 7. These conditions yield a value of $\bar{\nu} = 35$. The data were obtained at 23 °C.

an almost phospholipid-free rhodopsin species (Figure 2). In the present study, measurements of the dependence of the meta I to meta II transition kinetics on the level of rhodopsin-associated phospholipid were carried out by determining the rate of formation of meta II in solutions containing fixed amounts of disks and varying levels of micellar detergent. A typical oscillogram trace for an octyl glucoside solubilized disk sample is shown in the insert of Figure 1. The observed data were plotted as log $(A_\infty - A_t)$ vs. t to obtain first-order rate constants for the formation of meta II (Figure 1). The data for octyl glucoside solubilized rhodopsin are consistent with a first-order rate process and within experimental error correspond to a single-exponential decay. The rate constant for meta II formation is seen to increase with an increasing ratio of micellar detergent to disk phospholipid, conditions under which the amount of rhodopsin-associated phospholipid decreases (Figure 2). It is clear from Figure 2 that the level of rhodopsin-associated phospholipid is modulating the meta I to meta II transition kinetics.

The enthalpy of activation of the meta I–meta II transition was determined for several values of $\bar{\nu}$ by measuring the temperature dependence of the kinetics (Figure 3). The data yield linear Arrhenius plots, and the enthalpies of activation are summarized in Table I. $\Delta H^\ddagger$ shows a linear dependence on $\bar{\nu}$. Linear regression analysis of these data yields a slope of 0.50 kcal/mol of associated phospholipid and an intercept of 15.53 kcal/mol. The latter value should correspond to $\Delta H^\ddagger$ for the photolytic transition in a phospholipid free state in octyl glucoside. A value of $\Delta H^\ddagger = 19$ kcal/mol was reported by Applebury et al. (1974) for rhodopsin dissolved in dodecyldimethylamine oxide. The exact level of associated lipid is unknown in these experiments but lies in the range $0 < \bar{\nu} < 5$. A $\Delta H^\ddagger$ between 15.5 and 18 kcal/mol would be expected for octyl glucoside solubilized disks in this $\bar{\nu}$ range. Given the uncertainty in the value of $\bar{\nu}$ in the former experiments, the

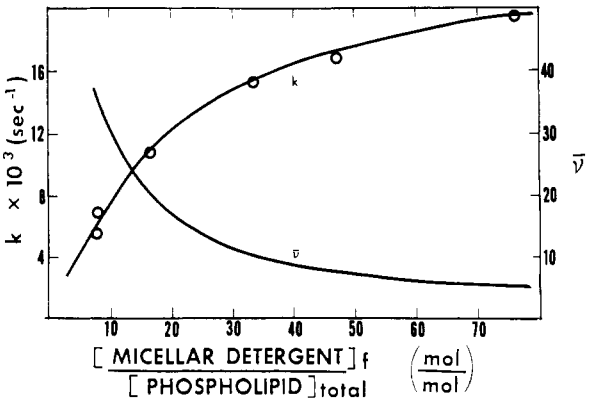

FIGURE 2: Rate of formation of meta II (k) as a function of the ratio of free, micellar detergent to disk membrane phospholipid. Also shown is the phospholipid binding curve, derived from eq 2, giving the molar ratio of phospholipid to rhodopsin ($\bar{\nu}$) in the rhodopsin–phospholipid–detergent micelles on which the kinetic measurements were obtained. Kinetic data were obtained at 23 °C.

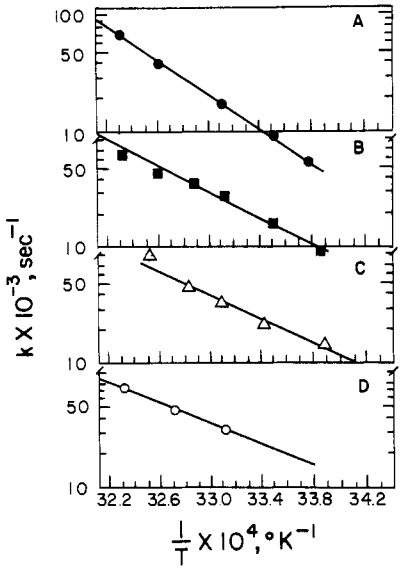

FIGURE 3: Arrhenius plots of the rate constants of the meta I–meta II transition for disks solubilized in octyl glucoside to yield rhodopsin with the following values of $\bar{\nu}$: (A) 7.9; (B) 10.9; (C) 20.2; (D) 34.3.

Table I: Enthalpy of Activation of the Meta I–Meta II Transition for Several Levels of Associated Disk Phospholipid

$\bar{\nu}$ (mol of phospholipid/ mol of rhodopsin)	$\Delta H^{\ddagger}$ (kcal/mol)
0	15.5[a]
7.9	18.7 ± 1.2
10.9	22.4 ± 2.4
20.2	25.1 ± 1.4
34.3	33.0 ± 1.4

[a] Value extrapolated by linear regression analysis of the accompanying data points in the table; this analysis yielded a correlation coefficient of 0.987 and a slope of 0.50.

agreement in $\Delta H^{\ddagger}$ is reasonably good for the two detergents.

Applebury et al. (1974) have reported that dodecyldimethylamine oxide solubilized disks, having approximately 75 phospholipids per rhodopsin, and column chromatographed rhodopsin, containing 5 phospholipids per rhodopsin, yielded similar meta I–meta II transition kinetics; this result implies a lack of dependence of the kinetics on the level of phospholipid in this detergent. An explanation of this apparent discrepancy with the observations reported here lies in the actual form in which rhodopsin solubilizes under the conditions employed in

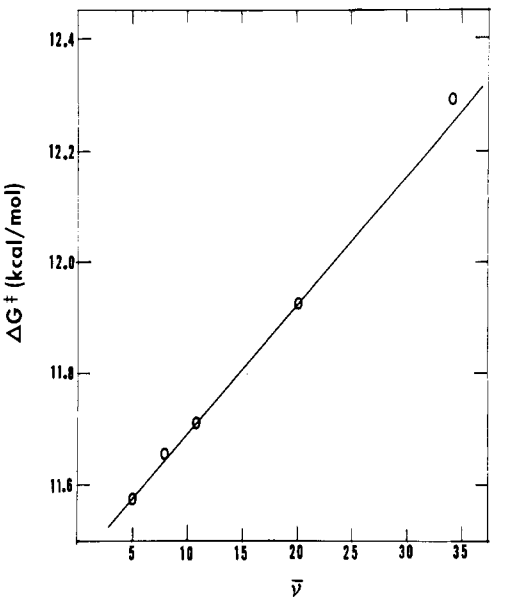

FIGURE 4: Activation free energy for the formation of meta II in octyl glucoside solubilized disks vs. the ratio of phospholipid to rhodopsin ($\bar{\nu}$) in the rhodopsin–detergent–phospholipid micelles. Values were calculated at 23 °C. Standard deviation for $\Delta G^{\ddagger}$ values is ±0.07 kcal/mol or less.

the measurements of Applebury and co-workers. These measurements were carried out at 100 mM dodecyldimethylamine oxide. Although a phospholipid association curve in dodecyldimethylamine oxide has not been determined, equivalent levels of octyl glucoside would be more than enough to yield essentially phospholipid-free, detergent–rhodopsin micelles upon disk solubilization, the phospholipid being distributed in detergent–lipid micelles. Therefore, the solubilized form of rhodopsin in both the disk and chromatographically prepared rhodopsin samples would likely be the same, resulting in an apparent lack of dependence of the observed kinetics on phospholipid level. These results point up the importance of characterizing the exact nature of the rhodopsin complex in solution in order to draw an unambiguous interpretation of experimental data. The effects discussed here could lead to apparent disagreements in rate measurements made in either the same or a different detergent solution due to variations in the composition of the rhodopsin–phospholipid–detergent micelles actually present in the solubilized disk solution.

As a further means of characterizing the interaction of rhodopsin with phospholipid, the dependence of the activation free energy for the meta I–meta II transition on the level of associated phospholipid was determined. The former quantity was found to have a linear dependence on the level of associated phospholipid (Figure 4), increasing by 24 cal for each additional mol of rhodopsin-associated phospholipid. These results are qualitatively similar to those obtained in studies of the phospholipid dependence of the unfolding of opsin (Stubbs & Litman, 1978b). In both studies, each additional protein-associated phospholipid molecule contributes the same increment of change to the activation free energy for the process, whether it be the fourth or fortieth molecule added.

A variety of data suggests that opsin undergoes a reversible conformation during the meta I to meta II transition (Liebman et al., 1974; Saibil et al., 1976; Ostroy, 1977; Downer & Englander, 1977). The kinetics of this transition shows a marked dependence on the level of rhodopsin-associated phospholipid. It has been estimated from geometric considerations that 30 molecules of phospholipid are required to form a boundary layer for rhodopsin (Stubbs et al., 1976). To the

degree that the activation free energy for the transition reflects the interaction between rhodopsin and disk phospholipid, one would expect that if a unique protein–phospholipid interaction occurred upon formation of a boundary layer for rhodopsin, either a leveling out or an inflection point would be observed for the dependence of the activation free energy on $\bar{\nu}$. The observed behavior of the activation free energy is, however, linear throughout the range of $\bar{\nu}$ studied; these data encompass the value of $\bar{\nu}$ associated with the formation of a phospholipid boundary layer around rhodopsin. The linear behavior of the activation free energy implies that both a noncooperative and nonspecific mode of interaction exists between rhodopsin and disk membrane phospholipids. Calculation of the activation free energy for rhodopsin with 75 associated phospholipid molecules, the ratio which exists in the disk membrane, yields a value of 13.2 kcal/mol, while the value for the disk membrane, as determined from the data of Applebury et al. (1974), is 14.6 kcal/mol. Hence, while there is no marked increase in the activation free energy associated with the formation of a phospholipid bilayer around rhodopsin, there does appear to be an increase of 1.4 kcal/mol associated with the insertion of rhodopsin into a phospholipid bilayer.

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Stimulation of Messenger Ribonucleic Acid Synthesis in Isolated Nuclei by a Protein That Stimulates RNA Polymerase II†

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ABSTRACT: A factor stimulating RNA polymerase II purified from Ehrlich ascites tumor cells was found to stimulate α-amanitin-sensitive RNA synthesis in nuclei isolated from spleen cells of anemic mice, though less than it stimulated purified RNA polymerase II. The fidelity of the resulting RNA synthesis was monitored by measuring the stimulation of globin mRNA synthesis. Globin mRNA was measured quantitatively by DNA–RNA hybridization by using plasmid DNA containing globin DNA sequences. Results showed that the synthesis of globin mRNA was enhanced in isolated nuclei in the presence of this factor coinciding with an increase of overall α-amanitin-sensitive RNA synthesis. Thus, it was concluded that an externally added factor did not stimulate random transcription but meaningful RNA synthesis in isolated nuclei.

Little information is available about proteins regulating eukaryotic gene expression. However, it is clear that RNA polymerase II or III alone is insufficient for faithful transcription in vitro (Weil et al., 1979a,b; Ng et al., 1979). There are several reports of proteins stimulating the activity of RNA polymerase II in vitro, though the biological functions of these proteins are unknown (Stein & Hausen, 1970; Seifart, 1970; Natori, 1972; Lentfer & Lezius, 1972; Sugden & Keller, 1973; Lee & Dahmus, 1973; Benson et al., 1978; Spindler, 1979; Revie & Dahmus, 1979).

We purified a protein, named S-II, from Ehrlich ascites tumor cells that exclusively stimulates the activity of RNA polymerase II (Sekimizu et al., 1979a). Studies using antibody against S-II showed that S-II is a nuclear protein that is localized in the nucleoplasm and that it is not a component

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