Deoxylysolecithin and a New Biphenyl Detergent as Solubilizing Agents for Bovine Rhodopsin. Functional Test by Formation of Metarhodopsin II and Binding of G-Protein†

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Received October 22, 1986; Revised Manuscript Received February 13, 1987

ABSTRACT: The protein–detergent interaction in rhodopsin–detergent micelles has been investigated by using formation of metarhodopsin II (MII) as a monitor. Two detergents of different structural rigidity have been applied. One of them is [3-(lauroyloxy)propyl]phosphorylcholine, which has a high conformational flexibility in its hydrophobic moiety like most of the known detergents for rhodopsin. This deoxylysolecithin was originally designed as a detergent for membrane proteins by Weltzien [Weltzien, H. U. (1979) *Biochim. Biophys. Acta 559*, 259–287]. The other detergent, which is highly rigid in its hydrophobic part, has been developed for this study. It consists of a biphenyl derivative and a hydrophilic octaethylene oxide group. Both the formation kinetics of MII and the position of its equilibrium with its tautomeric form, metarhodopsin I (MI), strongly differed in the deoxylysolecithin and biphenyl detergent. Deoxylysolecithin caused very fast MII formation and shifted the equilibrium strongly to MII, like other detergents with alkyl chains as the hydrophobic part. In the biphenyl detergent, however, formation of MII was slow and the MI/MII equilibrium similar to that in the native system. For rhodopsin reconstituted in lipid bilayers, normal MII formation requires a well-adjusted fluidity of the hydrocarbon environment of the protein [Baldwin, P. A., & Hubbell, W. L. (1984) *Biochemistry 24*, 2633–2639], which was explained by an appropriate interfacial pressure at the protein–lipid interface. Extension of this concept would indicate that in the micellar core a degree of fluidity comparable to that of the disk membrane is just achieved with the highly rigid biphenyl structure. The preservation of the normal MI/MII equilibrium in the biphenyl detergent allowed the study of G-protein binding by the accompanying equilibrium shift (so-called extra MII). The fast initial rise of free MII and the slow subsequent formation of the rhodopsin–G-protein complex are observed in two separate components.

Rhodopsin is the light-receptor protein of the vertebrate rod cell. The chromoprotein is the major integral protein of the rod outer segment disk membrane (Daemen, 1973). Photoexcited rhodopsin relaxes through a series of spectral intermediates into a temperature- and pH-dependent equilibrium of metarhodopsin I (MI)[1] and metarhodopsin II (MII) (Matthews et al., 1963). Formation of the spectroscopic species MII coincides with that of the enzymatically active conformation (R_M) which is able to bind G-protein (GTP-binding protein, transducin) (Kühn, 1980; Emeis et al., 1982; Longstaff et al., 1986) and to catalyze GDP/GTP exchange at the nucleotide binding site of G (Fung & Stryer, 1980). G-Protein in the GTP-binding form, in turn, dissociates from rhodopsin (Kühn, 1980) and activates a membrane-bound cyclic GMP phosphodiesterase (Fung et al., 1981).

During interaction of R_M with G, rhodopsin is forced into the MII conformation (Emeis & Hofmann, 1981; Emeis et al., 1982; Bennett et al., 1982; Hofmann, 1985, 1986). This enhanced MII formation, in the presence of excess G-protein, provides a fast spectroscopic monitor of the R_MG complex, the so-called extra MII (Emeis & Hofmann, 1981; Emeis et al., 1982).

A conformational change of the apoprotein during MII formation is not only obvious by the exposure of a G-protein binding site but also by the considerable volume change accompanying the MI/MII transition (Lamola et al., 1974a; Attwood & Gutfreund, 1980). There is evidence that the change of conformation affects the cytoplasmic surface as well as the hydrophobic part of rhodopsin which is embedded in the lipid bilayer of the disk membrane. Reconstitution of rhodopsin in different synthetic bilayers (Applebury et al., 1974; O'Brien et al., 1977; Baldwin & Hubbell, 1985a,b) and solubilization in detergents (Applebury et al., 1974; Williams et al., 1974; Lamola et al., 1974a,b; Steward et al., 1976; Baker et al., 1977) have shown that kinetics and equilibrium of the MI/MII reaction are drastically altered by changing the lipid or detergent host of rhodopsin.

The interaction of rhodopsin with the bilayer does not require specific lipids but rather depends on such integral parameters as membrane thickness and fluidity (Hong & Hubbell, 1973; Baldwin & Hubbell, 1985a,b). Litman et al. (1981) have shown that the activation free energy of the MII formation in the bilayer is determined by a phospholipid rearrangement accompanying the conformational change of rhodopsin during this transition.

† This work was financially supported by the Deutsche Forschungsgemeinschaft (SFB 60).

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[1] Abbreviations: R, rhodopsin; R*, photolyzed rhodopsin; R_M, enzymatically active rhodopsin; *r*, mole fraction of photoactivated rhodopsin per flash; G, G-protein; MI, metarhodopsin I; MII, metarhodopsin II; cmc, critical micelle concentration; WM, washed disk membranes; SMLE, sucrose monolauryl ester; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; PIPES, 1,4-piperazinediethanesulfonic acid; MES, 4-morpholineethanesulfonic acid; Con A, concanavalin A.

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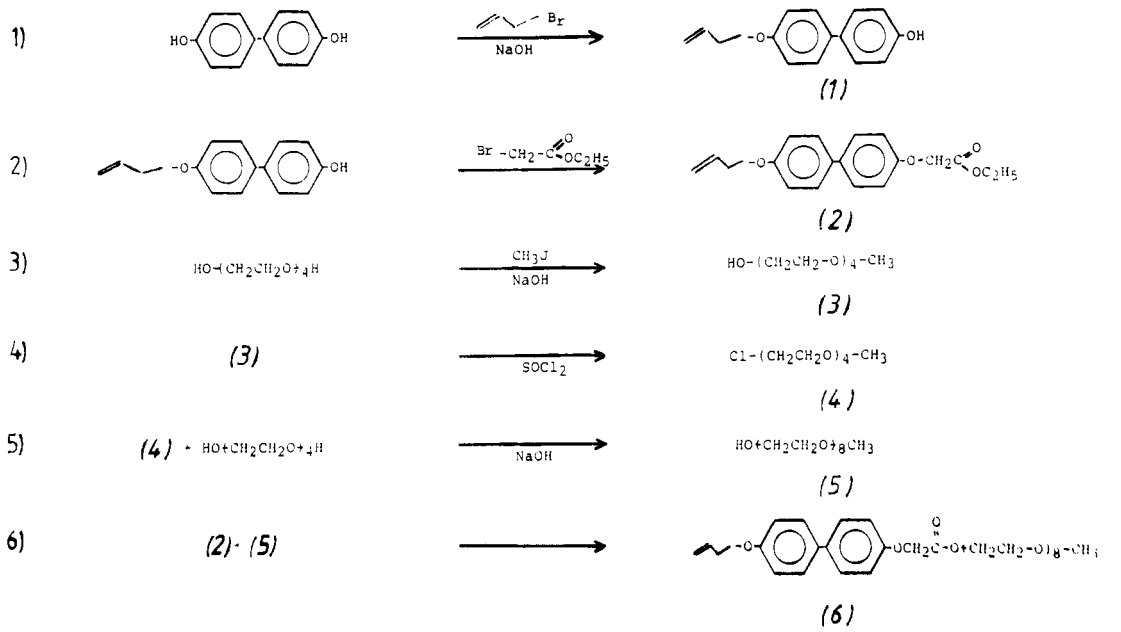

FIGURE 1: Reaction scheme for the synthesis of the biphenyl detergent 4-(allyloxy)-4′-[(2-oxo-3,6,9,12,15,18,21,24,27-nonaoxaoctacosyl)oxy]biphenyl.

A number of detergents have been tested for their ability to extract and to solubilize rhodopsin. The investigations included the test of thermal stability (Stubbs et al., 1976; Knudsen & Hubbell, 1978; Makino et al., 1980; De Grip, 1982; Fong et al., 1982), 11-*cis*-retinal regenerability (Hong & Hubbell, 1973; Stubbs et al., 1976; Knudsen & Hubbell, 1978; Makino et al, 1980; Fong et al., 1982), circular dichroism (Makino et al., 1980), and spectroscopic measurements of the MI/MII transition (Applebury et al., 1974; Lamola et al., 1974a,b; Steward et al., 1976; Baker et al., 1977). The MI/MII transition appears to be sensitive even to minor modifications of the rhodopsin environment that are not indicated by the other tests (Makino et al., 1980).

A good test for the functionality of solubilized rhodopsin has been provided by Kühn (1984), who loaded bleached rhodopsin, solubilized in sucrose monolauryl ester (SMLE), on a concanavalin A–Sepharose column and demonstrated binding of G-protein to this system. Okada et al. (1985), on the other hand, have reported that rhodopsin, when solubilized in the anionic detergent deoxycholate and reconstituted with G-protein, was able to catalyze GDP/GTP exchange and GTP hydrolysis only when the native phospholipid boundary of rhodopsin was preserved.

In this paper we have tested two new detergents as solubilizing agents for rhodopsin. [3-(Lauroyloxy)propyl]-phosphorylcholine is a deoxylysolecithin that was first synthesized and used as a detergent by Weltzien (Weltzien, 1979; Weltzien et al., 1979). This detergent, further on termed deoxylysolecithin, is chemically related to lecithin, one of the mayor lipid species of the disk membrane (Daemen, 1973). The other detergent belongs to a class of tensides, originally developed for the design of lyotropic liquid crystals by polymerization of monomeric tensides (Lühmann & Finkelmann, 1986, 1987). The members of this class all contain a rigid biphenyl group coupled via an ether bond to a short alkyl chain. Both these groups together form a hydrophobic part that is coupled to a hydrophilic ethylene oxide chain (see Figure 1). We have tested a number of such compounds as solubilizing agents for the B800-850 complex from *Rhodopseudomonas capsulata*. Only one of these, namely, 4-(allyloxy)-4′-[(2-oxo-3,6,9,12,15,18,21,24,27-nonaoxaoctacosyl)oxy]biphenyl (see Figure 1), was capable of solubilizing the B800-850 light-harvesting complex of *R. capsulata*, an integral membrane protein complex (Welte et al., 1985). The compound will be termed biphenyl detergent throughout this article.

Rhodopsin appeared appropriate as a test system for comparing the biphenyl detergent with a more conventional agent, deoxylysolecithin, because of the elaborate functional tests and the variety of detergents already applied. Both detergents were tested for their ability to extract rhodopsin from the disk membrane and for their influence on kinetics and extent of MII formation. The behavior of rhodopsin is quite different in these detergents. Strikingly, the micelle of the biphenyl detergent with its rigid hydrophobic structure seems to mimic the highly fluid native membrane environment of rhodopsin much better than the detergents with flexible alkyl chains. In this detergent the MI/MII equilibrium is similar to that in the native disk membrane so that the extra MII monitor could be applied. By this technique we demonstrate that the rhodopsin–G-protein interaction is well-preserved in the new detergent.

Materials and Methods

(*A*) *Preparation of the Biphenyl Detergent*. The biphenyl detergent (see Figure 1) was synthesized in the following way:

Synthesis of 4-(Allyloxy)-4′-hydroxybiphenyl (**1**). A solution of 4,4′-dihydroxybiphenyl (372 g, 2 mol), powdered sodium hydroxide (48 g, 1.2 mol), and a catalytic amount of KI in acetone (2 L) was heated at reflux for 2 h under a nitrogen atmosphere. Freshly distilled allyl bromide (121 g, 1 mol) was then added dropwise over 3 h, and heating was continued for 3 days. The acetone was distilled, and the solid residue was dissolved in hot ethanol (3 L). The solution was cooled, and the bietherified byproduct was removed by suction filtration. The filtrate was concentrated, and the solid residue was dissolved in 4 L of hot 25% (w/v) sodium hydroxide solution. On cooling, the sodium salt of the product crystallized. This material was dissolved in 25% sodium hydroxide solution (2 L), and the solution was neutralized with concentrated hydrochloric acid. The precipitated product was separated by suction filtration and dried in vacuum: yield 86.6 g (38.3%), white crystals; mp 119–121 °C.

Synthesis of [[4-(Allyloxy)-4′-biphenylyl]oxy]acetic Acid Ethyl Ester (**2**). To a stirred solution of 4-(allyloxy)-4′-

hydroxybiphenyl (86.6 g, 0.38 mol) in anhydrous ethanol (600 mL) was added a solution of 8.82 g of sodium (0.38 mol) in ethanol (100 mL). The ethyl bromoacetate (64 g, 0.38 mol) was added dropwise to the mixture over $^1/_2$ h. After a few minutes the product began to precipitate. Thereafter the mixture was heated at reflux for 3 h and then cooled, and the crystals were separated by suction filtration and washed with water. The product was dissolved in hot ethanol (3 L) and allowed to recrystallize on cooling to 0 °C: yield 74.6 g (63%), white crystals; mp 115–118 °C.

Synthesis of Tetra(ethylene glycol) Monomethyl Ether (**3**). Under a nitrogen atmosphere, sodium (9.5 g, 0.41 mol) was added to a solution of tetra(ethylene glycol) (200 g, 1.3 mol) in toluene (100 mL) and heated under reflux for 12 h. The mixture was cooled to room temperature, and CH_3I (59.5 g, 0.42 mol) was added dropwise over 2 h. After it was stirred for 1 h at room temperature, the mixture was heated under reflux for a further 12 h. The toluene was removed under vacuum, and the residue was fractionated over a 30-cm Vigreux column in the vacuum: yield 60.6 g (71%), colorless oil; bp 90 °C/0.0002 Torr.

Synthesis of 3,6,9,12-Tetraoxatridecyl Chloride (**4**). Tetra(ethylene glycol) monomethyl ether (60.6 g, 0.29 mol) was added to pyridine (25.5 mL) and anhydrous tetrahydrofuran (250 mL). The mixture was cooled to 0 °C and stirred under nitrogen while $SOCl_2$ (42 g, 0.35 mol) was added dropwise over 1 h. Thereafter the mixture was refluxed for 4 h. When it was cooled to room temperature, pyridinium hydrochloride precipitated and was separated by suction filtration. Excess $SOCl_2$ and tetrahydrofuran were distilled off from the filtrate at 15 Torr. The residue was fractionated over a 30-cm Vigreux column: yield 55.7 g (85%) colorless oil; bp 75 °C/10^{-4} Torr.

Synthesis of Octa(ethylene glycol) Monomethyl Ether (**5**). To a solution of tetra(ethylene glycol) (123 g, 0.63 mol) and toluene (100 mL) was added sodium (5.8 g, 0.25 mol). The mixture was heated at reflux and under nitrogen for 3 h. The solution was cooled to 90 °C and after a catalytic amount of KI was added, 3,6,9,12-tetraoxatridecyl chloride (55.7 g, 0.25 mol) was added dropwise at 2 drops/min to the solution. The mixture was stirred for another 5 days at 95 °C. Precipitated NaCl was separated by suction filtration, and toluene was removed from the filtrate by distillation. The residue was fractionated twice: yield 39 g (41.5%), colorless oil; bp 175–195 °C/0.0006 Torr.

Synthesis of 4-(Allyloxy)-4′-[(2-oxo-3,6,9,12,15,18,21,24,27-nonaoxaoctacosyl)oxy]biphenyl (**6**). To a mixture of octaethylene glycol monomethyl ether (5 g, 13.6 mmol) and [[4-(allyloxy)-4′-biphenylyl]oxy]acetic acid ethyl ester (2 g, 6.4 mmol) was added $Ti(OCH_2CH_3)_4$ (1.5 mL). The mixture was heated for 4 days at 130 °C under a nitrogen atmosphere. After it was cooled to room temperature, the mixture was hydrolyzed with 2 N HCl (100 mL), and the precipitated TiO_2 was removed by centrifugation. The supernatant was decanted, and the pellet was washed twice with water. The combined supernatants were neutralized with $NaHCO_3$ and then lyophilized. The residue was dissolved in ether–acetone (50:50) and purified by chromatography over silica gel. The pure fractions were concentrated, and the residue was crystallized from ethanol in the centrifuge at −20 °C. The crystals were dried under reduced pressure: yield 1.1 g (26%), colorless wax; mp ca. 20 °C.

(*B*) *Other Techniques.* The critical micelle concentration (cmc) of the biphenyl detergent was determined by the surface tension method with a tensiometer (digital tensiometer K10; Krüss, Hamburg, FRG). Nonanoyl-*N*-methylglucamide was purchased from Oxyl GmbH, Bobingen, FRG, and was used without further treatment. [3-(Lauroyloxy)propyl]-phosphorylcholine, termed deoxylysolecithin in the following, was synthesized and purified according to Weltzien et al. (1979a,b).

Thin-layer chromatography of the biphenyl detergent was performed on Merck TLC aluminum sheets precoated with silica gel 60, F_{254}. The substance was solubilized in 50% ether and 50% acetone, and the sheets were run in the same phase. Later the chromatograms were viewed in UV light, 354 nm, and stained with iodine.

SDS gel electrophoresis was performed according to the procedure of Laemmli (1970) with a 7–15% (w/v) acrylamide gradient gel.

(*C*) *Preparation of Washed Disk Membranes (WM) and Peripheral Proteins.* Bovine rod outer segments were prepared according to a standard procedure (Emeis & Hofmann, 1981). The retinae were shaken in isotonic saline (130 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 mM EDTA, 1 mM DTT, 10 mM PIPES, pH 7) and filtered through a nylon mesh. The resulting crude suspension was layered on a discontinuous sucrose gradient and washed in the isotonic buffer (as above).

Washed membranes divested of peripheral proteins and the protein extract were prepared from this rod outer segment suspension following the extraction procedure described by Kühn (1980). The rod outer segments were osmotically shocked in a low ionic strength buffer (1 mM DTT, 1 mM EDTA, 5 mM PIPES, pH 7.0), gently homogenized, and sedimented. The pellet yielded the washed disk membranes (WM) which were used for the solubilization and rhodopsin purification procedure described below. The supernatant was centrifuged again and yielded the extracted peripheral proteins. This protein extract was stored in liquid nitrogen.

The concentration of G-protein in the protein extract was estimated from saturation of the binding signal (P signal) (Kühn et al., 1981) on WM samples recombined with G-protein according to the method of Bennett and Dupont (1985).

All procedures involving rhodopsin were carried out under dim red light.

(*D*) *Rhodopsin Solubilization and Purification.* For disintegration of the disk membrane and solubilization of rhodopsin, we used the detergent nonanoyl-*N*-methylglucamide because solubilization with the biphenyl detergent at a concentration of 1.5% (w/v) was exceedingly slow (10 h).

Washed membranes were sedimented by centrifugation at 100000*g* for 30 min. The pellet was solubilized with a solution of 1.5% w/v nonanoyl-*N*-methylglucamide and 1 mM DTT at a final concentration of 100 μM of rhodopsin. After it was vortexed for 3 min, the solution was stored for 10 min in the refrigerator at 12–14 °C. The same volume of buffer (300 mM NaCl, 40 mM PIPES, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.2 mM EDTA, pH 6.5) was added, and after 15 min of centrifugation at 100000*g*, the clear supernatant was decanted and used later.

Binding of rhodopsin via its carbohydrate residues to an affinity column with immobilized concanavalin A (Con A–Sepharose) at 12–14 °C was done according to the procedure of DeGrip (1982). The column was subsequently washed with 100 mM nonanoyl-*N*-methylglucamide in the same buffer (8 column volumes). A gradient (100 mM nonanoyl-*N*-methylglucamide to 370 μM biphenyl detergent; 5 column volumes) was applied to the column, and then it was washed with biphenyl detergent in buffer solution (2 column volumes)

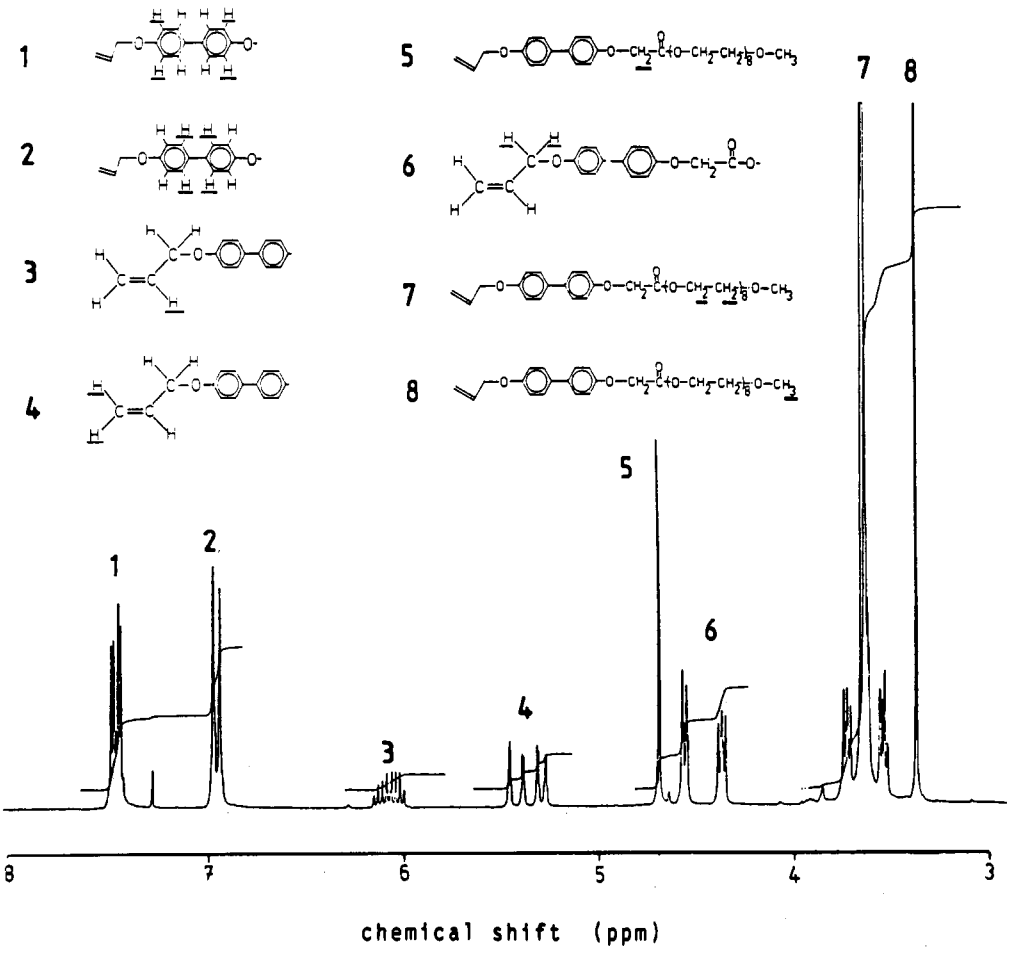

FIGURE 2: 250-MHz ^1H NMR spectrum of the purified biphenyl detergent. The interpretation of the spectrum by contributing protons is indicated. Chemical shifts are given relative to tetramethylsilane.

to complete the detergent exchange. With the detergent and *O*-methylmannose in buffer, the purified rhodopsin was eluted. Alternatively, rhodopsin was washed and eluted in an analogous way with deoxylysolecithin (460 μM). The purity was checked with SDS–polyacrylamide gel electrophoresis according to the procedure of Laemmli (1970). Detergent-solubilized rhodospin samples were stored at 4 °C.

(*E*) *Flash Photolysis Measurements.* All measurements were performed by using the fast two-wavelength spectrometer described by Hofmann and Emeis (1981). A neodymium–YAG laser YG580 (Quantel, Les Ulis, Orsay, France) equipped with a frequency doubler provided an actinic light flash (530 nm).

All measurements were done in isotonic saline (130 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 mM EDTA, 1 mM DTT, 10 mM PIPES, pH 7). For measurements at pH 6 the sample was buffered with 20 mM MES instead of 10 mM PIPES. The detergent concentration used in the sample was 3 times the critical micelle concentration. The rhodopsin concentration was as described in the legends of Figures 4–6.

MII formation was measured by comparing the light-induced changes in the difference of absorption at 380 and 417 nm (Hofmann & Emeis, 1981; Emeis & Hofmann, 1981). In such measurements the photocurrent at 417 nm (isosbestic point of MI to MII) serves as a reference for determining the level of MII (λ_{max} = 380 nm). In these records of the absorption difference changes ($\Delta Abs_{380} - \Delta Abs_{417}$) formation and decay of all photointermediates prior to MII give rise to a fast negative deflection which is followed by the slower positive signal of the absorption change at 380 nm related to the MII formation. The amplitude of the MII formation has therefore to be taken from the deepest point of the record.

The rhodopsin–G-protein interaction was investigated by using extra MII formation as a monitor. This technique [described by Emeis and Hofmann (1981) and Emeis et al. (1982)] profits from the shift of the MI/MII equilibrium toward MII during the interaction of rhodopsin with G-protein. The enhanced formation of stable extra MII in the absence of GTP was investigated by stepwise photolysis as described in detail by Emeis and Hofmann (1981). Briefly, each flash of a series of consecutive flashes photoexcites a fixed mole fraction *r* of rhodopsin. The absolute amount of rhodopsin that is photoexcited per flash thus decreases exponentially. An enhanced formation of MII in the presence of excess G-protein at the first flash appears as an enhancement over the normal exponential course of the signal amplitude. After a rhodopsin turnover of about 10%, all G-protein is complexed and further flashes yield a normal exponential decrease. In Figure 5 the enhanced MII formation (extra MII) is represented by a comparison of the measured MII formation at the first flash with the MII formation at a flash late in the sequence (normal exponential region) normalized by exponential extrapolation to the first flash.

RESULTS

Characterization of the Sample. The purity of the biphenyl detergent was demonstrated by thin-layer chromatography. The NMR analysis yielded correct signals, which are shown in Figure 2.

The critical micelle concentrations of the biphenyl detergent, the deoxylysolecithin, and the nonanoyl-*N*-methylglucamide were found to be 190 μM, 230 μM, and 50 mM, respectively.

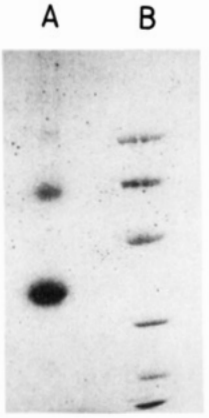

FIGURE 3: SDS–polyacrylamide gel electrophoresis of purified rhodopsin. Lane A was loaded with rhodopsin eluted from Con A–Sepharose. A large band originating from monomeric rhodopsin is seen as well as smaller bands at the dimer and trimer positions, as described previously (De Grip, 1982). Lane B shows marker proteins at 94, 67, 43, 30, 20, and 14 kDa.

We tried to solubilize disk membranes in these detergents with differing success. Fast and efficient solubilization was obtained with deoxylysolecithin. The biphenyl detergent was, however, much less effective in solubilization. Even after an incubation of 12 h in a 1% (w/v) solution of the biphenyl detergent, only a negligible fraction of the disk membranes was solubilized.

We therefore decided to use nonanoyl-*N*-methylglucamide, a commercially available and inexpensive detergent, as a solubilizing agent and to exchange it against the desired detergent during the subsequent rhodopsin purification step on a Con A–Sepharose column (see Materials and Methods). Nonanoyl-*N*-methylglucamide solubilizes the disk membrane very efficiently. This procedure allows the preparation of purified and delipidated rhodopsin in both detergents under the same solubilization conditions. A SDS gel of the eluate is shown in Figure 3.

Both samples of purified rhodopsin in deoxylysolecithin and the biphenyl detergent showed a normal rhodopsin absorption spectrum (not shown). After light exposure at pH 6 and 20 °C the absorption maximum was shifted from 500 to 380 nm, as in rod outer segment disk membranes. Both unphotolyzed samples showed a stable absorption spectrum when stored at 4 °C for at least 1 week. No precipitates or agglomerates were observed during this time.

Formation of Metarhodopsin II. The MI/MII transition was measured on both detergent samples and on suspensions of washed membranes at pH 6 and 21 °C. The recorded signal wave forms of the absorption difference at 380 and 417 nm are shown in Figure 4. The comparison of the signal kinetics shows that the reaction times of the MII formation in these samples differ drastically from each other. The obtained rate constants are given in Table I. In the deoxylysolecithin sample, MII formation is very fast (Figure 4b). The signal wave form is approximately monophasic with a rise time of about 0.6 ms (pH 6, 21 °C), which is ca. 10 times faster than the MII formation observed in washed membrane suspensions (Figure 4a). In the biphenyl detergent, however, a much slower MII formation is observed (Figure 4c). In this detergent the rise time is about 6–7 times longer than in WM suspensions.

A general view on the pH and temperature dependence of MII formation in these systems can be obtained by comparing the signal amplitudes measured at pH 6, T = 20 °C and pH 7.5, T = 8 °C. The final signal amplitudes measured under

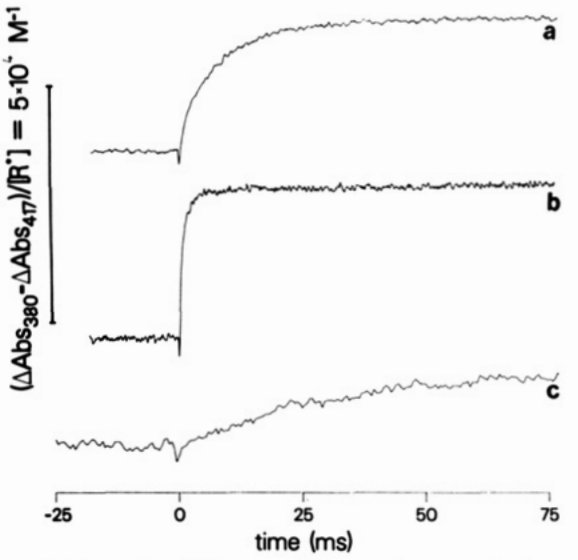

FIGURE 4: Formation of the spectral intermediate metarhodopsin II (MII signals) in different chemical environments: (a) washed disk membranes; (b) deoxylysolecithin; (c) biphenyl detergent. The short flash (532 nm) was applied at t = 0 and photoconverted a mole fraction of 0.05 of the rhodopsin. Each trace is the average of three recordings. Signals are the absorbance change at 380 nm (ΔAbs_{380}) minus the absorbance change at 417 nm (ΔAbs_{417}). The amplitudes are normalized to the amount of photolyzed rhodopsin [(R)r, concentration of rhodopsin times the mole fraction of photolyzed rhodopsin per flash]. Measuring conditions 21 °C, pH 6; concentration of rhodopsin in the sample 4 (first and second signals) and 1 μM (third signal).

Table I: Amplitudes and Time Constants of the Formation of MII in Different Chemical Environments[a]

	washed membranes	deoxylysolecithin	biphenyl detergent
pH 6, T = 21 °C			
A (M^{-1})	29×10^3	32×10^3	19×10^3
k_{obsd} (s^{-1})	160	1730	24
pH 7.5, T = 8 °C			
A (M^{-1})	9.5×10^3	31×10^3	4.2×10^3
k_{obsd} (s^{-1})	6.0	192	3.5

[a] A is the signal amplitude that was normalized to the amount of photolyzed rhodopsin; $A = (\Delta Abs_{380} - \Delta Abs_{417})/(R^*)$. k_{obsd} is the observed time constant of the apparent first-order signal time course.

both conditions in both detergent samples and WM suspensions are given in Table I. The signal amplitudes (determined as described under Materials and Methods) were normalized to the concentration of photolyzed rhodopsin [(R)r, concentration of rhodopsin times the mole fraction of photoactivated rhodopsin per flash]. These values can directly be compared with the change of the molar extinction coefficient ($\Delta\epsilon_{380}$) during the MI/MII transition.

WM suspensions showed the normal behavior of the MI/MII equilibrium. The amplitudes of MII formation for both conditions differ by a factor of 3, while at pH 6 and 21 °C the MI/MII equilibrium in WM suspensions is shifted almost completely to MII [(MII)/[(MI)+(MII)] = 0.95], in agreement with the results of Parkes and Liebman (1984).

In deoxylysolecithin the value of $(\Delta Abs_{380} - \Delta Abs_{417})/(R^*)$ is about the same as in WM suspensions at pH 6, T = 21 °C, which shows that the maximal amount of MII is formed and that the MI/MII equilibrium is completely shifted to MII under all conditions. In the biphenyl detergent, however, the MII formation shows a similar pH and temperature dependence as in WM suspensions with a slight shift of the MI/MII equilibrium toward MI. Ordering the systems in a sequence

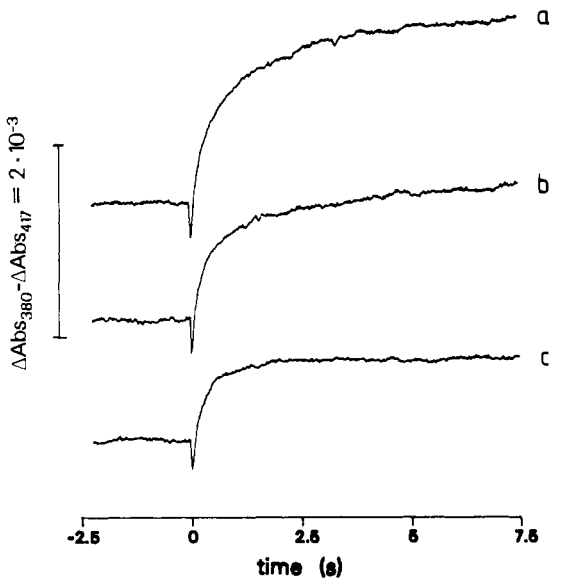

FIGURE 5: Formation of metarhodopsin II (MII) in a series of consecutive flashes, applied to rhodopsin in biphenyl detergent and in the presence of G-protein: (a) first flash; (b) second flash; (c) average of flashes 5 and 6. Each flash photoconverted a mole fraction of $r = 0.04$ of the remaining (nonphotoactivated) rhodopsin. Concentration of rhodopsin in the sample 2.6 μM. Mole fraction of G-protein relative to rhodopsin $g = (G)/(R) \approx 0.07$. All signal amplitudes are normalized by extrapolation to the first flash (see Materials and Methods). Under the conditions (15 °C, pH 7.5) MII rises in seconds. Determination of the MII levels from the absorption changes as in Figure 4. Note that MII formation is enhanced for the first flash (extra MII).

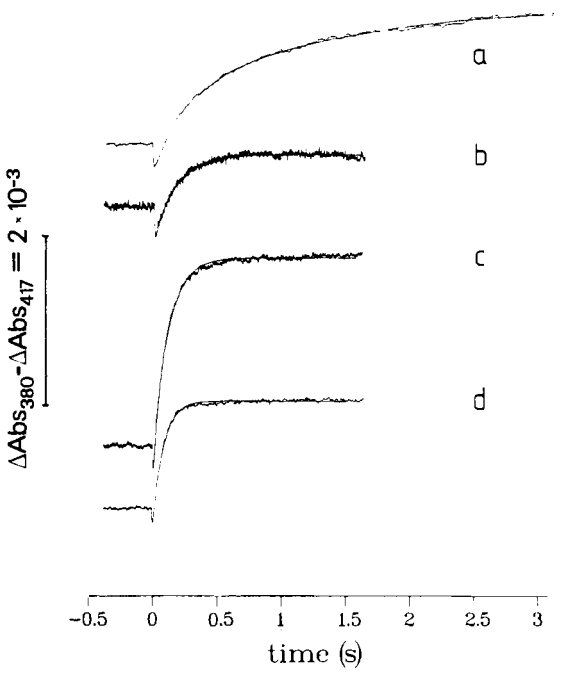

FIGURE 6: Formation of metarhodopsin II (MII) and of the MII–G-protein complex in biphenyl detergent and WM suspensions in the absence and presence of G-protein: (a) in biphenyl detergent with peripheral proteins; (b) in biphenyl detergent without peripheral proteins; (c) in WM suspensions with peripheral proteins; (d) in WM suspensions without peripheral proteins. Measuring conditions as in Figure 5. The signals were fitted by using a computer fit program. The fitted signal wave forms of a single first-order reaction [lanes b–d, $A[1 - \exp(k_{obsd}t)]$] and the sum of two first-order reactions [lane a, $A_1[1 - \exp(k_{obsd}t)] + A_2[1 - \exp(k_{ex}t)]$] are shown as solid lines in the noise of the signals. The constants obtained by the fit are (a) $A_1 = 0.9 \times 10^{-3}$, $k_{obsd} = 5.8\ s^{-1}$, $A_2 = 1.7 \times 10^{-3}$, $k_{ex} = 0.6\ s^{-1}$; (b) $A = 0.9 \times 10^{-3}$, $k_{obsd} = 5.6\ s^{-1}$; (c) $A = 2.9 \times 10^{-3}$, $k_{obsd} = 8.5\ s^{-1}$; (d) $A = 1.6 \times 10^{-3}$, $k_{obsd} = 12\ s^{-1}$. Comparison of lanes a and c shows that G-protein binding is much slower in the detergent than in WM suspensions.

of increasing favor of MII results in a succession of biphenyl detergent, WM, and deoxylysolecithin. The same is obtained for increasing rates of MII formation.

Binding of G-Protein. The similarity of the MI/MII equilibrium in rhodopsin–biphenyl detergent micelles to the one in the native disk membrane system encouraged us to investigate the rhodopsin–G-protein interaction in this preparation by means of the extra MII monitor (see Materials and Methods). Solubilized rhodopsin was recombined with protein extract in the absence of GTP, where a stable complex is formed between R_M and G-protein in its inactive GDP-binding form (Kühn, 1980). The mole fraction of G-protein relative to rhodopsin was about 0.07. Records of MII formation in this sample are shown in Figure 5. In this measurement a series of consecutive flashes was applied, in which each flash photoactivated a mole fraction of $r = 0.04$ of the remaining (nonactivated) rhodopsin. The signals from the first flash (Figure 5a) and second flash (Figure 5b) and the average of the fifth and sixth flashes (Figure 5c) were normalized by exponential extrapolation to the same amount of photoactivated rhodopsin in order to allow a direct comparison of the signal amplitudes. In this representation all signals would come to the same level if MII formation would only be determined by the photolysis of the pigment. This is not observed. The level of the signal from the first flash (upper trace) is much higher than that of the following signals, indicating extra MII formation in the presence of excess G-protein. The signal of the second flash shows only a little enhancement of MII formation compared to the lower trace. At these flashes most G-protein is already bound; the MII formation corresponds, therefore, to the normal equilibrium level. The enhanced MII formation in the presence of G-protein indicates well-preserved light-induced interaction of the solubilized rhodopsin with G-protein in the biphenyl detergent.

Analysis of such signals gives information about the reaction kinetics of the R_MG interaction. We compared the kinetics of extra MII formation at the first flash with the MII formation under the same conditions in the absence of protein extract (Figure 6a,b). We attempted to fit the signal wave forms in both cases by a first-order reaction or a sum of two first-order reactions, using a least-square fit program (based on the Marquart algorithm; Marquart, 1963). This analysis shows that MII formation in the absence of G-protein is monophasic, corresponding to a first-order reaction mode (Figure 6b). The apparent rate constant of this signal was determined to be $k_{obsd} = 5.6\ s^{-1}$, which is composed of the rate constants of the forward (k_1) and backward (k_{-1}) reactions of the equilibrium; $k_{obsd} = k_1 + k_{-1}$.

By contrast, the signals from the sample containing protein extract are biphasic (Figure 6a). The rate constant of the fast component corresponds to the MII formation measured in the absence of G-protein (rate constant $k_{obsd} = 5.7\ s^{-1}$). The superimposed slow component has a rate constant of $k_{ex} = 0.52\ s^{-1}$. There is good agreement of the fitted signal wave forms with the measured records (Figure 6, solid lines). The slow component (k_{ex}) reflects the formation of the extra part of MII due to the interaction with the G-protein arising after formation of an initial amount of free MII (fast component).

The complex formation in the detergent is much slower than that at the membrane. Figure 6c,d shows the formation of MII in WM suspensions in the absence and presence of protein extract measured under the same conditions as in Figure 6a,b. The wave form of the extra MII formation in WM suspensions is under these conditions monophasic (Figure 6c). In this system the forward reaction of the MII equilibrium is rate

limiting for the complex formation of membrane-bound G-protein (Emeis et al., 1981; Schleicher & Hofmann, 1987). The MII formation in the absence of protein extract (Figure 6d) has a rate constant of $k_{obsd} = k_1 + k_{-1} = 12\ s^{-1}$. The observed rate constant of the extra MII formation $k_{obsd} = 8.5\ s^{-1}$ corresponds to the forward reaction (k_1) of the MI/MII equilibrium.

Extra MII from the second flash is substantially slower than that from the first flash (compare parts a and b of Figure 5), corresponding to the decrease of excess G-protein from the first to the second flash due to the R_MG complex formation at flash 1.

Discussion

Metarhodopsin II in Biphenyl Detergent and Deoxylysolecithin. We have tested a couple of new detergents as solubilizing agents for rhodopsin. The spectroscopic intermediate metarhodopsin II (MII), which provides the binding conformation (R_M) for the G-protein, was used as a sensitive indicator of the effect of the detergent on the protein. We have analyzed the kinetics of formation of MII and its equilibrium with metarhodopsin I (MI). Both these features were very different for the two detergents.

Deoxylysolecithin was very efficient in dissolving the disk membrane. Rhodopsin, when solubilized in this detergent, showed a very rapid MII formation and a complete shift of the MI/MII equilibrium to MII. The biphenyl detergent, however, was very inefficient in extracting rhodopsin from the disk membrane. Purified rhodopsin solubilized in this detergent had to be prepared by treating rhodopsin with an efficiently solubilizing agent, for example, nonanonyl-*N*-methylglucamide, and a subsequent detergent exchange on a Con A–Sepharose column. In these samples MII formation was slower than in membrane suspensions (WM). The equilibrium showed a pH and temperature dependence similar to that observed in WM suspensions with a slight equilibrium shift toward MI.

By comparing these data with studies using other detergents, we are led to the conclusion that the behavior of rhodopsin in the detergent environment is related to the structure of the hydrophobic region of the detergent molecule. In all detergents whose hydrophobic region consists mainly of an alkyl chain, a very fast MI/MII reaction and a shift of the MI/MII equilibrium to MII are observed. This is the case not only for the deoxylysolecithin investigated in this study but also for lauryldimethylamine oxide, decyldimethylamine oxide, Emulphogene BC720, and Triton X-100. Fast MII formation in these detergents was reported in several studies (Lamola et al., 1974a,b; Applebury et al., 1974; Baker et al., 1977; Stewart et al., 1975), and an equilibrium shift toward MII in lauryldimethylamine oxide and Emulphogene was described by Lamola et al. (1974a,b). The only detergent that did not fit in this group was the steroid digitonin. On rhodopsin in digitonin micelles the overall rate of MII formation (Sengbusch & Stieve, 1971; Baker et al., 1977) and the MI/MII equilibrium (Lamola et al., 1974a,b) are similar to those in disk membranes. The deviation of MI/MII kinetics from the first-order time course (Erhardt et al., 1966; Shichi et al., 1977) suggests inhomogeneities in the preparation. This might partly be due to the variability of digitonin as a natural product (De Grip, 1982).

Digitonin and the biphenyl detergent have in common a rigid molecular structure of the hydrophobic region. Thus it appears that high rigidity of the molecules surrounding rhodopsin in a micelle is required for MII formation as in the native disk membrane. We shall compare this result with the properties of rhodopsin in recombinant phospholipid bilayers. Baldwin and Hubbell (1984a,b) have investigated MII formation in lipids of different unsaturation and length of the hydrocarbon chain. According to their data MII formation requires a certain degree of fluidity in the protein environment that can, for example, be produced by unsaturation of the alkyl chains. MII formation was generally prohibited for all tested membrane systems below their characteristic phase-transition temperature. The authors proposed a thermodynamic model in which the rhodopsin–lipid interaction is characterized by an interfacial free energy (interfacial tension) at the protein–lipid interface. In this model, a high degree of fluidity in the protein environment favors the solvation of the protein surface, thereby reducing the intrafacial tension at the protein–lipid interface. The resulting lower pressure onto the protein acts in favor of the MII conformation, which has a larger volume than MI (Lamola et al., 1974a; Attwood & Gutfreund, 1980).

An analogous view of the interaction of rhodopsin with its environment should also apply to the rhodopsin–detergent interaction in micelles. The observed shift of the MI/MII equilibrium to the right in detergents with saturated alkyl chains ($n = 12$) would then indicate a sufficiently high fluidity and low interfacial tension in the core of the micelles. This is in contrast to the effect of saturated and sufficiently short ($n \leq 14$) lipids forming bilayers in which MII is completely prohibited (Baldwin & Hubbell, 1985a,b). This discrepancy shows clearly that the interfacial tension is determined not only by molecular structure but also by packing constraints. Conceivably, the low curvature of the bilayer favors the alignment of extended hydrocarbon chains while the high curvature of micelles imposes packing constraints favoring a disordered fluid phase. The influence of increasing curvature on the phase behavior of hydrocarbon chains is documented in the literature. In highly curved small liposomes of a diameter of 150–250 Å, the gel-to-liquid phase transition is smeared out, indicating inhibition of the gel phase (Gruenewald et al., 1979; Schmidt & Knoll, 1985; Düzgünes et al., 1983). Because detergent micelles are even smaller (diameter around 60–100 Å; Tanford & Renolds, 1976), a high fluidity of the hydrocarbon core has to be expected and is indicated by experimental evidence (Wennerström & Lindmann, 1979; Lindmann & Wennerström, 1980).

Rhodopsin in micelles of the biphenyl detergent exhibited an MI/MII equilibrium similar to that in the native bilayer environment. We believe that the rigid biphenyl group imposes sufficient rigidity to the fluid hydrophobic core of the micelle to provide the interfacial tension that is necessary for the native free energy gap between MI and MII.

The biphenyl compound demonstrates that at complete sterol system (as in digitonin) is not necessary. On the other hand, the single phenyl group of Triton X-100 seems to be insufficient for creating a rigid micellar core, as indicated by the very fast MII formation in this detergent (Baker et al., 1977). The correct interfacial pressure of the hydrophobic environment could be important for preserving the native tertiary structure of the protein. This viewpoint should be considered in the search for new detergent molecules for membrane proteins.

Binding of G-Protein to Rhodopsin in the Biphenyl Detergent Micelles. Another aspect of this study is that it shows a way of investigating the kinetics of the R_MG interaction also in the absence of membranes. The membrane association of G-protein in the dark considerably enhances its effective local concentration, leading to a fast R_MG binding reaction. In

native membranes the complexation of membrane bound G-protein is rate limited by formation of the reactant MII (Emeis et al., 1982). Thus extra MII is formed with the rate of the MI/MII forward reaction (Emeis et al., 1982). Only at high bleaches or at low concentrations of G-protein does previously non-membrane-bound G-protein give rise to a slow component of a biphasic extra MII formation (Schleicher & Hofmann, 1987). In micellar solutions of rhodopsin the concentration of G-protein is much smaller and the formation of extra MII consequently much slower than for membrane-associated G-protein. This explains why formation of the normal equilibrium MII and that of enhanced MII are observed as two distinct superimposed signal components (Figure 6). For the initial concentration of G-protein in this experiment [$(G) \approx 1.8 \times 10^{-7}$] and the concentration of photoactivated rhodopsin at the first flash [$(R_M) = 1 \times 10^{-7}$], R_MG was formed with a reaction time of about 1.6 s. A lower limit for this time in the native system can be calculated from the known G-protein concentration in the cytoplasmatic space (500 μM; Chabre & Applebury, 1984). The 2800-fold concentration of G-protein in this compartment, compared to the homogeneous concentration in detergent, would yield a reaction time of 600 μs for R_MG formation.

This estimation, which is based exclusively on the effect of G-protein concentration, yields already a rate constant that is fast enough to explain the speed of G-protein activation in the native membrane system. In each elementary cycle of R_MG interaction, nucleotide exchange and G-protein dissociation are slower and therefore rate limiting (Hofmann, 1985; Kohl & Hofmann, 1987). Orientation of G-protein, which presumably results from its membrane association, does not appear to be required for the observed speed of sequential R_MG interaction.

It cannot be decided from these data whether G-protein is free in solution before illumination or associated to the rhodopsin micelles (similar to the membrane association of G-protein in membrane suspensions). At the mole fraction $g = (G)/(R)$ of about 0.07 in the samples, such a preformed structure would not express itself in the signal kinetics. This is obvious by the fact that, after a flash with a relative rhodopsin turnover $r = 0.04$, the instantaneous concentration of micelles containing one R_M and one G-protein is only $(R)gr = (R) \times 0.07 \times 0.04 = 0.0028(R) = 0.04(G)$. Thus, 96% of G-protein is bound to micelles with nonactivated rhodopsin and has to encounter its reaction partner by diffusion.

A rate constant similar to that for the rate of R_MG interaction in detergent has been measured for the transition of G-protein onto a membrane-bound dark binding site (Liebman & Sitaramayya, 1984; Schleicher & Hofmann, 1987). Thus the R_MG interaction rate determined in this study could be rate limited by the solution dark binding transition preceding the actual R_MG complex formation.

Due to the strong shift of the MI/MII equilibrium in deoxylysolecithin and other flexible detergents, the extra MII monitor cannot be used with these preparations, and the R_MG interaction has to be investigated by biochemical methods. Kühn (1984) has shown that G-protein is retained by a Con A–Sepharose column loaded with bleached rhodopsin solubilized in sucrose monolauryl ester (SMLE). The hydrophobic part of this detergent is a flexible alkyl chain. Thus conformational rigidity of the detergent (and a normal MI/MII equilibrium) does not seem to be required for undisturbed R_MG interaction. The recent finding by Okada et al. (1985) that photolyzed rhodopsin in deoxycholate needs a preserved phospholipid boundary to activate rhodopsin might be due to the negative charge of this detergent. The phospholipid presumably keeps the negative charges far enough from the G-protein binding site.

ACKNOWLEDGMENTS

We thank B. Lühmann for important contributions in the early stage of this work. We also thank H. U. Weltzien for providing us with deoxylysolecithin and H. Hamm for critical reading of the manuscript. We further thank I. Bäumle for expert technical assistance and M. Baumstark, K. Diederichs, and T. Willmann for providing us with the IPS computer plot program.

Registry No. **1**, 97344-30-4; **2**, 108970-87-2; **3**, 23783-42-8; **4**, 57722-04-0; **5**, 25990-96-9; **6**, 109011-11-2; [[4-(allyloxy)-4′-biphenylyl]oxy]acetic acid, 25800-40-2; 4,4′-dihydroxybiphenyl, 92-88-6; allyl bromide, 106-95-6; ethyl bromoacetate, 105-36-2; tetra(ethylene glycol), 112-60-7.

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Secondary Structure Determination of Human β-Endorphin by ^{1}H NMR Spectroscopy†

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Received March 11, 1987; Revised Manuscript Received April 16, 1987

ABSTRACT: The ^{1}H NMR spectra of human β-endorphin indicate that the peptide exists in random-coil form in aqueous solution but becomes helical in mixed solvent. Thermal denaturation NMR experiments show that in water there is no transition between 24 and 75 °C, while a slow noncooperative thermal unfolding is observed in a 60% methanol–40% water mixed solvent in the same temperature range. These findings are consistent with circular dichroism studies by other workers concluding that β-endorphin is a random coil in water but that it forms 50% α-helix or more in mixed solvents. The peptide in the mixed water–methanol solvent was further studied by correlated spectroscopy (COSY) and nuclear Overhauser effect spectroscopy (NOESY) experiments. These allow a complete set of assignments to be made and establish two distinct stretches over which the solvent induces formation of α-helices: the first occurs between Tyr-1 and Thr-12 and the second between Leu-14 and extending to Lys-28. There is evidence that the latter is capped by a turn occurring between Lys-28 and Glu-31. These helices form at the enkephalin receptor binding site, which is at the amino terminus, and at the morphine receptor binding site, located at the carboxyl terminus [Li, C. H. (1982) *Cell (Cambridge, Mass.) 31*, 504–505]. Our findings suggest that these two receptors may specifically recognize α-helices.

Because of the wide range of its pharmacological effects, the structure of β-endorphin is of interest. The sequence of this 31 amino acid endogenous opioid peptide, shown in Figure 1, is highly conserved throughout evolution (Li, 1982) and corresponds to the 61–91 C-terminal fragment of the hormone β-lipotropin (Li, 1964). Two of its fragments are also active neuropeptides: Met-enkephalin (Hughes et al., 1975) made up from the first five residues and dynorphin-(1–4) (Goldstein et al., 1979), which consists of the first four residues. β-En-

† This research was supported in part by Grants DA-03434 and GM-02907 from the USPHS to C.H.L. and by NIH Grants RR02300 and RR00711 and NSF Grant DMB84-02348 to O.L. and O.J.
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0006-2960/87/0426-5916$01.50/0