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Protein Nucleation and Crystallization by Homologous Protein Thin Film Template

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Abstract A new method of protein nucleation and crystallization based on Langmuir–Blodgett technology is here utilized for the template stimulation of crystal growth of so far non-crystallized proteins. Microcrystals (60–120 μm) of bovine cytochrome P450scc and human protein kinase CKII alpha subunit were obtained with use of the homologous protein thin film template by vapor diffusion modified hanging drop method. The induction of microcrystals nucleation by the thin template confirms in the two different important classes of proteins, until now never crystallized, the positive stimulatory influence for crystal formation of protein thin film template, which was observed in an earlier study with a model system (chicken egg white lysozyme) as an unexpected acceleration and enhancement in the crystal growth. J. Cell. Biochem. 85: 243–251, 2002. © 2002 Wiley-Liss, Inc.

Key words: protein crystallization; Langmuir–Blodgett protein thin film; template stimulation; protein nucleation

Interest in growing protein crystals is related to the need for protein structure determination at atomic resolution by X-ray diffraction methods [Giege et al., 1995; Rosenberger, 1996]. This need is becoming more and more severe with the rapid advances in biotechnology, bioelectronics, molecular pharmacology, and molecular medicine that require understanding of biological processes at the atomic level.

In spite of recent advantages in protein crystallography, a large number of proteins playing a critical role in living mechanisms is not so far crystallized. Protein crystallization techniques are usually based on vapor diffusion methods [McPherson, 1989], since they are easy to perform, require a small amount of sample, and permit easy variation of physical parameters during crystallization that allows flexibility during screening and optimization procedures [Ducruix and Giege, 1999].

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But this approach often has a random and irreproducible success, first of all for rather big and/or insoluble proteins. Moreover, to be crystallized, each protein requires its own specific conditions, which are often difficult to determine.

In the present work, we present a protein nucleation and crystallization by use of homologues protein LB (Langmuir–Blodgett) thin film template protein films [Langmuir and Schaefer, 1938; Nicolini et al., 1993; Nicolini, 1997]. In several cases it is shown that the deposition procedure does preserve protein structure and function, giving also rise to new useful properties in terms of stability [Nicolini et al., 1993; Nicolini, 1997]. Close packing and dehydration of the proteins is shown to be the reason of their heat proofness in thin films, namely the high thermal stability of their secondary and tertiary structure [Nicolini et al., 1993; Nicolini, 1997].

New LB-template protein crystallization methods helped us to generalize the protein crystallization procedure and allowed us to obtain protein microcrystals for the further crystal growth and 3D structure studies.

For this research, two proteins of different families have been chosen, namely human protein kinase CK2α and bovine cytochrome P450scc. Recombinant proteins seemed to be more adequate for technological reasons, as

they represent the homologous population of the molecules with controlled sequence, which can be ad hoc modified by site-specific mutagenesis. Furthermore, it can undergo cheap mass production and has the high level of purity. *Escherichia coli* is a desirable organism for the heterologous expression of proteins owing to its ease of manipulation, the availability of a variety of cloning and expression vectors, well-understood genetics, and the low cost of the culture [Porter and Larson, 1991]. In the case of expression of the metalloprotein (P450), the improvement of the purity level of the final product is possible to carry out not only by using specific detergents but also by applying the affinic resins (cholate-sefarose) to this protein during the chromatography purification [Kastner and Neubert, 1991].

Protein kinases are a large family of enzymes, critically involved in almost every regulatable cellular process [Hanks et al., 1988]. Protein Kinase CK2—formerly called casein kinase II or casein kinase 2—is ubiquitously distributed among eucariotic cells [Bossemeyer, 1995].

Protein kinase CK2 is a heterotetrameric serine/threonine protein kinase made by the association of the three dissimilar subunits [Chantalat et al., 1999]. Its quaternary structure is so stable that denaturing conditions are necessary for dissociation of CK2. Despite the stability of the tetramer, the CK2α subunit alone shows catalytic activity. The crystal structure of the recombinant catalytic subunit (α) of CK2 from *Zea mays* has been recently resolved, while human CK2α remains non-crystallized because of its drastic instability. Maize CK2α is significantly more stable than the human counterpart. Human CK2α shows only half of the specific activity of human holo-CK2 (1.2 vs. 2.4 U/mg), whereas the maize CK2α has a specific activity that is at least as high as the human holoenzyme, and often higher when determinated under optimal assay conditions. However, this data should be considered as a rough indication, because the activities of CK2α and CK2 depend strongly on the salt concentration and so they are difficult to compare [Nienfind et al., 1998].

For this study, human CK2α was produced by recombinant DNA from *Escherichia coli* cells. Recombinant maize CK2α consisted of 332 amino acids. The main difference between maize and human CK2α is found in the C-terminal, which is $\cong 60$ amino acids longer in recombinant human CK2α [Guerra et al., 1998]. For this reason we have prepared both entire and truncated forms of recombinant human CK2α (details in a separate study in preparation) with the aim to compare crystallization results. In the present work, crystallization of the catalytic subunit of human recombinant protein kinase CK2α was performed using protein thin film template method, recently described [Pechkova and Nicolini, 2001].

Cytochromes P450 are an ubiquitous class of *b*-type heme proteins that catalyze the hydroxylation of a vide variety of aliphatic and aromatic molecules. The cytochrome P450scc (Side-Chain Cleavage) is the heme containing membrane protein, structurally similar to cytochromes, but functionally is the typical monooxygenase [Hanukoglu and Hanukoglu, 1986]. P450scc is a steroidogenic enzyme associated with the inner-mitochondrical membrane of steroid producing tissues. It catalyses three oxidative reactions resulting on the cleavage of the side chain of cholesterol to produce pregnenolone. This step is important in steroid biosynthesis, since it is the first and rate-limiting step, and occurs in all steroidogenic tissues. Most of our understanding of the reaction mechanism of cytochrome P450scc comes from the numerous studies on the bovine enzyme [Williams et al., 2000]. Cytochrome P450 are of particular interest also because, on the one hand, they display multiple pathways and, on the other hand, P450scc is unusual among members of this class of enzymes in showing a high degree of substrate specificity. Its complex with its electron transfer partner protein adrenodoxin can find their application in biophysical nanotechology, namely in artificial nanocomputers and nanosensors realization [Nicolini, 1996]. For all above reasons P450scc structure resolution at the atomic level is of high importance. New protein crystallization method based on LB technique allows to obtain for the first time cytochrome P450scc microcrystals for the further X-rays diffraction or synchrotron radiation study with the aim of atomic resolution of the protein structure. It is indeed important to remember that while for the traditional X-ray diffraction crystals of rather big dimensions have to be obtained, new synchrotron radiation sources require quite smaller crystals. Focused X-ray beams from third generation synchrotron sources can be used for characterization of protein crystals of less than 30 μm in

size, perhaps down to 5–10 μm [Cusack et al., 1998].

In the following pages we present preliminary data of cytochrome P450scc and human CK2α kinase crystallization by means of a new LB thin film template crystallization method.

MATERIALS AND METHODS

Protein Expression and Purification

Human protein kinase CK2α. Human recombinant kinase CK2α cDNA was cloned in *E. coli*. Purification of CK2α involved centrifugation, solubilization in 25 mM Tris-HCl pH 8.5, containing PMSF 0.2 mM, 2-Mercaptoethanol 7 mM in the presence of a cocktail of protease inhibitors (Sigma); ultracentrifugation and several steps of affinity chromatography with use of gradient elution of NaCl. For each step of the purification, the presence of CKII was monitored by SDS–PAGE [Laemmli, 1970] and the total protein content by Bradford assay.

Finally, fractions containing alpha subunit of CKII were separated by gel filtration FPLC chromatography using a Sephacryl S-100 (Pharmacia) resin. The final preparation containing 1–3 mg of CK II was checked by SDS–PAGE and also by Western blotting. The purified protein was stored at −70°C in 25 mM Tris-HCl buffer with pH 8.5, containing 500 mM NaCl, 50% Glycerol, 0.2 mM PMSF, 7 mM 2-Mercaptoethanol, 1 nM ATP [Vergani et al., 2001].

Human protein kinase CK2α C-terminal truncated form. C-terminal truncated form of α subunit has been obtained by enzymatic reaction with tripsin. CK2α 0.27 mg/ml solution (in buffer 25 mM Tris-HCl pH 8.5, 25 mM NaCl, 7 mM β-mercaptoethanol, 0.2 mM PMSF, 1 nM ATP) was mixed with 0.25 μg/ml tripsin solution (enzyme/substrate ratio ≅ 1000/1) and incubated at 27°C for 20 min. The CK2α, treated with tripsin (hereafter referred as tCK2α) was further purified in the absence of any detergent. Final purification of tCK2α required anion exchange chromatography using a Mono Q HR 5/5 FPLC column.

Cytochrome P450scc. Bovine recombinant cytochrome P450scc was cloned in *E.coli* system expression: *cDNA* gene for mature form of P450scc was cloned in the pTrc99A vector [Amann et al., 1988] between the NcoI and KpnI sites to obtain bacterial expression of bovine P450scc (the product of *CYPA11A* gene). The *cDNA* gene of the mature protein was obtained by deleting the N-terminal mitochondrial targeting sequence coding the first 39 amino acid residues [Du Bois et al., 1981; Pernecky and Coon, 1996]. The correct orientation of the insert was confirmed by restriction mapping. The correct sequence of the insert was confirmed by the data of the sequence obtained with the automatic sequencer "Applied Biosystems" (Model 373A 1.2.0 version). *Escherichia coli* JM109 was transformed with pTrc99-P450scc plasmid using standard protocol according to [Sambrook et al., 1989]. After centrifugation, expressed cytochrome P450scc was purified by three different chromatographic steps: DEAE cellulose, hydroxyapatite and adrenodoxin-sepharose 4B columns. The sample was solubilized in 10 mM K phosphate buffer (pH 7.4) containing 0.1 mM EDTA, 0.2 sodium cholate, and 20% glycerine. Protein concentration was determined using the BCA assay (Pierce), using bovine serum albumin as a standard. SDS-polyacrylamide gel electrophoresis was performed as described [Laemmli, 1970]. Purified P450scc was stored at −20°C after adding cholesterol at final concentration of 20 μM [Nicolini et al., 2001; Vergani et al., 2001].

Protein Thin Film Preparation and Characterization

Protein thin films were engineered onto solid substrates using LB trough (MDT, Russia) [Facci et al., 1994]. Water purified with Milli-Q system (18.2 MΩ cm) was used as a subphase. Protein solutions were spread over the water surface with Hamilton syringes. The films were compressed immediately after spreading with a compression speed of 10 cm/min (the trough size was 100 cm × 30 cm, volume 250 ml).

Protein monolayer characterization. Human recombinant CK2α monolayers were formed in a Langmuir Teflon trough by spreading 500 μl of protein solution with a concentration 0.22 mcg/ml in 25 mM NaCl, 25 mM Tris-HCl, 7 mM β-mercaptoethanol pH 8.5 buffer, and 50% of glycerine. Distilled Milli Q water was used as a subphase. The subphase temperature was a 22°C. The formed film was compressed with a barrier speed of about 100 mm/min.

Monolayers of cytochrome P450scc were formed in a Langmuir Teflon trough by spreading 500 μl of protein solution with a concentration of 0.17 mcg/ml (or 250 μl with protein concentra-

tion 0.52 mg/ml) in 10 mM Potassium Phosphate buffer pH 7.4 which contains 0.1 mM EDTA and 0.2% Sodium Colate, and 20% of glycerine. Distilled Milli Q water was used as a subphase. The subphase temperature was 22°C. The formed film was compressed with a barrier speed of about 100 mm/min.

π-A Isotherms measurements were performed for a preliminary characterization of P450scc monolayers at air-water interface.

Supports preparation. Substrates, used for deposition, were: siliconized circle glass cover slide of 22 mm for modified hanging drop crystallization method, quartz resonators—for nanogravimetric measurements.

Circle glass cover slides supports (diameter 22 mm, Hampton Research, USA) were used as supports for the protein thin films deposition. Substrates were washed carefully and treated with heated concentrated sulphuric acid for 15 min. Then they were rinsed with distilled water, dried, and treated consequently with 5% demethyldichlorsilane solution hexane in for 30 min, with hexane for 15–20 min, with acetone for 15–20 min, and again with hexane for 15–20 min. After that they was dried in nitrogen flux, rinsed with water, and dried before use. For the hanging drop method modification, commercially available siliconized glass slides (Hampton Research, USA) were also used as supports for the protein thin film deposition.

Protein thin film formation. The transfer of protein monolayers from the subphase surface onto the previously siliconized solid (glass) substrate was performed by the Langmuir–Shaefer technique (variation of Langmuir–Blodgett method, horizontal lift) [Langmuir and Schaefer, 1938] by touching the support in parallel to the subphase surface at protein surface pressure 10 mN/m for CK2α and 15 mN/m for cytochrome P450scc. Excess water transferred with monolayers was removed by nitrogen flux. Thus substrate with different numbers (1, 2, 3) of protein monolayers were prepared. After film deposition, supports were dried in a nitrogen flux, incubated for a 8 h at +4°C, then rinsed carefully and dried again in nitrogen flux.

Nanogravimetric measurements. Quartz crystal nanobalance measurements were carried out by means of a homemade gauge with a sensitivity of 0.57 ± 0.18 ng/Hz using quartz oscillators with frequency of 10 MHz. Nanogravimetric measurements were carried out by means of a homemade gauge with a sensitivity of 0.57 ± 0.18 ng/Hz using quartz oscillators with frequency of 10 MHz. By the Sauerbrey equation [Sauerbrey, 1964], it is possible to correlate the frequency shift with the mass deposited onto the quartz oscillator. To obtain a direct connection between the frequency shift and the surface density, the Sauerbrey equation was changed as follows:

$$\Delta s = -K\Delta f$$

where Δs and Δf represent the surface density and the frequency shift, respectively, and K is a constant determined from the physical parameters of the utilized resonator. Calibration of the quartzes' balance was performed according to [Facci et al., 1993].

Protein Crystallization

Sample preparation. Human recombimant CK2α was concentrated in 25 mM Tris-HCl buffer with pH 8.5 containing 25 mM NaCl, and 7 mM β-mercaptoethanol pH 8.5 buffer by centrifugation using Centricon Centrifugal Membrane filter with MW Cut-off 10,000 Da, which allowed to eliminate glycerine. Protein purity was controlled by SDS–PAGE.

Cytochrome P450scc was concentrated in 10 mM Potassium Phosphate buffer pH 7.4 which contains 0.1 mM EDTA and 0.2% Sodium Colate by centrifugation using Centricon Centrifugal Membrane filter with MW Cut-off 10,000 D, which allowed to eliminate glycerine. Protein purity was controlled by SDS–PAGE.

Protein crystallization by classical hanging drop method. The hanging drop vapor-diffusion method was used for screening and crystallization procedures. For human protein kinase CK2α conditions of CK2α from *Zea mays* crystallization [Guerra et al., 1998] were initially utilized. The reservoirs contained a solution of 25% Polyethylene Glycol (PEG) 4000, 200 mM sodium acetate, and 100 mM Tris-HCl pH 8, 0.05% NaN_3; in the crystallization droplets 3 μl protein stock solution (8.5 mg/ml CK2α in 25 mM Tris-HCl, (pH 8.5) with 25 mM NaCl, and 7 mM β-mercaptoethanol) was mixed with equal volumes of reservoir. For the improvement of crystal formation, the percentage of precipitant in the reservoir was gradually increased from 25% to 30%.

For cytochrome P450scc crystallization, we utilized the hanging drop vapor diffusion method. Reservoirs contained 250 μl 0.1 M

ammonium sulfate solution (pH 7.5) with 18% PEG and pH 7.5. In the crystallization droplets, 2 μl protein stock solution (8 mg/ml P450scc in 10 mM potassium phosphate buffer (pH 7.4), which contained 0.1 mM NaCl, 0.2% Sodium Colate) was mixed with equal volumes of reservoir.

Protein crystallization by LB template protein crystallization method. The same conditions were used for CK2α and P450scc crystallization by the new LB template crystallization method, previously described as modified hanging drop method. In this innovative crystallization method, the small drop of protein solution with precipitants has to be settled on the protein thin film coated onto the glass circle cover slide [Pechkova and Nicolini, 2001]. Then the cover slide is to be inversed as in classical hanging drop method (Fig. 1). During the screening procedure the following parameters were varied: precipitant nature and concentration, number of the protein thin films monolayer.

RESULTS AND DISCUSSION

For each step of the purification, the presence of CK2α was monitored by SDS–PAGE and the total protein content by Bradford assay. The final preparation containing 1–3 mg of CK2α was checked by SDS–PAGE and also by Western blotting. The results show that alpha subunit was purified near to the homogeneity (Fig. 2). The result shows that alpha subunit was purified near to the homogeneity. The same results was obtained with CK2α truncated form (Fig. 3): SDS–PAGE of final fractions of cytochrome P450scc is shown on the Figure 4.

Preliminary structural characterization for the secondary structure of CK2α subunit was performed by Circular Dichroism spectroscopy. The protein was measured in buffer Tris-HCl 25 mM (pH 8.5), which contains 2-Mercaptoethanol 7 mM, ATP 1 nM. The following parameters were used for the acquisitions: path 0.05 cm, band width 2 nm, sensitivity 20 mdeg, response 4 s, scan speed 20 nm/min step resolution 1 nm [Vergani et al., 2001].

Circular Dichroism spectrum of CK2 alpha subunit (Fig. 5), purified as described above, shows that denaturation of the protein during all purification steps cannot be considered as a serious effect.

Pressure-Area Isotherm Measurements

π-A isotherms of CK2α subunit and cytochrome P450 monolayers are presented in Figure 6. X-axis is expressed in barrier coordinate units, as it is impossible to calibrate the axis in area per molecule units due to impossibility of calculating the actual surface concentration of the protein. This problem is general for protein monolayers and it results from some partial solubility of proteins in the volume of the subphase [Lvov et al., 1991]. Protein monolayers exhibit well-defined surface pressure dependence. This is reflected by a change of the molecular orientation of the proteins at the surface.

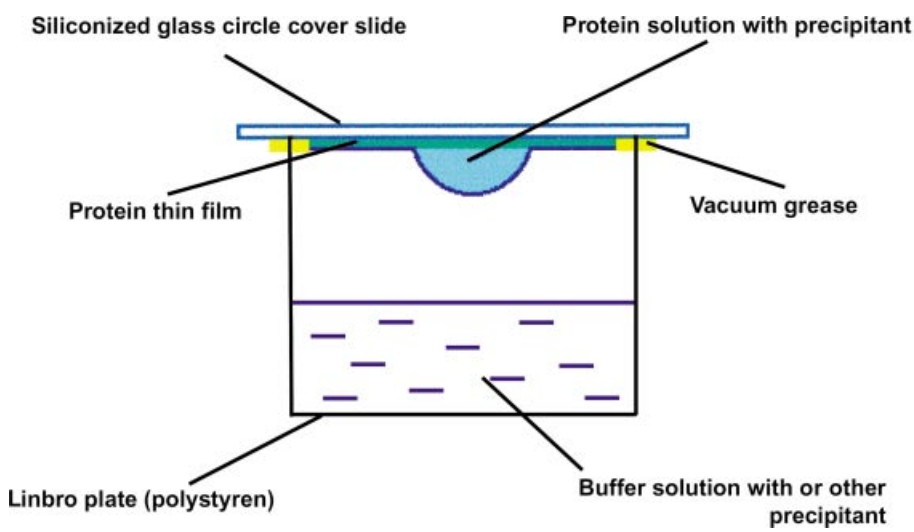

Fig. 1. LB template protein crystallization method. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

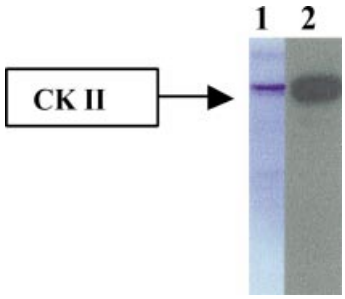

Fig. 2. Control of the purity of alpha subunit CK2 kinase recovered at the last step of purification, **lane 1** shows an SDS–PAGE of the sample after dialysis, and **lane 2** shows the same sample analyzed by Western Blotting technique. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Quartz Crystal Nanobalance

To further characterize thin film deposition, we have transferred the films on quartz oscillators and calculated the mass density as function of the number of layers. The results of the nanogravimetric study of deposited monolayers are presented in Figure 7 for both types of proteins. Linear dependence of the frequency shift upon the number of deposited layers indicates the reproducibility and homogeneity of the deposition. Knowing the molecular weight and dimensions of the protein, it is possible to compare the area per molecule in the film, calculated from nanogravimetric measurements, with that for the closely packed system. The area per molecule in the case of cytocrome (29.81 nm^2) is of the same order of magnitude as obtained by Nicolini et al. [2001] and corresponds well to the calculated area per molecule supposing the close packing of molecules and taking into consideration its sizes from the Protein Data Bank. The protein molecule can be estimated as an ellipsoid with the following sizes: 5 nm × 6 nm × 4 nm, giving, thus, in one cross section the area of about 30 nm^2.

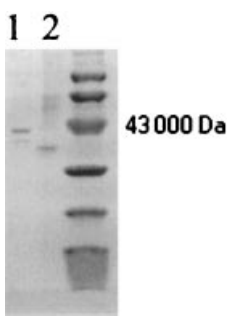

Fig. 3. SDS–PAGE of final fractions of CK2α entire (**1**) and truncated (**2**) form.

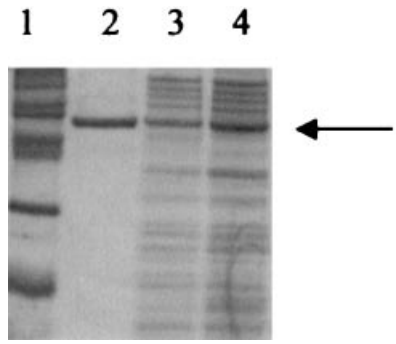

Fig. 4. SDS–PAGE of final fractions of cytochrome P450scc. **Line 1:** final fraction. **Lines 2 and 3:** intermediate fractions.

For the human recombinant CK2α subunit, mass density was about 3.68 ng/nm^2 which corresponds to the area of 20.36 nm^2 per one molecule of protein. The CK2 molecule can be estimated as a shear, which volume calculated knowing the molecular weight and average protein specific volume [Harpaz et al., 1994]. Its radius is about 23, 81 Å and one cross section area for the close film packing 17,81 nm^2.

Therefore the LB film used for the new protein crystallization method are closely packed, which could result from rather high molecule ordering in the film.

Crystallization Results

Preliminary concentration of protein buffer solution as well as glycerine removing have not resulted in significant protein denaturation as shown by gel electrophoresis (Fig. 8).

Traditional crystallization of human recombinant CK2α was unsuccessful since the protein is highly unstable. Using our previously described template crystallization method, we managed instead to obtain microcrystals of CK2α truncated form with dimensions of about

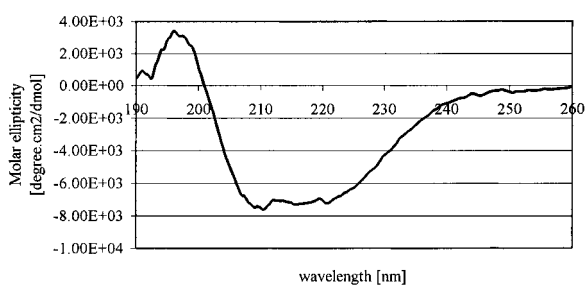

Fig. 5. CD spectra of human recombinant CK2α in solution.

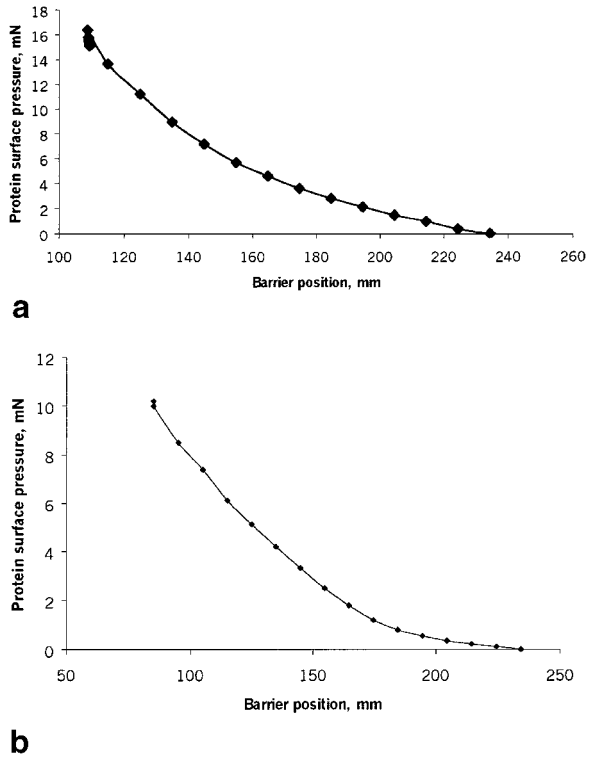

Fig. 6. π-A Isotherms of cytochrome P450scc (**a**) of protein kinase CK2α (**b**) monolayers.

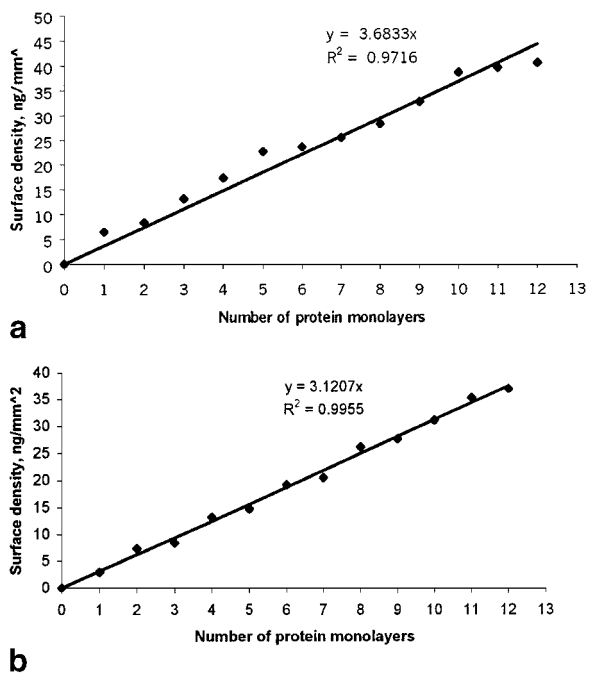

Fig. 7. **a**: The dependence of the surface density of deposited human protein kinase CK2α LB film upon the number of transferred layers. **b**: The dependence of the surface density of deposited cytochrome and reP450scc LB film upon the number of transferred layers.

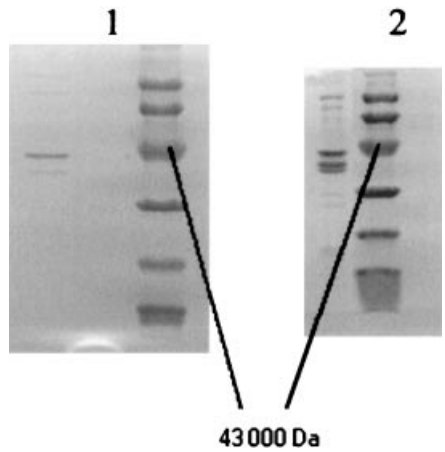

Fig. 8. SDS–Gel electoforesis. **Line 1** corresponds to CKα before the concentration 0.27 mg/ml in the following buffer: 25 mM Tris-HCl (pH 8.5), 25 mM NaCl, 7 mM β-mercaptoethanol, 0.2 mM PMSF, 0.2 mM PMSF, 1 nM ATP, 50% of glycerine. **Line 2**: CKα after the concentration 8.5 mg/ml, 0.18% of glycerine.

85 μm (Fig. 9). Crystal dimension distribution is presented in Figure 10. Microcrystals with average dimensions 10–15 μm were observed under the same conditions with non-truncated forms. In both cases (truncated and untruncated kinase), crystals were obtained in 200 mM sodium acetate, and 100 mM Tris-HCl (pH 8) with 28% PEG 4000, and 0.05% NaN_3 only in presence of the thin film template but not in solution.

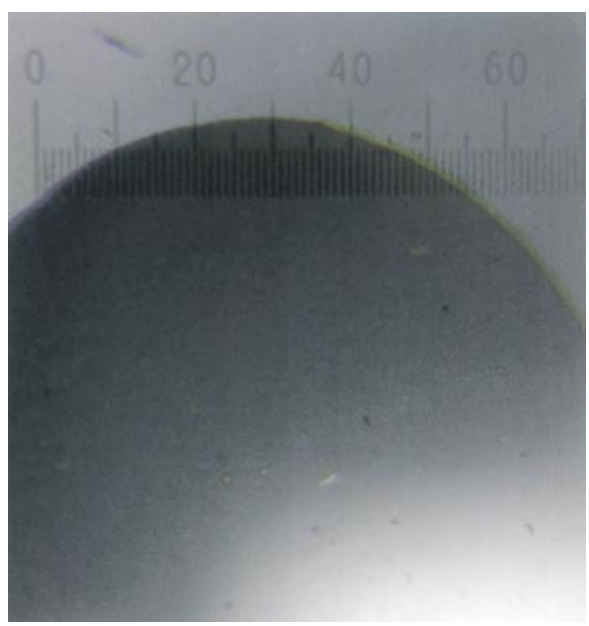

Fig. 9. Human recombinant protein kinase CK2α microcrystals obtained onto the CK2α thin film template.

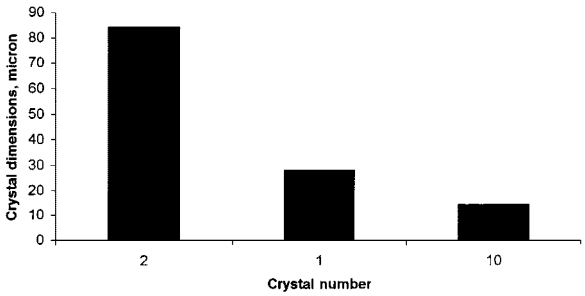

Fig. 10. Human protein kinase CK2α C-terminal truncated form crystals dimensions distribution.

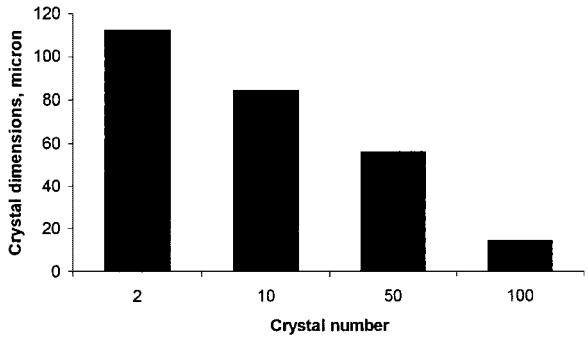

Fig. 12. Cytochrome P450scc crystals dimension distribution.

One can make the conclusion that when crystallized on the thin film template, protein stability does not take such a critical role as during classical crystallization.

Dimensions of cytochrome P450scc microcrystals obtained onto the homologous cytochrome thin film template is about 120 microns (Fig. 11). Crystal dimension distribution is shown in Figure 12. Similarly, cytochrome crystals was obtained in 0.1 M ammonium sulfate, pH 7.5 with 18% PEG 400 only the thin film template but not in solution.

As it was pointed out [Cusack et al., 1998], focused X-ray beams from third generation synchrotron sources can be used for characterization of protein crystals of less 30 μm in size, perhaps down to 5–10 μm. Therefore, protein crystals obtained should be big enough for the X-ray diffraction data collection using the synchrotron source for their 3D structure determination.

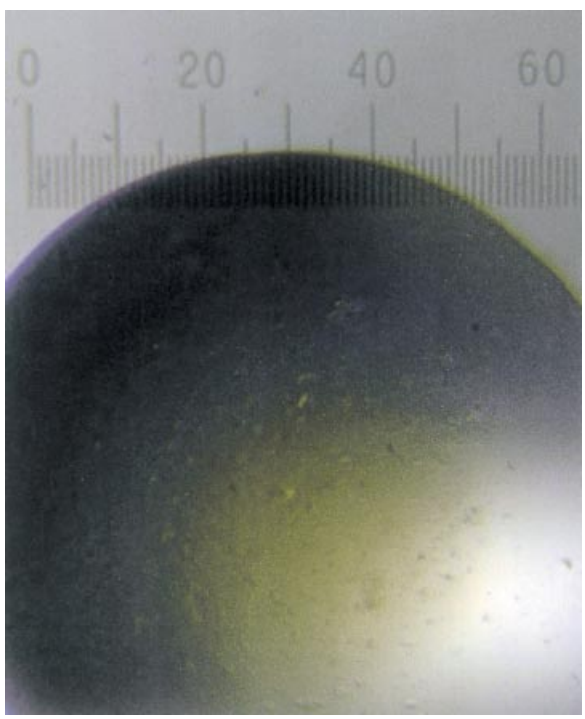

Fig. 11. Cytochrome P450scc microcrystals obtained onto the cytochrome thin film template.

CONCLUSIONS

Present study confirms the positive influence of protein thin film on the protein crystallization, namely induction of protein crystals nucleation. Rapid nuclei formation induced by the protein thin film template is a highly interesting scientific result by itself, requiring further investigation and theoretical interpretation. This fact is of great technological interest as it can become a new solution for so far unsuccessful protein crystallization, and therefore, protein unknown 3D structure investigation.

Microcrystals of two interesting classes of proteins, namely cytochromes and kinases, were obtained in a short period of time. The new method gives fast and reproducible results: microcrystals' shape and distribution observed after 6–8 h, remains the same for weeks, for both proteins. Moreover, in both cases, protein crystallization appears similar because of the template influence on the protein crystallization process. This invariant template influence, taking place at the different crystallization conditions and also for two different classes of proteins (cytochromes vs. kinase) and two different forms of proteins (truncated and entire form), can found the new approach for protein crystallization, namely general routine procedure with predictable results instead of random blind searches of the right crystallization conditions. Further work is however needed to understand how exactly the protein thin film influence on the protein crystallization proce-

dure. This investigation is to be continued along several directions, such as the study of:

- protein concentration influence
- film packing (protein plane concentration in the film) influence
- number of protein layers in the film template
- protein thin film—protein solution interface, and interaction, migration of protein molecules, dynamic equilibrium
- precipitant and salt concentration influence.

This research is indeed in progress in our laboratory, as well as the work on decreasing nuclei number in order to obtain rather big crystals, microcrystals use for seeding technique, protein crystal growth rate acceleration, crystal quality improvement, and protein crystal diffraction.

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