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Investigating crystal-growth mechanisms with and without LB template: protein transfer from LB to crystal

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Protein nanocrystallography has recently been introduced as a unique nanotechnology-based approach to forming stable protein crystals and to characterize them down to atomic resolution. In particular, a protein nanostuctured template appears to be capable of stimulating nucleation and crystal growth of so far unsolved proteins. In the present communication, aimed at investigating the lysozyme crystal-growth mechanisms with and without nanotemplate, the lysozymes appear to transfer directly from the nanostructured film into the drop to trigger the formation of the crystal, therefore highlighting the physical interpretation of the mechanism for nanotemplate-induced protein crystallization.

1. Introduction

Protein crystals obtained by nanotechnology-based protein template crystallization, as well as significant progresses in groundbreaking technologies such as atomic force microscopy (AFM; Li *et al.*, 1999; McPherson *et al.*, 2000, 2001), nanogravimetry (Facci *et al.*, 1993) and synchrotron microfocus (Cusack *et al.*, 1998; Riekel *et al.*, 2000) have enabled protein nanocrystallography to be defined as a unique technology capable of forming and characterizing stable protein crystals down to atomic resolution (Pechkova & Nicolini, 2004*a*). With respect to classical protein crystallography (Ducruix & Giegé, 1992; McPherson, 1999) and to its recent successful upgrade (Thorsen *et al.*, 2002; Rupp, 2003), protein nanocrystallography seems to represent a new approach for protein crystallization by providing a general routine procedure with predictable results instead of a random search of the correct crystallization conditions. So far, it has proved difficult to find the key to every existing protein by using a general procedure for protein crystallization. Indeed, to be crystallized, each protein requires its own specific conditions, which are often difficult to determine and require extensive empirical searching. For this reason, protein crystallography is often called an art rather than a science. Protein nanocrystallography, which uses the nanostructural protein template to stimulate nucleation and crystal growth for proteins of different families, could solve this problem by providing a general procedure with controllable results.

Moreover, the crystals grown with use of nanostructured template appear to be much more stable to synchrotron radiation from third-generation sources (for a review, see Pechkova *et al.*, 2004). The successes in protein crystallization by this new approach (Pechkova & Nicolini, 2001, 2002*a*,*b*, 2004*a*,*b*) bring us to the necessity to understand how the protein thin film influences the protein crystallization process. The present research was carried out in order to highlight how hte nanotemplate participates in the crystallization. The physical explanation of the phenomena becomes therefore possible.

The chicken egg-white lysozyme, a protein which over many years has remained the favorite model protein for crystallization mechanism studies (Judge *et al.*, 1999; Forsythe *et al.*, 1999; Kierzek *et al.*, 2000; Li *et al.*, 1999; Penkova *et al.*, 2002), has been chosen for these experiments. As was established in the previous communications (Pechkova & Nicolini, 2001, 2002*b*), the homologous nanotemplate causes the faster growth of lysozyme crystals. In order to discover the role of the nanotemplate in the lysozyme crystallization

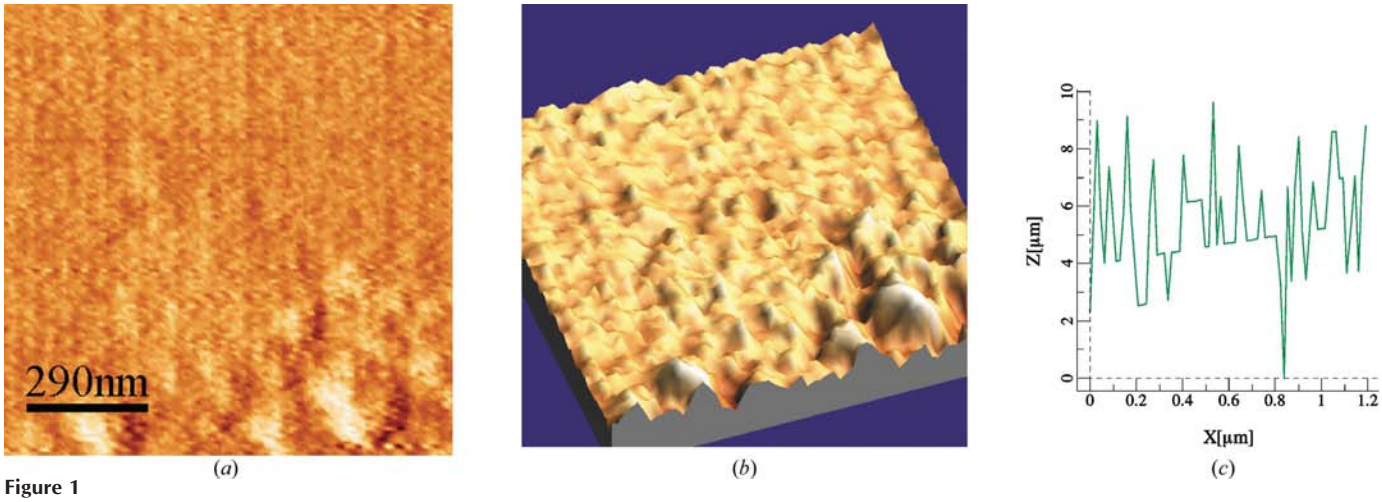

Figure 1
AFM images of lysozyme thin film in two dimensions (*a*) and three dimensions (*b*). The pixel height *versus* distance for one projection of the above AFM is also shown (*c*).

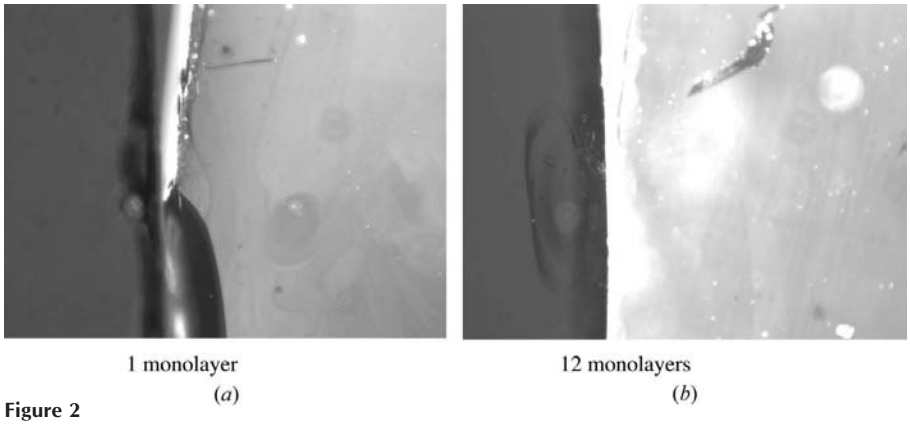

Figure 2
Fluorescence images acquired by CCD camera of CFSE-labelled lysozyme thin film. (*a*) right portion, one CFSE-labelled lysozyme monolayer; (*b*) right portion, 12 CFSE-labelled lysozyme monolayers. It should be noticed that as an internal standard the left part of each image refers to its corresponding unlabelled nanofilm counterpart.

process we used fluorescent labelled lysozyme for preparation of the protein nanotemplate. Fluorescent labelling permits the introduction of chromophoric groups into the protein molecule and the sample therefore becomes visible in fluorescence. We use carboxyfluorescein succinimidyl ester (CFSE), resulting in reliable labelling. Succinimidyl esters are indeed good reagents for amine modification since the amide products formed are very stable.

2. Experimental procedures

Chicken egg-white lysozyme protein was purchased from Sigma.

2.1. Fluorescein labelling of lysozyme

Solution of carboxyfluorescein succinimidyl ester (CFSE) with concentration 1.5 mg ml^{-1} in was prepared in anhydrous dimethylsulfoxide (DMSO). 100 µl CFSE solution was added to 1 ml protein lysozyme solution with concentration 1 mg ml^{-1}. The mixture was incubated for 90 min in the dark at room temperature with continuous gentle agitation. Labelled protein was separated from free Fluorescein compounds by extensive dialysis against PBS (overnight). Protein was concentrated with Centricon concentrators up to 40 mg ml^{-1}, centrifugated for 10 min at high speed and filtered through a 0.22 µm filter (http://flowcyt.salk.edu/protocols/flabel.html).

2.2. Nanotemplate crystallization

A nanotemplate-based crystallization method was utilized as described in Pechkova & Nicolini (2001). The important issue is that in present work the CFSE labelled proteins have been used for the nanotemplate preparation. CFSE-labelled lysozyme thin film were prepared by spreading 600 µl of 40 mg ml^{-1} CFSE-labelled lysozyme on the air–water interface of an LB Langmuir–Blodgett trough (Nicolini, 1997) and compressing it to a surface pressure 20 mN m^{-1}. A protein monolayer was deposited on a siliconized glass cover slide of 20 mm diameter (Hampton Research) by the Langmuir–Schaeffer method. The obtained protein template was analysed by AFM (see §2.3) to estimate the regularity and uniformity of deposition. A highly ordered protein nanotemplate was utilized in a modified hanging-drop protein crystallization method. A scheme of the protein hanging drop on the nanotemplate is shown in Fig. 4(*a*). The drop of protein solution and the precipitant (salt) was placed on the glass slide covered by thin film nanotemplate. Like in the classical hanging-drop method, the glass slide with the protein template and the drop was sealed on the crystallization plate (Limbro plate, Hampton Research) using vacuum grease. The crystallization conditions usually used for classical hanging-drop method were applied: 4 µl drop containing 20 mg ml^{-1} lysozyme in 25 m*M* sodium acetate buffer pH 4.5 and 0.45 *M* sodium chloride was placed on the siliconized glass slide covered with the CFSE-labelled lysozyme monolayer and stabilized over the reservoir containing 0.9 *M* sodium chloride in sodium acetate buffer. For comparison, the same conditions were used to obtained lysozyme crystals by the classical handing-drop crystallization method.

2.3. AFM measurements

Atomic force microscopy (AFM) was carried out by an 'in-house built' ELBATECH microscope operating in 'tapping' mode (Sartore *et al.*, 2000). In this 'non-contact mode', the AFM derives topographic images from measurements of attractive forces. Images of the lysozyme template have been grabbed with cantilever NSC14/Cr-Au MikroMash in a dry atmosphere. The typical resonance frequency of

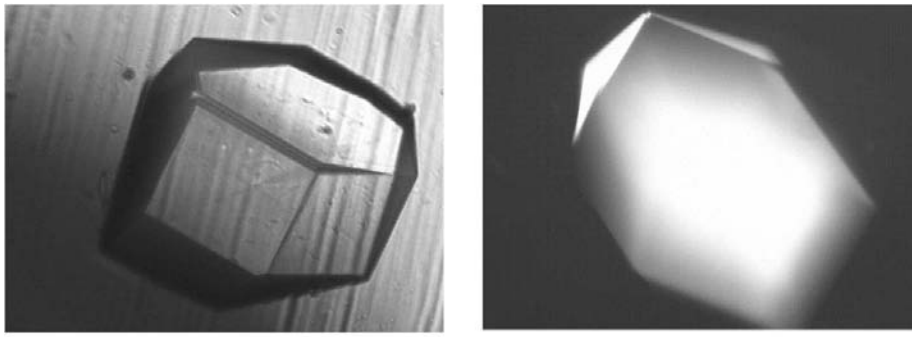

Figure 3
CCD images of lysozyme crystals produced by lysozyme thin-film template, either CFSE labelled (right) or unlabelled (left).

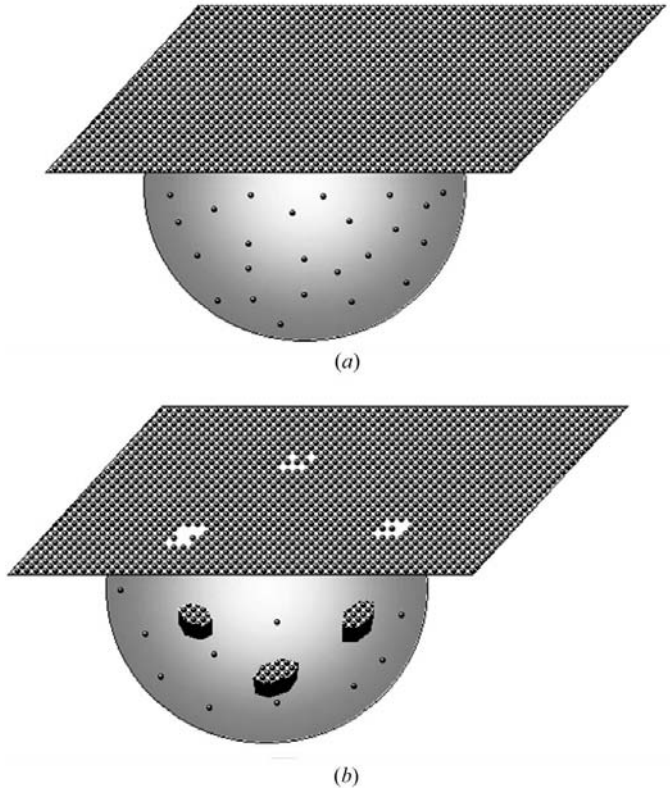

Figure 4
Model of nanotemplate-base hanging-drop method before (*a*) and after (*b*) crystal formation.

the cantilever tip is between 110 and 220 kHz and the proper positioning of the cantilever on the tip holder of AFM has been found at a frequency of 92 kHz with an intensity of 0.6 V. The set point value for loop control was set to 0.2 V.

The integral gain value during image grabbing was set at 4.4 (I gain) and the proportional gain value during image acquisition was set at 8.03 (P gain).

2.4. High-resolution fluorescence image acquisition

Fluorescence labelled and unlabelled lysozyme nanofilms were analyzed by a Zeiss microscope equipped with high special resolution and single-photon sensitivity CCD camera (Orca II, Hamamatsu, Japan). The utilization of the appropriate filter and proper calibration permit accurate measurements of the fluorescence intensity and wavelength (green emission).

3. Results and discussion

Fig. 1 shows an atomic force microscopy (AFM) image of the above lysozyme monolayer acting as a nanostructured template for subsequent crystal growth. The AFM image, both in the two dimensions (Fig. 1*a*) and three dimensions (Fig. 1*b*), displays an highly ordered and periodic organization of lysozyme molecules in the nanofilm template: this is made evident also by the pixel height *versus* distance along one line crossing the same AFM image (Fig. 1*c*).

High-resolution fluorescence images of the lysozyme nanofilm template display a significant difference between CFSE-labelled and unlabelled, even for a single monolayer template (Fig. 2*a*). Moreover, the intensity of fluorescence signal clearly increases with the number of CFSE-labelled lysozyme monolayers, being more fluorescent for the 12 lysozyme monolayers template (Fig. 2*b*). In order to allow a qualitative comparison, the corresponding unlabelled lysozyme nanofilm is kept in the left part of both Figs. 2(*a*) (unlabeled lysozyme monolayer) and 2(*b*) (unlabeled lysozyme multilayer).

Three parallel crystallization experiments were then carried out. CFSE-labelled nanostructured templates on the siliconized glass cover slide were used to stimulate protein crystallization by the protein thin-film template crystallization method (Pechkova & Nicolini, 2001, 2002*a*,*b*, 2003, 2004*a*,*b*; Pechkova *et al.*, 2003), as well as non-labelled template. The crystals grew up to 800 µm in 3 d. For comparison, the same proteins with the classical hanging-drop method yield a lysozyme crystal growth up to 650 µm in the same period of time. The crystals, grown with labelled nanotemplate demonstrate clear fluorescence as shown in Fig. 3. The crystal grown with or without non-labelled template which have no fluorescence signal are also shown for comparison.

In a separate communication (Pechkova *et al.*, 2004) it was indeed shown by mass-spectrometry and NMR that lysozyme crystals grown with CFSE-labeled template contains labeled proteins, which highlighted the role of template proteins in the crystal growth.

The model in Fig. 4 illustrates the possible physical explanation of the nanotemplate participation in the protein crystallization process. Since in-plane concentration of the lysozyme molecule in highly ordered monolayer is rather high, it is possible to conclude the film could provide the additional protein molecules to the drop solution. Taking into consideration the two-dimensional lysozyme nucleation confirmed by AFM studies (Li *et al.*, 1999), one can suppose that the thin protein film could be the source of two-dimensional islands of ordered molecules (Fig. 4*b*), detached from the template. These aggregates with periodic organization can become the new nucleation centres for the crystallization acting as seeding factor and trigger directly the construction of the lysozyme crystals. The crystals grown in this way are indeed larger and more stable to synchroton radiation as was discovered recently (Pechkova *et al.*, 2004*c*). Interestingly, the lysozymes in the film are similarly quite more stable than the same lysozymes in solution. This fact was recently confirmed by circular dichroism studies for lysozyme (Pechkova *et al.*, in press), though it is already well known for all types of proteins (Nicolini, 1997).

4. Conclusions

The dramatic role of the protein nanotemplate in the protein crystallization (Pechkova & Nicolini, 2004*b*) is confirmed by these

experiments outlining a physical basis of protein crystal growth in presence of homologous protein nanotemplate. Considering that all proteins, including those so far not crystallized as most membrane proteins, can be assembled in highly packed thin protein layers by a modification of Langmuir–Blodgett technology (Nicolini, 1997), the nanotechnology-based crystallization method appears capable of overcoming the major open problem in protein crystallography. The resulting mechanism adds very interesting promise for generalization to all classes of proteins so far not crystallized in order to bypass the bottleneck of crystallization by this new approach based on nanotechnology, with far reaching potential for applications in the life sciences and in the pharmaceutical industry (Nicolini & Pechkova, 2004).

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