Two-Dimensional Crystallization of Avidin on Biotinylated Lipid Monolayers

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ABSTRACT Two-dimensional crystals of avidin were obtained on mixed lipid monolayers containing biotinylated lipids (*N*-biotinyl-dipalmitoyl-L-α-phosphatidyl ethanolamine and dioleoyl phosphatidyl choline) by specific interaction. Image analysis of electron micrographs of these crystals revealed p2 symmetry with the unit cell parameters $a = 66 \pm 2$ Å, $b = 68 \pm 1$ Å, and $\gamma = 121 \pm 4°$. The projection map showed, at a resolution of about 27 Å, that the four subunits within one avidin molecule are separated into two parts. Comparison between avidin and streptavidin reveals that avidin molecule binds to the lipid monolayer in an orientation similar to that of streptavidin.

INTRODUCTION

Avidin is a protein from egg white (Wilchek and Bayer, 1988). The biotin-binding properties of avidin and streptavidin are remarkably alike. They are both composed of four identical subunits, each binding one biotin molecule. The affinity binding between biotin and avidin (or streptavidin) is so high that the process of complex formation can be regarded as irreversible. Thus the avidin- (or streptavidin)-biotin system has wide application in many fields, such as affinity chromatography, affinity cytochemistry, immunoassays, and gene probes (Wilchek and Bayer, 1990). Besides their similarities, there are also some significant differences between the two proteins. Avidin is a basic glycoprotein, each subunit containing an *N*-linked oligosaccharide side chain, whereas streptavidin is a non-glycosylated neutral protein. It has been proven that the oligosaccharide chains in avidin are heterogeneous (Bruch et al., 1982). These differences are believed to be the main reason why avidin is much more difficult to crystallize than streptavidin.

Two-dimensional (2D) crystals of macromolecules can be used for structure analysis by electron crystallography (Amos et al., 1982). A general method of forming 2D crystals of water-soluble proteins on lipid monolayers based on specific interactions between protein and lipid-bound ligand has been developed by R. D. Kornberg and colleagues (Uzgiris and Kornberg, 1983; Kornberg and Ribi, 1987). The method has been applied successfully in many cases (Kornberg and Darst, 1991). One of these was streptavidin, which revealed the orientation of streptavidin molecules on biotinylated lipid monolayers (Darst et al., 1991; Blankenburg et al., 1989).

In the present work, 2D crystals of avidin on mixed monolayers containing biotin-lipids were studied by electron microscopy with negatively stained specimens, and the projection structure of avidin at low resolution was determined by image analysis.

MATERIALS AND METHODS

Materials

Biotinyl-*N*-hydroxysuccinimide, biotin, DMPE, DPPE and DMPA were purchased from Sigma Chemical Co. (St. Louis, MO). DOPC was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Avidin was purified from egg white according the method of Melamed and Green (1963).

Preparation of biotinylated lipids

Two biotinylated phospholipids, B-DPPE and B-DMPE, were synthesized according the method described by Bayer et al. (1979)

Preparation of 2D crystal specimen

2D crystals were obtained using the lipid monolayer method with small Teflon wells (4 mm in diameter and 0.5 mm in depth; Kornberg and Ribi, 1987). In general, a solution of protein was placed in the wells. The lipid sample in solvent was spread on the surface of protein solution. The protein molecules were concentrated at the surface because of their affinity to the specific ligands, and the fluidity of the lipid allows the molecules to arrange themselves laterally into 2D ordered arrays.

In our experiments, usually 17.5 μl protein solution was placed in a Teflon well, and 1 μl of lipid solution was spread at the surface. The lipid sample was a mixture of B-DPPE/DOPC (1:4, M/M) at a total concentration of 0.7 mg/ml in chloroform/methanol (3:1, v/v). The protein solution contained 200 μg/ml avidin, 50 mM Tris-HCl, 150 mM NaCl, at pH 7.0. Incubations were carried out either at room temperature or at 4°C over periods ranging from 12 h to several weeks. The Teflon wells were always sealed in a humid chamber to prevent evaporation.

The amount of lipid sample we added to each well was much more than that required to form a monolayer. Thus, the lipid membrane was believed to be in the highest pressure state it could reach. This assumption was supported by the control experiments completed in a well with a larger surface (diameter 50 mm). The maximal surface pressure in the control experiments was obtained about 40 mN/m (see Fig. 1). The lipid packing density is an important factor in obtained crystals; however, it is hard to calculate the lipid density in the 4 mm diameter well directly from the initial lipid amount because of the edge effect (Mosser and Brisson, 1991, and Mosser et al., 1992).

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Abbreviations used in this article: B-DPPE, *N*-biotinyl-dipalmitoyl-L-α-phosphatidyl ethanolamine; B-DMPE, *N*-biotinyl-dimyristoyl phosphatiyl ethanolamine; DMPE, dimyristoyl phosphatidyl ethanolamine; DPPE, dipalmitoyl phosphatidyl ethanolamine; DMPA, dimyristoyl phosphatidyl acid; DOPC, dioleoyl phosphatidyl choline.

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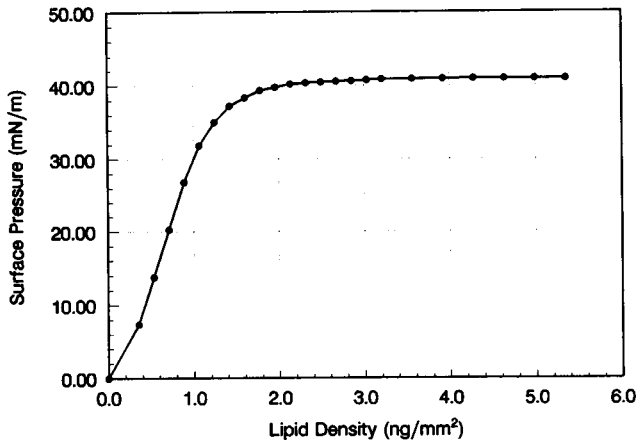

FIGURE 1 Surface pressure characteristics of B-DPPE/DOPC monolayer. The Teflon well was large in size (diameter 50 mm), and the surface pressure was measured by the Wilhelmy method. The subphase contained 200 μg/ml avidin, 50 mM Tris-HCl, 150 mM NaCl, pH 7.0 (same as the conditions to prepare the 2D crystal specimen with the small well). 0.5 μl of well mixed solution of B-DPPE/DOPC (1:4, molar ratio) was added onto the air/water interface each time. After about 10 min for equilibrium, the surface pressure was recorded, all experiments were performed at 20°C. The plot shows that the maximal surface pressure of the saturation stage of the B-DPPE/DOPC monolayer was ~40 mN/m, and the minimal quantity required was ~2.4 ng/mm².

After incubation in the small well, the lipid monolayers with ordered protein patches were first picked up on carbon-coated electron microscope grids. Then the grids were washed by one droplet of distilled water and then negatively stained with 1% (w/v) uranyl acetate for 30–60 s.

Image processing

The negatively stained samples were examined by a Hitachi H-800 electron transmission microscope. The quality of the images and the crystalline order of avidin protein were evaluated by optical diffraction. The best images were used for further analysis by computer after digitalization. The numeric images were processed using the MRC image-processing package (Amos et al., 1982). The lattice vectors (**a*** and **b***) of the diffraction pattern were refined by the coordinates of the visible peaks. Then the whole diffraction was filtered according to the refined vectors (**a*** and **b***), and the values of amplitude and phase were extracted by the program MMBOX. The signal-to-noise ratio of each peak was evaluated by IQ value (Henderson et al., 1990). In our work, the peaks with IQ <7 were used in final Fourier synthesis.

RESULTS

2D crystallization

Different lipid mixtures at different ratios, including B-DPPE/DOPC, B-DMPE/DMPE, and DMPA/DOPC, had been used to investigate the effects of lipids during the 2D crystallization process. Some pure lipids, such as DMPE, DMPS, and stearic acid, were also tried. Among those lipids used, only B-DPPE/DOPC could give the ordered arrangement of the protein layers. When using other lipids, whether containing biotinylated lipid or not, no satisfyingly ordered protein patch was found.

In the case of B-DPPE/DOPC, some larger areas of crystalline arrays of avidin protein were observed. Fig. 2 shows an electron image of a negatively stained 2D crystal domain. This crystal area was about 0.6 × 0.5 μm and belonged to a larger protein patch.

Structure of two-dimension crystals

The diffraction pattern (Fig. 3 *a*) of the obtained avidin 2D crystal showed that the crystal had an average unit cell $a = 66 \pm 2$ Å, $b = 68 \pm 1$ Å, and $\gamma = 121 \pm 4°$. The (2,1) reflection was clearly visible by the eye, corresponding to 27 Å resolution. When assuming p2 symmetry, analyses of phases of 10 selected peaks in the Fourier transform gave an average phase residual at about 20°. Fig. 3 *b* is the projection density map calculated in p2 symmetry. The distribution of stain-excluding density indicated that the avidin molecule was separated into two parts. It could be estimated from the map also that the individual molecule size of avidin was about ~60 Å × 45 Å. Eight different images were averaged to get the final result.

From the diffraction pattern, it looked as if it could have p3 or p6 symmetry. The refinement of the phase origin by the hexagonal lattice, however, gave a phase residue of about 38°, which was too big to be accepted.

DISCUSSION

It is believed that the fluidity of the lipid monolayer is a crucial factor during the 2D crystallization by the monolayer method. The results of our experiments showed that in the case of the lipid monolayer without any double bond in the

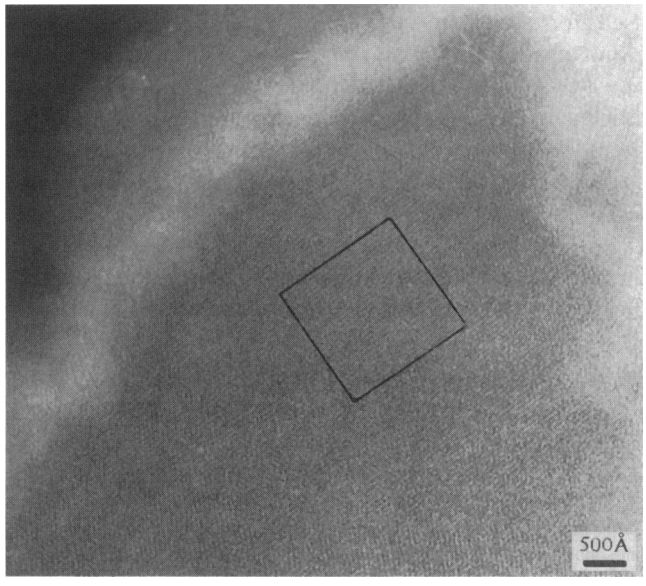

FIGURE 2 Electron micrograph of a negatively stained 2D crystal of avidin on B-DPPE/DOPC (1:4) monolayer. It was taken at 40,000× with the operation voltage of 100 kV. Scale bar represents 500 Å. The black box represents a selected area for computer calculation.

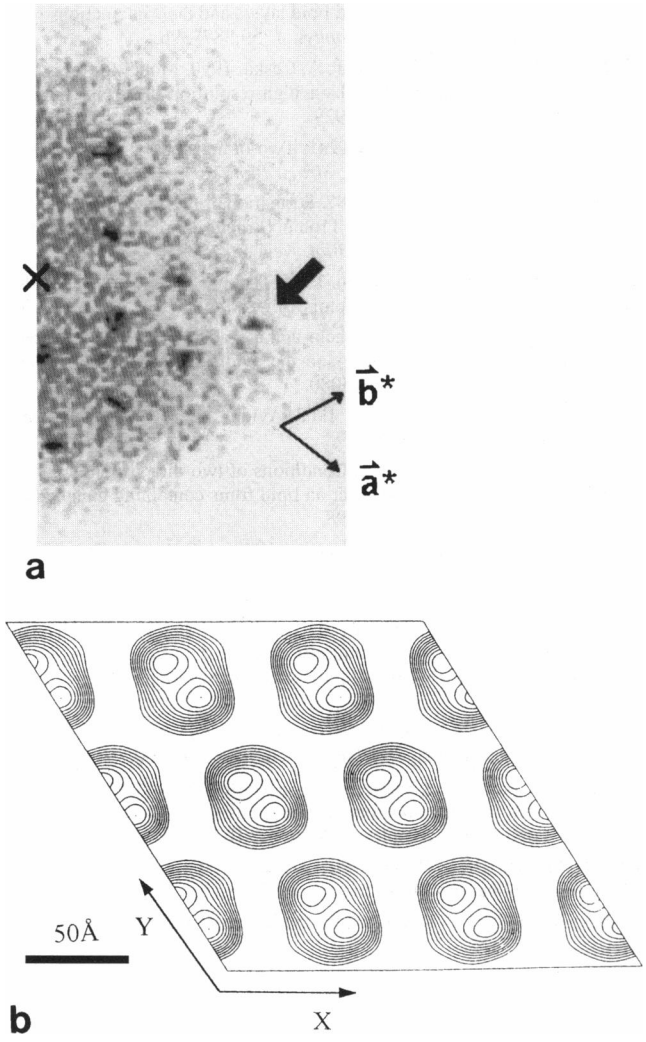

FIGURE 3 Project map of the avidin crystals. (*a*) Computed diffraction pattern. The (2,1) reflection, at 1/27 Å is indicated by an arrow. The X indicates the position of the origin peak, which has been removed to display the reflections more clearly. (*b*) Projection map of avidin crystal calculated in p2 symmetry. Scale bar represents 50 Å.

fatty chains, the protein could hardly form crystalline domains even after several weeks of incubation. This was in agreement with other reports (Kornberg and Ribi, 1987). The fluidity of the lipid monolayer could make the biotin group flexible, which may be of benefit to protein binding to the monolayer surface; moreover, because of the fluidity of the lipid layer the membrane-bound avidin preserved its lateral mobility to be settled in order on the surface.

The rapid 2D crystallization of protein at functionalized monolayer surfaces in the gas-analogous phase proceeds via diffusion-controlled nucleation and crystallization (Blankenburg et al., 1989). In the present work, crystals obviously grow at higher surface pressure, but, because of the high monolayer viscosities, their rates of growth are much slower (at least 12 h) than that at near zero surface pressures.

When the lipids with negatively charged heads were used, it was difficult to find any large patches in which protein molecules were densely arranged, although avidin was a positively charged protein at neutral pH (Wilchek and Bayer, 1988). In contrast, it was very easy to find many large protein patches if the lipid monolayer contained biotinylated lipids, although only B-DPPE/DOPC could give the ordered crystalline domains. This was consistent with a previous result we obtained with the surface plasmon resonance technique that avidin molecules were much more densely arranged on biotinylated lipid monolayer than non-biotinylated lipid monolayer (Qin et al., 1993). It had also been proven by our previous experimentation that nearly all the proteins bound to the monolayer by specific interaction, in the case of mixed lipid layer containing biotinylated lipids (Liu et al., 1994).

Studies of the monolayer properties showed that B-DMPE was a little unstable at air/water interface and was much more difficult to maintain at high pressure than B-DPPE. Because it was thought that high surface pressure was required for crystallization, most of the samples we used contained B-DPPE. Some of the avidin 2D crystals we obtained were obviously more than one layer. This supported the idea that 2D crystals could be used as nuclei for three-dimensional crystallization.

The three-dimensional structure of avidin has been resolved recently by x-ray crystallography (Livnah et al., 1993; Pugliese et al., 1993). When comparing the projection map of avidin we obtained with the published x-ray crystal structure, great similarity can be found. It can be assumed that four subunits within one avidin molecule are separated into two parts, corresponding to the two areas within one avidin molecule in the projection map. By comparing this with the result of streptavidin (Darst et al., 1991), it can also be found that avidin binds to the biotinylated membrane in a manner similar to that of streptavidin. Two biotin-binding sites of avidin are located on the side of the crystal abutting the lipid membrane, and were occupied by the biotin groups on the membrane, while the other two sites are on the opposite side of the crystal, exposed to the solution. The three-dimensional structure of avidin also reveals that the active binding site for biotin within each subunit is located in a deep pocket, at the center of a β-barrel. As the biotin-binding site is buried deep in the protein core a spacer is required for the valeryl side-chain carboxylate group exposed to solvent (Pugliese et al., 1993). In our experiment, biotin molecule was directly linked to the amino group of DPPE. Hence, avidin molecule possibly inserted a small amount into the membrane once it bound. This can explain, in another sense, why membrane fluidity is so crucial to ordered arrays in our experiment.

Although the four subunits of avidin molecule cannot be resolved in the final projection map, sometimes they can be observed clearly within some areas of the electron micrographs. The reason for loss of structure details, we think, is that the present quality of the avidin crystals is not good enough.

In contrast to streptavidin, avidin contains heterogeneous oligosaccharide chains. Avidin is also a positively charged protein because it contains many more positively charged amino residues than does streptavidin (Argarana et al., 1986), which merely contains net charges. It is believed that the

nonspecific adsorption originates from both the sugar content and the charge-charge interaction, especially the former. These reasons had prevented us from getting better ordered crystals than the present ones. Most of the crystals we obtained contained defects, and only a small number of them could be used for structure analysis. Several other reasons for the lower quality of the crystals were also considered. One possible source is their post-deposition rinses. However, this probably did not have much impact on the present low-resolution work.

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