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Rhodopsin in Dimyristoylphosphatidylcholine-Reconstituted Bilayers Forms Metarhodopsin II and Activates G_t[†]

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ABSTRACT: The photochemical intermediate metarhodopsin II (meta II; λ_{max} = 380 nm) is generally identified with rho*, the conformation of photolyzed rhodopsin which binds and activates the visual G-protein, G_t [Emeis, D., & Hoffman, K. P. (1981) *FEBS Lett. 136*, 201-207]. Purified bovine rhodopsin was incorporated into vesicles consisting of dimyristoylphosphatidylcholine (DMPC), and the rapid formation of a photochemical intermediate absorbing maximally at 380 nm was quantified via both flash photolysis and equilibrium spectral measurements. Kinetic and equilibrium spectral measurements performed above the T_m of DMPC showed that G_t, in the absence of GTP, enhances the production of the 380-nm-absorbing species while reducing the concentration of the 478-nm-absorbing species, metarhodopsin I (meta I), in a manner similar to that observed in the native rod outer segment disk membrane. This G_t-induced shift in the equilibrium concentration of photointermediates indicated that the species with an absorbance maximum at 380 nm was meta II. The presence of rho* in the DMPC bilayer was established via measurements of photolysis-induced exchange of tritiated GMPPNP, a nonhydrolyzable analogue of GTP, on G_t. Above T_m, the metarhodopsin equilibrium is strongly shifted toward meta I relative to the native rod outer segment disk membrane; however, at 37 °C, 40% of the photointermediates are in the form of meta II. The formation of meta II above T_m is slowed by a factor of ca. 2 relative to the disk membrane. Below T_m, the equilibrium is shifted still further toward meta I, and meta II forms ca. 7 times slower than in the disk membrane. Below T_m, photolyzed rhodopsin was still capable of activating G_t, although the rate was about 12× slower than that observed above T_m, confirming the presence of meta II in the gel-phase bilayer.

The visual pigment rhodopsin of the disk membrane of the vertebrate rod outer segment is an integral membrane protein consisting of hydrophilic domains linked by seven α-helices which span the disk membrane (Ovchinikov, 1982; Hargrave et al., 1983). Rhodopsin is representative of the class of integral membrane protein receptors which accomplish their signal transduction function by activating a specific G-protein. This combination of signal transduction by means of a ubiquitous mechanism and intimate contact with the phospholipid bilayer makes rhodopsin an ideal subject for studying the effects of bilayer composition and physical properties on signal transduction by integral membrane proteins.

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Within a few milliseconds of photon absorption, bleached rhodopsin forms a quasi-stable equilibrium between two photochemical intermediates: metarhodopsin I (meta I)[1] (λ_{max} = 478 nm) and meta II (λ_{max} = 380 nm) (Mathews et al., 1963). Meta II is spectrally identical with the form of rhodopsin (rho*) which catalyzes the exchange of GTP for bound GDP on G_t (Emeis & Hofmann, 1982; Kibelbek et al., 1990); thus, the degree to which this equilibrium favors meta II is considered functionally significant. Activation of G_t is the first step in the transfer of visual stimulus from rhodopsin to the rod cell plasma membrane sodium channels. Several previous studies have demonstrated that the phospholipid bilayer has a pronounced influence on the rate and extent of meta II formation (Applebury et al., 1974; O'Brien et al., 1977; Litman et al., 1981; Baldwin & Hubbell, 1985; Weidmann et al., 1989; Mitchell et al., 1990a). An understanding of the manner in which the physical properties of a phospholipid bilayer alter the meta I ↔ meta II equilibrium will enhance our knowledge of how cellular regulation of membrane lipid composition may affect signal transduction by integral membrane proteins.

It has previously been reported that when recombined with the saturated phospholipid DMPC photolyzed rhodopsin forms little or no meta II near neutral pH (Baldwin & Hubbell, 1985; O'Brien et al., 1977). This conclusion was partially contradicted by Ryba et al. (1986), who reported that in DMPC recombinants prepared with rhodopsin which was purified from fresh retinas, that had not been frozen, significant amounts of a species absorbing at 380 nm were formed. We have reinvestigated this question using the criteria of G_t binding (in the absence of GTP), resulting in enhancement of the equilibrium concentration of meta II and the level of G_t activation, measured as exchange of GTP for GDP on G_t, to ascertain the presence of meta II. Using these criteria, our results demonstrate that rhodopsin incorporated into DMPC bilayers forms meta II. Above the temperature of the gel-to-liquid-crystalline-phase transition, photolyzed rhodopsin in DMPC forms meta II to an extent and at a rate which are reduced compared to the native disk membrane, but shows ample ability to activate G_t. In the gel phase, the activation of G_t proceeds at least 12 times slower than above T_m.

Experimental Procedures

Recombinant Vesicle Preparation. DMPC and POPC were purchased from Avanti Polar Lipids Inc. and used without further purification. HPLC analysis carried out by Avanti Polar Lipids Inc. indicated no detectable impurities in these lipids. As carried out, the HPLC analysis would detect impurity levels of 0.1% or greater. Rhodopsin was purified from frozen bovine retinas via concanavalin A affinity chromatography, and small, unilamellar vesicles consisting of lyophilized lipid and purified rhodopsin in a molar ratio of 100 to 1, respectively, were prepared by using a dilution reconstitution method (Jackson & Litman, 1985). During the 20-min dilution reconstitution step involving DMPC, the lipid–OG–rhodopsin solution was maintained at 30 °C to ensure that the DMPC was in the liquid-crystalline phase. After the dilution step, the vesicle solution was dialyzed against three changes of 20-fold excess of buffer, using at least 12 h for each dialysis cycle. The vesicle sample was then concentrated about 5-fold in an Amicon ultrafiltration cell using a PM-10 membrane. The maximum residual OG concentration remaining after this process is on the order of 1 molecule of OG per 5000 PC molecules. Given that the partition coefficient for the distribution of OG between a PC bilayer and the aqueous buffer is 0.033 mM^{-1} (Almog et al., 1990), the actual OG/PC ratio in the bilayer would be substantially lower than the bulk ratio. Concentrated vesicle stock solutions were stored at 4 °C in an isotonic, pH 7.0, buffer consisting of 10 mM PIPES, 60 mM KCl, 30 mM NaCl, 2 mM $MgCl_2$, and 50 μM DTPA (PIPES buffer). Samples for spectral studies were prepared by diluting the vesicle stock solution with the appropriate temperature-compensated PIPES buffer to a rhodopsin concentration of 0.2–0.3 mg/mL. Samples for kinetic and equilibrium spectral measurements were sonicated on ice for 2 min in a bath sonicator at ca. one-third full power to increase optical clarity.

Flash Photolysis Measurements. The increase in absorbance at 380 nm following a laser flash (ca. 15% of the rhodopsin bleached) was monitored with a flash photolysis instrument of our own design (Straume et al., 1990). The meta I–meta II equilibrium constant, K_{eq}, was derived from the kinetic data via an analysis in terms of a branched photointermediate decay model which includes two spectrally identical, but kinetically distinguished, forms of meta II: meta II_{fast} and meta II_{slow} (Straume et al., 1990).

Equilibrium Spectral Measurements. Deconvoluted difference spectra of meta I–meta II equilibrium mixtures were derived from a series of four absorbance spectra acquired with a Hewlett-Packard 8452A diode array spectrophotometer (0.2-s measuring time yielded <0.3% bleach by measuring beam). These include (1) the starting rhodopsin suspension, (2) 3 s after a brief strobe flash which passed through a (520 ± 25)-nm band-pass filter (15–20% bleach), (3) following addition of 2 M hydroxylamine to a final concentration of 30 mM, and (4) after complete bleaching of the sample. Spectra were corrected for small differences in scattering, and difference spectra containing meta I and meta II contributions were constructed and corrected for unbleached rhodopsin. Individual meta I and meta II spectra were deconvoluted from the spectrum of their equilibrium mixture by using nonlinear least squares to fit the sum of two asymmetric quasi-Gaussians to the equilibrium spectral profile. Concentrations of meta I and meta II were calculated by using extinction coefficients of 44 000 cm^{-1} M^{-1} for meta I and 38 000 cm^{-1} M^{-1} for meta II (Applebury, 1984). Additional details regarding acquisition and analysis of both kinetic and equilibrium spectral data are given in Straume et al. (1990).

Preparation of G_t and Assay of GMPPNP Exchange. G_t was prepared by hypotonically washing bovine rod outer segments following rupture of the plasma membrane. This hypotonic extract was stored at −20 °C in 5 mM Tris buffer, pH 8.0, containing 1 mM EDTA and 30% glycerol, and was used without further purification. The concentration of active G_t was determined by using a tritiated, nonhydrolyzable analogue of GTP (GMPPNP) and a filter binding assay (Fung & Stryer, 1981). Binding of GMPPNP to G_t following exposure of the sample to room light for 3 min was determined by counting the radioactivity remaining bound to a nitrocellulose filter following repeated washing. Samples for the exchange assay were prepared by mixing [^{3}H]GMPPNP, hypotonic extract, concentrated DMPC vesicle stock suspen-

[1] Abbreviations: meta I, metarhodopsin I; meta II, metarhodopsin II; G_t, visual G-protein, GTPase of the rod outer segment, transducin; DMPC, 1,2-dimyristoyl-3-*sn*-phosphatidylcholine, di-14:0 phosphatidylcholine; GMPPNP, guanosine 5'-(β,γ-imidotriphosphate); PIPES, piperazine-*N*,*N*'-bis(2-ethanesulfonic acid); DTPA, diethylenetriaminepentaacetic acid; POPC, 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine; ROS, rod outer segment; PC, phosphatidylcholine; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; OG, octyl β-D-glucoside.

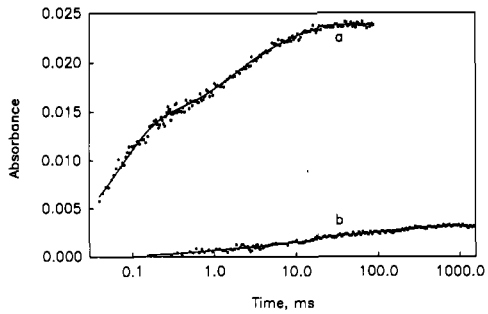

FIGURE 1: Increase in absorbance vs log time for rhodopsin in DMPC at 37 °C (a) and 15 °C (b). Solid curves are fits of the branched meta II model to the kinetic data.

sion, and the appropriate concentrated, temperature-compensated PIPES buffer to obtain rhodopsin, [^{3}H]GMPPNP, and G_t concentrations of 5, 10, and 1 μM, respectively. A portion of each sample was kept in the dark at the temperature of the measurement. Binding of GMPPNP to G_t in these dark samples served to correct the photolyzed samples in order to obtain the light-induced binding. Activation of G_t by rho* in DMPC and POPC vesicles at 5 and 30 °C following bleaching was measured with the filter binding assay.

RESULTS

The kinetics of the increase in absorbance at 380 nm following a photolyzing laser flash were measured for rhodopsin-containing DMPC vesicles at four temperatures above T_m (27, 30, 37, and 45 °C) and at three temperatures below T_m (5, 15, and 20 °C) (T_m = 23–24 °C; Hong & Hubbell, 1972). Examples of the rapid increase in absorbance at 37 and 15 °C are shown in Figure 1, curves a and b, respectively, and clearly demonstrate the large differences in absorbance kinetics at 380 nm at these two temperatures. The first step in determining whether or not this dynamic increase in absorbance at 380 nm corresponds to the formation of meta II was to verify that an equilibrium mixture of meta I and meta II is rapidly established following photolysis, as in the native disk membrane. Equilibrium spectral measurements of the mixture of photochemical intermediates present ca. 3 s after a brief strobe flash were made at 30 and 37 °C. An example of the resulting corrected, deconvoluted difference spectra at 37 °C is given in Figure 2A, and shows that within 3 s of photolysis the mixture of photochemical intermediates consists of meta I and meta II in apparent equilibrium. These kinetic and equilibrium spectral measurements strongly suggest that rhodopsin in DMPC vesicles forms a normal meta II state, in that the increase in absorbance at 380 nm has a rise time of less than 5 ms at 37 °C, and several hundred rise times later, the photointermediates present can be resolved as an absorption band centered at 380 nm (meta II) and a band centered at 478 nm (meta I). Both the observed rise time and the position of the deconvoluted bands are characteristic of the behavior of rhodopsin in disk membranes.

The second step in identifying the photochemical intermediate having maximal absorbance at 380 nm as meta II was to establish that the presence of G_t enhanced the 380-nm species at the expense of meta I, the 478-nm species, in the equilibrium mixture. The equilibrium spectral measurement shown in Figure 2A was repeated with G_t added to the sample at a ratio of one molecule of G_t per photolyzed rhodopsin molecule. Assuming that half of the photolyzed rhodopsin molecules are oriented with their cytoplasmic surface exposed to the exterior of the vesicle, this produced an effective ratio of two G_t per exposed, photolyzed rhodopsin. The population of molecules with their cytoplasmic surface exposed to the

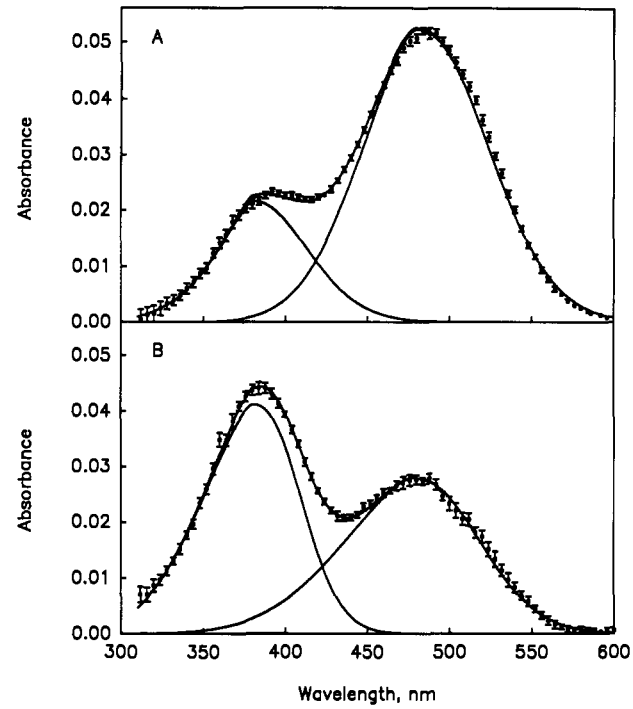

FIGURE 2: Meta I and meta II deconvoluted from equilibrium difference spectra acquired 3 s postflash, rhodopsin in DMPC vesicles at 37 °C in isotonic, 10 mM PIPES buffer, pH 7.0. (A) Meta I and meta II in the absence of G_t. (B) Meta I and meta II in the presence of excess G_t.

Table I: Percent of G_t Activated by Photolyzed Rhodopsin in DMPC and POPC

temp (°C)	time (min)	DMPC	POPC
30	3	97 ± 5	95 ± 5
5	3	7 ± 2	99 ± 1
	8	30 ± 2	
	13	55 ± 2	

interior of the vesicle cannot bind G_t, and thus the meta I–meta II equilibrium of this population is unperturbed. The large shift in the *observed* meta I–meta II equilibrium induced by the presence of G_t is shown in Figure 2B. After accounting for the population which cannot react with G_t, the increase in equilibrium meta II indicates that at least 95% of the molecules exposed to the extravesicular space contribute to the 380-nm peak. The fraction of the interior-facing population which is distributed in the meta I portion of the unperturbed equilibrium accounts for essentially all of the absorbance peak centered at 478 nm in Figure 2B. Therefore, Figure 2 demonstrates that the 380-nm species formed in a DMPC bilayer is in dynamic equilibrium with a species centered at 478 nm, and is stabilized by bound G_t. Both of these activities are fundamental properties of meta II. The G_t-induced enhancement of the 380-nm band at the expense of the 478-nm band provides strong evidence that the peak centered at 380 nm in DMPC consists of normal, functional meta II.

Meta II is generally identified with rho*, the form of photolyzed rhodopsin which catalyzes the exchange of GTP for bound GDP on G_t. Therefore, to further establish that the 380-nm species formed rapidly in DMPC consists of normal, functional meta II, the ability of this species to activate G_t was measured. Rhodopsin-containing POPC recombinant vesicles were prepared and analyzed in parallel with the DMPC samples to provide an independent assay of G_t activity. During a 3-min incubation in room light, at 30 °C, all of the G_t in both the DMPC and POPC samples was activated by

Table II: Values of K_{eq} in DMPC Vesicles and ROS Disk Membranes

temp (°C)	DMPC	ROS disk membranes[a]
5	0.03 ± 0.01	
15	0.05 ± 0.01	
20	0.064 ± 0.02	2.31 ± 0.13
27	0.28 ± 0.06	
30	0.35 ± 0.08	4.4 ± 0.7
37	0.66 ± 0.10	7.3 ± 0.6
45	1.0 ± 0.13	

[a] These values are taken from Straume et al. (1990).

Table III: Equilibrium Thermodynamic Properties of Meta I–Meta II in DMPC Recombinant Vesicles: Comparison with ROS Disk Membranes[a]

bilayer	parameter	meta II_{fast}	meta II_{slow}	meta II_{total}
DMPC	ΔH	17.0 ± 2.1	11.0 ± 1.9	13.7 ± 0.5
above T_m	ΔS	52.4 ± 4.9	31.6 ± 3.7	43.3 ± 1.7
	ΔG_{25}	1.43 ± 0.12	1.06 ± 0.06	0.82 ± 0.05
DMPC	ΔH	4.9 ± 1.4	7.7 ± 1.3	6.4 ± 0.6
below T_m	ΔS	9.8 ± 4.9	19.5 ± 4.3	16.2 ± 2.1
	ΔG_{25}	1.96 ± 0.17	1.92 ± 0.10	1.53 ± 0.07
disks[b]	ΔH	10.7 ± 2.0	10.2 ± 0.9	10.6 ± 0.5
	ΔS	33.7 ± 6.7	36.5 ± 3.0	37.9 ± 1.8
	ΔG_{25}	0.62 ± 0.10	−0.62 ± 0.04	−0.70 ± 0.03

[a] Values of ΔH, ΔS, and ΔG_{25} and uncertainties corresponding to one standard deviation derived by nonlinear least-squares fit to equilibrium constants as a function of temperature. [b] Values from Straume et al. (1990).

rho* (Table I). This result demonstrates that at temperatures above the gel-to-liquid-crystalline-phase transition photolyzed rhodopsin in DMPC is capable of activating G_t in a manner similar to that observed in other recombinant bilayers. In contrast, at 5 °C, a similar incubation yielded activation of all the G_t in the POPC sample, but only produced activation of 7% of the G_t in the DMPC sample. Continued incubation for a total of 8 and 13 min increased the level of activation to 30% and 55%, respectively (Table I). This result for rhodopsin in the gel-state bilayer is consistent with the small amount of equilibrium meta II observed in the flash photolysis measurements. The G_t activation measurements conclusively demonstrate that photolyzed rhodopsin in DMPC, both above and below T_m, forms meta II which is capable of activating G_t.

The meta I ↔ meta II equilibrium constant, K_{eq}, was derived via analysis of the flash photolysis data in terms of the branched meta II model (Straume et al., 1990) (Table II). At 30 and 37 °C, K_{eq} was also determined from analysis of equilibrium spectral data; these values agreed within experimental error with those derived from the kinetic data. Agreement between these two methods of determining K_{eq} was previously demonstrated for both ROS disk membranes (Straume et al., 1990) and egg PC–cholesterol recombinant vesicles (Mitchell et al., 1990b). The distinct effects of the gel-phase and liquid-crystalline-phase bilayers on the meta I ↔ meta II equilibrium are reflected in the wide range of values of K_{eq} above and below T_m, as shown in Table II. Previously determined values of K_{eq} for ROS disk membranes (Straume et al., 1990) are included in Table II to facilitate comparison with the formation of meta II in the disk membrane.

The temperature dependence of the separate meta I ↔ meta II_{fast} and meta I ↔ meta II_{slow} equilibria along with the overall meta I ↔ meta II_{total} equilibrium was examined in terms of ΔH, ΔS, and ΔG_{25}. The results of this analysis for DMPC in the gel and liquid-crystalline phases are given in Table III, along with the corresponding parameters for ROS disk membranes (Straume et al., 1990). Reduced values of K_{eq} in

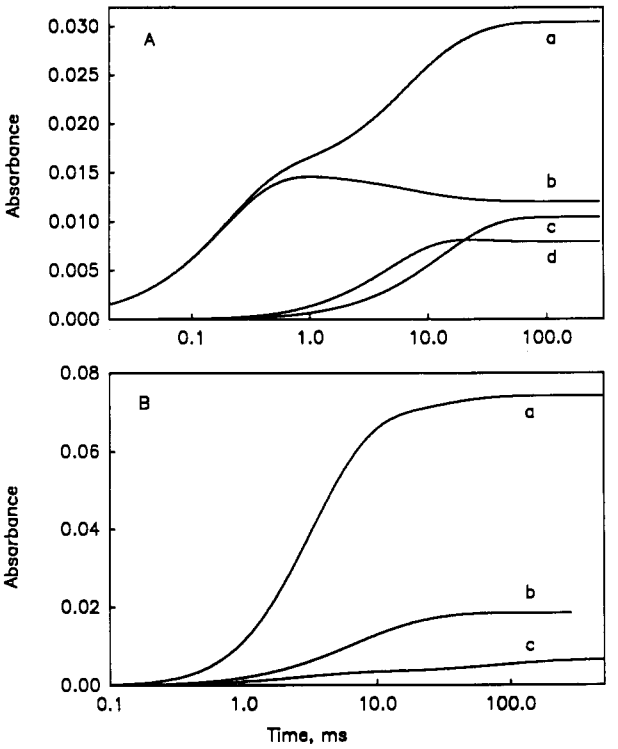

FIGURE 3: Deconvolution of observed absorbance kinetics at 380 nm. (A) Complete deconvolution for rhodopsin in DMPC vesicles at 25 °C: total absorbance change (a); meta I (b); meta II_{slow} (c); meta II_{fast} (d). (B) Kinetic behavior of normalized meta II_{total} for rhodopsin in ROS disk membranes (a), in liquid-crystalline-phase DMPC (b), and in gel-phase DMPC (c).

DMPC (Table II) are the result of increases in ΔG_{25}, but the thermodynamic changes which produce these increases are different for the two phases of DMPC. For meta II_{total} in the liquid-crystalline phase, ΔS and ΔH are both larger than in disks, resulting in an increase in overall ΔG_{25}, whereas in the gel phase, ΔH is *smaller* than in disks, but ΔG_{25} is much larger due to a sharp reduction in ΔS. Thus, the gel-phase bilayer reduces the equilibrium concentration of meta II due to a reduction in relative entropy between meta I and meta II, while the liquid-crystalline bilayer decreases meta II via an increase in relative enthalpy.

Analysis of the kinetic data in terms of the branched meta II model also makes it possible to separate the absorbance transient observed at 380 nm into the distinct contributions due to meta I, meta II_{fast}, and meta II_{slow}, and this type of separation for rhodopsin in DMPC at 25 °C is shown in Figure 3A. Extraction of the contribution due solely to meta II facilitates comparison of the kinetics of meta II formation in different lipid environments. The formation of meta II_{total}, deconvoluted from the total absorbance change observed at 380 nm, in DMPC above and below T_m and in ROS disk membranes at 25 °C is shown in Figure 3B. The rate constants used to obtain these curves were calculated by using values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ which had been derived via a transition-state analysis of the kinetic data. The large differences in steady-state absorbance between the three curves reiterate the differences in K_{eq} given in Table II. A convenient measure of the rate of meta II formation is the time it takes for the absorbance to increase to within $1 - 1/e$ of the steady-state value. In ROS disk membranes at 25 °C, this characteristic time is 4.0 ms, and in DMPC, it increases to 8.1 and 28.5 ms in the liquid-crystalline and gel phases, respectively. Thus, in terms of both kinetics and extent of formation, the gel-phase DMPC bilayer has a more pronounced negative effect on meta

II than the liquid-crystalline-phase bilayer.

Discussion

The results of the studies reported herein pertain to the ability of integral membrane proteins to undergo conformational changes associated with their functional activation in bilayers formed from lipids containing saturated acyl chains; this process is exemplified by the formation of meta II from photolyzed rhodopsin in DMPC bilayers. Previous investigations of meta II formation in DMPC bilayers have concluded that little or no meta II is formed in this system, near neutral pH, either above or below the T_m of DMPC (O'Brien et al., 1977; Baldwin & Hubbell, 1985). The implication drawn from these earlier studies is that rhodopsin is incapable of signal transduction activity in a DMPC bilayer. We have reinvestigated this question using criteria for meta II which were formulated in terms of properties of meta II which are directly related to its role in signal transduction and are characteristic of its behavior in disk membranes. Meta II is the last photointermediate which forms rapidly enough to participate in signal transduction. Therefore, one criterion for the formation of meta II is that it form on the millisecond time scale, as it does in disk membranes. Two additional criteria are (2) in the absence of GTP, G_t should bind to meta II and form a stable complex with meta II, thereby enhancing the equilibrium concentration of meta II, and (3) the functional expression of this binding should be observed in the presence of GTP as the catalyzed exchange of GTP (or GMPPNP) for GDP on G_t. The properties associated with these three criteria are a direct consequence of the functionally required change in protein conformation which is commonly associated with the role of rhodopsin in visual signal transduction.

The results presented in Figures 1 and 2 and Table I demonstrate that the behavior of rhodopsin in DMPC bilayers parallels its behavior in disks for the three stated criteria, and as such constitute incontrovertible evidence that rhodopsin photolyzed in a DMPC bilayer forms the functionally active photointermediate meta II. In DMPC, this photointermediate forms in a few milliseconds and then remains in a quasi-stable equilibrium with meta I for at least several seconds. In the presence of G_t, meta II formation is enhanced in DMPC vesicles; this photointermediate accounts for nearly all of the meta I $\leftrightarrow$ meta II equilibrium mixture associated with the photolyzed rhodopsin that has its G_t binding site oriented toward the outer surface of the vesicle bilayer. Furthermore, these results show that photolyzed rhodopsin in DMPC bilayers is capable of activating G_t, demonstrating the formation of rho* in this system. Although the equilibrium concentration of meta II in DMPC is severly reduced compared with the disk membranes (see Table II), at 37 °C the meta I $\leftrightarrow$ meta II equilibrium consists of approximately 40% meta II, demonstrating substantial formation of meta II at physiological temperature in a bilayer consisting of phospholipids with saturated acyl chains.

Earlier studies which reported little or no meta II formation in DMPC bilayers did not address the question of the production of a functional form of photolyzed rhodopsin and utilized very different experimental conditions, which did not allow a quantitative analysis of the observed spectral data. In the most recent of these, Baldwin and Hubbell (1985) bleached samples for 30 s (unspecified percent bleach), and their acquisition time for obtaining a complete spectrum on a conventional scanning spectrophotometer was 60 s. In contrast, equilibrium data acquired with a diode array spectrophotometer in the present investigation capture the meta I $\leftrightarrow$ meta II equilibrium 3 s after photolysis with a brief pulsed flash. In addition, the flash photolysis data demonstrate that the 380-nm species observed in rhodopsin-containing DMPC vesicles forms on the same time scale as does meta II in disk membranes. At a given temperature, independent analysis of kinetic and equilibrium spectral data gave the same value of K_{eq} for the DMPC system; this agreement between kinetic and equilibrium data is in accord with previous observations in disk membranes (Straume et al., 1990) and rhodopsin-containing egg PC vesicles (Mitchell et al., 1990b). Finally, our digitized spectra can easily be corrected for small differences in scattering and subtracted from one another with high precision, greatly enhancing the resolvability of small absorption changes observed with some of these samples.

In this study, we have demonstrated that rhodopsin, incorporated into a phospholipid bilayer containing saturated acyl chains, is capable of forming meta II and activating G_t. However, the data shown in Tables II and III and Figure 3B make it clear that the formation of meta II in DMPC is radically diminished when compared with the ROS disk membrane. This implies that while unsaturation of the acyl chains is not strictly required for functional activity, such unsaturation is clearly more permissive with respect to rhodopsin's functional ability than are the saturated acyl chains of DMPC. This observation may be related to the fact that the formation of the meta II state is associated with a positive volume change, which suggests that meta II represents an expanded structure with respect to meta I (Lamola et al., 1974; Attwood & Gutfruend, 1980). This property of meta II coupled with the rigid nature of a gel-phase bilayer accounts for the almost negligible amount of meta II formed below T_m. The results above T_m imply that even in the liquid-crystalline phase a DMPC bilayer is much less able to accommodate formation of meta II than is the disk membrane. It is reasonable to expect that the large number of highly unsaturated acyl chains of the disk membrane phospholipids plays a role in the higher level of meta II production in the disk membrane. Studies are underway to correlate the formation of meta II in DMPC with the physical properties of this bilayer as has been done for meta II formation in bilayers formed of unsaturated egg PC (Mitchell et al., 1990b).

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Mapping of Antigenic Epitopes on the α1 Subunit of the Inhibitory Glycine Receptor†

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ABSTRACT: The inhibitory glycine receptor (GlyR) is a ligand-gated chloride channel protein that occurs in developmentally regulated isoforms in the vertebrate central nervous system. Monoclonal antibodies (mAbs) against the GlyR distinguish neonatal and adult GlyR proteins by identifying distinct α subunit variants within these receptor isoforms. Here, bacterially expressed fusion proteins of the rat GlyR α1 subunit were used to localize the major antigenic epitopes of this protein within its N-terminal 105 amino acids. Synthetic peptides allowed further fine mapping of two mAb binding domains. MAb 2b, specific for the adult α1 subunit, bound to a peptide corresponding to amino acids 1-10, whereas mAb 4a, which recognizes both neonatal and adult GlyR isoforms, reacted with a peptide representing residues 96-105 of the α1 polypeptide. These data define unique and common antigenic epitopes on GlyR α subunit variants.

The inhibitory glycine receptor (GlyR)[1] of mammalian spinal cord is a ligand-gated chloride channel composed of two types (α and β) of homologous membrane-spanning subunits (Betz & Becker, 1988; Langosch et al., 1988; Betz, 1990). Immunological, pharmacological, and molecular cloning data indicate considerable subtype diversity of the ligand-binding α subunit of this receptor. Upon affinity purification from adult mammalian spinal cord, it displays an apparent molecular weight of 48K (Pfeiffer et al., 1982; Graham et al., 1985; Becker et al., 1986). In newborn rodents, a neonatal receptor species predominates which differs from the adult GlyR in binding affinity for the selective antagonist strychnine and apparent M_r (49K) of its α subunit (Becker et al., 1988). Recently, complementary and genomic DNAs encoding four variants of the α subunit have been isolated from rodents and man and shown to represent transcripts of distinct genes (Grenningloh et al., 1987, 1990a; J. Kuhse, V. Schmieden, and H. Betz, submitted for publication; B. Matzenbach, Y. Maulet, H. Betz, unpublished data). In case of the adult type α subunit [now termed α1; see Greenningloh et al. (1990a)], alternative splicing further extends GlyR α subunit heterogeneity (Malosio et al., submitted for publication).

Previous biochemical (Schmitt et al., 1987; Becker et al., 1988) and immunocytochemical (Triller et al., 1985, 1987; Altschuler et al., 1986; Araki et al., 1988; Van den Pol & Gorcs, 1988) studies of the postsynaptic GlyR complex have relied on the use of monoclonal antibodies (mAbs) for identifying specific receptor polypeptides. In particular, a quantitative dot receptor assay (DORA) has been employed to quantify α subunit levels in different brain regions and disease states (Becker et al., 1986, 1989). Moreover, differences in antigenic epitopes have been exploited to define neonatal and adult GlyR isoforms in primary cultures of spinal cord (Hoch et al., 1989) and mutant animals (C.-M. Becker and H. Betz, submitted for publication). Whereas on immunoblots the α1 subunit characteristic of the adult GlyR reacts with three mAbs (1a, 2b, and 4a) obtained by immunization with affinity-purified native receptor (Pfeiffer et al., 1984), the neonatal α subunit variant is only recognized by mAb 4a (Becker et al., 1988), suggesting variations in α subunit primary structure to underly these antigenic differences. To further define their molecular basis, we now have mapped the binding sites of different antibodies recognizing the α1 subunit of the adult rat GlyR by using bacterial fusion proteins and

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[1] Abbreviations: DORA, dot receptor assay; ELISA, enzyme-linked immunoassay; GlyR, inhibitory glycine receptor; mAb, monoclonal antibody; PNF, proximal N-terminal fragment; DNF, distal N-terminal fragment; ICF, intracellular fragment.

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