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Modulation of the metarhodopsin I/metarhodopsin II equilibrium of bovine rhodopsin by ionic strength
Evidence for a surface-charge effect
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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)/MII 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/MII 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.
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 rodouter-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, 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 A280/A560 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). Buffers Mes, Heps and 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 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 pM rhodopsin) in either Mes, Mops, Hepes or Bistris-propane and salts as indicated. A spectrum was recorded after bleaching the sample (10 s) with a light bulb and two or three spectra were taken to verify the stability of the photointermediate spectrum under the experimental conditions (spectrum 2). The relative amount of MI formed after illumination, ([MI]—[bleached rhodopsin]) was derived from the linear relationship between the λmax of the difference spectrum (spectrum 1—spectrum 2) and the amount of photoproduc (p). In these difference spectra, λmax varies linearly from 498 nm (0% MI, 100% MII) to 530 nm (100% MI, 0% MII). Assuming [bleached rhodopsin] = [MI] + [MII] (Parkes and Lieberman, 1984), the amount of MI was calculated from ([λmax—498]—[530—498])×100 (‘difference-spectrum approach’). The validity of this approach was verified by means of the classical approach, which requires a third spectrum after addition of hydroxyamine to convert all photointermediates in the sample into opsin and retinaloxime (DeGrip et al., 1983). The difference-spectrum approach, which was exploited for similar analyses in digitonin by Weitz and Nathans (1993), yields the same average values for MI but generally gives better reproducibility than the classical approach.

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 sample 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 (kapp) 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, photoconverted 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 MII → 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 MIII. 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 OG530 cut-off filter (Schott) in the transmission experiments, and a fiberoptics ring illuminator (Schott) in combination with an OG530 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 (Graeseby Specac) in the FTIR transmission experiments.

Static FTIR difference spectroscopy. For analysis of the rhodopsin to MI/MII transition, FTIR difference spectra were obtained in a similar way to that previously described (Rothschild 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 (Fisher Scientific Co.). The photoreceptor-membrane films were hydrated with about 2 µl 2×buffer 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 min/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
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 KCl, 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 MII.** The decay of MI was studied in the FTIR transmission mode at 15°C in spin-dried films that were hydrated with 2 mM buffer (20 mM buffer and 5 mM KCl); (▪), isotonic buffer A; (▲), buffer A + 1 M KCl; (●), buffer A + 2 M KCl; (♦), buffer A + 4 M KCl. For clarity, standard deviations (n = 3) are shown only for one curve (+ 1 M KCl). The SD in the other curves is comparable. Solid lines show fits to the Henderson-Hasselbach equation with two fixation points at pH 4 (0% MI) and at pH 12 (100% MI).

**RESULTS**

The pK\textsubscript{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 KCl); isotonic buffer A (without additional KCl) and with 1 M KCl, 2 M KCl and 4 M KCl (Fig. 1). Under isotonic conditions, we determined an apparent pK\textsubscript{a} of 7.3, which agrees well with the pK\textsubscript{a} reported by Parkes and Liebman (1984) for rod-disk-membrane suspensions under similar conditions. Under hypotonic conditions, the apparent pK\textsubscript{a} is shifted to 6.8. Increasing the concentration of KCl to 4 M shifts the apparent pK\textsubscript{a} of the equilibrium to about 9.5. Due to the instability of MI at high pH under high-salt conditions, no reliable measurements at pH ≥ 9 could be obtained. Except for the effect on the MI/MII equilibrium, the late photocascade in the presence of 4 M KCl is very similar to that under isotonic conditions: the absorbance maximum of rhodopsin is the same (498 nm) and, judging from to the absorbance changes in the ultraviolet/visible difference spectra, the same late intermediates (MI, MII and MI\textsubscript{M}) are formed upon illumination (data not shown). We did not observe significant effects of increasing salt concentration on the scattering, measured at 650 nm, of the membrane suspension, indicating that, under our experimental conditions, no significant aggregation occurs.

**FTIR difference spectroscopy at different KCl concentrations.** To establish whether the light-induced structural changes in the presence of 4 M KCl 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 KCl (middle spectrum) conditions and with Mes buffer (pH 6; lower spectrum). These 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 MI bands are indicated (arrows) in the lower spectrum.
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. The decay in these experiments is about 10% (n = 4). The inset shows typical curves for the rise in A_{350} at pH 6.1 and ~6°C for buffer A (solid line) and for buffer A with 2 M KCl (dashed line). Amplitudes were scaled for easier comparison. Mono-exponential functions were fitted to these data to obtain best estimates for k_{obs}.

High salt concentrations enhance the rate of MI formation. The formation of MI at ~6°C in photoreceptor-membrane suspensions was monitored from the rise in absorbance at 380 nm. Values for k_{obs} 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_{obs} reached a minimal value near neutral pH, in agreement with earlier reports (King and Gutfreund, 1984; Parkes and Liebman, 1984). At pH~7, the presence of 2 M KCl enhances k_{obs} about twofold.

The rate of MI 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-
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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 perfused 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-charge effects were put forward to explain the ionic-strength dependence of the purple-to-blue-transition in bacteriorhodopsin (Szundi and Steeckens, 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., 1993; DeGrip et al., 1983). These effects, however, involve 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., 1993; DeGrip 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 (succiinc anhdydride) rhodopsin suggests that changes in the cytosolic surface charge affect the MI/MII equilibrium (DeLange, F., Bovee-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 pKs 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^- and SCN^- (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 pKs 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 MI. Salt effects have been reported before only for rhodopsin in micellar solution. Matthews et al. (1963) observed that in digitonin micelles MI is favored in the presence of neutral salts such as lithium bromide, sodium phosphate or potassium phosphate, without affecting the apparent pKs of the MI/MII equilibrium. A more recent report describes ionic-strength effects on proton movements during the formation of MI (Arnis and Hofmann, 1993). In this study, it is shown that in dodecylmaltoside or nonylglycoside micelles, deprotonation of the retinal Schiff base (MI⇌MII) precedes proton uptake (MII + H+⇌MI). 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 pKs 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 pKs of the imidazole group (in water) is within the range of the apparent pKs 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 results in a complete blockade of the MI/MIII transition in digitonin solution. Since the pKs of the MI/MIII equilibrium can be shifted to at least 9 in the native photoreceptor membrane, we believe that residues other than histidines may participate in coupling of the deprotonation of the retinal Schiff base to the structural changes that result in MI formation. That the apparent pKs can shift from as low as pH 6.8 to over pH 9 suggests that not just a single residue is involved, but rather that a consortium of residues, like in a H-bonded network, may be responsible (DeGrip et al., 1993).

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 MI 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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