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SANS investigations of the lipidic cubic phase behaviour in course of bacteriorhodopsin crystallization

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Abstract

Detailed kinetics of a proteolipidic cubic phase was studied by neutron scattering in course of crystallization of the membrane protein bacteriorhodopsin. Initiation of the crystallization process by salt addition leads to a dramatic decrease of lattice constant, but no phase transition takes place. Cubic phase of Pn3m symmetry is observed during the entire crystallization process. No another phases are present in a macroscopic amount. Our observations show that process of the crystal growth in this case is similar to that for water soluble proteins.

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1. Introduction

Crystallization of membrane proteins is still a challenge in structural biology. Whereas the number of soluble protein structures has surpassed 10,000, and the rate of appearance of new structures is increasing steadily, general problem in attaining high-resolution structures of membrane proteins by X-ray crystallography is the reproducible growth of well-ordered three-dimensional crystals.

Landau and Rosenbush proposed a novel approach for crystallizing membrane proteins in a lipidic cubic phase [1]. This method allowed to obtain X-ray structures of important membrane

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protein bacteriorhodopsin and its photocycle intermediates at highest resolution [2–6]. Besides, the structures of some other membrane proteins were solved from crystals grown in cubic phase as well [7–9]. However, it is not yet clear whether crystallization of membrane proteins in the lipidic cubic phase is a general method. Efforts were undertaken to understand the mechanism of crystallization *in cubo* [10,11]. The phase state of crystallization samples has been studied by X-ray diffraction at initial and final stages of the crystallization process and a hypothetical mechanism of *in cubo* crystallization was proposed [11] and described as mathematical model [12]. However, behaviour of the lipidic phase during crystallization process has not been elucidated. This is an important part of the crystallization process because the way in which the properties of system are changing is often crucial for the results of crystallization [13].

In the present work detailed kinetics of the proteolipidic cubic phase behaviour in course of crystallization was studied by neutron diffraction. As far as neutrons penetrate deeply into matter and the aperture of neutron beam is large, these gave an important advantage: possibility of investigation of real probes used to grow BR crystals (in glass tubes with diameter of 3 mm). It was found that in spite of significant changes of parameters of the lipid cubic phase, caused by salt addition, the Pn3m symmetry of the cubic phase remains unchanged during the entire crystallization process. It was shown that coexistence of the macroscopic amount of the other phases as well as the presence of a phase transition is not necessary for the crystallization process.

2. Materials and methods

2.1. Sample preparation

1-monooleoyl-*rac*-glycerol (monoolein, C18:1c9) was purchased from Sigma Chemical and used without further purification. Bacteriorhodopsin (BR) was solubilized from purple membranes in phosphate buffer: 20 mM Na/K-P_i, pH 6.9, 3.75% (w/v) *N*-octyl-β-glucopyranoside (OG). Protein solution was diluted three times by 370 mM Na/K-P_i buffer, pH 5.6 [14]. Then it was concentrated to final BR concentration of ca. 7.5 mg/ml in Amicon filters with 30 kDa cut off. Samples were prepared by using the standard method of BR crystallization [1] in glass capillaries with a diameter of 3 mm. BR solution was added to melted MO in proportion 50/50. Then a centrifugation was performed for better mixing of MO with BR solution and for homogeneity of the cubic phase. Prepared samples were equilibrated during 24 h at 22 °C in darkness.

Protein crystallization was initialized by addition of phosphate salt (Na/K-P_i, pH 5.6) to lipid/protein solution mixture (final salt concentration in the samples varied from 0.7 to 2.4 M) and the samples were centrifuged again. Crystals grew in darkness at 22 °C.

2.2. Neutron scattering

Neutron experiments to study the behaviour of BR/MO/buffer system in course of the protein crystallization were carried out at small momentum transfer neutron diffractometer D16 (ILL, Grenoble, France) in the range of scattering vector Q ($Q = 4\pi \sin\theta/\lambda$) from 0.05 to 2.4 Å^{-1} and at small angle neutron scattering spectrometer YuMO (FLNP, JINR, Dubna, Russia) in the range from 0.01 to 0.4 Å^{-1}.

At D16 diffractometer the samples were measured firstly without precipitant, then every two hours after salt addition during eight hours and, finally, after two days. The measurement time at D16 was 10 min.

The measurements at YuMO spectrometer were carried out also firstly without the precipitant, then immediately after the salt addition and then the kinetics of the behaviour of BR/MO/buffer system was monitored every 20 min during first hours after the precipitant addition. The measurement time at YuMO was 5–10 min. The samples were investigated at 22 °C in darkness.

3. Results and discussion

3.1. Determination of cubic phase parameters

MO/water system is able to form cubic phases of Pn3m and Ia3d symmetries [15], which are

characterized by the following ratios for the reflection positions $\sqrt{2}$:$\sqrt{3}$:$\sqrt{4}$:$\sqrt{6}$:$\sqrt{8}$:$\sqrt{9}$ and $\sqrt{3}$:$\sqrt{4}$:$\sqrt{6}$:$\sqrt{8}$:$\sqrt{10}$:$\sqrt{11}$, correspondingly [16].

Diffraction spectrum measured at D16 diffractometer contained 2–3 clearly distinct peaks (Fig. 1), which were fitted by Lorentzians to determine exact reflection positions. According to the ratio of the diffraction peaks positions the symmetry of the formed cubic phase was determined.

On diffraction curve obtained at YuMO spectrometer two overlapping peaks were clearly observed (Fig. 2). In some spectra they merged into one obviously asymmetrical peak (Fig. 4b). Diffraction peaks obtained in these measurements were fitted by Gaussians. An example of a scattering curve and of fitting of two peaks is presented in Fig. 2.

For all measured samples the ratio of reflection positions corresponded to that of Pn3m symmetry with accuracy of 1%. Indeed, the accuracy of the peak position is 0.2% for the first peak and is about 1% for the second one. Thus, the precision of the peak ratio determination is within 1%. The peak ratio for measured spectra coincides with theoretical value $(3/2)^{1/2} = 1.225$ for Pn3m phase with accuracy of 0.01, that is within experimental error, and is clearly distinct from the value $(4/3)^{1/2} = 1.155$ for Ia3d phase. After the determination

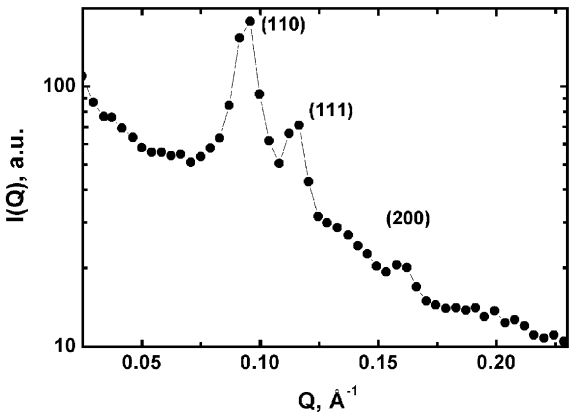

Fig. 1. Diffraction spectrum measured at D16 diffractometer, ILL. Three low-resolution diffraction peaks are observed. The symmetry of the cubic phase was determined from the peak positions.

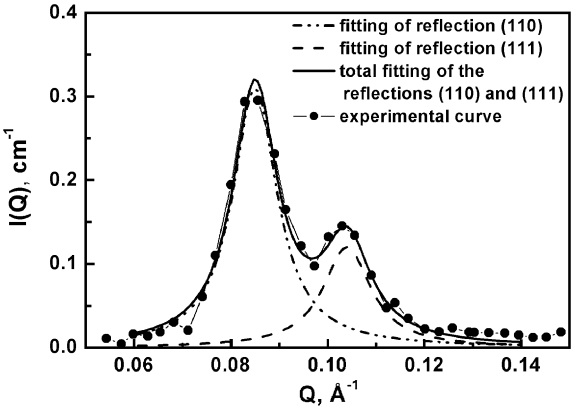

Fig. 2. Example of the scattering curve from a lipidic cubic phase measured at YuMO and the fitting for two peaks by Gaussians.

of cubic phase symmetry the lattice constant values were calculated.

Reliability of the symmetry assignment was tested with MO/water system. The phase assignment and its lattice parameter value determined from our experimental data were similar to the reported earlier [15]. Hypothetical possibility of the presence of Ia3d phase simultaneously with Pn3m phase was estimated by computer simulations of neutron diffraction data (lattice constants known for cubic phase of MO/water system at 22 °C in the region of Ia3d–Pn3m transitions were used for these simulations). Besides, X-ray tests of the lipidic phase in some of the crystallization probes were carried out to check the presence of the Ia3d phase. Due to higher resolution of X-ray instrument the separation of the diffraction peaks is more distinct and the sensitivity of this method is higher. In all tests the presence of Ia3d phase has not been observed. Thus, we conclude that the amount of Ia3d phase in crystallization probes studied by neutron diffraction does not exceed a few percent.

Unambiguous assignment of the cubic phase symmetry for data measured at YuMO was possible only for the points in which system was stable. Behaviour of diffraction pattern in the points where parameters of cubic phase are not stable is discussed in Section 3.2.2.

3.2. Phase behaviour of monoolein/detergent/bacteriorhodopsin/buffer system

3.2.1. Slow part of kinetics

Crystallization procedure described in Section 2.1 allows to obtain the first visible crystals (~5–10 μm) usually on the second or third day after precipitant addition. The time scale for the study of mesophase kinetics was chosen on the basis of this fact. The key step of the crystal formation—nucleation should occur within this time interval.

Thus, the phase behaviour of the MO/BR/OG/buffer (250 mM Na/K-P_i in D_2O, pH 5.6) system was studied at the following stages of the crystallization process: (a) MO/BR/OG/buffer mixture equilibrated during 24 h at 22 °C in darkness before the precipitant addition, (b) each second hour during first eight hours after addition of dry salt and (c) in about two days after addition of the precipitant.

It was found that at our crystallization conditions MO/BR/OG/buffer system forms the cubic phase of Pn3m symmetry (Table 1, Fig. 3). Thus, the initial phase state of the system is the same as that observed in the MO/water system, but with lattice constant increased by 15–17 Å. This effect is due to the presence of detergent, as was studied in details [17]. Detergent concentration in our samples was estimated from the linear dependency of detergent amount on the lattice constant of cubic phase expressed as $C_{OG} = (d_{001} - 106)/3.3\%(w/v)$ [17] and is equal to $4 \pm 1.5\%(w/v)$.

A dramatic decrease of the lattice constant is observed after initiation of the crystallization process by the salt addition. All substantial changes of cubic phase parameters are completed within two hours. As seen in Fig. 3 the decrease of lattice constant strongly depends on the concentration of the added salt. It decreases with the increase of salt amount. The cubic phase conserves the Pn3m symmetry in the whole range of the salt concentrations from 0.7 to 2.4 M in all investigated time moments and presence of another phases was not detected (Fig. 3b).

Control experiments to study the behaviour of the cubic phase of MO/water and MO/crystallization buffer systems were carried out also. The behaviour kinetics of these systems is similar to that observed for the crystallization samples. Only initial and final states depend on composition of the system, but not the kinetics.

Table 1
Dependence of the lattice constant of the lipid cubic phase of Pn3m symmetry for MO/BR/OG/buffer (250 mM Na/K-P_i in D_2O, pH 5.6) system on added salt concentration (C_{salt}) at the studied crystallization conditions at different stages of the crystallization process, and results of crystallization

C_{salt} (M)	Lattice constant[a] (Å)			Final size of crystals
	Before salt addition	2 h after salt addition	2 days after salt addition	
0.7	114	97	102	—
0.9	114	95	98	40
1.1	115	94	97	40
1.2	115	92	94	40
1.3	117	93	96	30
1.4	117	92	94	30
1.5	120	95	97	50
1.6	114	92	94	40
1.7	118	90	92	20
1.8	118	91	94	40
2.0	115	91	93	25
2.2	115	87	89	—
2.4	116	89	91	20

The data are average of several experiments
[a]The accuracy of the lattice constant determination is ±1 Å.

3.2.2. Fast part of kinetics

The kinetics of the MO/BR/OG/buffer system during the first two hours following salt addition was investigated at the YuMO spectrometer (Fig. 4a and b).

Immediately after the salt addition a nonequilibrium state of MO/BR/OG/buffer system is observed. At this time the changes of cubic phase occur and diffraction peaks are substantially broader than in equilibrium (Fig. 4b). A direct determination of cubic phase symmetry is not possible in this case. But taking into account reliably determined symmetry before and after salt addition, and also monotonous change of diffraction peak position during the whole process we conclude that the symmetry is conserved within the nonequilibrium period. The reason for the

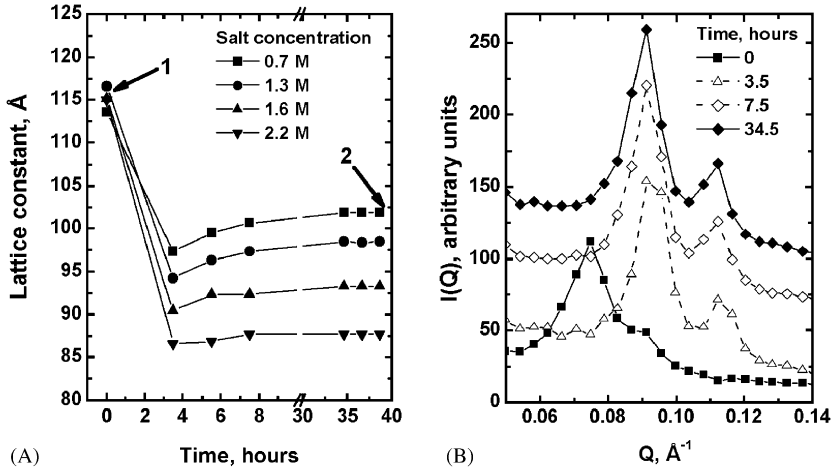

Fig. 3. (A) The time dependence of the lattice constant of MO/BR/buffer system at different salt concentrations (D16, Grenoble). Time is counted from the moment of salt addition. Arrow 1 indicates the state of equilibrated cubic phase just before addition of the precipitant. Arrow 2 shows characteristic time of appearance of the first crystals visible in optical microscope. (B) Diffraction curves of a sample without salt and at different time moments after salt addition. Final salt concentration in the sample is 1.5 M.

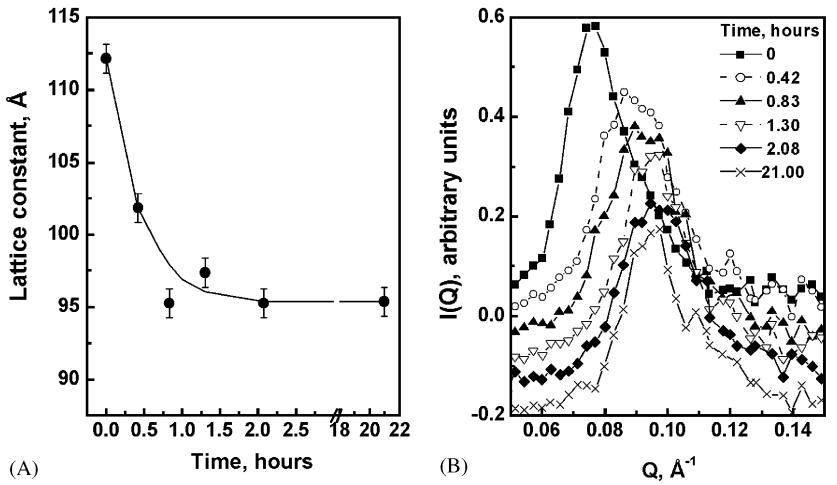

Fig. 4. (A) Characteristic time dependence of the lattice constant for salt concentrations of 1.4 M, obtained for the MO/BR/buffer system behaviour immediately after the salt addition (YuMO, Dubna). (B) Diffraction pattern behaviour induced by salt addition at the same time scale.

broadening of the diffraction peaks is the gradient of the conditions (hydration and salt concentration) along the crystallization probe.

Thus, this detailed study of mesophase behaviour has shown that the changes take place during first 1.5–3 h after the salt addition (Fig. 4a and b). Addition of dry salt induces decrease of the lattice constant by ca. 10–30 Å, but other phases (except cubic phase of Pn3m symmetry) were not observed. The decrease of the lattice constant is at least one order of magnitude faster than time of formation of the first visible crystals. Thus, we conclude that the kinetic processes induced by addition of precipitant do not influence growing of

the crystals, but one cannot exclude its importance for the nucleation. No phase changes of lipidic phase on macroscopic scale are necessary for crystallization.

It was demonstrated [11] that growth of crystals is accompanied by formation of very small local regions of lamellar phase around growing crystals. The size of these regions is comparable with the size of the crystals and is approximately 50 μm. We consider these regions as microscopic and such amounts of another phase were not detectable in our neutron diffraction experiments.

3.3. Crystal growth

The probes studied by SANS were regularly checked with optical microscope during three month period after salt addition. Hexagonal plate-like crystals grew in the samples with salt concentration from 0.9 to 2.6 M. Crystals appeared in two to ten days after salt addition and reached maximal size in one month. The induction period (time required for crystal nucleation), growth rate and average size of the crystals decrease with increasing salt concentration. The information on the final size of the crystals is summarized in Table 1 and Fig. 5. Qualitatively the results of crystallization in cubic phase are similar to that observed for crystallization of water soluble proteins [13], i.e. the number of crystals in a probe rises while their size decreases with the increasing concentration of precipitant. Apparently, growth of precipitant concentration increases the number density of nucleation centers, leading to decrease of average crystal size.

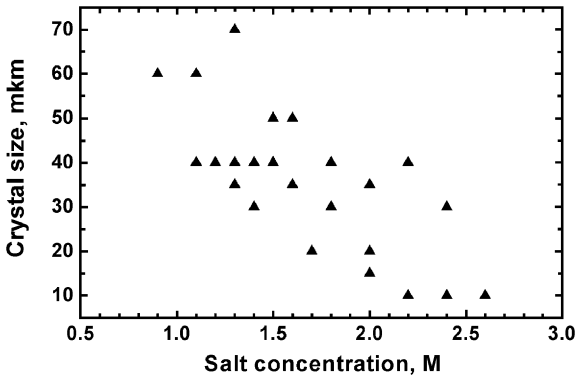

Fig. 5. Dependence of the final maximum size of the crystals on the salt concentration in probes.

4. Conclusions

A neutron scattering study of the detailed kinetics of the lipidic cubic phase during crystallization process of the membrane protein bacteriorodopsin is presented. The MO/BR/OG/buffer system, as well as MO/water system, forms the cubic phase of Pn3m symmetry [15], but with increased lattice constant value due to detergent presence [17]. It was shown that the symmetry of cubic phase remains unchanged at investigated crystallization conditions during the whole crystallization process. Initiation of the crystallization process by salt addition leads to dramatic decrease of the lattice constant, but no phase transition takes place and only the cubic phase of the Pn3m symmetry with smaller lattice constant was always observed. Hence, we conclude that presence of macroscopic amount of an additional phase, as well as a phase transition are not necessary conditions for the crystallization of BR molecules in the lipidic cubic phase.

Addition of precipitant induces a number of changes, which play a role of factors influencing on the crystallization process. The first one is the increase of ionic strength; a factor commonly used to initiate crystallization. Secondly, there are the changes of properties of hydrophobic component of crystallization mixture. Salt addition leads to the decrease of the lattice constant of cubic phase, and hence, to the change of curvature, that could be a factor triggering crystallization [12]. The third factor is an increase of effective protein concentration due to the shrinkage of cubic phase. Addition of salt removes part of water from the cubic phase, thus, the volume of matrix with protein decreases by ca. 10%.

BR crystals belong to the type I of membrane protein crystals [18]. In such crystal the molecules are assembled in 2D crystal layers where proteins contact by membrane embedded parts. These layers are stacked forming 3D periodic structure. Organization of BR molecules in 2D layers is

similar to that in purple membrane (PM), natural 2D crystals BR packed in vivo. Studies of PM showed that it is hydrophobic interactions that provide stability of this 2D crystalline ensemble [19]. Hence, we suggest that in-plane association of BR molecules is rather due to the changes of lipidic cubic phase properties than due to increased salt concentration. However, creation of 3D crystals (multilayer structure) requires interaction between layers through the polar parts of BR molecules. The increased salt concentration favours contact of water exposed parts of BR. Apparently, these observed structural changes of cubic phase and the increase of salt concentration lead to crystallization of BR molecules. A further systematic study of the *in cubo* crystallization are necessary to reveal the mechanism of crystallization.

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