Protein crystallisation on chemically modified mica surfaces

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Chemically modified mica sheets have been tested as heterogeneous nucleant surfaces for lysozyme, concanavalin A and thaumatin. Smooth mica surfaces with reduced hydrophilic properties and different density of ionisable groups have been prepared by a silanisation reaction using mixtures of n-propyltriethoxysilane and 3-aminopropyltriethoxysilane in different percentages starting from 0 to 100% of aminosilane. The crystallisation experiments were carried out with the hanging drop vapour diffusion technique. The results suggest that these mica surfaces act as heterogeneous nucleant agents, whose effectiveness is due to non-specific attractive and local interactions between charged residues of the protein and the ionisable groups on the mica surfaces.

Keywords: mica; protein crystallisation; lysozyme; concanavalin A; thaumatin; heterogeneous nucleation

1. Introduction

The success of structural analyses of biological macromolecules by X-ray crystallography depends on the availability of suitable single crystals. Crystallisation of proteins is usually carried out by trial-and-error procedures that frequently require large amounts of product. Since heterogeneous nucleation could be very useful for protein crystal growth at lower supersaturation, a wide range of heterogeneous nucleants have been tested for model proteins. An epitaxial growth of three dimensional protein crystals has been observed on mineral surfaces (McPherson & Schilichta, 1988 and 1989, Kimble *et al.*, 1998), lipid layers (Kornberg *et al*., 1991, Ku *et al*., 1993, Darst & Edwards, 1995, Edwards *et al*., 1995, Hemming *et* al., 1995), and templates of poly-L-lysine surfaces (Rong *et al*., 2001, Tsekova *et al*., 1999, Nanev *et al*., 2000). Heterogeneous nucleation on poly(vinylidene fluoride) films (Punzi *et al*., 1991), protein thin film templates (Peckova & Nicolini, 2001), porous silicon (Chayen *et al*., 2001) and a variety of potential nucleants (Chayen & Saridakis, 2001) has been studied. Our previous studies (Fermani *et al*., 2001) have shown that polymeric films containing ionisable groups are suitable for protein crystallisation at a lower starting protein concentration and with shorter crystallisation time than those required using siliconised cover slips. To avoid possible effects due to the roughness of the polymeric film surfaces, chemically modified mica surfaces were tested. Mica is a natural mineral characterized by a layered structure. This feature allows the cleavage of the mineral along the basal plane (0 0 1) forming thin, rigid and smooth sheets widely used as flat reference surfaces at atomic level (Nishimura *et al*., 1994). In water, the interstitial ions of the superficial regions are partially lost leaving negative charges on the surface. These surfaces with a reduced wettability have been prepared by a silanisation reaction (Luda *et al*., 1999) using mixtures of n-propyltriethoxysilane and 3-aminopropyltriethoxysilane in different percentages starting from 0 to 100% of aminosilane to modulate the density of ionisable groups on mica surface. The crystallisation experiments carried out with the hanging drop vapour diffusion technique for three model proteins, lysozyme, concanavalin A and thaumatin, indicate that these mica sheets can act as efficient heterogeneous nucleant surfaces.

2. Materials and methods

2.1. Materials

Chicken egg-white lysozyme (E.C. 3.2.1.17), concanavalin A from Jack Bean (type V), thaumatin from arils of the African shrub *Thaumatococcus danielii* (T-7638) and Sigmacote used to siliconise the cover slips, were purchased from Sigma Chemical Co. Mica (muscovite) samples were purchased from Electron Microscopy Science. The n-propyltriethoxysilane and 3-aminopropyl-triethoxysilane were purchased from Sigma-Aldrich. All other chemicals were high-grade reagents (Merck).

2.2. Preparation of mica surfaces

The hydrophilic properties of the mica samples were reduced by modifying their surfaces with a silanisation reaction (Luda *et al.*, 1999). The reaction was carried out in vapour phase for 18 hours. Before each reaction the mica was carefully cleaved in air along its basal plane. To vary the density of the ionisable groups on the mica surface mixtures of two silanes, n-propyltriethoxysilane and 3-aminopropyltriethoxysilane, in different percentages starting from 0 to 100% of aminosilane, were used in the reaction. The composition of the five mixtures used for the silanisation reaction are reported in Table 1. These mica sheets were incubated at least 3 hours with the buffered solutions used for the crystallisation trials. No materials were released in solution as checked by atomic absorption for metal ions and chromatographic analyses.

2.3. Contact angle measurements

Contact angles were determined using a sessile drop method at room temperature. A volume of 5 µl of pure water was dropped onto the surface of the mica samples and onto the surface of the siliconised cover slip, respectively. The drop was left undisturbed for about 1 min and then its shape was recorded with a digital camera. The contact angle values reported in Table 1 were the average of at least three measurements.

2.4. Roughness measurements

The surface roughness was evaluated by means of atomic force microscopy. A Digital Instruments Nanoscope III atomic force microscopy (AFM) was used to observe the topography of the mica surfaces, chemically modified mica and siliconised cover slip. All images were obtained in tapping mode by using micro-fabricated silicon nitride cantilevers (Digital Instruments). The mean roughness, defined as the arithmetic average of the absolute values of the surface height deviation measured from the mean plane surface, was calculated over a square of 1 µm using the Nanoscope software. The values reported in Table 1 were the average of at least four measurements.

2.5. Crystallisation experiments

Crystallisation trials were carried out at 20 ± 1 °C by the hanging drop vapour diffusion technique using a 24 well tissue culture tray. This method allows the direct comparison of the crystallisation process on control siliconised cover slips and on mica sheets stuck to the cover slips acting as supports. Aliquots (2.5 µl) of protein solution at different concentrations were added to 2.5 µl of reservoir

solution in a hanging drop. No alteration of the surface was observed during the crystallisation time or due to the crystallisation conditions. The starting conditions of crystallisation reported in Table 2 were those found in the literature adapted to our experiments. In a set of successive experiments the protein concentration was lowered to examine the differences in term of crystallisation time between the control drop and the drops on mica surfaces.

The crystallisation time was determined visually by optical microscopy, measuring the elapsed time between the microdroplet deposition and the appearance of the first visible crystal under polarizing light at a magnification of 320x. The crystallisation experiments were repeated at least three times in order to test the reproducibility of the results.

Table 1

Mixture compositions for silanisation reaction, contact angles and roughness of mica sheets. The standard deviations are reported in parentheses.

	3-apes (% v/v) *	Contact angle (°)	Roughness (nm)
Control[#]	-	104.4 (1.5)	0.50 (0.06)
Mica	-	<10	0.11 (0.05)
Mica A	0	104.4 (1.5)	0.25 (0.09)
Mica B	30	86.9 (1.9)	0.38 (0.17)
Mica C	50	83.0 (2.3)	0.29 (0.07)
Mica D	70	81.7 (2.5)	0.30 (0.12)
Mica E	100	81.3 (2.7)	0.29 (0.07)

* 3-apes: 3-aminopropyltriethoxysilane. The values indicate the percentage of 3-apes in the binary mixture 3-apes/ n-propyltriethoxysilane.
[#] Siliconised cover slip.

Table 2 Starting experimental conditions used for the protein crystallisation.

Protein	Conc. (mg/ml)	MW* (kDa)	pI*	Conditions
Lysozyme	15	14.3	9.3	Sodium chloride 1M, sodium acetate 0.1 M (pH 4.5)
Concan. A	15	25.6	5.8	Ammonium sulphate 1.5 M, sodium citrate 0.1 M (pH 6.0)
Thaumatin	35	22,2	8.3	Sodium-potassium tartaric acid 1 M Pipes 0.1 M (pH 6.8)

* The MW and the classical pI values were calculated using a ExPASy server tool.

3. Results

Contact angle measurement is an effective way to assess the hydrophilic properties of a given surface. The results are reported in Table 1. The untreated, freshly-cleaved mica yields a low contact angle indicating a most complete wettability. The mica silanised with neutral silane shows the same contact angle as the siliconised cover slip, used as control. An increase of the percentage of aminosilane in the reaction mixture causes a decrease of the contact angle, in agreement with the increase of the surface wettability due to a higher density of amino charged functional groups on the surface.

In a heterogeneous crystallisation experiment the roughness of the surfaces is a crucial parameter to address the crystal nucleation mechanism. The data reported in Table 1 show that the silanisation process increases the roughness of the mica surfaces, however this is always lower than that of the siliconised cover slip.

Lysozyme was crystallised on mica surfaces starting from a protein concentration of 15 mg/ml and then lowered to 10 mg/ml and 5 mg/ml. A slight decrease of the crystallisation time was observed with the increase of the ionisable group's density on the mica surface (Table 3). In all the experiments only two or three crystals were observed in each drop. At a minimum concentration of 5 mg/ml no crystal growth occurred in span of weeks on the control siliconised cover slip. The presence of positively charged groups on the mica surface does not affect the crystal dimensions nor the nucleation density.

Concanavalin A behaves in a different way. At starting protein concentrations of 15 mg/ml and 10 mg/ml a decrease in the crystallisation time is observed going from the control cover slip to the mica silanised with 100% of the aminosilane (Table 4). The increasing of the number of ionisable groups on the substrate surface causes a reduction of crystal dimensions and an increase of the nucleation density. When the starting protein concentration is lowered to 5 mg/ml, a crystalline precipitate, which completely covers the mica surface, is observed in all the drops, in agreement with the results obtained on polymeric films containing ionisable groups (Fermani *et al.*, 2001).

Thaumatin shows a behaviour close to lysozyme for protein concentration values in a range from 35 mg/ml to 5 mg/ml (Table 5). A slight decrease of the crystallisation times going from the control to mica E, does not induce a change in crystal dimensions nor in nucleation density (Fig. 1). At lower protein concentrations (2.5 and 2 mg/ml) a trend, similar to that found for concanavalin A, is observed (Table 5) (Fig. 1). Analogous results were obtained using polystyrene sulfonated films as heterogeneous nucleant surfaces (unpublished results).

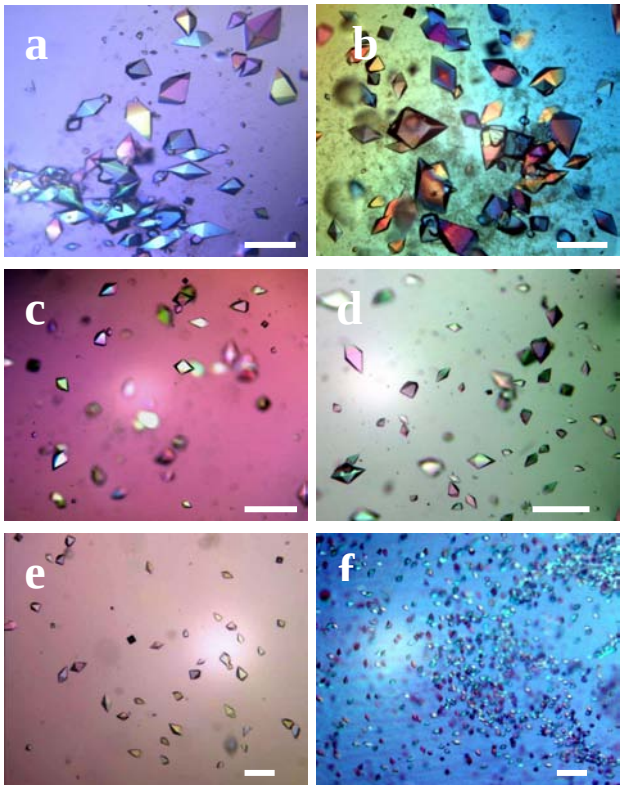

Figure 1

Cross polar optical micrographs of thaumatin crystals grown at different starting protein concentrations: 35 mg/ml on control siliconised cover slip (a) and mica E (b); 5 mg/ml on control siliconised cover slip (c) and mica E (d); 2.5 mg/ml on control siliconised cover slip (e) and mica E (f). Scale bars: (a) and (b) 0.5 mm; (c) and (d) 0.2 mm; (e) and (f) 0.1 mm.

Table 3 Crystallisation times and crystal sizes for lyzozyme at three different starting protein concentrations.

	15 mg/ml		10 mg/ml		5 mg/ml	
	Time (days)	D* (mm)	Time (days)	D* (mm)	Time (days)	D* (mm)
Control[#]	4	0.22	3	0.44	-	-
Mica A	4	0.15	2.5	0.28	3	0.34
Mica B	4	0.27	2	0.37	3	0.46
Mica C	3	0.29	1.5	0.34	3	0.34
Mica D	3	0.25	1.5	0.25	2	0.28
Mica E	2	0.34	1.5	0.45	2	0.33

* D: the value refers to the average length of the longest axis of the crystal calculated on a set of several dozen crystals.
[#] Siliconised cover slip.

Table 4 Crystallisation times, crystal sizes and nucleation densities for concanavalin A.

	15 mg/ml			10 mg/ml		
	Time (h)	D* (mm)	d[$] (n. c./mm^2)	Time (h)	D* (mm)	d[$] (n. c./mm^2)
Control[#]	22	0.26	6	26	0.17	6
Mica A	22	0.23	5	26	0.17	6
Mica B	22	0.18	31	22	0.14	48
Mica C	16	0.15	29	22	0.09	52
Mica D	16	0.08	48	22	0.05	120
Mica E	16	0.03	Mass.	8	0.03	Mass.

* D: the value refers to the average length of the longest axis of the crystal calculated on a set of several dozen crystals.
[$] Nucleation density expressed as number of crystals in mm^2.
[#] Siliconised cover slip.
Mass.: massive crystallisation.

Table 5 Crystallisation times, crystal sizes and nucleation density for thaumatin.

	35 mg/ml			5 mg/ml			2.5 mg/ml			2 mg/ml		
	Time (h)	D* (mm)	d[$]	Time (h)	D* (mm)	d[$]	Time (h)	D* (mm)	d[$]	Time (h)	D* (mm)	d[$]
Control[#]	2	0.37	16	4	0.16	32	38	0.13	13	38	0.07	32
Mica A	2	0.33	22	4.5	0.13	40	38	0.09	19	38	0.06	19
Mica B	2	0.24	26	2	0.17	22	24	0.10	22	24	0.06	22
Mica C	2	0.27	24	2	0.16	41	24	0.07	38	18	0.06	38
Mica D	1.5	0.34	22	2	0.13	36	22	0.09	113	18	0.05	255
Mica E	1.5	0.31	26	1.5	0.14	32	16	0.09	120	18	0.03	302

* D: the value refers to the average length of the longest axis of the crystal calculated on a set of several dozen of crystals.
[$] Nucleation density expressed as number of crystals in mm^2.
[#] Siliconised cover slip.

4. Discussion

Contact angle and roughness measurements show that the silanisation reaction of mica sheets using mixtures of n-propyltriethoxysilane and 3-aminopropyltriethoxysilane, is a suitable tool to prepare smooth heterogeneous nucleant surfaces for proteins.

The wettability and the density of ionisable groups on the mica surface can be tuned using different molar ratios of the two silanes in the reaction mixture.

As the percentage of 3-aminopropyltriethoxysilane increases, the induction time and the starting protein concentration necessary for the crystallisation of concanavalin A decrease. On the contrary the crystallization of lysozyme is not affected by the mica surface even with the highest density of ionisable groups. The thaumatin behaves similarly to lysozyme at high starting protein concentration (Table 5) but for concentration values lower than 5 mg/ml, the surface effect on the crystallisation becomes evident with a reduction of the induction time and crystal dimensions, and with an increase of the nucleation density. A similar behaviour could not be observed for lysozyme because for protein concentration values lower than 5 mg/ml, no crystallisation occurs either on the control or on mica surfaces.

When both the protein and the mica surface have a net electrical charge of the same sign, as for lysozyme at pH 4.5 (net charge about +12, calculated using the pI, MW, titration curve tool from ExPASy server), the crystallization is not influenced by the surface and a homogeneous nucleation occurs.

The buffer pH used for crystallization of concanavalin A is not far from its pI (Table 2) and the electrical net charge is near to zero. The remarkable influence of the mica surface on the concanavalin A crystallisation indicates that the heterogeneous nucleation prevails over the homogeneous one even when the $\Delta S_{vibr} > 0$. If this ΔS_{vibr} is comparable in magnitude to the $\Delta S_{vibr} > 0$. If this ΔS_{vibr} is comparable in magnitude to the the starting protein concentration.The homogeneous nucleation dominates when the protein concentration is high, while the heterogeneous one dominates at low protein concentration. These results suggest that for thaumatin and concanavalin A, which are bigger and more flexible than lysozyme, local attractive interactions between exposed charged residues and the ionisable groups on the mica surface can be more important than the net charge of the protein.

These results obtained on chemically modified mica surfaces, which are in agreement with those obtained for lysozyme and concanavalin A on polymeric films containing ionisable groups (Fermani *et. al.*, 2001), represent a further support of the mechanism previously proposed. Non-specific attractive and local interactions, between exposed charged residues of the protein and the ionisable groups on the nucleant surfaces, might promote molecular collisions and cluster formation raising the probability of nucleation. The effectiveness of these nucleant systems can be modulated varying several properties such as: chemical nature and density of ionisable groups on the surface; surface roughness and wettability; pH and precipitant concentration of the mother solution. This could allow to design nucleant surfaces for the control of the crystallisation process in order to induce the nucleation at low supersaturation and growth of crystals of suitable size for X-ray diffraction studies.

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