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Absorption spectroscopy of three-dimensional bacteriorhodopsin crystals at cryogenic temperatures: effects of altered hydration

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A comparative study of absorption spectroscopy at 100 K has been performed on three-dimensional crystals of bacteriorhodopsin extracted from a lipidic cubic phase and on native purple membrane. A modified microspectrophotometer has been designed which yields absorption data with a high signal-to-noise ratio and remarkable reproducibility. Excellent agreement of the absorption spectra of the three-dimensional crystals and the purple membrane is observed provided that a rigorous crystal-handling procedure is followed. This result supports the equivalence of the protein structure in both the cubic phase crystals and the native purple membrane. On the other hand, it is shown that dramatic deviations of the crystal spectrum can be induced by minor changes in the extraction method. Exposure to air at room temperature can lead within a short time to an irreversible dehydration manifested by a distinct species with an absorption maximum at 500 nm. Exposure of the crystals to a buffer with lower ionic strength than the crystallization solution produces a different spectral form with an absorption maximum at 477 nm, which was assigned to a distorted protein conformation induced by osmotic stress. The extreme sensitivity of these crystals to experimental conditions is relevant for X-ray structural studies, in particular as different experimental treatments are implemented to trap the intermediates of the protein's photocycle.

1. Introduction

Since the development of the lipidic cubic phase (LCP, also known as '*in cubo*') crystallization method (Landau & Rosenbusch, 1996), the high-resolution structure of five membrane proteins and of one membrane-protein complex crystallized *in cubo* have been elucidated (Landau *et al.*, 2003). The method has proven particularly successful in yielding high-quality crystals of archaeal retinal proteins. Of these, bacteriorhodopsin (bR) constitutes a paradigm for its stability and for yielding the best resolved structures so far (Luecke *et al.*, 1999). bR is the light-driven proton pump present in the purple membrane (PM) of the archaeal bacterium *Halobacterium salinarum*. It was the first membrane protein to be crystallized in LCP (Landau & Rosenbusch, 1996). Using time-resolved Fourier transform infrared spectroscopy (FTIR), Heberle and coworkers showed that bR undergoes a photocycle in the crystals (Heberle *et al.*, 1998). This work was followed by X-ray diffraction experiments determining the structures of the various intermediates of the photocycle, which can be trapped in the crystals by flash-freezing under specific conditions of illumination. In the last few years, the structures of the ground state and of key intermediates have

been unravelled for bR (Schobert *et al.*, 2002; Sass *et al.*, 2000; Rouhani *et al.*, 2001; Takeda *et al.*, 2004; Kouyama *et al.*, 2004; Matsui *et al.*, 2002), as well as for the phototactic receptor sensory rhodopsin II (SRII; Landau *et al.*, 2003; Neutze *et al.*, 2002; Royant, Nollert *et al.*, 2001).

Concomitant with X-ray diffraction experiments, which allow the determination of the protein structure and of the structural changes that follow photoexcitation, optical spectroscopy is needed to evaluate the state of the protein molecules within the crystal, as it enables a direct comparison between the protein molecules in the crystal and in their native environment. Absorption spectroscopy has been widely used to investigate retinal proteins during the last three decades. For bR in particular, it constitutes a very accurate probe of the retinal chromophore and its immediate protein environment. Owing to long-range electrostatic interactions, spectroscopy is also sensitive to external parameters such as pH (Ludmann *et al.*, 1998; Groma *et al.*, 2001; Ohtani *et al.*, 1986), relative hydration (Lazarev & Terpugov, 1980; Renthal & Regalado, 1991; Varo & Lanyi, 1991), lipidic environment (Fitter *et al.*, 1998; Fischer & Oesterhelt, 1979), ion binding (Eliash *et al.*, 2001; Jonas & Ebrey, 1991) and oligomeric state of the protein (Becher & Ebrey, 1976; Wang *et al.*, 2002). Some of these factors might differ drastically between the crystal and the native environment, with possible alterations of the protein function and structure. Other important modifications may be induced in the crystal during exposure to X-ray radiation (Matsui *et al.*, 2002) or even as a result of the necessary cooling procedure that precedes the diffraction experiment (Halle, 2004). In order to discard these effects, it is necessary to ascertain the occurrence of a native-like absorption spectrum in the crystals ideally before and during data acquisition in the synchrotron beam.

Although simple in concept, absorption measurements present several difficulties when applied to microcrystals, mainly owing to the small size of the sample, its relatively high optical density and the intensity limitations imposed by the photosensitivity of retinal proteins. While a few reports have shown high-quality absorption spectra from bR crystals (Matsui *et al.*, 2002; Sakai *et al.*, 2002; Schertler *et al.*, 1991), reproducibility is often a problem (Royant, Edman *et al.*, 2001) and optical artifacts sometimes interfere with the measured spectra (Schobert *et al.*, 2002). In order to overcome these difficulties, we devised a micro-absorption setup which yields high reproducibility rates (up to 90%) thanks to the utilization of a very sensitive detection system and the possibility of inspecting the crystal in the probe beam. In addition, precise control of the beam spot size allows us to prevent light from bypassing the sample and to avoid scattering at the crystal edges. Under these conditions, we conducted a systematic study of the optical properties of bR crystals at a cryogenic temperature (100 K) typical for X-ray diffraction experiments. We establish a sample-handling protocol that makes the observation of PM-like absorption spectra possible. Deviations from this protocol are easily observed in the case of non-intentional dehydration and when a non-saturated buffer is used. The extreme sensitivity of bR crystals to the external conditions tested in this work contrasts with the relative stability and reversibility of hydration effects on PM. This situation is discussed in the framework of the special environment that the crystal confinement constitutes for the proteins.

2. Experimental section

2.1. Sample preparation: standard protocol

bR crystals belonging to space group $P6_3$ were obtained by crystallization in 60%(*w*/*w*) monoolein (MO) LCP as described elsewhere (Landau & Rosenbusch, 1996). Small aliquots of MO gel (<1 µl) containing ten to 15 crystals each were extracted from the vials where crystallization took place and immediately immersed in a 1 ml reservoir of a damping solution adjusted to precisely match the crystal's pH of 5.6. The ratio of LCP to damping solution enables us to control parameters in the bath that can affect the quality and composition of the crystals. The solution consisted of saturated Sørensen buffer, 20%(*v*/*v*) glycerol and *Candida rugosa* lipase (Sigma-Aldrich) as a hydrolyzing agent for the MO (Nollert & Landau, 1998). Sørensen buffer is a mixture of 94.8% KH_2PO_4 and 5.2% Na_2HPO_4. The saturated solution of these salts guarantees high ionic concentration in the bath. Glycerol, acting as a cryoprotectant, facilitates crystal handling with the cryoloops.

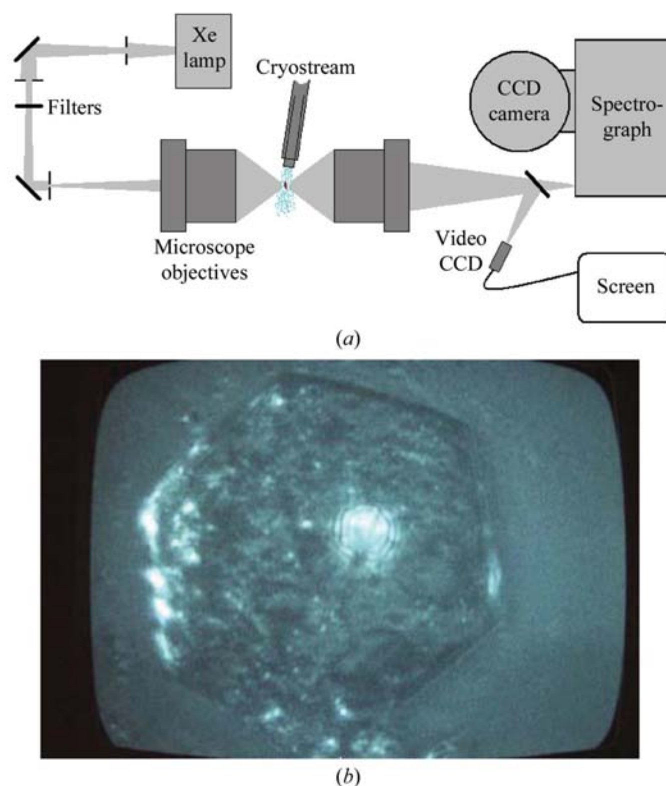

Figure 1
(*a*) Diagram of the experimental setup for low-temperature absorption microspectroscopy. (*b*) Photograph of a bR crystal in the probe beam as is directly seen on the monitor.

After immersion of the LCP droplets in the damping solution, the samples were left in the dark at room temperature for more than 6 h, allowing complete hydrolysis of the MO. Single hexagonal plate crystals with dimensions ranging from 50 × 5 to 120 × 10 µm (limiting diameter × thickness) were removed from the buffer with Hampton Research cryoloops and immediately immersed in liquid nitrogen, where they were stored in the dark until the measurements were carried out. Illumination with a tungsten lamp for over 10 min ensured the light-adaptation of the crystals prior to freezing. Once in liquid nitrogen, further illumination was avoided to exclude the generation of low-temperature trapped intermediates in the crystals.

The procedure described above yields the samples that show PM-like absorption spectra. In the following we refer to it as the 'standard protocol'.

2.2. Sample preparation: alternative protocols

In order to test the influence of room-temperature handling on the crystals, we modified the standard protocol in the way described below as alternative protocol 1. Alternative protocol 2 is meant to increase the free water content of the crystals in order to avoid dehydration artifacts.

2.2.1. Alternative protocol 1. Crystals were treated following the standard protocol, but instead of being stored in liquid N_2 immediately after being fished out, they were kept overnight in closed caps containing water reservoirs. The crystals were not directly in contact with the water, but were close to its surface in order to ensure vapour equilibrium. Next, they were immersed in liquid N_2 and their absorption spectra at 100 K were recorded under the same conditions as for every sample described in this paper.

2.2.2. Alternative protocol 2. The saturated Sørensen buffer was substituted by a 20 m*M* solution of the same salts adjusted to the same pH. The rest of the preparation and measurement strictly follows the procedures of the standard protocol.

2.3. Low-temperature absorption measurements on bR crystals.

Individual crystals were removed from the liquid-nitrogen bath and quickly placed with a Cryo Tong (Hampton Research) into the micro-spectrophotometer, where an Oxford Cryostream (500 series) kept the sample at 100–110 K throughout the measurements. The spectrophotometer (Fig. 1) is an improved version of the home-built setup described in Schenkl *et al.* (2003).

The light of a 300 W Oriel Xe lamp was collimated through a series of pin-holes into a Cassegrain objective (magnification ×15, focal distance 13.35 mm, Ealing) and focused onto a spot of 15–20 µm measured size. An identical Cassegrain objective collected the light and focused it directly on the entrance slit of the spectrograph. A Schott KG5 and quartz neutral density filters were used to adjust the intensity in the spectral range 300–800 nm. The probe light is unpolarized and the crystal axis *c* (*i.e.* the axis perpendicular to the hexagonal plane) is oriented along the light-propagation direction. The measured intensity on the focus was ~40 µW cm^{-2} and the total sample illumination time was 20–30 s (10 s for data acquisition). The detection system comprises a Jobin–Yvon Spectrograph (Spex 270 M) with a liquid-nitrogen-cooled CCD camera (Spectrum One, Jobin–Yvon). The wide dynamic range of this device allowed us to work with samples of OD up to 2.5 without loss of linearity. The wavelength scale was calibrated using an Hg-Ar 'pen-ray' Oriel lamp.

To overcome the difficulty of positioning the microcrystals in the light beam, we installed a mini video camera without objective lens. By means of a flipping mirror we could alternatively send the focused beam towards the entrance of the spectrograph or towards the camera's CCD, making a direct magnified image of the setup's focal plane on it, which allowed us to correctly position the crystal in the focus of the beam (see Fig. 1*b*).

In order to cross-check the reliability of the results, several spectra were measured for each crystal. Using the video image, we were able to select different spots. In all cases the resulting spectra showed identical features, except for some slight variations of the scattering offset.

2.4. Absorption spectra of PM suspension at 100 K

In order to compare the absorption properties of bR in the crystals with those of bR in its native environment, a PM suspension was prepared in a buffer with a composition as described in Loppnow *et al.* (1992). PM patches of *H. salinarum* from a commercial suspension paste (MIB) were suspended in a HEPES buffer/glycerol mixture consisting of 12 m*M* HEPES, 162 m*M* NaCl, 50%(*v*/*v*) glycerol, pH adjusted to 7.0. The concentration of PM was adjusted for OD values of ~0.5 cm^{-1} in the maximum of the absorption band.

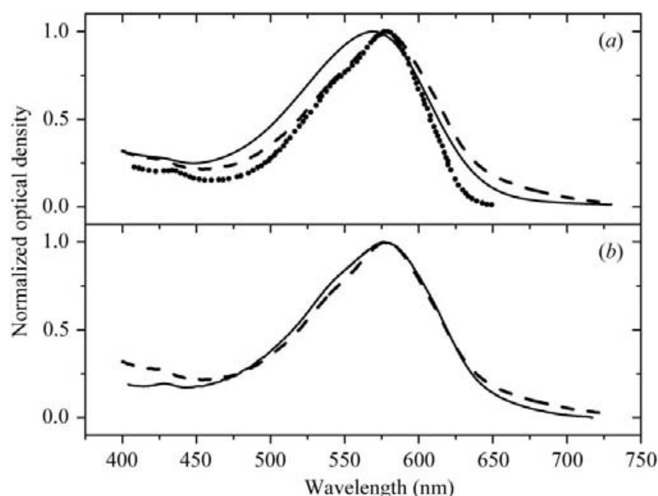

Figure 2
(*a*) Normalized absorption spectra of a PM suspension at 100 K (dashed line) and at room temperature (solid line). The 100 K spectrum published in Litvin & Balashov (1977) is reproduced for comparison (dots). (*b*) Normalized absorption spectrum of a bR crystal at 100 K (solid line) compared with the spectrum of a PM suspension at the same temperature (dashed line).

The optical path in the sample was 1 mm. We found that the use of plastic windows hinders the formation of cracks, which tend to form under the present conditions with glass windows. Measurements were performed at 100 K in an Oxford continuous-flow exchange-gas cryostat, mounted in a Shimadzu UV-3101PC spectrophotometer. The spectra were recorded by scanning from red to blue within ~2 min and the excitation intensity was less than 50 nW cm^{-2}, thus preventing the formation of K intermediates during the scan.

3. Results

Fig. 2 shows the absorption spectrum of the PM suspension measured at 100 K, which is used as a reference for the crystal spectra. A spectrum of PM suspension at room temperature is also depicted (Fig. 2*a*). To facilitate comparison, the spectra are normalized to an OD of 1 at the maximum of the band. At room temperature the absorption band of PM is centred at 569 nm and exhibits a full-width at half-maximum (FWHM) of 108 nm, while the maximum of the low-temperature spectrum is red-shifted to 577 nm and the band, with a 99 nm FWHM, presents a sharper structure. The low-temperature spectrum shows some absorption at $\lambda \geq 650$ nm, in contrast to spectra reported by others for T = 100 K (Litvin *et al.*, 1975; Litvin & Balashov, 1977; Lozier *et al.*, 1975; Stoeckenius & Lozier, 1974). The spectrum of Litvin & Balashov (1977) is reproduced for comparison. As the sample was free of cracks or other sources of light scattering, we believe that this absorption may arise from a small amount of K intermediates, which may be formed owing to spurious background illumination during sample cooling.

In the same figure, the normalized spectrum of a typical crystal treated with the standard protocol and measured at 100 K is presented (Fig. 2*b*, solid line). For $\lambda \geq 650$ nm, the absorption does not decrease as sharply as reported by others (Schobert *et al.*, 2002; Sakai *et al.*, 2002), suggesting that some amount of K intermediate is present but to a much smaller extent than for the PM. Given the present illumination conditions (see above), a steady-state mixture of BR ground state and K may have been reached within a few seconds, but with a negligible amount of K. The spectral similarity of PM and crystals in the main BR absorption band (500–620 nm) indicates that one species, with the same spectroscopic properties as the proteins in PM, is mainly contributing to the spectrum. In the following, this species is named bR_{577}, corresponding to the band maximum at 100 K. The crystal spectrum presented in Fig. 2 is representative of 90% of the crystals treated under these conditions. Thick crystals with OD > 2.5, which naturally led to distorted absorption spectra, were excluded from these statistics.

In previous work (Schenkl *et al.*, 2003), we reported the systematic presence of the dehydrated species bR_{500} in crystals studied at room temperature. It could be possible that this species disappears at low temperature by conversion to an energetically favoured conformation that is entropically unstable in a naturally water-lacking crystal. In order to study the origin of the occurrence of bR_{500}, a batch of crystals were treated following alternative protocol 1, which keeps the low-temperature conditions for the absorption measurement. However, it includes a prior exposure of the crystal to a water vapour-saturated atmosphere, which was the typical environment of storage and measurement in the room-temperature experiments.

Fig. 3 shows a typical low-temperature absorption spectrum of a crystal treated with alternative protocol 1. For comparison, a crystal spectrum measured at room temperature and the spectrum of a crystal treated with the standard protocol

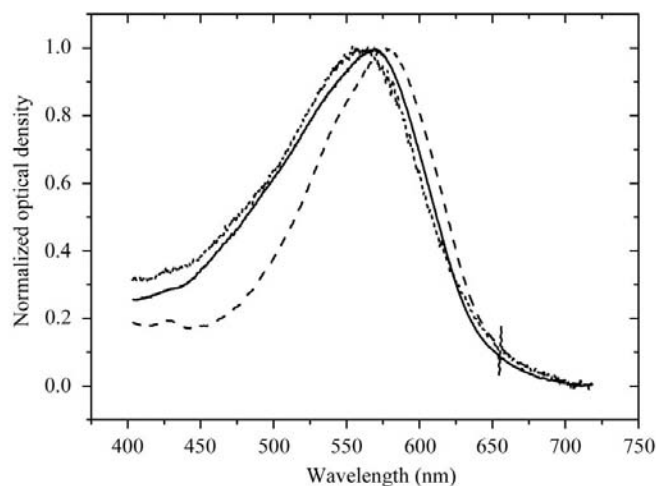

Figure 3
Low-temperature normalized absorption spectrum of a crystal treated with the standard protocol but stored overnight at room temperature in a closed cap with saturated water vapour (solid line). Also shown for comparison are a typical spectrum of partially dehydrated crystals from previous work (dots) and a low-temperature spectrum of a crystal treated with the standard protocol (dashed line).

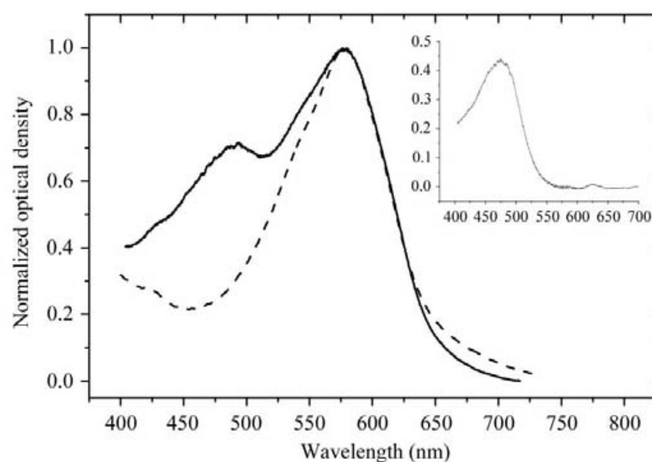

Figure 4
Normalized absorption spectrum of a crystal at 100 K after undergoing a modified protocol in which the saturated buffer was substituted by a 20 m*M* Sørensen buffer (solid line) compared with the spectrum of PM suspension at the same temperature (dashed line). The inset shows the difference between this spectrum and that of a crystal showing the typical bR_{577} spectrum.

are also depicted. The shape and larger width of the absorption band deviate from those of a low-temperature PM spectrum qualitatively in the same way as the room-temperature data (Schenkl *et al.*, 2003). The only feature we can ascribe to a temperature effect is the $\sim$10 nm red shift of the maximum (Fig. 2). This constitutes evidence for the presence of bR_{500} at 100 K. A band-shape analysis shows that 30% of the proteins are in the bR_{500} form. We conclude that the absence of this species in the crystals treated with the standard protocol is not a consequence of the thermal stabilization of the native bR form at cryo-temperatures. The standard protocol and the manipulation under cryogenic conditions can only guarantee that the dehydrated species does not form in the crystal. Handling at room temperature even in a water-enriched atmosphere is responsible for the origin of bR_{500}.

As opposed to dehydration, water availability within the crystals can be increased by exposing them to a buffer with lower ionic strength. A typical spectrum of crystals treated with alternative protocol 2 is shown in Fig. 4. The spectrum exhibits two absorption bands. One is centred at 577 nm, in good agreement with the suspension spectrum at low temperature, while the other is located on the blue side of the spectrum. Further insight into this additional band is gained by taking the difference spectrum (shown in the inset). Here, the normalized spectrum of a crystal treated with the standard protocol was subtracted from the present spectrum. The difference clearly corresponds to a distinct absorption band centred at 477 nm. We assign this band to a new spectroscopic species of bR that we subsequently call XbR_{477}.

Assuming a similar extinction coefficient for bR_{577} and XbR_{477}, we deduce a composition of 25–35% XbR_{477} for crystals treated in the diluted (20 m*M*) Sorensen buffer. The rest of the proteins maintain their natural PM-like absorption spectrum (bR_{577}).

4. Discussion

The above results complement those obtained previously by other groups (Sakai *et al.*, 2002), who showed that PM-like absorption spectra can be obtained in bR crystals. We show in addition that a strict sample-handling protocol is required to observe a single spectroscopic species that corresponds to the protein in the native membrane. Such a homogeneous composition was measured with a reproducibility close to 90%. Although this does not prove a complete equivalence of the protein structure and functionality in both PM and three-dimensional crystals, it is a very sensitive indication of a similar chromophore conformation and a conserved electrostatic environment. One may then expect the photocycle intermediates to be similar in the crystals and in PM.

Spectroscopic or structural studies on individual crystals at room temperature are confronted with handling limitations owing to possible dehydration. Even in the presence of a humid atmosphere, dehydration of the crystals at room temperature (in alternative protocol 1) cannot be avoided. Water evaporation from a crystal surface with a high salt content into an atmosphere that is in vapour equilibrium with a reservoir of pure water is rather unlikely. We therefore suggest that dehydration actually takes place during the few minutes that the vapour equilibrium takes to be re-established within the cap after introduction of the crystal. The opposite process, *i.e.* rehydration of the dried crystal from the water-saturated atmosphere, was never observed, in keeping with our previous observations (Schenkl *et al.*, 2003). This may be owing to a stable lipidic rearrangement on the dry surface of the crystals that isolates the bulk from the surrounding medium, thereby hindering further diffusion. A simple experiment supporting this explanation was conducted by exposing both freshly fished out and intentionally dried crystals to a solution of HCl at pH 2.0. In this case the 'drying process' consisted of exposing the crystals for a few minutes to the ambient air. Acidification of PM results in colour change from purple to deep blue owing to the protonation of Asp85 (Metz *et al.*, 1992). As observed under an optical microscope, fresh bR crystals turned dark blue immediately after contact with the acidic solution. The same process took about 5 min for the intentionally dried crystals (Fig. 5), revealing a structural modification that effectively hinders diffusion upon dehydration.

Concerning the nature of the new spectroscopic species XbR_{477}, various possibilities can be discussed. Retinal absorption is widely tunable in this spectral region depending on protein conformation, bonding or isomerization state (Schreckenbach *et al.*, 1977; Friedman *et al.*, 2003; Tallent *et al.*, 1998). It is noteworthy that the appearance of the 477 nm band is specific to the crystalline environment. A suspension of PM in 20 m*M* Sørensen buffer at pH 5.6 exhibits no trace of the XbR_{477} species, which we verified both at room and cryogenic temperature (data not shown). Only in the crystals does the ionic strength gradient between the external buffer and the crystal inner bulk create osmotic stress. Such stress may ultimately deform the proteins, affecting the neighbourhood of the retinal chromophore and leading to the new species.

Treating the crystals in diluted buffers presents the advantage of avoiding the formation of salt crystals that obstructs

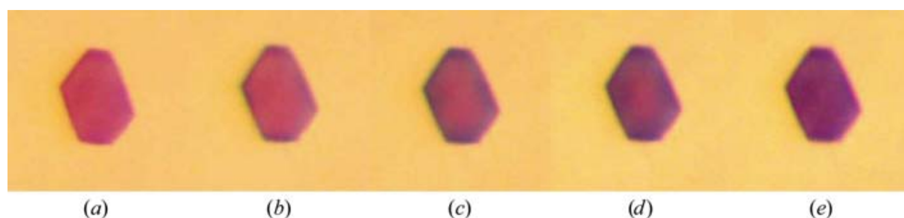

Figure 5
Time progression of bR conversion into blue species for a crystal intentionally dried in air. The crystal is exposed here to a pH 2.0 solution of HCl. The pictures were taken (*a*) in pH 5.6 Sørensen buffer, (*b*) immediately after buffer-exchange to the pH 2.0 solution, (*c*) after 1 min, (*d*) after 3 min and (*e*) after 10 min.

mounting the bR crystals on cryoloops from the dissolved LCP. Washing individual crystals in pure water has also been proposed as a means of removing residual lipids and lipase from the crystal surface (Nollert & Landau, 1998). Our results, however, advise against these procedures and any other treatment that could induce an osmotic stress in the crystals to avoid artifacts such as the formation of the XbR_{477} species.

5. Conclusions

In this study, we obtained spectroscopically homogeneous bR crystals whose absorption spectra correspond to the bR_{577} form found in the native PM of *H. salinarum* under natural conditions at low temperatures. No traces of other absorbing species of bR were found when crystal handling was performed following the standard protocol described in §2. At the same time, we find these crystals to be critically sensitive to environment: the spectral properties of the proteins can be drastically modified by conditions that hardly affect the native PM. In the case of dehydration, a few seconds exposure to air are sufficient to induce the dry species bR_{500}. For PM, vacuum pumping is needed to obtain a similar concentration of bR_{500} (Lazarev & Terpugov, 1980). In addition, exposure to a standard biological buffer that induces no change in the spectral properties of natural PM can convert 25–35% of the proteins in the crystal into a species with drastically modified absorption properties (XbR_{477}). We suggest this effect to arise from the structural stress induced in the protein by the osmotic pressure applied to the lipidic layers in the presence of a strong ionic gradient.

Such protein modifications are readily avoidable during crystal handling as long as a rigorous procedure is carried out. However, as various treatments are designed to optimize the excitation and trapping the proteins in specific stages of their photocycle, special care must be taken to clarify the nature of the resulting species. Light-induced structural changes and sudden freezing under the constraints of crystalline packing constitute a combination of critical processes that might easily lead to the generation of species that differ from the natural bR/PM intermediates. A rigorous analysis of the absorption properties of these crystals is mandatory to establish the nature of the trapped species. So far, the most commonly used method has been to check for the occurrence of PM-like difference spectra in off-line absorption setups, which is a valid but limited approach, in particular when spectral reproducibility is low. In this case, on-line absorption measurements of the specific diffracting crystal are required, allowing, in addition, direct evaluation of the degree of radiation damage suffered by the sample during X-ray exposure (Matsui *et al.*, 2002).

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