Physical Detwinning of Hemihedrally Twinned Hexagonal Crystals of Bacteriorhodopsin

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ABSTRACT Hexagonal crystals of the membrane protein bacteriorhodopsin of space group $P6_3$ grown in lipidic cubic phase are twinned hemihedrally. It was shown that slow changes of salt concentration in the mother liquor lead to a split of crystals so that the split parts preserved high diffraction quality. Analysis of diffraction data from split crystals by Yeates statistic and Britton plot showed that the split parts are free of twinning. It is concluded that crystals of bacteriorhodopsin are composed of several macroscopic twinning domains with sizes comparable to the original crystal. The appearance of twinning domains during crystal growth and the mechanism of splitting are discussed.

INTRODUCTION

Twinning is a defect of crystal growth. A twinned crystal consists of several domains related to each other by specific symmetry operation, so that reciprocal lattices of twin domains overlap at least in one dimension (Parsons, 2003). Twinning can be classified as merohedral or nonmerohedral. In the case of nonmerohedral twinning only some subsets of diffraction reflections from different domains overlap, whereas for merohedrally twinned crystal diffraction patterns from different domains overlap exactly in three dimensions (Dauter, 2003). Hemihedral is the most common type of merohedral twinning when only two different orientations of twin domains are present (Parsons, 2003; Terwisscha van Scheltinga et al., 2003). Without further analysis diffraction patterns of hemihedrally twinned crystals are indistinguishable from crystals without twinning. The intensity, I_{obs}, of each reflection of the twinned crystal, is a sum of crystallographic intensities, I_{cr}, from different domains:

$$I_{obs}(\mathbf{h_1}) = \alpha I_{cr}(\mathbf{h_1}) + (1-\alpha) I_{cr}(\mathbf{h_2})$$
$$I_{obs}(\mathbf{h_2}) = (1-\alpha) I_{cr}(\mathbf{h_1}) + \alpha I_{cr}(\mathbf{h_2}), \quad (1)$$

where $\mathbf{h_1}$ and $\mathbf{h_2}$ are twin related reciprocal lattice vectors and α the twining ratio, a volume fraction of equally oriented domains. Equation 1 implies that the domain size is larger than the coherent length of the x-ray beam (Yeates, 1997). The twinning is perfect when the value of the twin ratio is close to 0.5. In this case, Eq. 1 is confluent and crystallographic intensities, I_{cr}, cannot be extracted from diffraction data. The structure can still be solved (Breyer et al., 1999; Dauter, 2003; Terwisscha van Scheltinga et al., 2003) and Protein Data Bank contains dozens of protein structures derived from perfectly twinned crystals, however the data set of a perfectly twinned crystal contains two times fewer independent observations.

The shape and optical properties of twinned crystals are not necessarily different from those of nontwinned crystals; therefore inspection of a crystal by optical microscopy is not a reliable check of twinning (Yeates, 1997). Recent examples of such crystals are reported by Contreras-Martel et al. (2001) and Terwisscha van Scheltinga et al. (2003). This is one of the reasons why information on the size and organization of twinning domains in protein crystals is rather poor. In some cases, twinning can be discerned under polarized light. There were observations of crystals composed of just a few twin domains as well as composed of a great number of domains (Sieker, 1988; McPherson, 1999). Commonly it is supposed that the size of twin domains is at least in the order of micrometers, otherwise the domain size is smaller than the coherent length of x rays, which would violate the basis of crystallographic treatment of twinning.

A common way to detect and characterize twinning is the analysis of diffraction intensities (reviewed by Dauter, 2003). One of the indications of twinning is the presence of peaks in the self-rotation map showing unfeasible noncrystallographic symmetry. In addition, the second-order moment of the intensity distribution should be analyzed (Stanley, 1972). Its values for nonsymmetric reflections in the case of nontwinned and perfectly twinned crystals equal 2.0 and 1.5, respectively.

For the determination of the twinning ratio statistical methods like Yeates statistics and the Britton plot were developed. The basic principles of these methods are different, therefore they give independent estimates of α. The Britton plot (Fisher and Sweet, 1980) is based on the assumption that the observed and crystallographic intensities are positive and reflections of a nontwinned crystal are independent. In turn, the Yeates statistics introduces a function for twin related intensities

Submitted May 27, 2004, and accepted for publication August 9, 2004.

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0006-3495/04/11/3608/06 $2.00

doi: 10.1529/biophysj.104.046573

$$H = [I_{obs}(\mathbf{h_1}) - I_{obs}(\mathbf{h_2})]/[I_{obs}(\mathbf{h_1}) + I_{obs}(\mathbf{h_2})]$$

(Yeates, 1988). When crystallographic intensities obey the Wilson statistics, a cumulative distribution, $S(H)$, is a function sensitive to α so that for noncentrosymmetric reflections,

$$S(H) = [1 + H/(1 - 2\alpha)]/2.$$

These methods applied to bacteriorhodopsin (bR) crystals are reviewed by Royant et al. (2002).

bR is a small (26 kDa) proton pump of the *Halobacterium salinarium* (for review, see Lanyi, 2000). bR contains the chromophore retinal, which gives the purple color to the protein. bR is spanning the membrane in seven transmembrane helixes connected by loops protruding into the water phase. It was the first protein crystallized in the lipidic cubic phase (Landau and Rosenbusch, 1996). Among its different crystalline forms (Essen et al., 1998; Takeda et al., 1998; Faham and Bowie, 2002) the highest resolution was obtained from platelike hexagonal crystals of space group $P6_3$. These crystals belong to class I of membrane protein crystals (Michel, 1991) in which two dimensional crystalline protein layers are staked in a 3D crystal formed by the contacts of the water protruding protein parts.

Hexagonal bR crystals of space group $P6_3$ often exhibit a perfect hemihedral twinning (Luecke et al., 1998). The highest resolution of the bR ground state structure and structures of some of its photocycle intermediates available at the moment (1.43–1.47 Å) were obtained from nearly perfectly twinned crystals (Schobert et al., 2002; Lanyi and Schobert, 2002). Twinning in bR crystals indicates the presence of a twofold noncrystallographic axis parallel to the *a* axis, which corresponds to the existence of two orientations of crystal domains with Bragg reflections *hkl* and *kh-l*, respectively. The corresponding orientations of the twin domain crystalline lattices and the putative organization of domains in the crystal are depicted in Fig. 1.

Here we describe the splitting of hexagonal hemihedrally twinned bR crystals which was achieved by a slow reduction of the salt concentration in the mother liquor. The split parts displayed high diffraction quality comparable to the original crystal. Checking of the split parts with respect to twinning by the Yeates statistics and the Britton plot showed that they are free of twinning. Twinned hexagonal bR crystals consist of several macroscopic not twinned domains. Appearance of twinning domains during crystal growth and the mechanism of the splitting are discussed.

MATERIALS AND METHODS

Crystallization and splitting

bR crystals were grown in the lipidic cubic phase of monoolein as described in detail elsewhere (Gordeliy et al., 2003). The crystals grew in probes where concentration of dry salt was in the range 1.5–4.0 M (per volume of protein solution). Then crystals were soaked in 3 M sodium phosphate buffer (Na-Pi) pH 5.6 with 0.1% octylglucoside for several days to dissolve the cubic phase (Schobert et al., 2002).

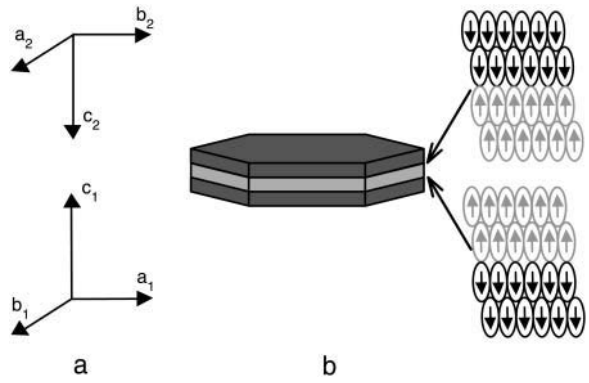

FIGURE 1 (*a*) Mutual orientation of crystallographic axes of twin domains in a hexagonal crystal of bR. (*b*) Domain organization and schematic packing of bR molecules on the surfaces of twinning domains contact. Shown two possible surfaces of domains contact.

To determine twinning ratio crystals were mounted in cryoloops and flash-cooled to 100 K. After diffraction experiments on a rotating anode x-ray generator crystals were warmed to room temperature and subjected to a splitting procedure.

This procedure is similar to the sitting drop crystallization. In a cell of Falckon crystallization plate of ∅18 mm a crystal was placed onto a microbridge in 10 μl 3 M Na-Pi buffer pH 5.6 and equilibrated with 2 M Na-Pi pH 5.6 well solution. Every second day the salt concentration of the reservoir solution was decreased by 0.5 units until the crystal split. After splitting, crystals were equilibrated with 3 M Na-Pi well solution for one day to bring crystals back to the conditions providing cryoprotection.

Successfully split crystals were flash-cooled again and used for diffraction measurements on rotating anode generator. Crystals which diffracted well were used to collect a complete data set on synchrotron.

Determination of twinning ratio

Diffraction data were collected on a rotating anode x-ray generator (Bruker-Nonius FR 571) at 40 kV/50 mA, $\lambda = 1.5418$ Å, equipped with a Mar Image Plate detector and on beamline ID 14-1 of the European Synchrotron Radiation Facility, Grenoble, France.

To determine the twinning ratio diffraction data from three to five images, were collected on the rotating anode generator for the crystal oriented with *ab* plane roughly perpendicular to the x-ray beam in the resolution range 30–2.0 Å. The data were integrated using MOSFLM and SCALA (Collaborative Computational Project, 1994). Integration of data measured from several diffraction images ended up with several hundred twin related pairs as summarized in Tables 1 and 2.

Statistical analysis was performed for nonsymmetric reflections using the twinning server at the University of California, Los Angeles (www.doe-mbi.ucla.edu/Services/Twinning/, Yeates, 1997) and DETWIN routine of CCP4 (Collaborative Computational Project, 1994).

The values of twinning ratios calculated from several hundred reflections were compared with values determined from the complete data set measured from the same crystal (Table 1). The congruity of twinning ratios between both sets of data suggests that there is no bias due to incompleteness of the data. All values of twinning ratios calculated for each crystal by the Britton plot and Yeates statistics are congruent within 5% accuracy and the second moments of intensities also agree with these values (Table 2).

TABLE 1 Comparison of crystallographic parameters and twinning fractions for two sets of data measured from the same crystal: 1), data measured on rotating anode generator (procedure of measurement is described in Materials and Methods)—partial data set, and 2), data measured on a synchrotron source — complete data set; statistics were calculated for noncentrosymmetric reflections

	Partial data set	Complete data set
Lattice constants, *a*,*b*,*c*, (Å)	60.77, 60.77, 110.24	60.77, 60.77, 110.49
Multiplicity	1.2	5.7
Rsym (%)	4.8 (17.2)	10.6 (29.6)
$\langle I/\sigma \rangle$ (last shell)	11.8 (4.2)	5.4 (2.6)
Number of reflections*	1416	46572
Resolution range, (Å)	19.9–2.0	47.6–1.35
Twin fraction determined from:		
Yeates statistics plot	−0.03	−0.03
$\langle H \rangle$	0.03	0.03
$\langle H^2 \rangle$	0.02	0.03
Britton plot	0	0
$\langle I^2 \rangle / \langle I \rangle^2$ averaged on resolution shells	2.0	2.1

*Number of nonsymmetric twin related reflections used for calculation of statistics.

RESULTS

Trials successful in cubo crystallization contain plenty of crystals of different sizes and quality. The shapes of some crystals are different from the normal, hexagon plate, and resemble two agglutinated hexagonal plates (Fig. 2). The mutual orientation of agglutinated plates is similar to the putative organization of twin domains in a crystal (Fig. 1 *b*); therefore it was supposed that agglutinated and twinned crystals have similar nature.

At first, the attempts to separate agglutinated plates applying mechanical force were undertaken. However, the crystals cracked across the *ab* plane before the hexagonal plates were separated. Therefore another approach based on gradual changes of mother liquor properties was used. It was observed that whenever a bR crystal was transferred from 3M sodium phosphate buffer to the solution with low salt concentration it cracked across the hexagonal plane into small segments and at the same time the hexagon laminated. This observation suggested that a gradual change of salt concentration can induce a crack along the surface between twin domains before the domain destruction. Indeed, it was found that a slow reduction of mother liquor salt concentration from 3 to ~1 M (see materials and methods) induces splitting of agglutinated plates. Some of the split crystals diffracted well enough to determine the twin ratio which in all cases was equal to zero within the experimental error.

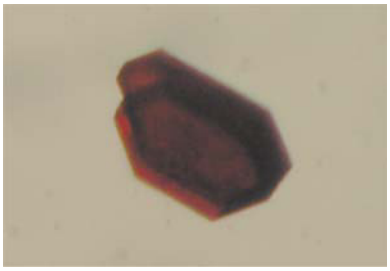

FIGURE 2 Photography of a crystal that looks like two agglutinated hexagonal plates. The crystal size along hexagon is 150 μm and thickness ~45 μm.

The same technique was applied to well diffracting bR crystals which had no visible defects and gave normal diffraction images, i.e., without signs of the existence of distinct domains. Fifty-two crystals with twin ratios >35% (almost all crystals meet this condition) diffracting beyond 2.5 Å on the rotating anode generator were selected. The crystals had the shape of single hexagon plates with sizes, 150–250 μm in diameter and 20–40 μm in thickness, as determined with the help of optical microscopy.

TABLE 2 Crystallographic parameters of crystals before and after splitting

Crystal number	1		2		3		
	Original	Split part	Original	Split part	Original	Split part 1	Split part 2
Crystal size*, (μm)	200		250		300		
$\langle I/\sigma \rangle$	12.2 (3.1)	9.7 (3.7)	16.6 (3.5)	11.8 (4.2)	12.1 (4.2)	13.8 (2.4)	10.3 (3.9)
Number of reflections†	208	528	824	1416	350	1112	1148
Resolution range, (Å)	25.6–2.2	30.3–2.5	11.4–2.0	19.9–2.0	26.3–2.3	30.4–2.4	30.6–2.4
Twin fraction determined from:							
Yeates statistics plot	0.38	−0.03	0.40	−0.03	0.39	0.01	0.01
$\langle H \rangle$	0.40	−0.02	0.42	0.03	0.45	0.02	0.03
$\langle H^2 \rangle$	0.40	−0.01	0.41	0.02	0.44	0.03	0.02
Britton plot	0.39	0	0.40	0	0.41	0	0
$\langle I^2 \rangle / \langle I \rangle^2$ averaged on resolution shells	1.5	2.1	1.6	2.0	1.6	1.9	2.1

The crystals have space group $P6_3$ with lattice parameters $a = b = 60.8 \pm 0.1$Å, $c = 108.9 \pm 1.4$ Å, $\alpha = \beta = 90°$, $\gamma = 120°$. The values of corresponding parameters for the last resolution shell are shown in parentheses.

*The crystal sizes are given for diameter of hexagonal plate, whereas thicknesses of the crystals estimated with optical microscope were in the range of 20–30 μm.

†Number of nonsymmetric twin-related reflections used for calculation of statistics.

As a result 17 crystals split parallel to *ab* plane. A majority of crystals split into two parts of roughly equal thickness (Fig. 3). Three crystals split into three and more parts, but none of them diffracted as well as the original crystal. Fig. 3 *d* shows the initial stage of the splitting process into three parts. Most of the split crystals lost diffraction properties.

As listed in Table 2, in the case of two crystals (Nos. 1 and 2) one of the split parts diffracted to the resolution as the original crystal. Another crystal (No. 3) split into two parts along the plate of hexagon. The diffraction quality of these two resulting parts is conserved and they show no twinning. These data together with the Yeates statistics and Britton plots (Fig. 4) demonstrate that although the original crystals have twinning ratios of ~0.4, the split parts showed no twinning.

The control experiments where flash-frozen crystals after diffraction experiments were placed in 3M Na-Pi buffer pH 5.6 and then flash-cooled again did not show substantial deterioration of crystal diffraction properties nor did they induce splitting.

DISCUSSION

Structure of twinned crystals

Hemihedrally twinned crystals of bR were split along *ab* plane into parts of approximately the same thickness and it was demonstrated that each of them or at least one has no twinning. This result directly shows that twinned crystals of BR are composed of macroscopic domains that have the shape of hexagonal plates with the size in *ab* plane equal to that of the whole crystal and their thickness comparable to the original crystal. It was explicitly demonstrated for one of the crystals that it is composed of two twinning domains. For the other crystals presented in Table 2 one of the domains composes roughly half of crystal volume. Taking into account that in all cases of crystal splitting the resulting part (if diffracting) had no twinning, we suggest that crystals split along the contact plane of the twinning domain. Thus, the splitting of crystals into three and more parts may indicate the existence of more than two twinning domains in the bR crystals. It is important to stress that split domains always have the size comparable to that of the original crystal.

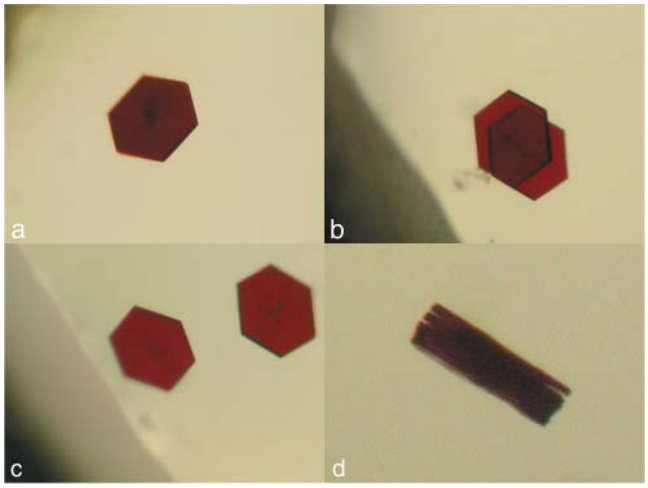

FIGURE 3 Photography of bR crystal at different stages of splitting (*a*) crystal has no visual defects. (*b*) Hexagonal plates are shifted to each other using a nylon cryoloop. (*c*) Finally separated crystals. Crystal's size is 300 μm, twinning ratio 45%. (*d*) Initial stage of crystal splitting into three parts, side view. Crystal size is 200 μm × 40 μm.

Nature of twinning

The hexagonal plane of bR crystals is perpendicular to the *c* axis, since the growth along this direction occurs most probably due to two-dimensional nucleation (McPherson, 1999), which is in accordance with a model of in cubo crystallization proposed by Caffrey et al. (2000; Gordeliy, unpublished). Twinning domains are in contact with each other along the same plane and hence, as we assume, appear due to two-dimensional nucleation as well.

There are two possibilities for the domains to contact each other: by cytoplasmic to cytoplasmic (CPtoCP) or by extracellular to extracellular (ECtoEC) surfaces of bR (Fig. 1 *b*). The hexagonal crystals of bR normally have twinning ratio exceeding 0.4 and they are composed of two twinning domains. Such organization of the crystal may originate from the difference in attraction of twin-domains for the contact of ECtoEC and CPtoCP surfaces. The difference in this interaction may arise due to the difference of charge distribution on the water-exposed surfaces of BR. The extracellular surface of BR is neutral, whereas the overall charge of the cytoplasmic surface is negative (Fig. 5). The interaction of short loops inside a crystal provides a relatively weak vdW contact between the layers (crystallographic structure reveals only vdW contacts between two amino-acid residues per molecule). Hence, even a weak electrostatic repulsion of cytoplasmic 2D crystalline surfaces may have noticeable impact on the interdomain interaction.

Finally, a scenario of crystal growth appears to be as follows: the second twinning domain appears soon after or together with the nucleation. These domains contact each other by EC surfaces. Both hexagonal surfaces of the growing twinned crystal are CP surfaces which are not favorable for an additional twinning domain nucleation, although nucleation is not entirely impossible. Thus, such a crystal with high probability continues its growth without the appearance of new twinning domains.

Splitting of crystals

Another question is why the crystals split along the twinning domains upon change of salt molarity? We suppose that the splitting may be driven by two concurrent mechanisms.

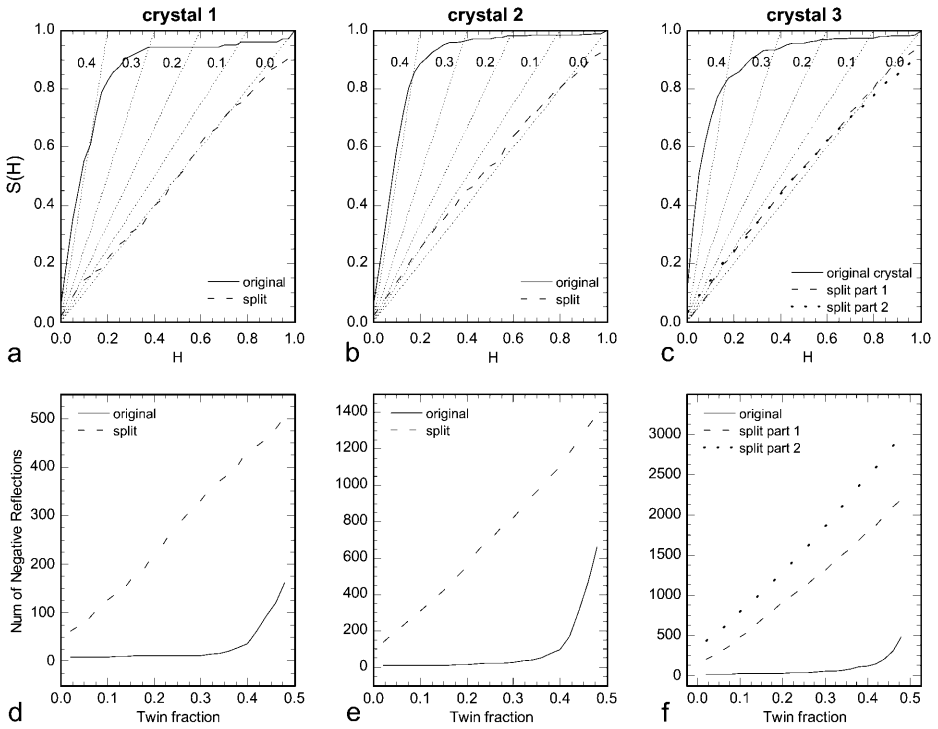

FIGURE 4 (*a–c*) Cumulative distribution $S(H)$ for crystals 1, 2, and 3 from Table 2. Dashed lines show theoretical cumulative distribution of H for twinning fractions 0, 0.1, 0.2, 0.3, and 0.4. (*d–f*) Britton plots, dependence of number of negative intensities after detwinning, as a function of twinning ratio, for original and split crystals No. 1, 2, and 3, respectively (Table 2).

The addition of dry salt as precipitant agent induces crystallization, hence salt creates energetically favorable conditions for bR molecules to contact. The energy of the contact between domains is lower than the energy along the corresponding plane inside a domain. Hence, a gradual reduction of salt concentration diminishes energy profit of the domain contact, and the energy of the domain contact vanishes at a higher salt concentration than that of inter-domain contact. This is the reason why domains separate before the crystal is damaged.

The second possible reason for splitting is a stress in the crystal created by the salt concentration gradient during vapor diffusion. The crystal split as a result of stress relaxation by cracking along an imperfect surface which is the surface of twinning domains contact. In this case it is more difficult to control conditions necessary for splitting and this would explain poor reproducibility of the results.

Finally we would like to mention that the understanding of the origin of twinning in a crystal can result in the discovery of better procedures to grow untwinned crystals. Different approaches, which result in the modification of the properties of bR's hydrophilic surfaces, should influence interaction between layers, which might prevent the formation of twin domains. Such approaches may involve utilization of molecules which bind specifically to one of bR surfaces or, e.g., genetic modification of the protein.

Crystals of membrane proteins grown in lipidic cubic phase are quite specific with respect to their layered membranelike structure, which probably made the separation of twin domains possible. However, we believe that whenever twinning is encountered it is worth trying the separation of domains.

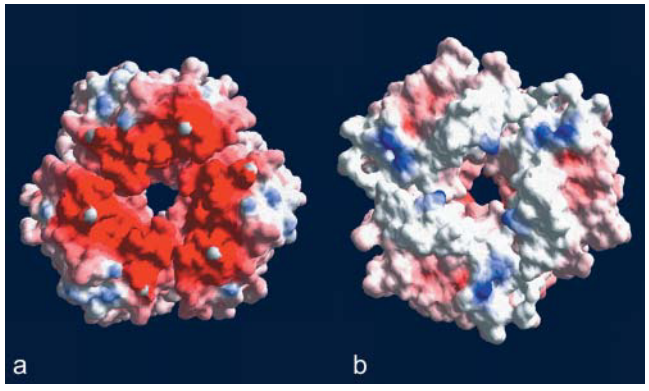

FIGURE 5 Electrostatic surface potential of bR trimer: (*a*) cytoplasmic surface negatively charged and (*b*) neutral extracellular surface. Surface potentials are generated in Swiss-PDBViewer 3.7.

We thank Jörg Labahn for stimulating discussions and Christian Baeken for technical assistance. We are also indebted to the staff of beamline ID 14-1, European Synchrotron Radiation Facility, Grenoble, France, for their support.

This study was supported by the Alexander von Humboldt Foundation.

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