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Evidence for a surface-charge effect

Modulation of the metarhodopsin I/metarhodopsin II equilibrium of bovine rhodopsin by ionic strength

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The effects of ionic strength on formation and decay of metarhodopsin II (MI), the active photointermediate of bovine rhodopsin, were studied in the native membrane environment by means of ultraviolet/visible and Fourier-transform infrared (FTIR) spectroscopy. By increasing the concentration of KCl in the range from hypotonic to 4 M, the apparent \( pK_a \) of the metarhodopsin I(MI)/MIII equilibrium is shifted by approximately pH three, in favor of the MI intermediate. In addition, the apparent rate of MI formation is enhanced by an increase in ionic strength (about twofold in the presence of 2 M KCl). MI decay is independent of the salt concentration. Attenuated-total-reflectance/FTIR data show that the high-salt conditions have no effect on the rigidity of the membrane matrix and do not induce structural changes in the intermediates themselves. Different salts were tested for their ability to shift the MI/MIII equilibrium; however, no clear ion dependence was observed. We interpret these results as an indication for direct involvement of the cytosolic surface charge in the regulation of the photochemical activity of bovine rhodopsin.

Keywords: bovine rhodopsin; metarhodopsin I/metarhodopsin II equilibrium; \( pK_a \); ionic strength; surface charge.

Rhodopsin is the photoreceptor protein located in the disk membranes of retinal rod photoreceptor cells, and is considered to be a model for the superfamiliy of guanine-nucleotide-binding regulatory protein (G protein)-coupled receptors (DeGrip et al., 1983; DeGrip et al., 1985), of which Asp83 has been identified (Rath et al., 1993; Fahmy et al., 1993), and sulfydryl groups (Rath et al., 1994), and (c) a transient change in charge distribution (Bennett et al., 1980; Rüppel and Hagins, 1983). The MI/MIII equilibrium has been shown to be sensitive to temperature, pH (Matthews et al., 1963) and pressure (Lamola et al., 1974; Atwood and Gut Freund, 1980). Environmental properties, such as lipid composition, membrane fluidity (Applebury et al., 1974; DeGrip et al., 1983; Wiedmann et al., 1988; Mitchell et al., 1990; Gibson et al., 1993; Brown, 1994), the degree of hydration (Rafferty and Shichi, 1981; Ganter et al., 1988), and the presence of detergents (Lamola et al., 1974; König et al., 1989) also affect the equilibrium. Furthermore, several mutations involving polar amino acid residues in rhodopsin have been reported that shift the MI/MIII equilibrium (Weitz and Nathans, 1992, 1993; DeCaluwé et al., 1995). These and other studies on rhodopsin analogs or mutants (Longstaff et al., 1986; Sakmar et al., 1989; Robinson et al., 1992; Zvyaga et al., 1993) suggest that the MI/MIII transition is at least partially controlled by local electrostatic interactions and/or charge distributions. In general, it is evident that the MI/MIII transition involves the largest structural change in the protein and that it strongly responds to the environment. It is therefore essential to study local effects in the native environment, the photoreceptor membrane.

If the MI/MIII transition were controlled basically by electrostatic factors, it should respond to the ionic strength of the solvent. Salt effects on the kinetics of rhodopsin bleaching have been reported for rhodopsin in detergent solution (Arnis and ...
Hofmann, 1993). However, since almost all detergents dramatically alter the pH dependence of the MI/MII equilibrium (Matthews et al., 1963; Lamola et al., 1974; König et al., 1989), effects of ionic strength on the apparent pK of this equilibrium have not been investigated. We have analyzed these aspects using ultraviolet/visible and Fourier-transform infrared (FTIR) spectroscopies on the native rod-outlet-segment (ROS) membrane. While ultraviolet/visible spectra primarily contain information about the chromophore and its electrostatic environment, FTIR spectra contain vibrational information of the entire complex and can be used to monitor conformational changes in the rhodopsin molecule. Here, we demonstrate that several properties of the rhodopsin photocascade respond to an increase in ionic strength, without a marked ion dependence: the formation but not the decay of MII is accelerated; and the pK of the MI/MII equilibrium is shifted upwards, increasingly favoring MII at increasing salt concentrations. We will discuss the implications of these findings for a role of the protein-surface charge in the regulation of rhodopsin function.

MATERIALS AND METHODS

ROS were prepared as previously described (DeGrip et al., 1980). The resulting photoreceptor membranes had an A380/A500 ratio of 2.0—2.2. All manipulations were performed under dim red light (RG645 cut-off filter; Schott). Standard solutions contained 20 mM buffer, 130 mM NaCl, 10 mM KCl, 3 mM MgCl₂, 2 mM CaCl₂, and 0.1 mM EDTA (buffer A) and 0.1 mM Bistris-propane were used to cover the pH range 5.5—9.0.

Ultraviolet/visible spectroscopy. All ultraviolet/visible analyses were performed on a Perkin Elmer Lambda 215 double-beam spectrophotometer equipped with an end-on photomultiplier detector. A circulating bath connected to the cuvette holder was used to control sample temperature.

Calculation of the amount of MI formed after illumination. The measurements were routinely performed at 10°C, since at this temperature the decay of MII is negligibly slow and does not interfere with our analyses. Samples contained a ROS-membrane suspension (≈2 μM rhodopsin) in either Mes, Mops, Hepes or Bistris-propane and salts as indicated. A spectrum was recorded between 750 nm and 250 nm (170 s) of the initial, unbleached sample (spectrum 1). The sample was illuminated for 10 s (OG530 filter; Schott) and two or three spectra were recorded to verify the stability of the photointermediate spectrum under the experimental conditions (spectrum 2). The relative amount of MI formed after illumination, [(MII) - (MII) at 500 nm] / [bleached rhodopsin] = [MII] - [MII] (Parkes and Liebman, 1984). Buffers Mes, Mops and Bistris-propane were used to cover the pH range 5.5—9.0.

Kinetic analysis of the formation of MII. The formation of MII was studied at —6°C, with a time resolution of 0.1 s, using the rise in absorbance at 380 nm upon bleaching the samples with a short photoflash in the spectrophotometer. Photoreceptor-membrane suspensions (≈2 μM rhodopsin) were studied in buffer A (with or without 2 M KCl) containing 20% glycerol (mass/vol.) to prevent freezing. Flash illumination, using a conventional photoflasher equipped with a Kodak 32 filter, resulted in approximately 40% bleaching/flash. Each sample was illuminated at least twice to evaluate reproducibility. Although the photomultiplier was protected from the photoflash by a Kodak 58 filter, reliable absorbance data could only be collected after 2 s. The apparent rate constants (kₚ) were obtained by fitting a mono-exponential function to the absorbance data.

Kinetic analysis of the decay of MII. The decay of MII to metarhodopsin III (MIII) was analyzed at pH 6, 15°C, by measuring 50 spectra (170 s/spectrum) after bleaching the sample (10 s illumination). We selected 15°C and pH 6 because, under these conditions, photoreconverted rhodopsin decays almost fully and within several milliseconds to MII, while MII decay is dominated by the transition of MII to MIII. Other processes, such as MII → opsin + retinal and MIII → opsin + retinal, proceed only very slowly under these conditions, compared with MII → MIII (Blazynski and Ostro, 1981, 1984; Klinger and Braiman, 1992). Buffer A (Mes) with or without 4 M KCl was used. We took the decrease in the absorbance difference between 380 nm and 418 nm (isosbestic point for the transition; Van-Breugel et al., 1979) as a measure of the decay of MII. Similarly, the rise in absorbance difference between 455 nm (MIII) and 418 nm was used to measure the formation of MII. Fits of a mono-exponential function to these absorbance data were used to calculate the rates of MII decay and MIII formation under both experimental conditions.

FTIR spectroscopy. FTIR analyses were performed on a Mattson Cygnus 100 spectrometer equipped with a liquid-nitrogen-cooled narrow-band HgCdTe detector. The operation of the spectrometer and spectral manipulations were carried out by means of the Expert-IR software package (Mattson). All spectra were taken at 8 cm⁻¹ resolution. Samples were illuminated in the spectrometer for 20—30 s by means of a 20-W halogen lamp equipped with a KG1 infrared filter and an OGS30 cut-off filter (Schott) in the transmission experiments, and a fiberoptics ring illuminator (Schott) in combination with an OGS30 filter in the attenuated total reflectance (ATR)/FTIR experiments. Sample temperature was controlled by means of a circulating bath in the ATR/FTIR experiments and an immersion cooler in combination with a computer-controlled variable-temperature cell (Graseby Specac) in the FTIR transmission experiments.

Static FTIR difference spectroscopy. For analysis of the rhodopsin to MII/MIII transition, FTIR difference spectra were obtained in a similar way to that previously described (Rothchild et al., 1987; DeGrip et al., 1988). Samples were prepared by isopotential spin drying of an aqueous suspension of photoreceptor membranes (containing 2—3 nmol rhodopsin) on an AgCl window (Fishier Scientific Co.). The photoreceptor-membrane films were hydrated with about 2 μl 2Xbuffer A, with or without 4 M KCl, and sealed by means of a rubber O-ring spacer and a second AgCl window. The concentration of buffer A was doubled to enhance its buffering capacity. Difference spectra were obtained at 10°C by subtracting the spectrum (256 scans, 1/m/spectrum) just before illumination from the spectrum immediately after sample illumination. Under the various experimental conditions, the shape of the amide-I band in the absolute

values for MII but generally gives better reproducibility than the classical approach.
infrared dark spectra did not change significantly, indicating that no significant changes in rhodopsin conformation had occurred.

To allow better control of pH and ionic strength, ATR/FTIR experiments were performed. A suspension of photoreceptor membranes (containing ~40 nmol rhodopsin) was dried under a gentle stream of nitrogen to form a film on a horizontal trough-plate germanium ATR accessory (Spectra-Tech). The ATR accessory was mounted with a home-built perspex-flow setup allowing in situ illumination. Buffer A (Mes, pH 6, or Bistris-propane, pH 8.8), with or without 2 M KC1, cooled to 10°C, perfused the film at a rate of 12 ml/min. Difference spectra were calculated by subtracting blocks of spectra (1800 scans, ~7 min) to improve the signal-to-noise ratio.

Kinetic analysis of the decay of MI. The decay of MI was studied in the FTIR transmission mode at 15°C in spin-dried films that were hydrated with 2 M buffer (20 mM Mes, pH 6.0, or Bistris-propane, pH 8.8) and with or without 4 M KC1. The decay was analyzed by taking 60 subsequent spectra (256 scans, 1 min/spectrum) after illumination of the sample. Decay rates of the peak absorbance of various typical MII bands in these difference spectra were obtained by fitting a mono-exponential function to the data. We monitored the absorbance decay of four bands: the negative peaks at 1390 cm\(^{-1}\) and 1435 cm\(^{-1}\), the positive peak at 1687 cm\(^{-1}\), and the sum of the positive peak at 1750 cm\(^{-1}\) and the negative peak at 1768 cm\(^{-1}\) [these can be added because they were shown (Rath et al., 1993; Fahmy et al., 1993) to originate from a frequency shift of the C=O vibration of the carboxyl group in the Asp83 residue and appear in the MII/rhodopsin difference spectrum only].

RESULTS

The pK\(_a\) of the MI/MII equilibrium shifts to higher values at higher salt concentrations. We determined the relative amount of MI formed after bleaching ROS-membrane suspensions at 10°C as a function of pH at five salt concentrations: hypotonic buffer (20 mM buffer and 5 mM KC1); isotonic buffer A (with

FTIR difference spectroscopy at different KC1 concentrations. To establish whether the light-induced structural changes in the presence of 4 M KC1 are similar to those under isotonic conditions, we applied FTIR difference spectroscopy. Fig. 2 shows the ATR-FTIR difference spectra of photoreceptor-membrane films perfused with Bistris-propane buffer A (pH 8.8) under isotonic (top spectrum) and 2 M KC1 (middle spectrum) conditions and with Mes buffer A (pH 6; lower spectrum). The spectra were scaled with respect to the 1238-cm\(^{-1}\) band, since the amplitude of this chromophore band does not change significantly during the MI/MII transition. Typical MII bands are indicated (arrows) in the lower spectrum.
In principle, the KCl effect on the MI/MII equilibrium may either be the result of binding of $K^+$ or $Cl^-$ at specific binding sites or an effect of the high ionic strength of the bulk solution on the local pH at the membrane surface and/or on the intramolecular charge distributions. In the case of specific binding (i.e. $K_c \leq 1$ mM), saturation of the binding sites is likely to occur at relatively low salt concentrations (isotonic) and one would not expect to observe the large difference in apparent $K_c$ between isotonic solution and 4 M KCl shown in Fig. 1. Hence, we investigated the ability of other salts and of the zwitterions betaine and NDSB195, a sulfobetaine derivative (Vuillard et al., 1995), to shift the equilibrium towards MII. This shift was assayed by measuring the amount of MI formed at pH 8 and 10°C in the presence of 0.5 M divalent salts, 1 M monovalent salt or 2 M zwitterions (Fig. 3). Most salts induce a comparable shift in equilibrium towards MII. However, the two zwitterions and tetrathylammonium chloride had no significant effect. 1 M KCl, NaCl, RbCl, CsCl, or 0.5 M CaCl$_2$, or MgSO$_4$ all cause about the same shift (45 ± 5%) towards MII, so the effect is not highly cation specific. Changing the anion in the series KCl, KNO$_3$, KBr, KI and KSCN shows that there is some anion selectivity. The shift induced by KI or KSCN is significantly larger than that induced by the other salts (1 M KSCN being about as effective as 2 M KCl, Figs 1 and 3). These results nevertheless indicate that the shift in the MI/MII equilibrium is primarily dictated by the ionic strength of the membrane suspension.

High salt concentrations enhance the rate of MII formation. The formation of MII at ~6°C in photoreceptor-membrane suspensions was monitored from the rise in absorbance at 380 nm. Values for $k_{on}$ were obtained under isotonic conditions and in the presence of 2 M KCl at various pH between 5 and 8.5 (Fig. 4). Under isotonic conditions, $k_{on}$ reached a minimal value near neutral pH, in agreement with earlier reports (King and Gulfreund, 1984; Parkes and Liebman, 1984). At pH = 7, the presence of 2 M KCl enhances $k_{on}$ about twofold.

The rate of MII decay is not affected by high salt concentrations. The decay of MI was analyzed at 15°C and pH 6.0 by means of ultraviolet/visible and FTIR spectroscopy (transmission mode). The FTIR difference spectra we obtained were very similar to those presented in other FTIR studies on MI decay (Rothschild et al., 1987; Klinger and Braiman, 1992; data not shown). All FTIR difference bands analyzed decayed at essentially the same rate. No significant differences were ob-
Fig. 5. Effect of 4 M KCl on the decay of MI and formation of MII as measured by ultraviolet/visible spectroscopy. $A_{450\text{-nm}} - A_{445\text{-nm}}$ and $A_{435\text{-nm}} - A_{430\text{-nm}}$ (change in absorbance), representing MI decay and the formation of MII, respectively, were measured at 15°C and pH 6.0 (Mes buffer A), under isotonic (solid line) and 4 M KCl (dashed line) conditions. Amplitudes were scaled for easier comparison.

**DISCUSSION**

In this study we investigated the effect of ionic strength on the late photocascade of bovine rhodopsin in the photoreceptor membrane. The most striking observation was that the apparent $pK_a$ of the MI/MII equilibrium strongly depends on the ionic strength of the membrane suspension. The shift in equilibrium position is primarily due to an ionic-strength-dependent increase in the rate of the MI→MII transition. For instance, from the data presented in Fig. 4 and the shift in position of MI, under these conditions, we calculated that at pH 7, assuming a pseudo-first-order reaction scheme and defining $k_2 = k_{+2}$, the rate of MI formation (M$\text{I}$$\rightarrow$MII; $k_{-2}$) increases about tenfold in response to an increase in ionic strength of 2M KCl, while the reverse reaction (MII→MI; $k_{+2}$) is only accelerated about twofold. Because the equilibrium shift is not highly ionic specific, we conclude that it is not primarily caused by specific binding of ions, but that it rather depends on the bulk ionic strength. A possible explanation for the different behavior observed with KI, KSCN and tetraethylammonium chloride is that the $I^-$, SCN$^-$ and tetraethylammonium ions are more lipophilic than the other ions tested. It seems unlikely that higher ionic strength causes structural changes in rhodopsin or its photointermediates, considering that their visible and infrared spectral properties are independent of ionic strength. Small structural changes in the retinal-binding pocket would have already influenced $A_{450}$ and the characteristic band patterns in the FTIR difference spectra (DeCaluwe et al., 1995). The implications of the observed salt effects for models of the MI/MII transition will be discussed below.

Membrane properties. Biomembranes and lipid-protein interactions are known to be sensitive to the ionic environment. For instance, several salts can influence membrane proteins indirectly by affecting the fluidity of the membrane matrix (Yang et al., 1993; DeGrip et al., 1983). These effects, however, involve binding of ions to the lipid headgroups and therefore always show a preference for divalent and trivalent cations (e.g. Ca$^{2+}$, Mg$^{2+}$, and La$^{3+}$), which we did not observe here. Binding of divalent cations, such as Ca$^{2+}$, to phosphatidylserine bilayers, for example, has been shown to rigidify the membrane, which results in a shift of the MI/MII equilibrium towards MI (DeGrip et al., 1983; Gibson and Brown, 1993). The fluidity of the photoreceptor membrane, however, seems to be unaffected by high salt concentrations, as we noticed that the frequency of the methylene C-H symmetric-stretch vibration remains at 2854.2 ± 0.2 cm$^{-1}$ in ATR/FTIR spectra of ROS films permed with Bistris-propane buffer with and without 2 M KCl (DeLange, F. and DeGrip, W. J., unpublished data). The frequency of this vibration is a good indicator of membrane packing (Cameron et al., 1980; Lamba et al., 1994).

Effects on surface pH. Another explanation might be that the ionic strength of the bulk membrane suspension affects the surface pH. Because of the relatively high local charge density, the pH at the membrane surface usually differs from bulk pH. Surface-charged effects were put forward to explain the ionic-strength dependence of the purple-to-blue transition in bacteriorhodopsin (Szundi and Stoeckenius, 1989). Alexiev et al. (1994) showed that the charge density calculated from the ionic-strength dependence of the purple-to-blue transition in a bacteriorhodopsin mutant reconstituted in detergent/lipid mixed micelles equalled the surface-charge density calculated from the ionic-strength dependence of the apparent $pK_a$ of a pH indicator dye attached to the extracellular side of the protein, thereby showing a direct relationship between the purple-to-blue transition and the surface potential on the extracellular side of bacteriorhodopsin. Another example of such a phenomenon is the ionic-strength dependence of the equilibrium between acid and alkaline metarhodopsin in octopus photoreceptor membranes, which was interpreted to be due to screening of net negative charges at the extracellular membrane surface (Koutalos et al., 1990). In these two systems, raising the ionic strength results in a decrease of the apparent $pK_a$, indicating that the MI/MII equilibrium is towards MII, the side from which we are titrating the MI/MII transition should have a net positive charge. As the cytosolic side of rhodopsin contains more basic than acidic residues, this side is probably positively charged (Ovchinnikov et al., 1982; Hargrave et al., 1983). Hence, part of the shift in $pK_a$ of the MI/MII equilibrium we observe might be explained by an ionic-strength dependence of the surface charge and hence of the surface pH at the cytosolic side of the photoreceptor membrane. Screening of the net-negative lipid-headgroup charges is not expected to result in an altered equilibrium position, since it is possible to recover full photochemical function of rhodopsin upon reconstitution in a neutral lipid environment alone (Brown, 1994). The results for the two zwitterions may be interpreted to be due to less effective screening of these surface charges. Preliminary evidence from comparative titration studies, as in Fig. 1, of ROS membranes and partially digested (protease K) or modified (succinic anhydride) rhodopsin suggests that changes in the cytosolic surface charge affect the MI/MII equilibrium (DeLange, F., Bovec-Geurts, P. H. M. and DeGrip, W. J., unpublished data).

However, a shift in the surface pH can only explain part of the observed shift in $pK_a$. The cross-sectional area of rhodopsin is known to lie in the range of 8–12 nm$^2$ (Tsui et al., 1990).
From the folding model of bovine rhodopsin (Ovchinnikov et al., 1982; Hargrave et al., 1983), we deduce that, in our experimental pH range, there is a maximum of five net positive charges on the cytosolic side of the protein. Applying the Gouy-Chapman and Boltzmann equations, in modified forms (Koutalos et al., 1990), one can calculate that under such conditions this surface charge would be effectively screened at 2 M monovalent electrolyte. This calculated concentration probably represents an upper estimate, since Tsui et al. (1990) claim that 0.2 M monovalent salt is enough to screen the surface potential of disc vesicles. The apparent pK<sub>a</sub> of the MI/MII equilibrium, however, is still considerably shifted upwards upon raising the KCl concentration from 2 M to 4 M. Therefore, high ionic strength might also affect buried residues that are involved in tuning the pH sensitivity of the MI/MII equilibrium. This could also explain the stronger effect on the MI/MII equilibrium of the lipophilic anions I<sup>-</sup> and SCN<sup>-</sup> (Fig. 3), because they might get closer to these buried residues. The suggestion that a high ionic strength of the bulk membrane suspension can influence residues in the interior of rhodopsin is supported by the observation that at pH > 9 the absorbance maximum of rhodopsin is blue-shifted by 8 nm in rod-disc membranes at 4 M KCl, while this shift does not occur under isotonic conditions (Koutalos, 1992).

This study reports ionic strength effects on the apparent pK<sub>a</sub> of the MI/MII equilibrium of bovine rhodopsin in the native photoreceptor membrane. Almost all detergents dramatically alter the MI/MII transition. In most detergents, the transition is no longer a pH-dependent equilibrium in the experimental pH range (pH 5 — 9), but fully proceeds to MII. Salt effects have been reported before only for rhodopsin in micellar solution. Matthews et al. (1963) observed that in digitonin micelles MII is favored in the presence of neutral salts such as lithium bromide, sodium phosphate or potassium phosphate, without affecting the apparent pK<sub>a</sub> of the MI/MII equilibrium. A more recent report describes ionic-strength effects on proton movements during the formation of MII (Arnis and Hofmann, 1993). In this study, it is shown that in dodecylmaltoside or nonylglucoside micelles, deprotonation of the retinal Schiff base (MI<sub>a</sub> ⇌ MII<sub>a</sub>) precedes proton uptake (MII<sub>a</sub> + H<sup>+</sup> ⇌ MII<sub>a</sub>). Schiff-base deprotonation was found to be accelerated at high ionic strength. This agrees with our observation that MI formation is accelerated at 2 M KCl in ROS membranes.

Our finding that the pK<sub>a</sub> of the MI/MII equilibrium is sensitive to the ionic strength of the membrane suspension up to very high KCl concentrations, supports the concept that the MI/MII equilibrium is at least partially controlled by electrostatic factors (Robinson et al., 1992; Weitz and Nathans, 1993; Zvyaga et al., 1993). It has been proposed that the pH dependence of the MI/MII equilibrium is mainly regulated by a histidine residue, because the pK<sub>a</sub> of the imidazole group (in water) is within the range of the apparent pK<sub>a</sub> of the MI/MII equilibrium in digitonin solution (6.4 at 3.2°C; Matthews et al., 1963). Weitz and Nathans (1992) suggested that this histidine is His211, as they found that its replacement by either cysteine or phenylalanine has been reported before only for rhodopsin in micellar solution. Nathans (1992) suggested that this histidine is His211, as they found that its replacement by either cysteine or phenylalanine.

In conclusion, we believe that the observed effects can be attributed to ionic-strength effects on the protein itself, and do not involve the lipid matrix as much. While the ionic strength will vary only very little under physiological conditions, these studies seem to indicate that the charge asymmetry in bovine rhodopsin is finely tuned so as to produce significant amounts of the active MII intermediate under physiological conditions. It would be interesting to investigate whether this concept of charge-driven activation modulation also occurs for other members of the family of G-protein-coupled receptors.

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