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Articles

Conformation and Stability of α-Helical Membrane Proteins. 1. Influence of Salts on Conformational Equilibria between Active and Inactive States of Rhodopsin†

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ABSTRACT: We studied the influence of salts on the pH-dependent conformational equilibria between the active and the inactive photoproduct states of rhodopsin, Meta II and Meta I, respectively, and between the active and inactive conformations of the apoprotein opsin. In both equilibria, the active species is favored in the presence of medium to high concentration of salt. The ion selectivity for the Meta I/Meta II equilibrium is particularly pronounced for the anions and follows the series trichloroacetate > thiocyanate > iodide > bromide > sulfate > chloride > acetate. The Hill coefficient of this salt-induced transition is close to 2.0. Both ion selectivity and Hill coefficient suggest that the transition is mainly regulated by ion binding to two specific charged binding sites in the protein with smaller contributions being due to the Hofmeister effect. We propose that these putative ion binding sites are identical to those sites that are titrated in the corresponding pH-dependent conformational transition. They presumably function as ionic locks, which keep the receptor in an inactive conformation, and which may be disrupted either by pH-dependent protonation or by salt-dependent ion binding.

The visual pigment rhodopsin belongs to the large class of G protein-coupled receptors (GPCRs),[1] which play a considerable role in signal transduction processes (*1*−*4*). It is, as the other members of this family, a transmembrane protein consisting of a bundle of seven membrane-spanning α-helices, which are interconnected by cytoplasmic and extracellular loop regions.

In contrast to other GPCRs that are activated by diffusible ligands, rhodopsin has covalently bound its chromophore 11-*cis* retinal via a protonated Schiff base linkage to Lys^{296} on helix 7, lending it a visible absorption maximum at 500 nm. After photon absorption as the initial event of signal transduction, the chromophore undergoes a *cis* to *trans* isomerization. Within milliseconds, the resulting structural changes within the chromophore binding pocket, which is situated in the transmembrane region of the receptor apart from the solvent, are propagated to the cytoplasmic surface of the protein (*1*, *3*, *4*). The induced conformational changes on the cytoplasmic side then allow the docking of its cognate G protein transducin and the activation of the visual signal transduction cascade (*1*). The formation of the active receptor

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[1] Abbreviations: FTIR, Fourier transform infrared spectroscopy; GPCR, G protein-coupled receptor; Meta I and Meta II, metarhodopsin I and II; MES, 2-*N*-morpholino ethanesulfonic acid; BTP, bis-tris propane; TCA^-, trichloroacetate, TEA^+, tetraethylammonium; TMAO, trimethylamine *N*-oxide.

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state, metarhodopsin II (Meta II), is accompanied by a shift of the absorption maximum to 380 nm, reflecting the deprotonation of the retinal Schiff base. Meta II is in a conformational equilibrium with its still inactive immediate predecessor Meta I (λ_{max} 480 nm), which is pH dependent with the active state being formed at the acidic side of the equilibrium (*5*).

The Meta II state is intrinsically unstable and decays at 30 °C within minutes by hydrolysis of the retinal Schiff base linkage and dissociation of all-*trans* retinal from the apoprotein opsin. It was shown recently that opsin also forms a pH-dependent conformational equilibrium between an active and an inactive conformation, similar to Meta I/Meta II (*6*). Yet the p*K* of this transition is shifted to more acidic values by more than 4 units as compared to that of the Meta I/Meta II equilibrium.

While salt effects on soluble proteins have been investigated for a long time, only a little information is available about the influence of salts on membrane proteins. We reported recently on salt effects on the synthetic visual pigment 9-demethyl rhodopsin (*7*, *8*), which has an abnormally reduced ability to form the active state Meta II upon photon absorption as compared to the native pigment rhodopsin (*9*, *10*). This low activity could be counteracted to some extent by chaotropic salts, which also had some influence on stability and decay pathways of the active state. Using UV−visible and FTIR spectroscopy (*11*), we considerably extend our previous findings in an investigation of similar properties of native rhodopsin. It is shown that the dependence of this salt-induced transition to the active state Meta II on salt concentration is characterized by a Hill coefficient close to two, reflecting the existence of two specific ion sensitive sites controlling the receptor conformation. Such sites are presumably defined by clusters of charged amino acids as the highly conserved E(D)RY motif of class A GPCRs at the cytoplasmic end of helix 3, which is likely to interact with residues on helix 6 (*12*). The receptor is thereby constrained in an inactive conformation, as shown recently as well for the β_2-adrenergic receptor (*13*) and the rat μ opioid receptor (*14*). In addition, also the conformational equilibrium between the active and the inactive opsin states shows a salt dependence, which is somewhat weaker as compared to that of Meta I/Meta II, possibly reflecting the decreased accessibility of the presumably involved charge bridge between the residues Glu-113 and Lys-296 in the transmembrane core (*6*, *15*).

EXPERIMENTAL PROCEDURES

Preparation of the Pigment. Bovine rhodopsin in washed disk membranes was prepared from the rod outer segments of cattle retinae after established methods (*9*). All experiments involving pigment were carried out under dim red light. Pigment was stored in distilled water at −20 °C at a concentration of ∼50 μM.

UV−Visible Spectroscopy. UV−visible spectroscopy of suspensions of disk membranes was performed with a Perkin-Elmer Lambda 17 UV−vis spectrophotometer equipped with thermostated cuvette holders in 100 μL microcuvettes with 10-mm path length (Hellma, Müllheim, Germany). To account for the considerable light scattering of the turbid membrane suspensions, spectra were recorded in double-beam mode with an identical but bleached sample in the reference beam. Scanning speed was 960 nm/min allowing a time resolution of 1 min.

For an analysis of the Meta I/Meta II equilibrium, membrane suspensions (typically 2 μM rhodopsin) were photolyzed at 10 °C in 20 mM BTP buffer, pH 8.5, for 20 s through a fiber optic and a long-pass filter (> 530 nm) fitted to a 150 W slide projector. The difference spectrum "photoproduct minus dark state" was determined, and the stability of the photoproduct was verified by taking several subsequent spectra. The Meta II content was evaluated by determining the position of the dark state depletion peak from the second derivative of the smoothed difference spectra as described previously (*16*). In the absence of Meta I, the position of the depletion peak, λ_D, is in membranes slightly above 500 nm and is shifted to longer wavelength by increasing contributions of the positive Meta I peak. The Meta II content was calculated from the linear interpolation Meta II/(Meta I + Meta II) = (λ_D − 504 nm)/(535 − 504 nm).

FTIR Spectroscopy. FTIR spectroscopy was performed in transmission mode with a Bruker IFS 28 FTIR spectrometer equipped with a Hg−Cd−Te detector. As sample type, we used 4 μm sandwich samples, which show a similar position of the Meta I/Meta II equilibrium as diluted membrane suspensions as verified by UV−visible and FTIR spectroscopy (R. Vogel and F. Sieber, unpublished results). These samples were prepared on specially designed CaF_2 windows as described previously (*6*). To ensure accurate pH adjustment, the dried sample films were preequilibrated with 20 μL of 200 mM buffer for 30 s before squeezing out the excess buffer by sandwiching the sample window with a top window. We used citrate buffer (for pH < 6.0), 2-*N*-morpholino ethanesulfonic acid buffer (MES, pH 6.0−7.0), and bis-tris propane buffer (BTP, pH > 7.0), all supplemented with 200 mM NaCl. The samples were thermostated (stability 0.1 °C) in the dry air purged sample chamber. Spectra were recorded in blocks of 512 scans at 4 cm^{-1} resolution with 1 min acquisition time. The samples were photolyzed for 30 s by a fiber optic connected to a 150 W slide projector and a long-pass filter (> 530 nm).

For an analysis of the conformational equilibrium of opsin, samples were photolyzed at 30 °C, and the decay of the resulting Meta II photoproduct was followed over 30 min, as described previously (*6*). The spectrum obtained 15 min after photolysis was considered to be the final opsin spectrum, as no further spectral changes occurred afterward.

RESULTS

NaCl Shifts the Meta I/Meta II Equilibrium to the Active State Meta II. In the native membrane environment, the rhodopsin photoproducts Meta I (478 nm) and Meta II (380 nm) form a pH-dependent steady-state equilibrium with a p*K* around 7.0 at 10 °C and Meta I being formed at higher pH (*17*). At pH 8.5, the photoproduct consists therefore almost entirely of the inactive Meta I state. In the light induced UV−visible difference spectra (Figure 1A), we observe consequently no absorption increase at 380 nm in the absence of NaCl, but only a difference band due to the shift of the absorption maximum from 500 nm for the dark state to 478 nm for Meta I. As evident from the same figure,

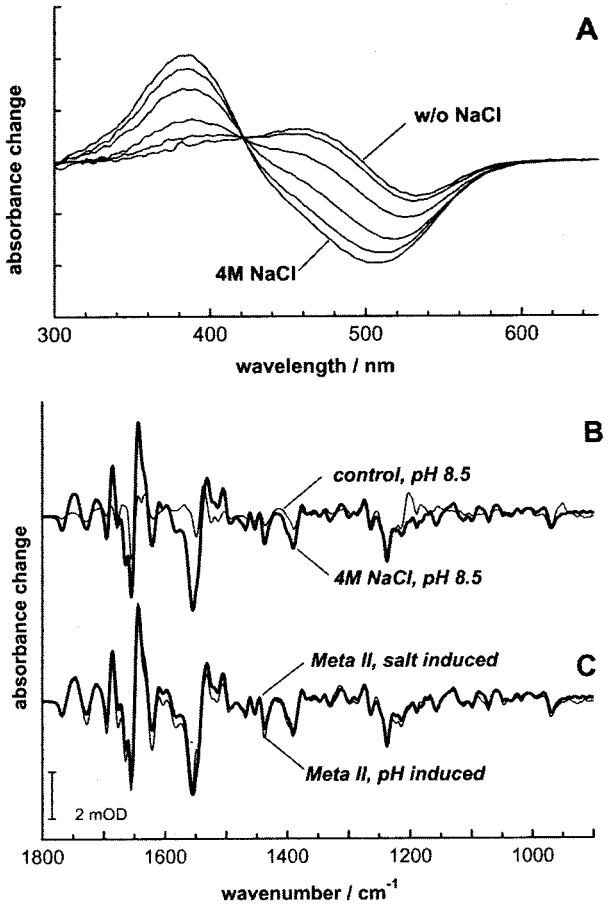

FIGURE 1: NaCl shifts the Meta I/Meta II equilibrium toward Meta II. (A) UV−visible difference spectra "photoproduct minus dark state" of rhodopsin membrane suspensions (~2 μM) at 10 °C and pH 8.5 in the absence of NaCl and at 0.5, 1.0, 2.0, 3.0, and 4.0 M NaCl; tickmarks are 50 mOD. (B) FTIR difference spectra "photoproduct minus dark state" from sandwich samples at pH 8.5 and 10 °C, in the presence of 4 M NaCl (thick line), corresponding to Meta II, and with only 200 mM NaCl (control, thin line), corresponding mostly to Meta I. (C) Comparison of the salt-induced Meta II state obtained at pH 8.5 in the presence of 4 M NaCl (thick line, spectrum as in B) with the classical, pH-induced Meta II state obtained at pH 6 with 200 mM NaCl (thin line).

however, the Meta I/Meta II equilibrium can be shifted to the active conformation Meta II at still the same pH by increasing the concentration of NaCl. The obtained UV−visible difference spectrum obtained at 4 M NaCl is indistinguishable from a difference spectrum, where the active Meta II conformation is stabilized by low pH (not shown), with the typical difference bands due to the absorption shift from 500 to 380 nm. This behavior is paralleled in infrared difference spectra, which reveal at pH 8.5 a Meta I photoproduct state at 200 mM NaCl, and a Meta II photoproduct at 4 M NaCl (Figure 1B). As in the UV−vis, the difference spectrum of the salt induced Meta II state corresponds to that of a pH-induced Meta II state (Figure 1C). We can therefore conclude that presence of NaCl facilitates the transition to the active state Meta II under conditions otherwise favoring Meta I.

Ion Selectivity of the Salt Induced Shift of the Meta I/Meta II Equilibrium. We extended our investigations also to other salts and found a quite distinct specificity of the salt-induced transition to Meta II for anions and, in part, also for cations (Figure 2A and B). In the case of the anions (all tested as

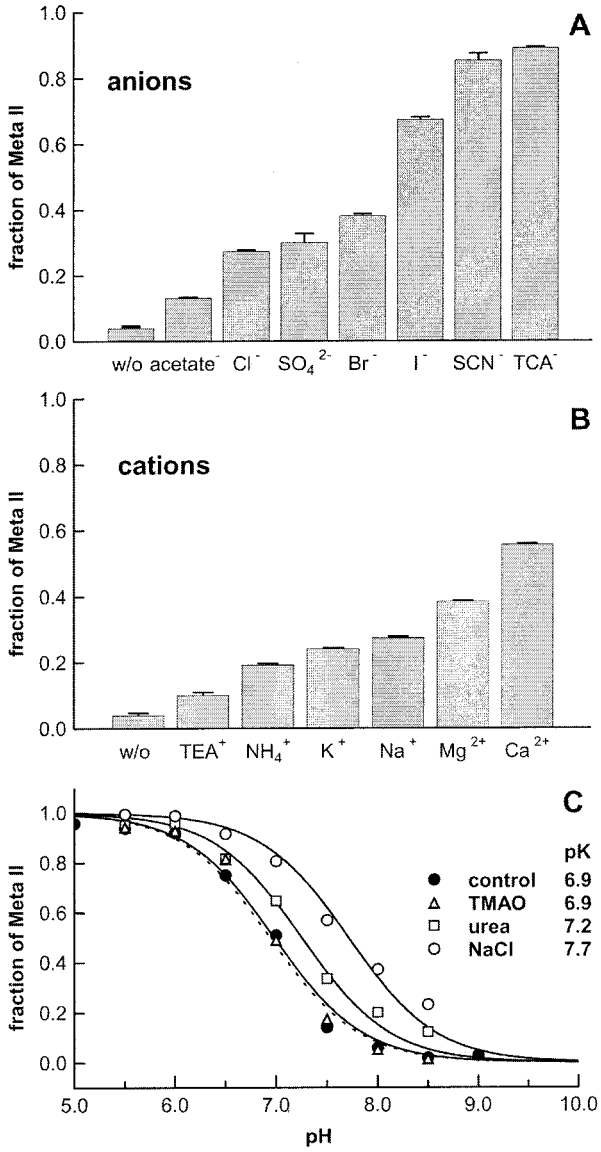

FIGURE 2: Ion selectivity for the salt-induced Meta II shift. The fraction of Meta II in the Meta I/Meta II photoproduct equilibrium at pH 8.5 with several sodium (A) and chloride salts (B) at 1 M anion concentration. (C) The protein stabilizer trimethylamine *N*-oxide (TMAO) shows at 1 M concentration no influence on the p*K* of the Meta I/Meta II equilibrium, and the strong neutral destabilizer urea leads only to a relatively small shift compared to NaCl. The controls without salt contains 20 mM buffer. All data were determined by UV−visible spectroscopy with membrane suspensions of rhodopsin (~2 μM) in 20 mM buffer at 10 °C; the data in A and B are mean values ± SD of 2−4 experiments.

sodium salts at 1 M anion concentration), the effect is most pronounced for trichloroacetate (TCA), thiocyanate, and iodide, and it decreases with bromide, sulfate, and chloride. With acetate, we observe only little effect on Meta II formation (13% Meta II) as compared to the controls with only 20 mM buffer (4% Meta II), while the Meta II formation increases successively with the chlorinated, increasingly larger derivatives monochloroacetate (33% Meta II), dichloroacetate (48%), and trichloroacetate (89%) (not shown). Among the cations, divalent calcium and magnesium cations are the most efficient as compared to the monovalent sodium, potassium, ammonium, and TEA^+ (tetraethylammonium) (all tested as 1 M chloride salts). Most efficient is the guanidinium cation with full Meta II formation already below 200

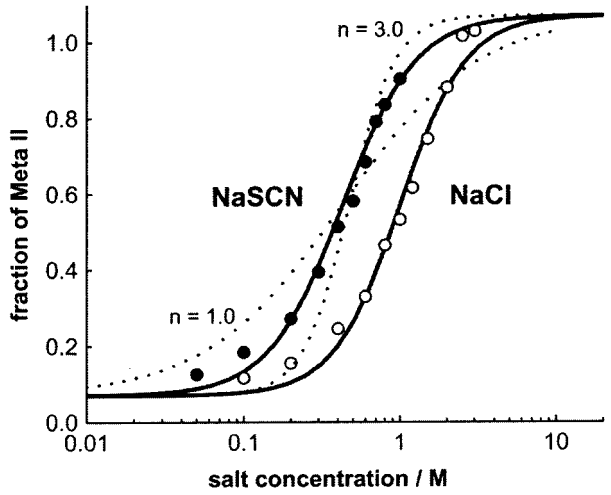

FIGURE 3: Concentration dependence of salt-induced Meta II formation. The fraction of Meta II in the Meta I/Meta II photoproduct equilibrium was determined as a function of concentration of NaSCN (closed symbols) and NaCl (open symbols). The solid lines are fits to a Hill equation with a dissociation constant k_d = 0.21 M^n and a Hill coefficient n = 1.9 for NaSCN, and k_d = 1.00 M^n and n = 2.1 for NaCl (r^2 = 0.99 and 0.98, respectively). The broken lines are fits with a fixed Hill coefficient of 1.0 and 3.0 to the NaSCN data for comparison. The data were obtained in the presence of 500 mM sodium acetate to saturate potential salt effects on the local membrane surface pH as described in the text.

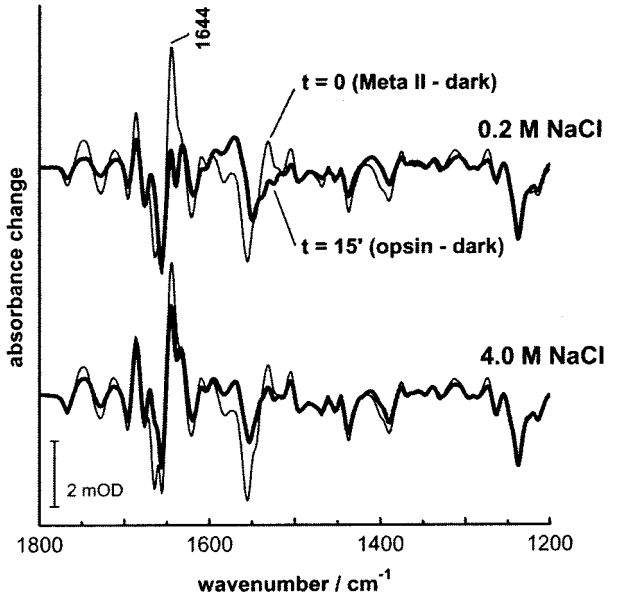

FIGURE 4: Salt dependence of the conformational equilibrium of the Meta II decay product opsin in membranes. Meta II was allowed to decay to opsin at 30 °C and pH 5.0 in the presence of 200 mM (upper) and 4 M NaCl (lower spectra). Shown are the FTIR difference spectra "Meta II minus dark state" (thin line) obtained immediately after sample photolysis and "opsin minus dark state" (thick line) obtained after complete decay of Meta II (15 min after photolysis).

mM concentration. A quantitative analysis was prevented as Meta II was slowly destabilized already at very low (150 mM) concentration of guanidinium salts, leading to the formation of a 370 nm photoproduct indicative of a non-native state (not shown).

We further investigated the influence of the nonionic strong chaotrope urea and the nonionic kosmotrope trimethylamine *N*-oxide (TMAO) on the Meta I/Meta II equilibrium (Figure 2C). While there is no significant shift of the p*K* of the Meta I/Meta II equilibrium in the presence of 1 M TMAO, there is a small upshift with 1 M urea from 6.9 to 7.2. This shift, however, is still small as compared to that induced by 1 M NaCl (p*K* 7.7). We can therefore conclude that the observed effect of salts on the Meta I/Meta II equilibrium depends largely on their ionic nature.

The Hill Coefficient of the Salt Induced Transition to Meta II Suggests Two Specific Ion Binding Sites. In Figure 3, we analyze the concentration dependence of the salt-induced transition to Meta II for NaCl and NaSCN. The amount of Meta II as a function of salt concentration at constant temperature and pH can be satisfactorily fitted (r^2 = 0.98) by the equation

$$[\text{Meta II}]/([\text{Meta I}] + [\text{Meta II}]) = c^n/(c^n + k)$$

where c is the salt concentration and n and k are adjustable parameters. This equation corresponds to the reaction Meta I + n·salt ⇌ Meta II·salt$_n$, with n as the Hill coefficient and k as the dissociation constant of the reaction. For NaCl, we obtain n = 2.1 and k = 1.00 M^n. For NaSCN, we observe a similar Hill coefficient of n = 1.9, but a considerably smaller dissociation constant k = 0.21 M^n (r^2 = 0.99), reflecting the stronger effect of the thiocyanate anion compared to chloride. As the Meta I/Meta II equilibrium was recently shown to be influenced at low salt concentration by an ionic strength dependent shift of the local pH on the disk membrane surface (*18*), we conducted these experiments in the presence of 500 mM sodium acetate, where this effect should be saturated. A similar Hill coefficient close to 2 was obtained as well in the absence of acetate for NaCl concentration above 100 mM. The results strongly suggest that the mechanism of the salt-induced transition from Meta I to Meta II is similar for both NaCl and NaSCN and that it is at least for these two salts largely dependent on the binding of two anions. NaCl and NaSCN merely differ by the considerably higher affinity of the thiocyanate anion as compared to chloride for the putative binding sites.

Influence of NaCl on the Conformational Equilibrium of Opsin. At 30 °C, Meta II is no longer stable, but decays with a half-time of around 3 min via hydrolysis of the retinal Schiff base and dissociation of the receptor into retinal and the apoprotein opsin. At neutral to high pH, the conformational changes involved in formation of the active Meta II state are reverted during the decay to the inactive opsin conformation (*19*). We could show recently that this reversal is inhibited at very low pH and that an active conformation similar to that of Meta II is preserved during the decay (*6*). The resulting active opsin state shows therefore essentially all conformational features of Meta II.

Similar as for the Meta I/Meta II equilibrium, high concentration of NaCl shifts the conformational equilibrium of opsin from the inactive to the active conformation. At pH 5.0, the conformational features of Meta II are mostly reverted during the decay to opsin in the presence of 200 mM NaCl, but not at 4 M NaCl (Figure 4). This is particularly pronounced in the amide I and amide II range around 1650 and 1550 cm^{-1}, respectively, and is most obvious for the strong amide I difference band of Meta II at 1644 cm^{-1}, which is preserved during the decay at 4 M, but not at 200 mM NaCl. Careful comparison of the opsin spectra

obtained in the presence of 4 M NaCl to those obtained with 200 mM NaCl in the pH range from 4.0 to 8.0 indicate a salt-dependent shift of the pK_a of the opsin conformational equilibrium from 4.3 (with 200 mM NaCl at 30 °C) by approximately 1.2 p*K* units toward more alkaline values in the presence of 4 M NaCl. In comparison, the corresponding shift of the p*K* of the Meta I/Meta II equilibrium is more than 2 units (not shown). A detailed analysis of the ion selectivity and the concentration dependence of the opsin equilibrium was hampered by the influence of salts on the stability of opsin (*20*).

DISCUSSION

In this study, we report on the influence of salts on the conformational equilibria of the light receptor rhodopsin. The rhodopsin photoproducts form a conformational equilibrium between the active and the inactive states Meta II and Meta I, respectively. A similar equilibrium is observed for the apoprotein opsin after decay of the photoproduct states and dissociation of the receptor into retinal and opsin. Both equilibria are known to be pH dependent with the active species being favored at lower pH. We show in this study by both UV−visible and FTIR spectroscopy that presence of salt favors the active state conformation by shifting the p*K* of the transition to more alkaline values.

In principle, salts may influence properties of proteins by a variety of effects. The three major mechanism are (a) ionic strength dependent electrostatic screening of surface potentials (Debye−Hückel effect), (b) salt dependent changes of the protein−solvent interface reflecting preferential interactions of the ion with the solvent or the protein (Hofmeister effect), and (c) direct binding of ions to charged residues.

A major involvement of the Debye−Hückel effect in the salt-induced formation of Meta II can be excluded as this effect depends only on the ionic strength of the surrounding medium and saturates already at low salt concentrations. It cannot account for the pronounced ion selectivity observed in this study and for the persistence of the effect at medium to high concentrations of salt. The Debye−Hückel effect was, however, shown to be involved in a slight salt-dependent shift of the Meta I/Meta II equilibrium at low salt concentrations (<200 mM KCl) (*18*). Under these conditions, salt shifted the Meta I/Meta II equilibrium toward Meta I by electrostatic screening of the net negative surface potential of the cytoplasmic side of the disk membrane and thereby influencing the local membrane surface pH.

The Hofmeister series classifies ions according to their ability to interact with water (*21*−*23*). Ions that interact strongly with water are termed kosmotropic ions and are generally small or divalent ions with a high surface charge density (*24*). Large monovalent ions, on the other hand, are termed chaotropes, as they preferentially bind to the peptide backbone of the protein and may thus induce unfolding of the protein. The Hofmeister effect is therefore characterized by preferential binding to or preferential exclusion from the protein−solvent interface, depending on the nature of the salt (*25*, *26*). Thereby, either an increase or a decrease of this interfacial area is favored in the presence of salt, leading consequently either to protein unfolding in the case of chaotropes or to the stabilization of a more compact state in the case of kosmotropes. In this framework, the underlying principles are not restricted to ions and can as well be extended to neutral cosolvents. In the case of anions, the Hofmeister series follows in general the classification trichloroacetate > thiocyanate > iodide > bromide > chloride > acetate > sulfate in order of their chaotropic properties. The strong effect of the chaotropes trichloroacetate, thiocyanate, or iodide on the Meta I/Meta II equilibrium may therefore suggest that chaotropic ions favor Meta II by favoring a slight unfolding of the protein to a more open structure. Such a proposal seems plausible, as the transition from Meta I to Meta II is known to involve an increased accessibility and disordering of the cytoplasmic side of rhodopsin (*27*, *28*). If the salt-induced formation of Meta II were mainly due to the action of chaotropic cosolvents according to the Hofmeister effect, we would, however, expect kosmotropic protein stabilizers to favor consequently Meta I. Such an effect was not observed in this study. The neutral stabilizer trimethylamine *N*-oxide (TMAO) shows no significant influence on Meta I/Meta II, while the strong kosmotrope sulfate clearly favors Meta II. On the other hand, the strong chaotrope tetraethylammonium (TEA^+) chloride and the neutral protein destabilizer urea, which would be expected to favor strongly Meta II, have both only a weak influence on the equilibrium and are considerably less efficient than, for instance, chloride.

We must therefore conclude that the salt-induced Meta II formation may not be entirely due to Hofmeister effect. The relative inefficiency of the neutral cosolvent urea suggests that the ionic nature of the cosolvent is important. Therefore, binding of ions to charged amino acid side chains as specific binding sites may be the predominant mechanism. Such a mechanism is also in full agreement with the Hill coefficient around 2 of the salt-induced transition. A much higher Hill coefficient and therefore a much steeper concentration dependence would be expected if nonspecific binding of ions to a continuum of binding sites, as suggested in the framework of the Hofmeister effect, were involved. In the case of ion binding to charged residues, the ion selectivity is similar to the so-called electroselectivity series for ion binding to ion-exchange resins (*29*). In this series, anions are listed according to their affinities in the order sulfate > thiocyanate > iodide > bromide > chloride > acetate (*30*). In the case of monovalent anions, this series resembles the Hofmeister series, while divalent ions due to their higher charge density tend to have a higher affinity as compared to monovalent ions, which is opposite to the Hofmeister series. If we consider the possibility that potential binding sites are not freely accessible from the solvent, but may be somewhat buried within the protein, we would expect weakly hydrated ions to penetrate easier to this site. Such a behavior was observed previously for anion binding to the Schiff base in rhodopsin (*16*) and also to other proteins (*31*, *32*) and follows in the case of anions the Hofmeister series. A combination of both effects, electroselectivity and hydration dependent partitioning into the protein interior may ultimately lead to an ion selectivity with divalent sulfate somewhere in between chloride and bromide as observed in this study.

The cation selectivity is not as pronounced as the anion selectivity. The divalent cation calcium shows a relatively strong effect on the Meta I/Meta II equilibrium, which may reflect its high affinity for binding to carboxylates (see ref *24* and references therein). TEA^+ on the other hand is a

strong chaotrope and should easily penetrate into less polar environments. Its only marginal activity may therefore be only explained by a very weak affinity to negatively charged groups due to its very low surface charge density, that may be accompanied already by steric exclusion.

The ion selectivity found here is in line with that found in a previous study that also examined the influence of salts on the Meta I/Meta II equilibrium of rhodopsin in membranes (*33*). In that study, the observation was interpreted mainly as a surface charge screening, which seems unlikely considering recently published results (*18*). In a recent study by our group on a similar salt effect on the abnormal Meta I/Meta II equilibrium of the artificial pigment 9-demethyl rhodopsin, we interpreted the salt-induced formation of Meta II on the basis of the presented data to be predominantly due to the Hofmeister effect, a phenomenon arising from the action of salts at the protein−solvent interface, which influences protein stability (*20*, *21*). We suggested that a salt-induced partial unfolding of solvent-exposed protein domains upon binding of chaotropic ions to the peptide backbone were mainly responsible for the observed effect (*28*). The complex spectral properties of that modified pigment, however, hampered a clear analysis of the salt effects. Regarding the results presented in this study, particularly the ion selectivity and the Hill coefficient around 2, the molecular basis for the salt-induced shift to Meta II seems to involve primarily the binding of ions to specific charged amino acid side chains, resulting in a weakening of specific electrostatic constraints, which otherwise stabilize the inactive Meta I conformation. The Hofmeister effect, involving the binding of cosolvent to the large number of binding sites offered by the protein backbone, seems to be only secondary, but may account for the relatively small, but significant effect of urea.

The recently published 3D structure of the dark state of rhodopsin (*12*) as well as biochemical data on constitutively active opsin mutants (see, e.g., ref *34*) suggest that the inactive states of rhodopsin and its Meta I photoproduct are, in addition to the charge bridge between the Schiff base and its counterion, to a large extent maintained by interhelical electrostatic and hydrogen bonding networks between cytoplasmic sections of the transmembrane helices, as e.g., between the conserved E(D)RY motif on helix 3 and Glu^{247} and Thr^{251} on helix 6 (*1*, *12*) and between Met^{257} on helix 6 and the NPxxY motif on helix 7 (*35*). Formation of Meta II involves relative movements between helices 3, 6, and 7 (*36*−*40*) and therefore alterations of these networks. Vice versa, alteration of the networks by site-directed mutagenesis may lead to constitutive activity or a shift in the Meta I/Meta II equilibrium toward the active state Meta II. Particularly the ERY network between helix 3 and 6, which is in part solvent accessible and known to be involved in the pH-dependent regulation of the Meta I/Meta II equilibrium (*41*), seems therefore to be a likely candidate for an ion binding site. This is further underlined by the observation that proton uptake during the transition to Meta II is abolished both by replacement of Glu-134 of the ERY motif by a neutral glutamine (*42*), as well as by the presence of high concentrations of sodium chloride (*43*).

As compared to the Meta I/Meta II equilibrium, the influence of NaCl on the opsin equilibrium is less pronounced, suggesting a lower affinity of the involved binding sites for solute ions. Previous studies on opsin suggest that opsin is kept in an inactive conformation mainly by the electrostatic interaction between the negative charge of Glu-113 and the positive charge of Lys-296 (*6*, *15*). The observed lower affinity may therefore be explained by the decreased solvent accessibility of this charge bridge in the transmembrane core of the protein compared to ERY network, which is localized close to the cytoplasmic surface.

In summary, we have shown that the conformational equilibria of rhodopsin, which are regulated by charged groups and thus accessible to pH titration, are as well susceptible to salts. In regard of the high degree of conservation of the presumably involved protein domains within class A GPCRs, a similar susceptibility seems plausible also for other receptors and for pH-dependent transitions of membrane proteins in general.

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