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Overcoming merohedral twinning in crystals of bacteriorhodopsin grown in lipidic mesophase

Valentin Borshchevskiy,[a,b,c]‡ Rouslan Efremov,[b,c]‡§ Ekaterina Moiseeva,[a,b,c]‡ Georg Büldt[b] and Valentin Gordeliy[a,b,c]*

[a]Laboratoire des Protéines Membranaires, Institute de Biologie Structurale J.-P. Ebel, UMR5075 CEA–CNRS–UJF, Grenoble 38027, France, [b]Institute of Structural Biology and Biophysics (ISB-2), Forschungszentrum Jülich, Jülich 52425, Germany, and [c]Centre of Bionanophysics, Moscow Institute of Physics and Technology, Dolgoprudniy, Russia

‡ These authors equally contributed to the work.
§ Current address: Mitochondrial Biology Unit, Medical Research Council, Cambridge CB2 0XY, England.

Correspondence e-mail: valentin.gordeliy@ibs.fr

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Twinning is one of the most common crystal-growth defects in protein crystallography. There are neither efficient rational approaches for the growth of nontwinned protein crystals nor are there examples of systematic studies of the dependence of the twinning-ratio distribution on crystallization conditions. The description of the twinning phenomenon has been covered even less for membrane-protein crystals and is nonexistent for crystals grown using lipidic phases (*in meso*). In the present work, possibilities for overcoming merohedral twinning are investigated for crystals of the membrane protein bacteriorhodopsin (bR) grown *in meso*. It is shown that traditional crystallization additives are not effective in the case of the *in meso* crystallization of bR. The twinning ratio was determined for 310 crystals grown under different crystallization conditions. A correlation of the twinning ratio with the growth rate of the crystals was observed. Slow growth indicated that crystals had a noticeable chance of avoiding twinning. Model calculations were performed in order to rationalize this observation. The calculations confirmed the experimental observation that most crystals consist of two twin domains and showed that under this condition small changes in the probability of twin-domain formation lead to dramatic changes in the number of nontwinned crystals, which explains why slow crystal growth results in a considerable number of nontwinned crystals.

1. Introduction

Twinning is one of the most common pathologies of protein crystal growth. It occurs when several crystals with differently orientated lattices grow together. The gravest cases are those of perfect merohedral twinning. In this case, the nodes of the reciprocal lattices of differently oriented domains overlap in three dimensions. In addition, the volume fractions of the domains are similar, which means that the observed intensities are the sums of crystallographic intensities with Miller indexes related by the twin operator and true crystallographic intensities cannot be extracted from the data. Generally it is difficult to overcome twinning, although a number of structures have been solved from twinned crystals. Experimental phasing is particularly challenging for perfectly twinned crystals and requires very high-quality diffraction data (Dauter, 2003).

In this work, we consider a case of merohedral twinning in $P6_3$ crystals of the membrane protein bacteriorhodopsin (bR) grown *in meso*. *In meso* crystallization is a rapidly growing field of structural biology which has proved to be efficient for several membrane proteins (Cherezov *et al.*, 2007; Gordeliy *et al.*, 2002; Moukhametzianov *et al.*, 2006; Pebay-Peyroula *et al.*, 1997) and has the potential to become more widely used as a method of choice for membrane-protein crystallization

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(Cherezov *et al.*, 2004). During *in meso* crystallization, membrane proteins are reconstituted into the lipid bilayer of the mesophase, from which they crystallize into type I protein crystals formed by stacks of layers of two-dimensional membrane-protein crystals (Michel, 1991).

bR can be crystallized by different methods and in various crystal forms (Faham & Bowie, 2002; Landau & Rosenbusch, 1996; Schertler *et al.*, 1993; Takeda *et al.*, 1998); of these, the crystals belonging to space group $P6_3$ diffract to the highest resolution but simultaneously suffer from hemihedral twinning. Of 28 structures solved from $P6_3$ crystals of bR and its mutants, 19 are from perfectly twinned crystals.

The hemihedral twinning of the $P6_3$ crystals implies the presence of domains with two orientations interrelated by a twofold axis, so that the hkl and $kh{-}l$ reflections overlap. Hence, the experimentally observed intensity is the weighted sum of two reflections,

$$\begin{aligned} I_{hkl}^{\rm obs} &= (1-\alpha)I_{hkl} + \alpha I_{kh-l} \\ I_{kh-l}^{\rm obs} &= (1-\alpha)I_{kh-l} + \alpha I_{khl}. \end{aligned}$$

Here, α is the twinning ratio: the fraction of the domains in one of the orientations. Through analysis of intensity distribution statistics, merohedral twinning can be recognized and α can be estimated (Yeates, 1997). Since the twinning ratio is known, I_{hkl} can be calculated from the equation system and the usual tools can be applied for crystallographic analysis. The detwinning procedure introduces additional noise into the data, which increases and finally tends to infinity as the twinning ratio reaches 50%.

Structures of bR have been solved at resolutions as high as 1.43 Å. However, all bR structures that have been reported to date with resolutions better than 1.9 Å were obtained from perfectly twinned crystals, *i.e.* with α close to 50%. The only exceptions are the 1.55 Å resolution ground-state structure obtained using a bR crystal with a 24% twinning ratio (Luecke *et al.*, 1999) and the 1.9 Å resolution ground-state structure obtained using an untwinned bR crystal (Belrhali *et al.*, 1999). In the case of perfect twinning the equation system cannot be solved, I_{hkl} are not available and special care needs to be taken in structural analysis. Moreover, in the case of perfect twinning the number of independent observations is reduced by a factor of two.

Since bR is a light-driven proton pump, its mechanism is of great interest in bioenergetics. The structural changes that take place during its pumping cycle (photocycle) are relatively small (Edman *et al.*, 1999, 2004; Kouyama *et al.*, 2004; Lanyi & Schobert, 2003, 2007; Matsui *et al.*, 2002; Royant *et al.*, 2000; Sass *et al.*, 2000; Schobert *et al.*, 2002). Hence, the availability of high-quality diffraction data is required in order to resolve the structures of intermediate states. This implies that the data should not be twinned, because only then can difference Fourier electron-density maps be calculated between the experimental structure factors of the intermediate and ground states of the protein, which are the most objective guide for the model building of intermediate states (Henderson & Moffat, 1971).

Numerous efforts have been made to elucidate the structural changes accompanying the bR photocycle using cryotrapped intermediate states. However, all of the published K, L and M intermediate-state structures have either relatively low resolution (>2.1 Å; Sass *et al.*, 2000; Takeda *et al.*, 2004; Edman *et al.*, 1999, 2004; Kouyama *et al.*, 2004; Matsui *et al.*, 2002; Royant *et al.*, 2000) or almost perfect twinning (Lanyi & Schobert, 2002, 2003, 2007; Schobert *et al.*, 2002, 2003; Facciotti *et al.*, 2001). The models built for these intermediates by different groups and from different crystal forms are inconsistent with each other (Lanyi, 2004; Matsui *et al.*, 2002; Takeda *et al.*, 2004). Together with the low occupancy of the intermediate states, radiation damage to the crystals and crystal space-group-related differences, the twinning problem has also contributed to this controversy. Thus, highly diffracting untwinned crystals belonging to space group $P6_3$ are crucial for elucidation of the molecular mechanism of proton transport by bR.

Some empirical approaches have been used to avoid twin formation in the crystallization of soluble proteins. Either small-molecule additives are used for protein crystallization or new crystallization conditions that produce crystals that belong to a different space group are searched for (Yeates, 1997).

In the present work, various approaches to obtaining crystals without twinning have been explored. The twinning ratio was determined for several hundred crystals with different rates of growth and it is shown that when bR crystals grow slowly they have a noticeable chance of avoiding twinning. In addition, model calculations of the growth of twinned crystals were performed to explain the observed phenomenon.

2. Materials and methods

Purple membranes were extracted from *Halobacterium salinarium* S9 (Oesterhelt & Stoeckenius, 1974), purified and crystallized as described in detail in Gordeliy *et al.* (2003). Together with the described crystallization conditions, trials were set up in which detergents from the CYMAL, FOS-choline, glucopyranoside, maltopyranoside and MEGA detergent kits from Anatrace were used in the crystallization. A dry mixture of Na_2HPO_4 (5%) and KH_2PO_4 (95%) was used as precipitant. Crystallization probes were inspected every 14 d during the course of crystallization and the time of growth was determined as the time in which the crystals achieved their maximum size. Crystals were separated from the lipidic phase in a 3 *M* solution of sodium phosphate buffer pH 5.6 containing 0.1% *n*-octyl-β-D-glucoside (Schobert *et al.*, 2002).

To determine the twinning ratio, three to five diffraction images were collected from crystals on a rotating-anode X-ray generator (Bruker–Nonius FR751) equipped with a MAR image plate. The images were collected with an exposure time of 5 min per image and an oscillation angle of 1°. The crystals diffracted to approximately 2 Å resolution and had a mosaic spread of 0.5°. Integration and scaling of the data using *MOSFLM* and *SCALA* (Collaborative Computational Project, Number 4, 1994) resulted in several hundred twin-related reflections. The twinning ratio was determined using the merohedral crystal twinning server (Yeates, 1997) and the *DETWIN* routine of *CCP*4 (Collaborative Computational Project, Number 4, 1994). The same sampling, with the same experimental and crystal parameters, was used as in our previous study (Efremov *et al.*, 2004). It was demonstrated that the twinning ratio calculated from such a sampling is in agreement with the value calculated from the complete data set. The congruity of the twinning ratios excludes bias from the presence of partially recorded reflections and incompleteness of the data. All values of the twinning ratios for each crystal from the Britton plot and Yeates statistics are congruent within 5% precision.

The simulation of crystal growth was performed using *MATLAB* (The MathWorks Inc., Natick, USA).

3. Results and discussion

3.1. Direct observation of twinning domains

It has been shown previously that twinned crystals of bR are formed as two or more domains (Efremov *et al.*, 2004). The domains are hexagonal plates whose crystallographic *a* and *b* axes are parallel and whose *c* axes are antiparallel. The domains can be physically detwinned by the procedure described in Efremov *et al.* (2004), which provides an original way of obtaining untwinned crystals. The salt concentration in the mother liquor was decreased by vapour diffusion until the crystal split. The crystals split along the *ab* plane and disjointed into two or more parts of similar thickness (see Fig. 3 in Efremov *et al.*, 2004). Each of these parts possessed no twinning.

Using different detergents as additives for *in meso* crystallization, we observed that crystals grown in the presence of CYMAL-5 at concentrations of several percent had a shape consisting of two domains in the form of truncated hexagonal pyramids which are joined by their smallest hexagonal surfaces (Fig. 1*a*). The relative volume of the domains varied greatly within one crystallization probe. The twinning ratio of these crystals was determined by analysis of their diffraction intensity statistics. The twinning ratio corresponded to the volume ratio of the observed domains, as estimated from inspection of the crystals under a stereomicroscope, *i.e.* the twinning ratio was close to zero if one part was much smaller that the other and twinning was almost perfect when the parts were of comparable size. Some of these crystals cracked into two fragments when separated from the lipid phase and the resulting fragments showed no twinning, which proved them to be twin domains orientated as illustrated in Fig. 1(*b*). Thus, for crystals grown in the presence of CYMAL-5 it was possible to select crystals with low twinning by visual inspection of their shape, with no need for analysis of the diffraction intensity distribution.

The composition of these crystals supports our previous conclusion about the morphology of bacteriorhodopsin crystals: the twinned bacteriorhodopsin crystals comprise only a few large-scale twin-related domains.

3.2. Crystallization with small-molecule additives

A number of small-molecule additives are known to modulate crystal growth and to have an influence on twin formation in water-soluble protein crystals (Sauter *et al.*, 1999). We studied the influence of the additives glycerol, dioxane and dimethylsulfoxide on the crystal growth and twinning of bR crystals. Additives influence *in meso* crystal growth at concentrations exceeding 1%(v/v) in the protein solution. Upon an increase in the additive concentration, reduction of crystal size, deterioration of crystal shape and even complete inhibition of crystallization were observed (Table 1). Addition of glycerol accelerated *in meso* crystal

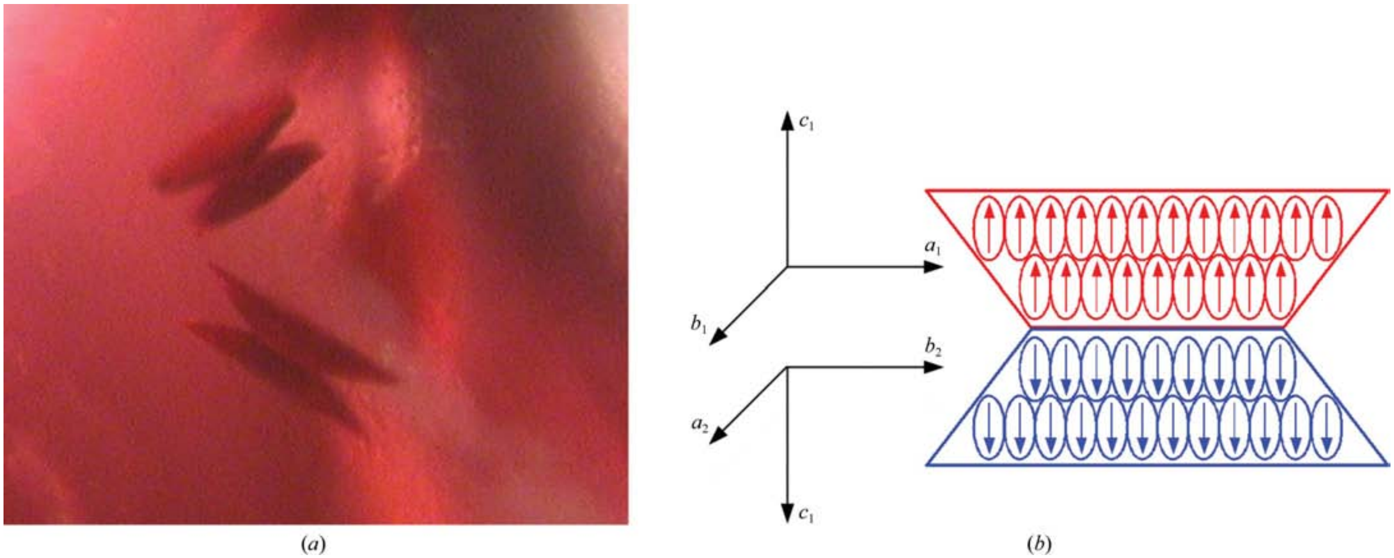

Figure 1
(*a*) Photograph of bacteriorhodopsin crystals with two twin domains. The crystal dimensions are ~200 × 60 µm. (*b*) The mutual orientation of the crystallographic axes of the twin domains and a schematic representation of the protein packing in the crystals shown in (*a*).

growth by more than twofold and yielded essentially thinner crystals. This can be interpreted as a reduction of the interlayer interactions induced by glycerol (McDaniel *et al.*, 1983). For crystals that diffracted to sufficiently high resolution, the twinning ratio was determined. Its value for all crystals was close to 50%. The additives used did not reduce the twinning ratio of $P6_3$ bR crystals, but rather influenced the crystallization process more generally and decreased the crystal diffraction quality.

This observation can be rationalized as follows. All three additives are amphiphiles, with a high ability to modify lipid-bilayer properties (Gordeliy *et al.*, 1998). We suggest that at the concentrations used in this study the additives have a significant effect on the mesophase properties of the crystallization, but only minor effects on the interactions between twin domains. As a result, additives disturb crystal growth more strongly than they influence twin-domain interaction and formation. We conclude that 'classical' additives are not efficient against twinning in $P6_3$ bR crystals grown *in meso*.

Table 1
Influence of additives on bR crystallization.

Additive	Concentration [%(*v*/*v*)]	Salt (*M*)	Crystal size (μm)	Visual estimation of crystal quality
Control	0	1.8–2.6	70–120	Well shaped hexagonal plates
Dioxane	0.5	1.8–2.6	80–100	No effect
	0.9	2.2	∼60	No effect
	1.8	N/A	N/A	No crystals
	3	N/A	N/A	No crystals
Glycerol	0.5	1.8–2.6	50–90	No effect
	1.0	1.0–1.8	70–100	Thin
	2.0	1.2–2.6	70–100	First crystals appear more than twofold faster
	3	1.6–1.8	100	First crystals appear more than twofold faster; crystals are thinner
Dimethyl sulfoxide	0.5	1.4–1.8	20–40	Smaller
	1.0	1.2	70	Badly shaped
		1.6–2.8	∼10	Very small
	2.0	1.6–2.6	∼60	Well shaped
	3.0	2.0–2.8	20–50	Badly shaped

3.3. Twinning fraction and rate of crystal growth

In the course of the study, bR crystals grown under a variety of crystallization conditions, varying the salt and protein concentrations and the detergent type (as additive) and concentration, were tested on the X-ray generator in order to determine the twinning ratio and to select crystals with a low twinning ratio for structural analysis.

We found that independently of crystallization conditions, crystals with low twinning ratio (<20%) were found in probes where crystals appeared later, after 2–3 weeks rather than 2–3 d, and grew relatively slowly, *i.e.* over a period of ten weeks. In Fig. 2 each chart represents the distribution of the twinning ratio for crystals from a single crystallization trial. In these trials crystals grew to their final size in a period of time between two weeks (the fastest) and six months (the slowest).

The distributions show that the twinning ratios for all crystals which grew in less than 1.5 months (Figs. 2*a* and 2*b*) were higher than 20%. In contrast, some crystals from the probes where crystals grew for longer than 1.5 months had a low twinning ratio or even no twinning at all (Figs. 2*c*–2*h*).

To corroborate the observation that slower crystal growth increases the probability of crystals having low twinning, the twinning ratio was determined for 83 crystals that grew in less than 1.5 months and for 227 crystals which grew in longer than 1.5 months. The distributions of the twinning ratio for these two classes of crystals (Fig. 3) demonstrated that 11% of the slowly grown crystals had a twinning ratio below 10%. Of the rapidly grown crystals, none had a twinning ratio below 10% and only a 5% fraction had a twinning ratio in the range 10–20%.

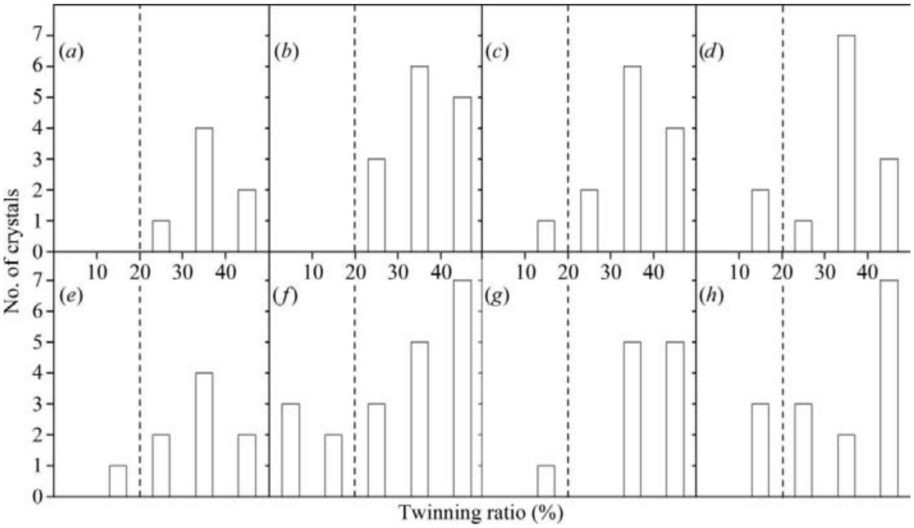

Figure 2
Twinning-ratio distribution in individual crystallization probes with the following characteristic times of crystal growth: (*a*) ∼2 weeks, (*b*) <1.5 months, (*c*) >1.5 months, (*d*) and (*e*) ∼4 months, (*f*) ∼5 months, (*g*) and (*h*) ∼6 months.

bR crystals are not the only crystals that display a dependence of the twinning ratio on the growth rate. It has previously been noted for the water-soluble protein plastocyanin that variations in crystallization conditions which lead to slow crystal growth may be associated with low twin formation (Redinbo *et al.*, 1993). This phenomenon might be common for twinned protein crystals and attention should be paid to the rate of crystal growth whenever twinning is encountered.

Surprisingly, the twinning-ratio distribution for slowly grown bacteriorhodopsin crystals is wave-like. It peaks below 10% and above 35%, with a gap in between. In an attempt to explain this non-intuitive distribution, model calculations have been performed.

3.4. Model of twinned crystal growth

bR crystals belong to class I of membrane-protein crystals (Michel, 1991). They have a layered structure in which each layer is a two-dimensional crystal of the membrane protein. From crystal structure and AFM studies of bR crystal growth (Qutub *et al.*, 2004), it was found that growth occurs by the sequential formation of two-dimensional nuclei on surfaces of the growing crystal parallel to the *ab* crystallographic plane. One of the surfaces is exclusively formed by the hydrophilic cytoplasmic (CP) surface of the bR molecules, while another surface is formed by the extracellular (EC) surface. The two-dimensional nucleus forms contacts with the crystal *via* CP-to-EC interactions during normal crystal growth. Nucleation *via* CP-to-CP or EC-to-EC interactions leads to the origin of a new twin domain.

A one-dimensional model was used to simulate crystal growth in the direction perpendicular to the *ab* crystallographic plane (Fig. 1*b*). Crystal growth begins from a single layer and proceeds by consecutive addition of new layers to each surface of the crystal alternatively (Supplementary Fig. 1[1]). A new layer is added to the CP surface by forming either CP–EC or CP–CP contacts and to the EC surface by forming either EC–CP or CP–CP contacts. The EC–CP contact corresponds to normal crystal growth and has a relative probability of 1, while the formation of CP–CP or EC–EC contacts corresponds to nucleation of the twin domain and has probabilities relative to normal crystal growth of P_1 and P_2, correspondingly. It was assumed that the probabilities remain unchanged during crystal growth. A total number of 4000 layers were used in the simulation, which corresponds to the experimentally observed average crystal thickness of ~20 µm.

There are two variables in the model: the probabilities P_1 and P_2. The behaviour of the model was explored in an attempt to find combinations of P_1 and P_2 which produce twinning-ratio distributions similar to those observed experimentally (Fig. 3). It was noticed that two independent runs simulating the twinning-ratio distribution for 100 crystals with identical parameters displayed essential variations arising from poor statistics (compare Figs. 4*a* and 4*b*). This indicates that for our experimental sampling of 83 and 227 crystals the statistical noise is quite large. However, the principal features of the distributions are still distinguishable.

Under symmetrical conditions, when $P_1 = P_2$, the distributions show a peak at zero twinning ratio. The height of the peak decreases as the probability of twin formation increases. The distribution over the nonzero range remains quite flat until the peak at zero vanishes (Supplementary Fig. 2[1]).

When an asymmetry in the probabilities is introduced, the peak at zero value changes very little, while the rest of the distribution has low values at low twinning ratios which gradually increase towards higher twinning ratios (Supplementary Fig. 3[1]). The distributions under asymmetrical conditions resemble the experimentally observed distribution.

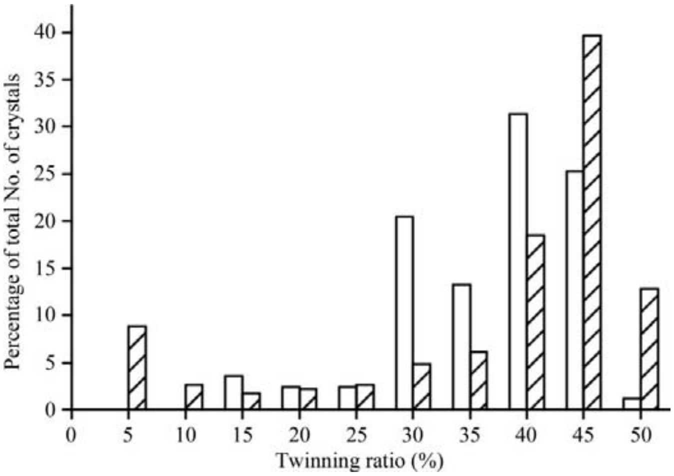

Figure 3
Twinning-ratio distribution for crystals with two different characteristic growth times: less than 1.5 months (83 crystals), empty bars; more than three months (227 crystals), shaded bars. For the latter distribution the appearance of almost untwinned (<10%) crystals is evident.

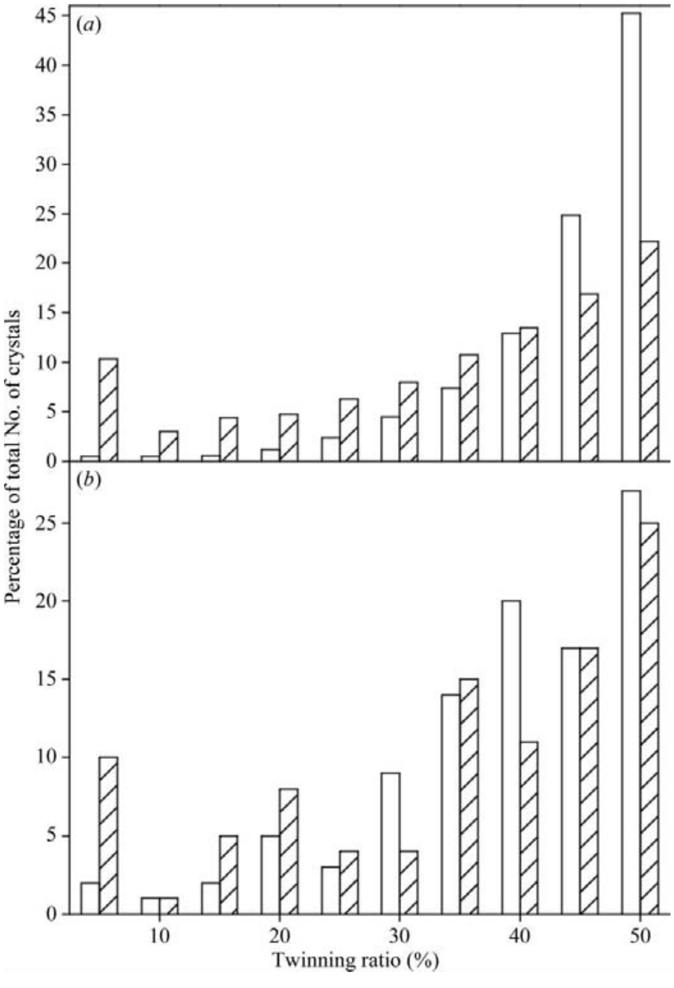

Figure 4
Modelled distributions of twinning ratios simulating experimental distributions for slow (shaded bars) and fast (empty bars) crystal growth. The P_1 probabilities for the models are 1.25×10^{-3} and 3×10^{-5} correspondingly; $P_2 = 0$. Distributions were calculated for (*a*) 5000 crystals and (*b*) 100 crystals.

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[1] Supplementary material has been deposited in the IUCr electronic archive (Reference: YT5018). Services for accessing this material are described at the back of the journal.

Simultaneously, the introduction of asymmetry leads to a shift in the peak of the number of twin domains distribution (compare Supplementary Figs. 2*b* and 3*b*) from six domains (for $P_1 = P_2 = 10^{-3}$) to two domains ($P_1 = 10^{-3}$, $P_2 = 0$), which is in accordance with the experimental results. When P_2 is much smaller then P_1, changes in P_2 affect the twinning-ratio distribution very little; however, they do lead to noticeable changes in the distribution of the number of twin domains. Further model analysis was performed with $P_2 = 0$ (Supplementary Fig. 4). Under these conditions, small changes in P_1 lead to dramatic changes in the fractions of nontwinned and perfectly twinned crystals, while the fraction of crystals with an intermediate twinning ratio changes much more slowly. The best fit of the experimentally observed distributions corresponds to a probability P_1 of 3×10^{-3} for fast crystal growth, where less than 1% of crystals grow without twinning, and of 1.25×10^{-3} for slow growth, where 10% of crystals have no twinning (Fig. 4*a*).

The model grasps the principal features of the experimentally observed distributions. Even taking statistical noise into account, the region of experimental distributions close to the twinning ratio of 50% is not described by the simulations (compare Figs. 4*a* and 3). There are several possible reasons for this discrepancy. One of the these may be related to the underestimation of the twinning ratio by computational procedures owing to noise in the diffraction intensities. Another possible reason is the heterogeneity of the crystallization medium, which is responsible for the inevitable differences in the growth rates of different crystal surfaces. For a crystal with two twin domains this will result in different sizes of the domains and consequently a twinning ratio that is lower than 50%. The protein concentration and crystal-growth rate change during the crystallization process, which was not taken into account during modelling. Despite this, the model describes the principal features of the experimentally measured data surprisingly well.

The proposed model of twinned crystal growth explains how the relative probabilities P_1 and P_2 influence the shape of the twin-fraction distribution. However, the connection between the overall rate of crystal growth and P_1 and P_2 remains uncertain. The theory of *in meso* crystallization is not complete and not directly able explain this connection. We provide our plausible hypotheses concerning this phenomenon in the Supplementary Material.

4. Conclusions

We have shown that there is no simple way to grow nontwinned $P6_3$ crystals of bR *in meso*. However, the rate of crystal growth strongly affects the twinning-ratio distribution of the crystals. Searching for crystallization conditions leading to slow crystal growth, it was possible to select crystallization trials that contained up to 10% nontwinned crystals. Our model calculations show that to satisfy the observed twinning-ratio distribution the twin-domain formation has to be asymmetrical with respect to crystal surface growth, which simultaneously leads to predominant growth of crystals with only two domains. Under these conditions, a small decrease in the probability of twin-domain formation dramatically increases the proportion of nontwinned crystals.

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