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ORIGINAL PAPER

Hydroxylamine as a thermal destabiliser of bacteriorhodopsin

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Abstract The light-catalysed reaction of hydroxylamine (HA) with retinal is one of the basic features of bacteriorhodopsin (BR). Surprisingly, according to recent results, neither the photocycle and proton pumping of BR, nor the *trans–cis* isomerisation of retinal is prerequisite for photobleaching of BR in the presence of HA. How, then, is the accessibility of retinal to HA enhanced on illumination? We studied whether local thermal denaturation of BR, proposed recently, could provide an explanation for HA-promoted bleaching. According to our results, HA does not alter the absorption spectrum and the photocycle kinetics of BR substantially at room temperature, even at molar concentrations, but grossly affects the temperature of thermal denaturation. At pH 7, the presence of 0.5 M HA reduces the denaturation temperature from 100°C to as low as 72°C. The decrease is proportional to the logarithm of the HA concentration over more than three orders of magnitude, and even 0.5 mM HA has a significant effect. In addition, photobleaching becomes considerably faster with increasing temperature in the presence of HA, it takes a few seconds at 50–60°C. Our results suggest that photobleaching of BR in the presence of HA can be explained by overall destabilisation of the structure of the protein and local thermal denaturation that has already accounted for the photobleaching of the HA-free BR at elevated temperatures. These results further support the importance of thermooptic effects in protein photoreactions and identify HA as a thermal destabiliser of BR.

Keywords Spectroscopy · Bleaching · Protein denaturation · Technical application · Photothermal effect · Data storage in biomaterials

Abbreviations

BR	Bacteriorhodopsin
HA	Hydroxylamine

Introduction

Hydroxylamine (HA) is an important compound used for studying retinal proteins. A protein is identified as a retinal protein if it is bleached in HA solution and regenerated subsequently by addition of a suitable retinal analogue (Oesterhelt et al. 1974; Oesterhelt and Schuhmann 1974). For BR, illumination is necessary for efficient bleaching (Yokoyama et al. 2002), thus it was assumed that HA reacts with retinal in an intermediate state of the photocycle. In earlier studies, various photo-intermediates had been assumed to exclusively host the retinal bond cleavage reaction of HA (Oesterhelt et al. 1974; Oesterhelt 1998; Subramaniam et al. 1991; Iwamoto et al. 2001). Such conclusions were based on the fact that replacement of some residue in BR affected (or even removed) both some photo-intermediates and the light-dependent reaction of retinal with HA. However, this does not mean that BR has to populate those photo-intermediates for bleaching by HA, because the use of non-isomerisable chromophores proved that measurable photochemistry (photocycle) of BR was not a prerequisite

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for photobleaching in the presence of HA (Rousso et al. 1998). Further, not even a chromophore–protein covalent bond was a prerequisite for structural effects after light absorption by BR (Aharoni et al. 2000). Similar observations were explained by structural changes induced by charge re-distribution in the non-isomerisable chromophores after absorption of a photon (Aharoni et al. 2000, 2002).

BR is a non-degrading protein in the dark and on illumination at room temperature. Bleaching is slow in the presence of even 1 M hydroxylamine in the dark. According to Sanz et al. (1999) and Perálvarez et al. (2001), the bleaching can be characterised by a time constant larger than 200 h in a suspension of purple membranes. However, in some studies non-negligible bleaching of BR was observed, especially in liposomes and detergents, where the two-dimensional (2D) para-crystalline PM structure was disrupted (Subramaniam et al. 1991; Sonoyama et al. 2004), suggesting that the protein–protein contacts in the trimer are stabilising factors for BR.

BR also has high thermal stability. Its thermal denaturation and bleaching occurs only at approximately 100°C at pH 7 and 85°C at pH 9 (Jackson and Sturtevant 1978; Tristram-Nagle et al. 1986; Brouillette et al. 1987; Dancsházy et al. 1999; Perálvarez et al. 2001; Dér et al. 2007). Recently, we discovered the light-promoted form of the thermal denaturation of BR that manifests itself in photobleaching due to photodenaturation at elevated temperatures (Dancsházy et al. 1999). These experiments indicated that BR is an unstable protein on illumination even at 40°C, especially at high pH. Under prolonged and intense illumination, the temperature dependence of photodegradation/bleaching is similar to that in the dark, but is shifted to lower temperature (Dancsházy et al. 1999). These findings were explained by the so called local thermal denaturation model, introduced recently by us, in which the absorption of light by retinal induces local warming by approximately 40°C in a volume surrounding the retinal that is approximately one tenth of the volume of BR. The spectra of the product states formed in the photodegradation pathway were also determined (Dancsházy et al. 1999; Dancsházy and Tokaji 2000). Another observation was that the increase of photobleaching with increasing pH can be explained by the decrease in the thermal denaturation temperature caused by increasing pH (Dancsházy et al. 1999).

These observations led us to hypothesise that HA has an overall destabilising effect on BR, even in the dark, that is independent of its interaction with the retinal–Schiff-base bond. In this way HA reduces the energy barrier towards thermal denaturation and bleaching. This is in addition to a decrease of the barrier via the local thermal denaturation phenomenon, manifested under strong light conditions. The predicted consequence is that the temperature of both the thermal unfolding and the light -dependent bleaching decreases with increasing HA concentration. This paper is devoted to testing this destabilising effect of HA on BR by studying the photobleaching and thermal denaturation of BR over a wide range of HA concentrations.

Materials and methods

Halobacterium Salinarum strain S9 cells were grown and the purple membranes were prepared according to standard procedures (Oesterhelt and Stoeckenius 1974). The purple membranes containing BR were then incorporated for absorption and absorption kinetic measurements into 1.5 mm thick slices of 10% polyacrylamide gel. The BR containing gels had absorption of 0.37–0.39 at 570 nm at room temperature, equivalent to ~40.2 μM (1.05 mg/ml) BR concentration (Oesterhelt and Stoeckenius 1971). The same gels were used in the bleaching experiments. The samples were buffered either to pH 7 or 9 at different HA concentrations by immersion of the gel slices into solutions of the desired pH. The composition (citrate, borate, phosphate, and diethyl barbiturate) and concentration (30 mM of each component) of the universal buffer were the same as those used in our other studies (Dancsházy et al. 1999; Dancsházy and Tokaji 2000, 1993; Tokaji 1993, 1998). The gel slices were incubated for at least 2 h at room temperature (20–22°C) in the bleaching experiments. No bleaching due to HA was observed during this period. The rate of heating was 1°C min^{-1} in the bleaching experiments. For the calorimetric and fluorescence measurements, the same buffering solution but suspensions of the purple membranes were used. The optical density in the fluorescence studies was the same as in the gels. It is important to note that the pH was stable over a tested 20–80°C range and that the pH was set after addition of HA in the samples containing HA.

The absorption spectra were measured with a Shimadzu (Kyoto, Japan) UV-160 spectrophotometer. The absorption changes were recorded using our custom absorption kinetic measuring system (Dancsházy and Tokaji 1993). The excitation light source was a Nd–YAG laser (Continuum, Surelite I-10) emitting at 532 nm.

Differential scanning calorimetry studies of purple membranes dispersed in the desired buffer were performed using a VP-DSC calorimeter (MicroCal, LLC Northampton, MA, USA), equipped with 0.5-ml sample and reference cells. The instrument's baseline was determined before each sample scan. Samples were scanned between 10 and 110°C, at a rate of 0.5°C min^{-1}, using the passive feedback mode of the VP-DSC instrument. Immediately after completion of the heating scan, a cooling scan was performed at the same rate. The reversibility and repeatability of the unfolding

process was checked by the cooling and a second heating scan, respectively. Before the start, and the repeated heating cycle, the samples were allowed to equilibrate for 1 h at 10°C. The protein concentration was determined spectrophotometrically, using the standard estimated values of 63,000 M^{-1} cm^{-1} for the molar extinction coefficient (at 568 nm) and 26 kDa for the molecular weight of bacteriorhodopsin (Oesterhelt and Stoeckenius 1971). Samples of typically 4 mg/ml protein were used in the DSC experiments. Heat-capacity data were corrected for the instrument's baseline, and normalised for the scan rate and the protein concentration. Excess heat capacity profiles were analysed by use of the Origin software provided with the instrument. A progress baseline was fitted to each profile and subtracted from the whole curve. The heat of denaturation (ΔH_{tot}) and the transition temperatures (T_m) were estimated by integrating over the temperature range corresponding to the full transition.

Fluorescence emission spectra were measured by use of a Quanta Master QM-1 fluorescence spectrometer (Photon Technology International, Princeton, USA) with a 10 mm × 10 mm path-length cuvette and excitation and emission slits of 2 nm.

Results

The absorption spectra of BR in the presence and absence of 1 M HA at pH 7 and 20°C are shown in Fig. 1. The spectra are practically identical in the visible range, and are very similar to spectra recorded at a five-fold lower HA concentration and somewhat higher temperature as reported by Sonoyama and Mitaku (2004). A small increase in the absorption of the sample containing HA can be seen below 400 nm. The absorption in this spectral region originates mainly from the aromatic amino acids and from the higher energy transitions of the retinal. In order to test whether this small change (4%) had some significance, we measured the fluorescence of tryptophan with a 295 nm excitation, and observed an approximately 20% increase in the emission (in the 320–340 nm region) because of the presence of 1 M HA (data not shown). This observation suggests that HA affects the BR structure to some extent even at room temperature.

Surprisingly, however, this effect is not reflected by the photocycle kinetics. The kinetics of the absorption changes at 412 and 570 nm at different flash intensities, and in the presence and absence of 1 M HA, are shown in Fig. 2. The curves are normalised at their maxima and minima. The kinetics of the photocycle and their dependence on the intensity of the actinic flash are practically independent of the presence of HA. This means that HA does not affect the kinetics of the intermediates and the cooperative interaction

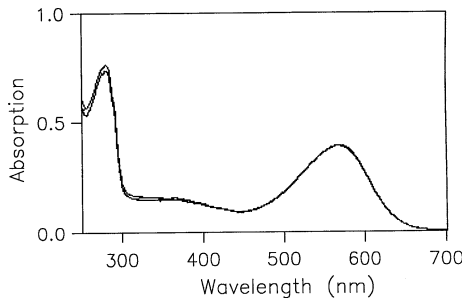

Fig. 1 Absorption spectra of BR (~1 mg/ml), in the form of purple membrane fragments in polyacrylamide gel, in 1 M hydroxylamine and in 1 M NaCl. Note the absence of difference in the visible region and the slight increase in the UV region because of hydroxylamine

of the BR molecules (Tokaji 1993, 1998) at room temperature. Thus the vicinity of retinal and the proton translocation pathway are likely to be unaffected by HA.

The denaturation (bleaching) temperature in the dark as a function of the HA concentration is shown in Fig. 3. At pH 7.0, the denaturation temperature is a closely linear function of the logarithm of the HA concentration over more than three orders of magnitude (correlation coefficient, $r = 0.994$). At 0.5 M HA, the denaturation temperature is reduced to 72°C, and HA is effective even below millimolar concentrations. These observations suggest that the effect of HA is not restricted to a few hypothetical specific (HA-binding) sites.

Thermal denaturation in the absence of HA occurs at a lower temperature at pH 9 (86°C) than at pH 7 (100°C) (in agreement with Brouillette et al. 1987; Kahn et al. 1992). This is probably the reason why the effect of small concentrations of HA at pH 9 is negligible, as seen in Fig. 3. From about 5 mM to higher concentrations, the effect of HA is similar to that at pH 7. However, HA reduces the denaturation temperature to even lower values at pH 9 than at pH 7. At an HA concentration of 0.5 M this temperature is as low as 67°C.

The relationship between thermal bleaching and unfolding (denaturation) of BR was tested by differential scanning calorimetry. Different concentrations of HA were examined. The DSC profiles of BR obtained during the first and second heating scans in the absence (control conditions) and in the presence of 10 mM HA are shown in Fig. 4. Under both conditions, neither the down-scans (not shown), nor the second heating scans (dashed lines) contain any transition peaks, which indicates that thermal unfolding (denaturation) of the BR is irreversible. The midpoint temperature, T_m, of the thermal unfolding of the BR under control conditions is 99°C, with a pre-transition located at 78°C. The total calorimetric heat of unfolding, ΔH_{tot} is 529 kJ/mol. Both the transition temperatures and the heat of transition are in good agreement with previous data obtained for the unfolding of BR (Jackson and Sturtevant

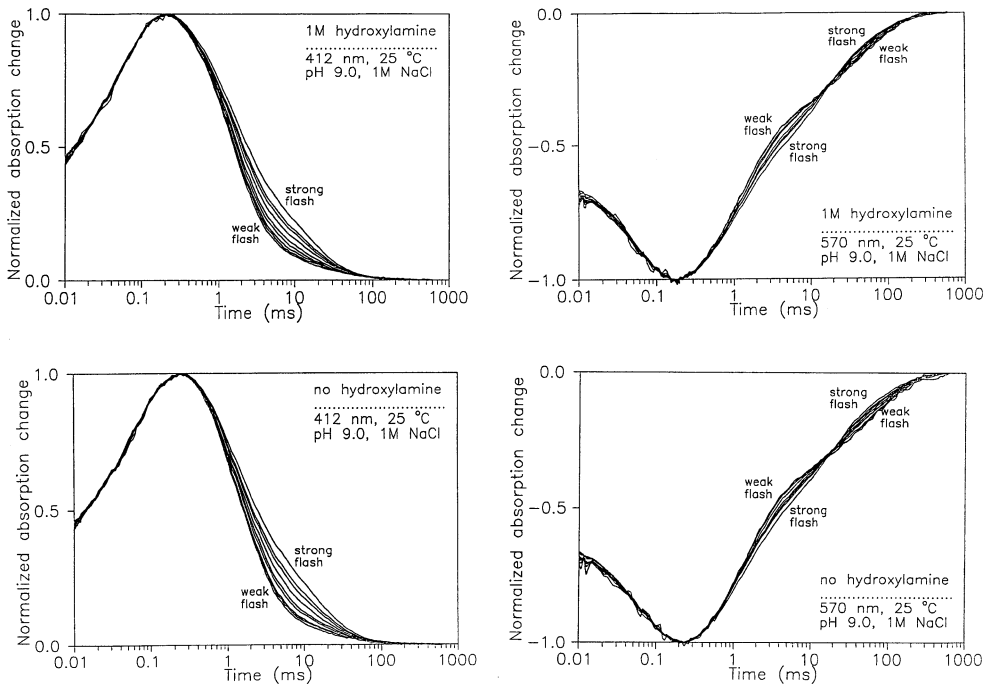

Fig. 2 The absorption changes accompanying the photocycle of BR (purple membrane fragments in polyacrylamide gel) at 412 and 570 nm, at different flash intensities and under different conditions as shown. Note the lack of alteration by hydroxylamine

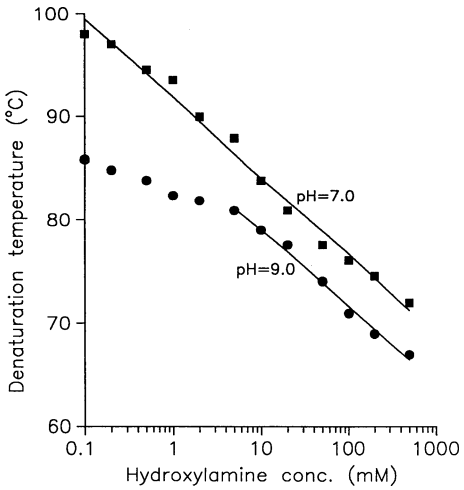

Fig. 3 The bleaching temperature of BR (purple membrane fragments in polyacrylamide gel) in the dark as a function of hydroxylamine concentration. The sum of the concentrations of hydroxylamine and NaCl was kept at 1 M. Comparison with Fig. 4 confirms that the bleaching and the thermal denaturation temperatures shift together

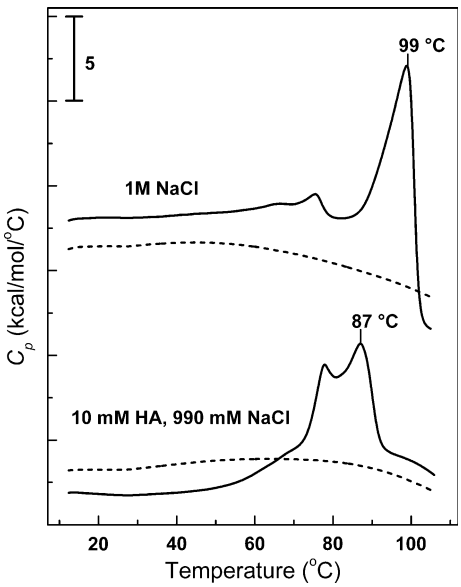

Fig. 4 Excess heat-capacity profiles of purple membrane dispersions (containing ~4 mg/ml BR) during the first (*solid lines*) and second (*dashed lines*) heating scans, at pH 7, in the presence of 1 M NaCl (*top two traces*), and in the presence of 10 mM hydroxylamine and 990 mM NaCl (*bottom two traces*). The position of the midpoint temperature of the mean unfolding transitions, T_m, is shown for both conditions

1978; Tristram-Nagle et al. 1986; Perálvarez et al. 2001; Dér et al. 2007), taking the different conditions into account. When the buffer contains 10 mM HA, the midpoint of the main transition peak shifts to lower temperature by about 12°C compared to the control, for which T_m is 87°C. The pre-transition DSC feature, often seen as two small transitions in the range 50–80°C in the calorimetric scan of BR (Taneva et al. 1995), was reported to be sensitive to different ionic conditions (Jackson and Sturtevant 1978; Dér et al. 2007) and was attributed to the helix alpha

II-to-alpha I conformational change of the BR (Krimm and Dwivedi 1982; Wang and El-Sayed 2000) and might also be related to the melting of the hexagonal para-crystalline arrangement of the trimeric BR units (Perálvarez et al. 2001). The position of the pre-transition features changed relatively little but they became more marked on addition of HA. The total calorimetric heat of unfolding in the presence of 10 mM HA is ΔH_{tot} = 508 kJ/mol, which is close to the value for the control. The midpoint temperatures obtained by DSC for control and HA-treated samples are in close accordance with the denaturation temperature values obtained from the bleaching experiments, 99 and 84°C for the mean and pre-transition, respectively, at pH 7. These results prove that BR indeed unfolds (denatures) in the presence of HA in the dark at the temperatures of the bleaching (indicated in Fig. 3) and that HA reduces the temperature of thermal bleaching and denaturation of BR substantially, and to the same extent (within experimental error).

The photobleaching phenomenon described in our previous papers (Dancsházy et al. 1999; Dancsházy and Tokaji 2000) needs relatively strong illumination intensity (~200 mW × h) and long duration to be effective at temperatures below 60°C. In the presence of HA, a much smaller dose is effective. Absorption of BR at 570 nm after illumination at different temperatures in the presence of 1 M HA is shown in Fig. 5. The fact that the shape of this curve is similar to that of the curve observed at high exposure and in the absence of HA (Dancsházy et al. 1999), again suggests that HA does not alter the function (or structure) of BR significantly until the bleaching reaction starts.

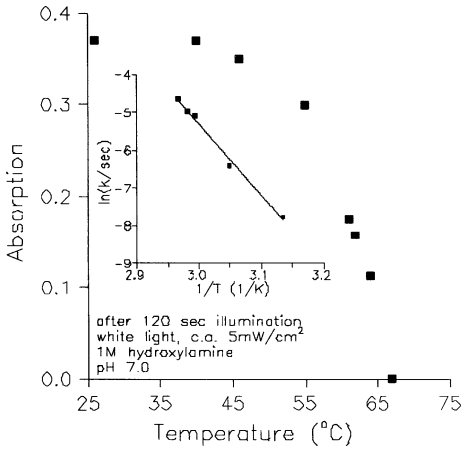

Fig. 5 The temperature dependence of the bleaching of BR (purple membrane fragments in polyacrylamide gel), i.e. the absorption at 570 nm, in hydroxylamine at moderate light exposure. The *inset* shows the corresponding Arrhenius plot of the calculated rate constants, which reflects a single transition. The second, hydroxylamine–retinal reaction is probably fast and thus invisible

Discussion

Various factors stabilise BR against bleaching and thermal denaturation, including the bound retinal and the retinal cavity (Sonoyama and Mitaku 2004; Sugiyama et al. 2006), certain regions and residues of the BR sequence (Subramaniam et al. 1991; Sanz et al. 1999; Perálvarez et al. 2001; Yokoyama et al. 2004; Perálvarez-Marín et al. 2006), protein–protein interaction in the 2D para-crystalline BR trimer (Tokaji 1993, 1998; Sasaki et al. 2005) and hence the membrane environment (Mukai et al. 1999; Sonoyama et al. 2004), optimum pH (this study and, for example, that of Dancsházy et al. 1999). Most of these depend on others, and obviously, perturbing them reduces the energy barrier towards bleaching and unfolding of BR. The retinal cavity, with its H-bonding network and ion-pairing interactions, and the retinal molecule bound covalently to a Schiff-base are probably the most important stabilising factors of BR, because this and other studies (Sugiyama et al. 2006) show that the thermal denaturation (unfolding) of BR and the light-dependent and HA-dependent cleavage of the retinal correlate strongly (at low light intensity). In addition, protection of the retinal–Schiff-base bond (from HA and water) assumes a folded protein with a tight retinal cavity (Sanz et al. 1999), and unfolded bacterio-opsin, i.e. bleached and denatured BR, refolds in the presence of all-*trans* retinal (Ghimire et al. 2005; Sugiyama et al. 2006).

Our results on the photocycle kinetics suggest that the presence of HA alone does not alter the key parts of the proton translocation pathway, the retinal cavity of BR and the 2D para-crystalline structure of the purple membrane significantly. Accordingly, the reversible loss of crystallinity of the purple membrane on photobleaching in the presence of HA (Möller et al. 2000) seems to have no substantial contribution (e.g., loosening of the para-crystalline structure) from an effect of the HA in the dark. The similarity of the dependence of the absorption kinetics on temperature in the absence (Dancsházy et al. 1999) and presence (this study) of HA can be explained by the local thermal denaturation model of photobleaching: In the presence of HA a substantially smaller light dose, 0.17 mW × h, was enough to bleach BR. This is more than three orders of magnitude below the value that had been used for HA-free samples (Dancsházy et al. 1999). Because of the low exposure, the energy barrier is reduced to a lesser extent (hence the bleaching temperature is not as down-shifted) than at high light intensity.

Our results from photobleaching and thermal denaturation of BR (in the dark) clearly show that HA has a dual nature as a destabiliser of BR. The first is its well known interaction with the retinal–Schiff-base bond, leading to bleaching of BR. This reaction is often used as an indicator of access of water to the interior of the retinal cavity

(Sonoyama et al. 2004). In its second nature, observed in this work for the first time, HA is an overall perturbant and destabiliser of BR, which is not necessarily related to its interaction with the Schiff-base and the retinal. Our results do not enable identification of the underlying molecular mechanism by which HA induces a down-shift of the denaturation temperature in the dark, although the high-temperature intermediate state of BR reported recently (Sonoyama and Mitaku 2004) might be important here, but the observation that this down-shift is biphasic at pH 9 (Fig. 3) is in keeping with a dual nature of HA as a BR destabiliser.

We believe that this new role of HA is related to its ability to perturb the structure of (interfacial) water (Yeo and Ford 1992; Vizoso et al. 1994; Vizoso and Rode 1995), because the structure of interfacial water has recently been shown to be an important determinant of protein conformations, also in the case of BR (Dér et al. 2007). Indeed, it should be noted that the absorption and fluorescence spectra of BR change in the UV region in the presence of HA, suggesting that tryptophans (and possibly other aromatic amino acids) might reflect the overall destabilisation of the protein. Note that we have also detected effects of HA on BR with DSC in the pre-transition region, but further studies (e.g. HA concentration dependence) are needed to separate changes in the pre-transition itself from the effect of the down-shift of the main, unfolding transition (hence the enhanced overlap with the pre-transition peaks). Following the above reasoning, altering the water structure reduces the energy barrier to (heat) denaturation, hence the temperature of denaturation (and bleaching) decreases with increasing HA concentration. This effect, which is monotonic and smooth over a large concentration range (three orders of magnitude in this study; Fig. 3), is in keeping with HA being highly water-soluble and the assumption that there are no specific (HA titration) sites on the protein for this kind of action. It is likely that this structural effect of HA on BR varies both in space (over the protein–water interface) and time (over different photo-intermediates of the photocycle), so different regions and photo-intermediates of BR might have different sensitivities to changes in the water structure. Maglova et al. (1989) studied the unfolding of monomeric BR, complexed in a detergent, in water–urea solution at room temperature. High urea concentration (above 3 M) was needed to denature BR under those conditions, and the process was slow. The authors concluded that urea denatured BR via hydrophobic effects, hence the main denaturation step was related to changes in the structure of the interfacial water. Interestingly, it was preceded by a small change in the spectral properties of BR, interpreted as increased accessibility of the retinal moiety to the polar solvent.

The retinal cavity and its neighbourhood is a high sensitivity, critical region. Illumination reduces the energy barrier to (heat) denaturation (and bleaching), as also suggested by Sonoyama and Mitaku (2004) on the basis of kinetic studies on the HA–BR reaction, via the thermooptic effect (Dancsházy et al. 1999), i.e. conversion of most of the photon energy to heat, hence raising the temperature of the protein around the retinal cavity. This model also explains why lower light intensity is needed to bleach BR in the presence of HA, and why a lower HA concentration is effective when BR is under high light intensity conditions and can absorb photons (even with a non-isomerisable chromophore). This explanation is strongly supported also by the observation that some BR mutations and solubilisation of BR in lipid–detergent micelles, i.e. altering its lipid environment, can reduce the energy barrier towards bleaching so much that a reaction between HA and monomeric BR is detectable even in the absence of illumination (Subramaniam et al. 1991). Further, it is reasonable to assume that light-dependent effects observed in BR with a non-isomerisable chromophore, even at sites remote from the retinal (Aharoni et al. 2000, 2002), are caused not only by charge re-distribution in the retinal after photon absorption (Groma et al. 2004) but also by the subsequent conversion of 100% of the photon energy to heat. It should be noted that the thermooptic contribution to unfolding and bleaching of BR depends on the photon flux, whereas that of HA increases with its concentration. In addition, if other stabilising factors are perturbed, e.g. by residue replacements, the heat dissipation pathway might become less effective, leading to more local heating and to less stable BR.

Finally, acceleration of the photothermal bleaching of BR by HA may have technological importance in, for example, use of BR as a data-storage medium (Birge 1995), as an alternative to known procedures. This might be especially true if further studies prove that, similarly to photobleaching in HA at room temperature (Oesterhelt and Schuhmann 1974), the colour loss caused at higher temperatures can be made reversible either in the traditional way (by adding free all-*trans* retinal to the medium; Ghimire et al. 2005; Sugiyama et al. 2006), or by an alternative procedure.

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