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Two-Dimensional Crystallization of Streptavidin by Nonspecific Binding to a Surface Film: Study with a Scanning Electron Microscope

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ABSTRACT A two-dimensional (2D) crystal of streptavidin has been obtained by a nonspecific binding method. The protein molecules were bound and formed a dense packing on the film of poly(1-benzyl-L-histidine) spread at the surface of protein solution. The surface film was moderately heated to stimulate crystallization of bound streptavidin. A potential of this method for obtaining 2D crystals of soluble proteins is demonstrated. The present 2D crystal structure of streptavidin resembles that previously obtained by specific binding to biotinylated lipid. We show in addition that the 2D array of protein with usual size ~50 Å can be imaged using a high resolution scanning electron microscope (HR-SEM) and subject to structural analysis at low resolution. Various limitations in HR-SEM degrade considerably the image quality. However, the usability of a bulk plate as specimen support would make HR-SEM a convenient tool, when such a substrate must be considered in application of protein arrays, and if an intrinsic low resolution is acceptable.

INTRODUCTION

The general techniques to form two-dimensional (2D) crystals of protein are of great interest in such fields as structural biology and nanotechnology. In a nanotechnological world, a 2D crystal of protein would be one of the basic structures toward protein-based devices. Beyond 2D crystals, more complex structures of protein assembly, e.g., layered structure or mosaic of multiple protein species, would be required for future device applications. Although little is known about the mechanism of 2D crystallization of soluble proteins at interfaces, examples are being accumulated from which we may be able to infer physical and chemical principles for the crystallization. Among these (for a review see Kornberg and Darst (1991)) are a specific binding between protein and ligand molecule incorporated into a lipid monolayer (Uzgiris and Kornberg, 1983; Ludwig, 1986) and a nonspecific binding to a charged lipid layer (Darst et al., 1991b). A direct spreading of a concentrated protein solution on a clean mercury surface has also obtained successful results (Yoshimura et al., 1990). The 2D crystal of streptavidin (Green, 1975) has been obtained by specific binding to alkylated biotin molecules in a lipid monolayer (Blankenburg et al., 1989; Darst et al., 1991a). Our method has already been applied to obtain densely packed arrays of ferritin (Furuno et al., 1989) and catalase (Furuno et al., 1992), where a nonspecific binding of dissolved protein molecules to an interfacial layer of synthetic polypeptide, poly(1-benzyl-L-histidine) (PBLH), is utilized and a heating of the interfacial film stimulates crystallization (Sato et al., 1993). The size of the ordered array of ferritin was also enhanced considerably by the similar thermal treatment (unpublished results). This paper suggests a generality of this method by demonstrating the third 2D crystal prepared under the similar procedures.

Two modern techniques, scanning tunneling microscopy (STM) (Binnig et al., 1982) and atomic force microscopy (AFM) (Binnig et al., 1986), are currently advancing toward high resolution imaging of biological macromolecules on a bulk substrate (Guckenberger et al., 1989; Butt et al., 1990; Ohnishi et al., 1992). A classical technique using a focused electron beam as probe, scanning (secondary) electron microscopy (SEM), has also made a remarkable progress in resolution to better than 10 Å (Nagatani et al., 1987). Several proteins with relatively large molecular mass have been observed on electroconductive bulk plates with the high resolution SEM (HR-SEM) (Furuno et al., 1989, 1992; Nakadera et al., 1991). Well contrasted raw images of 2D crystal of streptavidin (~50 Å, ~60 kDa) on a silicon wafer are presented in this paper. In the previous paper (Furuno et al., 1992), we have imaged the top layer of a thin three-dimensional crystal of catalase with the HR-SEM. The present study, therefore, suggests that the HR-SEM can be used to image the surface of the layered film of protein with usual molecular mass as streptavidin. Thus the HR-SEM would become a useful and straightforward tool in the nanotechnology for biological molecules on a semiconductor wafer.

MATERIALS AND METHODS

Streptavidin and poly(1-benzyl-L-histidine) were purchased (from Sigma Chemical Co., St. Louis, MO). A small trough (20 × 35-mm^2 area, 2-mm depth, 1-mm sole thickness) was filled with a solution of streptavidin (concentration 15 μg/ml, 20 mM Tris, pH 7.0, 20 mM NaCl). A surface film of PBLH was spread at the surface of the protein solution at a density of ~1/20 residue/Å^2 (for spreading PBLH (Furuno et al. 1989)). After 1–3 h of incubation at room temperature, the trough was heated to 37°C and stood still for 30 min. A heating unit with an aluminum block (11 × 11 × 8 cm^3) was used. The trough was placed in a well (2-cm depth) milled in the aluminum block, on top of which was placed an aluminum plate (1-cm thick) for air

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tightening and for ensuring thermal uniformity. After the heating, the block was cooled down below 25°C naturally or with a stream of nitrogen gas. The interfacial film was transferred to a silicon wafer with hydrophobic surface (~6 × 10 mm^2) by placing it horizontally onto the interfacial film. The wafer was recovered from the surface, rinsed with pure water, and stained in phosphotungstate (2.5%, pH 4.8–5.4) for 30–60 s. It was placed in an eppendorf tube (1.5 ml) with the wetted face down, and the excess solution on the surface was finally spun down or blown off with a microcentrifuge at 6000 rpm (×2000 g). During observation with a high resolution scanning electron microscope (S900; Hitachi, Ltd.) the specimen temperature was kept at ~70°C to reduce surface contamination. Several technical aspects in HR-SEM for observation of protein molecules on a bulk plate are discussed in more detail in our previous paper (Furuno et al., 1992).

RESULTS AND DISCUSSION

Streptavidin is the third protein crystallized in two dimensions by means of the PBLH method. Fig. 1 *a* shows a scanning electron micrograph of the negatively stained 2D crystal of streptavidin, which extends over an area of 1 × 1 μm^2 (Fig. 1 *b* is a higher magnification image). Fig. 1 *c* shows an area near crystal boundary where the molecules are deposited randomly on the silicon wafer. The protein molecules are seen dark, as previously discussed (Furuno et al., 1992), while the stain which accumulates on the surface of the array appears bright. We recognize each dark particle (Fig. 1 *c*) to be about 50 Å, although this shape is not well resolved. The images are noisy due mainly to the nonuniformity of the stain (see Fig. 1 *b*), but they exhibit relatively high contrast which would be a merit in a routine use of the HR-SEM for characterizing the surface of flat substrate deposited with protein molecules.

The experimental conditions were not widely surveyed for optimization of crystallization. However, several clear results were obtained. Without the thermal treatment the order of the protein assembly was poor at most areas. We could find out a packing of small 2D crystals (~0.1 μm) with a low probability under a scan for wide area. On the other hand, the thermal treatment of the interfacial film remarkably increased the probability to find large areas where 2D crystals were aggregated. The size of each crystal domain was 0.3–2 μm in diameter. The reproducibility of the crystallization was sufficiently high to find 2D crystals in any specimen prepared under the same condition as for Fig. 1 *a* (see Materials and Methods). The 2D crystal was formed in a relatively wide range of pH (6.5–7.5), ionic strength (10–30 mM NaCl), treating temperature (28–38°C), and surface density of PBLH (one-tenth to one-twentieth residue per $Å^2$). The method and experimental conditions were similar to those for 2D crystallization of catalase, except that the optimum subphase pH was in a rather narrow range for catalase (Sato et al., 1993).

Fourier filtering was applied to the negatively stained image of the 2D crystal with a personal computer system as previously reported (Furuno et al., 1992). The structural analysis for the image taken by HR-SEM is restricted to a rather low resolution because of the intrinsic difficulties for obtaining a high resolution images with the present HR-SEM, where the resolution limit would be about 30 Å for protein arrays (Furuno et al., 1992). In the Fourier transform, only (10), (01) and ($\bar{1}1$) diffractions (and occasionally (11)) were visible on the computer display. The ($\bar{1}1$) diffraction corresponds to a spacing of 44 Å. Higher order spots, if present, appear around or exceed the above 30-Å limit. Therefore, some other factors, e.g., nonuniformity of the staining of the specimen surface, lattice distortion, or intrinsically weak structure factor, would directly lead to the disappearance of

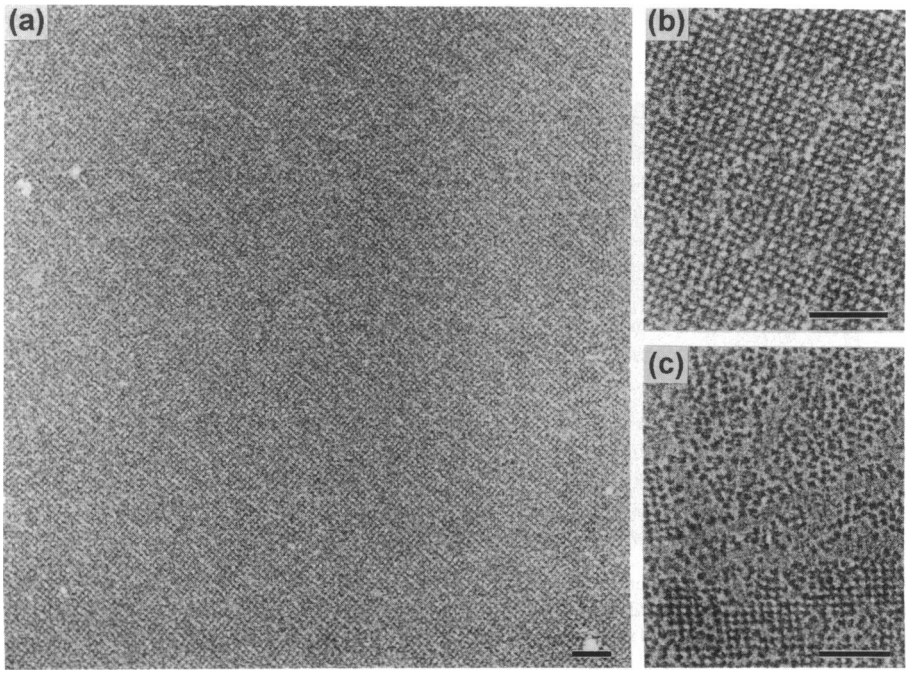

FIGURE 1 Scanning electron micrographs of negatively stained 2D crystals of streptavidin on a silicon wafer. (*a*) (taken at 100,000 ×) shows a large monodomain area; (*b*) was taken at twice as high magnification 200,000 ×; (*c*) shows a region near crystal edge (taken at 180,000 ×). Scale bar = 500 Å.

such spots near the limit. However, it is possible to extract a low resolution filtered image by a back-Fourier transformation and to know the characteristics of the crystal structure. A precise evaluation for the crystallinity of the present crystal is, of course, left for transmission electron microscopy.

Fig. 2 shows the noise-filtered images of an area in the 2D crystal (Fig. 1 *a*). We can determine the lattice parameters from the low resolution images. The unit cell parameters were determined by extracting lattice vectors from a translational cross-correlation pattern between the reference and the entire image obtained by Fourier filtering (Fig. 2), where a weak filtering was applied to make lattice distortion appear clearly. The translationally correlation-averaged image (*inset* in Fig. 2) was obtained by superimposing images with unit area centered at the cross-correlation peaks above a certain threshold value. The present crystal form, with unit cell parameters of $a = 60 \pm 2$ Å, $b = 64 \pm 3$ Å, and $\gamma = 91 \pm 2°$ (average of 10 samples), differs apparently from that prepared by specific binding to biotinylated lipid (Darst et al., 1991a), in which two molecules are in a square lattice with unit cell parameters $a = 84 \pm 1$ Å, $b = 85 \pm 2$ Å, and $\gamma = 90 \pm 1°$ (3600 Å^2/molecule and with space group C222; hereafter we call this structure simply as square lattice or square cell). One molecule occupies 3840 Å^2 in our crystal which is comparable to that in the square-lattice crystal. It is recognized, however, that the features in the filtered images (Fig. 2) closely resemble the projection map of the square-lattice crystal (obtained by transmission electron microscopy, where the indicated molecular size is ~55 × 45 Å). Fig. 2 indicates that the stain, which appears bright, accumulates at relatively large spaces between the protein molecules.

Fig. 3 shows schematically the difference in the unit cell structure between the two crystal forms. A skew of the square lattice by 4° in γ and by 2–4 Å in the unit cell length leads

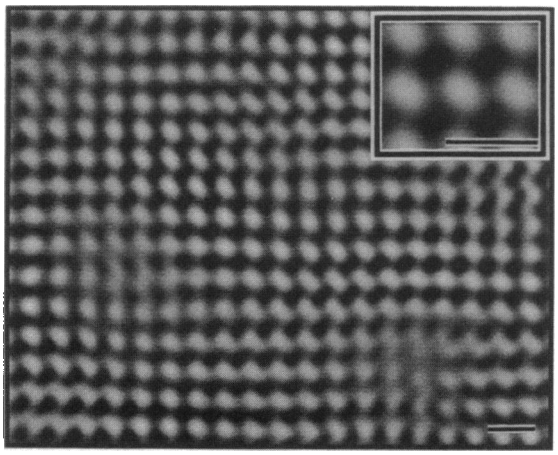

FIGURE 2 Fourier-filtered and correlation-averaged (*inset*) images of negatively stained 2D crystal of streptavidin (Fig. 1 *a*). The bright region corresponds to the accumulated stain, and the dark region corresponds to the protein molecule. Scale bar = 100 Å.

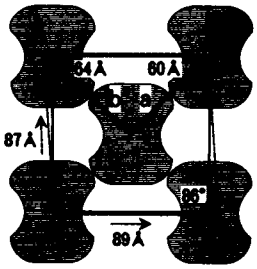

FIGURE 3 Comparison of the crystal structure between the square lattice (Darst et al., 1991a) and that in the present study. The cell introduced by the two vectors **a** − **b** and **a** + **b** is drawn by broken line and compared with the face-centered square cell drawn by real line. The hatched dumbbells schematically represent the streptavidin molecules arranged in the square cell.

to the unit cell dimensions in the present crystal. A close resemblance in both lattice parameters and filtered image suggests that the packing of the streptavidin molecules is similar between the two 2D crystal forms. Here we must remember that the determination of our crystallographic parameters was rather preliminary due to the poor diffraction orders. The present crystal may be almost isomorphous to the square-lattice crystal.

It is considered that, since PBLH is a polymer, its layer at the air/water interface is not so fluid as that of unsaturated phospholipid used to prepare the square-lattice crystal. Then, a stronger asymmetry in the boundary condition at two faces of the bound protein layer, i.e., air and water sides, will be present, which would lead to a hindrance of the intrinsic square-lattice formation. It is thought rather that the above close resemblance in the crystal structure is itself a surprising matter, because one is due to specific binding and the other is presumably based on nonspecific binding. This suggests, on the contrary, that the protein-protein interaction governs the 2D crystal formation, and the spread interfacial layer provides a sticky plane which interferes less with the in-plane diffusion and rotation of the bound protein molecules.

Similar crystallization experiments were carried out for inactive streptavidin, i.e., biotin-bound form. The binding of the inactive streptavidin to the PBLH layer was quite poor, and no arrays were found in the transferred films. Thus, a possibility is suggested that the interaction between PBLH and streptavidin is confined to the biotin binding region in the streptavidin molecule. If this is the case, the close resemblance of our crystal structure to the square cell would be convincing.

REFERENCES

Binnig, G., H. Rohrer, C. Gerber, and E. Weibel. 1982. Surface studies by scanning tunneling microscopy. *Phys. Rev. Lett.* 49:57–61.

Binnig, G., C. F. Quate, and C. Gerber. 1986. Atomic force microscope. *Phys. Rev. Lett.* 56:930–933.

Blankenburg, R., P. Meller, H. Ringsdorf, and C. Salesse. 1989. Interaction between biotin lipids and streptavidin in monolayers: formation of oriented two-dimensional protein domains induced by surface recognition. *Biochemistry.* 28:8214–8221.

Butt, H.-J., K. H. Downing, and P. K. Hansma. 1990. Imaging the membrane protein bacteriorhodopsin with the atomic force microscope. *Biophys. J.* 58:1473–1480.

Darst, S. A., M. Ahlers, P. H. Meller, E. W. Kubalek, R. Blankenburg, H. O. Ribi, H. Ringsdorf, and R. D. Kornberg. 1991. Two-dimensional crystals of streptavidin on biotinylated lipid layers and their interactions with biotinylated macromolecules. *Biophys. J.* 59:387–396.

Darst, S. A., E. W. Kubalek, A. M. Edwards, and R. D. Kornberg. 1991. Two-dimensional and epitaxial crystallization of a mutant form of yeast RNA polymerase II. *J. Mol. Biol.* 221:347–357.

Furuno, T., H. Sasabe, and K. M. Ulmer. 1989. Binding of ferritin molecules to a charged polypeptide layer of poly-1-benzyl-L-histidine. *Thin Solid Films.* 180:23–30.

Furuno, T., K. M. Ulmer, and H. Sasabe. 1992. Scanning electron microscopy of negatively stained catalase on a silicon wafer. *Microsc. Res. Tech.* 21:32–38.

Green, N. M. 1975. Avidin. *Adv. Protein Chem.* 29:85–133.

Guckenberger, R., W. Wiegrabe, A. Hillebrand, T. Hartmann, Z. Wang, and W. Baumeister. 1989. Scanning tunneling microscopy of a hydrated bacterial surface protein. *Ultramicroscopy.* 31:327–332.

Kornberg, R. D., and S. A. Darst. 1991. Two-dimensional crystals of proteins on lipid layers. *Curr. Opinion Struct. Biol.* 1:642–646.

Ludwig, D. S., H. O. Ribi, G. K. Schoolnik, and R. D. Kornberg. 1986. Two-dimensional crystals of cholera toxin B-subunit-receptor complexes: projected structure at 17-Å resolution. *Proc. Natl. Acad. Sci. USA.* 83: 8585–8588.

Nagatani, T., S. Saito, M. Sato, and M. Yamada. 1987. Development of an ultra high resolution scanning electron microscope by means of a field emission source and in-lens system. *Scanning Microsc.* 1:901–909.

Nakadera, T., A. Mitsushima, and K. Tanaka. 1991. Application of high-resolution scanning electron microscopy to biological macromolecules. *J. Microsc.* 163:43–50.

Ohnishi, S., M. Hara, T. Furuno, and H. Sasabe. 1992. Imaging the ordered arrays of water-soluble protein ferritin with the atomic force microscope. *Biophys. J.* 63:1425–1431.

Sato, A., T. Furuno, C. Toyoshima, and H. Sasabe. 1993. Two-dimensional crystallization of catalase on a monolayer film of poly(1-benzyl-L-histidine) spread at the air/water interface. *Biochim. Biophys. Acta.* 1162: 54–60.

Uzgiris, E. E., and R. D. Kornberg. 1983. Two-dimensional crystallization technique for imaging macromolecules, with application to antigen-antibody-complement complexes. *Nature (Lond.).* 301:125–129.

Yoshimura, H., M. Matsumoto, S. Endo, and K. Nagayama. 1990. Two-dimensional crystallization of proteins on mercury. *Ultramicroscopy.* 32: 265–274.