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Specific binding of avidin to biotin containing lipid lamella surfaces studied with monolayers and liposomes

Zheng Liu, Hong Qin, Caide Xiao, Chenhui Wen, Shaopeng Wang, Sen-fang Sui

Biophysics Laboratory, Department of Biological Sciences and Biotechnology, Tsinghua University, Beijing 100084, People's Republic of China

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Abstract. The interaction of avidin (from egg white) with phospholipid (monolayer and bilayer) model membranes containing biotin-conjugated phospholipids has been studied. In the first part, using surface sensitive techniques (ellipsometry and surface plasmon resonance) we demonstrated that the nonspecific adsorption of avidin to phospholipid lamella could be abolished by adding an amount of Ca^{2+}, Mg^{2+} or Ba^{2+} that led to an electrostatic interaction. The specific binding of avidin to lipid mixtures containing biotin-conjugated phospholipids was obviously composition dependent. The ratio 1:12 of a B-DPPE/DPPE mixture was found to be the optimum molar ratio. When we compared the results from the surface sensitive techniques with those from the electron micrographs of a two dimensional crystal of avidin (obtained in our laboratory), the optimum ratio was found to be determined by the effect of lateral steric hindrance. In the second part, we observed the pattern of the layers of fluorescently labeled phospholipid and adsorbed proteins with a home-made micro fluorescence film balance. The fluorescence images showed that avidin was preferentially bound to the receptors that were in the fluid domains. Further, with a sensitive fluoresence assay method, the effect of the phase behavior of liposomes on the specific binding of avidin was measured. This showed that avidin interacted with biotin-lipid more weakly in the gel state liposome than in the liquid state liposome. The major conclusion was that the binding of avidin to a membrane bound model receptor was significantly restricted by two factors: one was the lateral steric hindrance and the other was the fluidity of the model membrane.

Abbreviations: B-DPPE, Biotinyl dipalmitoylphosphatidyl ethanolamine; B-DMPE, Biotinyl dimyristoylphosphatidyl ethanolamine; BNHS, d-biotin-N-hydroxysuccinimide ester; DMPA, dimyristoylphosphatidyl acid; DMPC, dimyristoylphosphatidyl choline; DMPS, dimyristoylphosphatidyl serine; DOPC, dioleoylphosphatidyl choline; DPPC, dipalmitoylphosphatidyl choline; DPPE, dipalmitoylphosphatidyl ethanolamine; FITC, fluorescein isothiocyanate; RDB-DOPE, N(Lissamine rhodamine B sulfonyl) dioleoyl phosphatidylethanolamine; SPR, surface plasmon resonance

Correspondence to: S. F. Sui

Key words: Molecular assembly – Artificial membrane – Two-dimensional crystallization of protein

Introduction

In recent years, interest in the interaction between ligands and membrane bound receptors using artificial membrane systems has increased considerably. In one example, the incorporation of the insulin receptor into liposomes and supported lipid lamellae was thoroughly investigated (Sui et al. 1988). In another example, a membrane bound model receptor, the Fab'-fragment, linked covalently to a phospholipid was studied (Egger et al. 1990). The high-affinity binding system, streptavidin/biotin, was studied at an air-water interface using various biotin lipids (Blankenburg et al. 1989). Two-dimensional crystals of streptavidin were obtained by specific binding on the lipid layer (Darst et al. 1991). Assembling the membrane bound model receptor was also stimulated by the potential applications in the field of biosensors (Zhao and Reiterate 1992; Kuhn 1989), as well as by the use of surface sensitive techniques to study the events on the cell membrane (McConnel 1986).

In the present study, a model system was set up using the high affinity system of avidin as a ligand, and biotin as a model receptor. Avidin is a glycoprotein purified from egg white. Under neutral conditions, the affinity of avidin for biotin is one of the highest known, 10^{15} M^{-1}. It is just one order of magnitude less than the chemical bonds, but much stronger than noncovalent bonds (Bayer and Wilchek 1990). Another characteristic of the avidin-biotin complex is its stability. The complex is stable over a broad range of pH values and in the presence of high concentrations of denaturanats (Bayer and Wilchek 1990). Although the biotin can be covalently linked to many chemical compounds by its carboxyl group, this does not affect the affinity of the cyclic ureido group for the avidin. Here, we attached the biotin covalently to the head group of appropriate phospholipids, and then proceeded to assemble artificial membrane systems containing biotin-lipid.

We used avidin rather than streptavidin as a ligand in this paper since avidin from egg white is readily obtainable, whereas the isolation of streptavidin from *Streptomyces avidinii* is much more complicated and expensive. The remarkable nonspecific adsorption of avidin to phospholipid bilayers appeared to be a problem, and this is not the case for streptavidin (Blankenburg 1989). This seemed to make avidin unfavorable for model system studies of ligand-receptor interactions, but we solved this problem by adding a suitable amount of bivalent alkaline-earth metal ions (Qin et al. 1992). Thus avidin is also a good model for studying the differentiation between specific binding and nonspecific adsorption, which is central to a molecular understanding of biological recognition and molecular engineering applications.

Materials and methods

1. Materials and sample preparation

DMPA, DMPS, DMPC, DOPC, DPPC, DPPE and BNHS were commercial products from SIGMA. RDB-DOPE was purchased from Avanti Polar Lipids, Inc. Biotin, FITC and other chemicals were purchased locally. B-DPPE and B-DMPE were prepared according to Bayer (1979), by linking BNHS to the head group of DPPE or DMPE.

Avidin was purified from egg white by following the procedure of Melamed and Green (Melamed and Green 1963; Green 1963). Its specific activity was assayed by UV spectrometric titration and the purity of the avidin was more than 90%. Avidin-biotin complex (inactive avidin) was prepared by adding biotin to avidin solution until the fluorescence intensity (EX = 280 nm, EM = 350 nm) of the tryptophan of avidin ceased to decrease. (Lin and Kirsch 1977).

Silica slides were the product of the Beijing Nonferrous Metal Institute. In order to prepare supported monolayers on a silica surface, it is essential to treat the substrate in such a way that it is highly hydrophobic. The silica slide was first cleaned in plasma cleaner (Sterilizer PDC-3 XG) and then washed with ethanol. After being air dried, the cleaned silica slide was put into 10% dichlorodimethylsilane in trichloroethylene solution for 20–30 min and subsequently rinsed with trichloroethylene and ethanol.

To form lipid monolayers at an air/water interface a computer controlled LB film balance was used, the total volume of the trough is 250 ml and the total area 370 cm^2. The area covered by the lipid monolayer was changed by a movable barrier; and the surface tension was measured with the Wilhelmy system. The subphase we used was pure water, the lipid monolayer itself was prepared by deposition of about 20 μg of the lipid in about 20 μl of 3:1 chloroform/methanol mixture in small droplets at different positions on the water surface on one side of the trough. In order to form a supported planar film, the lipid monolayers were first compressed to a pressure of 35 ~ 40 mN/m, and then horizontally transferred onto the pretreated substrate by hand. Observations with fluorescence microscopy showed that an area with a diameter larger than 8 mm was totally covered by lipid monolayer around the central part of the substrate, which was large enough for the available optical reflection detection (the diameter of the cross section of the laser beam is about 0.5 mm).

To prepare biotin-phospholipid containing liposomes, the mixture of lipid DPPC with B-DPPE or B-DMPE was dissolved in chloroform. After drying under nitrogen in a vacuum desiccator, the mixture was suspended in PBS buffer (100 μl buffer containing 1.36 μmol DPPC with different molar ratios of B-DPPE or B-DMPE), and then sonicated for about 20 minutes in a 50 °C water bath until the solution turned clear.

2. Ellipsometry

The principle of in situ ellipsometry in the study of the interaction between avidin and a supported planar lipid monolayer is shown schematically in Fig. 1. The ellipsometer (model TP-77) was made by Beijing Photo Machinery Institute and uses a He-Ne laser (wavelength of 632.8 nm) as the light source. The sample cell is made from teflon and has a volume of 3.0 ml. To displace the bulk solution there were two small holes on the two side walls that were connected to two separate pumps. The monolayer covered silica slide was placed at the bottom of the chamber, and then the buffer was slowly directed in. For observation of protein adsorption a suitable amount of avidin was added (to an ultimate concentration of 30 μg/ml). After 60 min for equilibration, the parameters of the ellipsometry were detected. The ellipsometry gave two readings, which were converted into changes in amplitude Ψ and phase Δ of the light upon reflection. If the tickness of the membrane is very thin (less than 10 nm), then the change of the phase parameter Δ is approximately proportional to the thickness of the membrane. Since the protein adsorption satisfied this requirement, we used the parameter Δ to describe the change of the thickness of the adsorption layer in this paper. For further information about theory, instrumentation and calculations see Azzam and Bashara (1977).

3. Surface plasmon resonance

The home-made surface plasmon resonance (SPR) apparatus used is shown schematically in Fig. 2. A He-Ne laser (wavelength of 632.8 nm) is the incident light source. The measurement was performed by varying the angle of incidence Θ. In order to characterize the interaction between avidin and the lipid monolayer, the measurements were made as follows: after transferring the lipid monolayer onto the fresh surface of the gold coated cover slide, the slide was carefully stuck with index matching fluid onto the bottom surface of the triangular prism (as shown in Fig. 2). Then the sample cell was installed and the buffer was slowly pumped into it. The initial peak position of the SPR curve is buffer was recorded. Then the protein solution was pumped into the cell and the second SPR curve was measured after equilibrium. As with the treatment of the ellipsometry measurement, if the tickness of the film

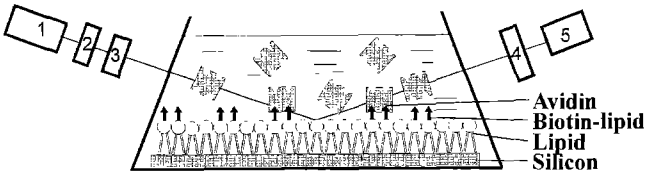

Fig. 1. Schematic illustration of ellipsometry. The lipid monolayer was deposited onto a hydrophobic surface of silicon wafer by horizontal transfer from the air/water interface. A cuvette was fitted into the holder of the ellipsometer. This was assembled in such a way that the two side walls (perpendicular to the direction of incident light) consisted of optically polished faces. The bulk solution could be exchanged continuously through the tubes on the walls of the cuvette. The incident angle of light is 70° and the wavelength 632.8 nm. 1. He-Ne laser, 2. polarizer, 3. compensater, 4. analyzer, 5. photodiode

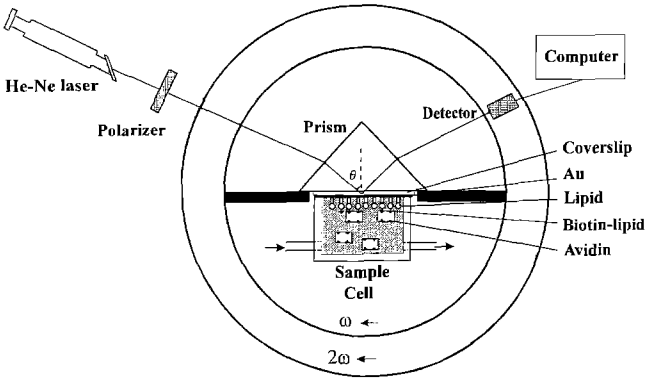

Fig. 2. Schematic illustration of the arrangement of the apparaturs for surface plasmon resonance. Using a 632.8 nm laser, the measurement was performed by varying the incidence angle θ. The plane of polarization of the incident beam was controlled with a polarizer. To stabilize the laser amplitude fluctuation a high quality laser power supply with electronic stabilizer was used. Before measurement the laser tube was preheated for about 1 hour until the light intensity become stable. The triangular prism (refractive index 1.8) was fitted onto the small rotating-stage of the "θ–2θ" angle-measuring apparatus. As the small rotating-stage moves with an angular velocity of ω (0.5 °/sec), the photodiode fitted on the big rotating-stage moves at 2ω (1.0 °/s). The SPR curves are recorded as the photodiode moves following the reflection light beam. The angular accuracy of the "θ-2θ" angle-measuring apparatus is 0.01 °. The sample cell was made from teflon, its volume was 2.0 ml

is very thin (for example, less than 10 nm), the displacement of the SPR peak position is linearly related to the layer tickness (Raether 1988). Thus we used the shift of the peak position as a quantitative measure of the tickness of the adsorption layer in the present work. To observe the desorption effect, a suitable amount of Ca^{2+} was added to the desired concentration (5 mM). Both techniques, i.e. ellipsometry and SPR, were used to measure the protein adsorption layer. A direct comparison of the two measurement methods was made under identical conditions with a model system as shown in Fig. 3. Figure 3 shows that the layer numbers of stearic acid are 1, 2 and 3 respectively, the measurement parameters in dΔ with ellipsometry and dΘ with SPR display a satisfactory linear correlation. This examination provided a basis for the comparison of measurements of the two related techniques.

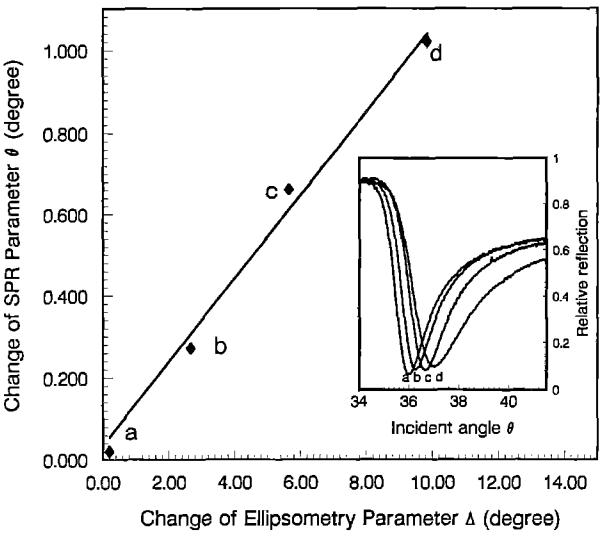

Fig. 3. A direct comparison of ellipsometry and SPR measurements under identical conditions. dθ, the displacement of the SPR minimum; dΔ, the change of the parameter Δ of ellipsometry. (*a*), without film, (*b*), (*c*) and (*d*), with 1, 2 and 3 layers of stearic acid respectively. The substrate was the cover slide coated with gold film (about 50 nm). The surface plasmon spectra are shown in the insert

4. Microfluorescence film balance

The computer controlled microfluorescence film balance system was constructed using a *Nikon DIAPHOT-TMD inverted epifluorescence microscope* and a computer controlled film balance. The area of the rectangular Teflon trough was 140 cm^2 with a 40 mm-diameter quartz window; which was at the bottom of the trough above the microscopic objective. The temperature was controlled by a water channel in the base of the trough. The excitation of the fluorescent label in the monolayer was performed by illumination with a 100 W high-pressure mercury lamp. Several filter combinations for different wavelength ranges between near UV and IR were available. Long working distance objectives could be used for different scales. The highest scale of the system was 400×. The trough was fixed onto the movable stage and the observing position could be changed. The fluorescence images of monolayers were recorded by Nikon F 301 camera with high speed film (Kodak TMAX-P 3200). Exposure time varied from 0.25 S to 10 S owing to variations in the sample fluorescent density. The pictures were transferred to a computer by a digital scanner for further calculations.

Results

1. Influence of receptor density on avidin binding

The non-specific adsorption of avidin to the lipid monolayer is a serious problem in the study of the specific binding of avidin, which must be resolved. In previous work, we solved this problem by adding a suitable amount of Ca^{2+} to the subphase (Qin et al. 1992). In this investiga-

tion, using ellipsometry, we confirmed the feasibility of this method and have shown that some other ions can be useful. The results are shown in Fig. 4. From Fig. 4 we deduce that other bivalent alkaline-earth metal cations such as Mg^{2+} and Ba^{2+} also had the detachment effect. However, the univalent alkaline metal cations Na^+ and K^+ and the bivalent cation Zn^{2+} did not have this function.

After solving the problem of nonspecific adsorption, the specific binding of avidin could be investigated. To analyze the influence of receptor density on ligand binding, the molar ratios of B-DPPE/DPPE mixtures were varied in a rather narrow composition interval with the LB film technique. The interactions of avidin with these monolayer surfaces were precisely analyzed with ellipsometry and surface plasmon resonance. The ellipsometry results shown in Fig. 5 displayed a significant membrane composition dependence of protein specific binding.

Figure 5 shows an optimum molar ratio of B-DPPE/DPPE at 1:12. Around this ratio the amount of protein sepcifically bound to the membrane surface is the highest; and the non-specific adsorption may be negligible. Higher or lower than the ratio of 1:12, the total amount of avidin specifically bound to the monolayer would decrease. On the pure B-DPPE lipid monolayer, the biotin groups packed very closely. There was little free-space between biotin groups, and the lateral steric hindrance restricted avidin to specific binding to the receptor. For DPPE, the head group was very small. When DPPE was added, the free-space between biotin groups increased. Greater free-space was advantageous to the biotin/avidin binding, which increased the total amount of protein binding to the monolayer. However if the percentage of DPPE was too high, the model receptors on the monolayer became relatively rare, and the total amount of membrane bound protein decreased again. One can calculate the thickness of the film from the ellipsometer parameters. In the case of protein adsorption, however, only the average thickness can be calculated and this can not be translated unambiguously into molecular dimensions (Jönssen et al. 1982). When the adsorbed protein layer was modeled as a homogeneous layer we estimated the complex refractive index as $\hat{n} = \{1.55, 0\}$ (Schmidt et al. 1990). The effective complex refractive index of the silanzied solid substrate obtained in air was $\hat{n} = \{4.0, 0.12\}$ and the refractive index of the lipid layer measured in air was $\hat{n} = \{1.54, 0\}$. Thus an end value in thickness of (4.7 ± 0.2) nm of the avidin layer was obtained in the case of lipid monolayers with the optimum molar ratio. This value was a little less than the molecular dimension (~5.0 nm) observed directly from the electron micrograph (shown in discussion section below). This evaluation led us to conclude that the adsorbed avidin formed a submonolayer. Similar observations were obtained also by the SPR method (as shown in Fig. 6). Figure 6 clearly displays an optimum composition ratio of B-DPPE/DPPE located between 1:10 and 1:15.

Another intriguing suggestion from the results of the ellipsometry and SPR experiments was that the non-specific adsorption was almost eliminated if the monolayer composition was around the optimum molar ratio. Thus, if one organizes a functional monolayer is such a way that the composition is chosen just as the optimum ratio, the nonspecific adsorption of avidin is of minor importance. This implies a possible approach to overcoming the problem of nonspecific adsorption, which would help to improve the molecular engineering applications of the avidin/biotin-lipid system.

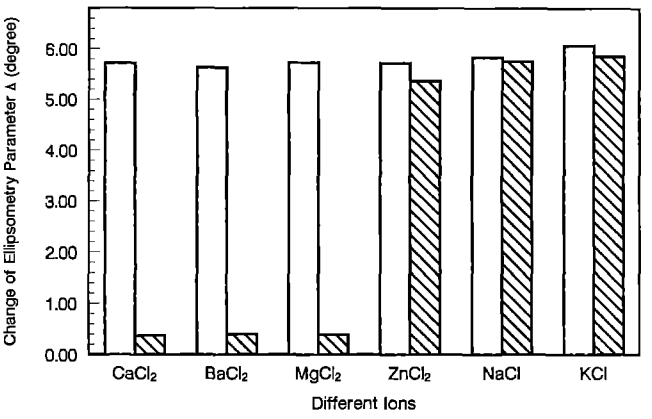

Fig. 4. The effects of metal ions on the detachment of non-specific adsorption of avidin was determined with ellipsometry at 20 °C. The supported planar monolayer consisted of a mixture of B-DPPE/DPPE (1:5). For the experiments of non-specific adsorption, inactive avidin (avidin-biotin complex, which can not specifically binding to biotin-lipid, 1.0 mg/ml in water, pH 6.5) was added into the cuvette. The ultimate concentration of protein in the cuvette was 50 μg/ml. After 60 min for equilibriation, the parameter Δ was recorded; then the bulk solution was exchanged. For desorption experiments, the ion containing solution flowed through the chamber for 60 min until the parameter Δ did not change. Ion concentrations in water (pH=6.5): 5 mM for $CaCl_2$, $BaCl_2$, $MgCl_2$ and $ZnCl_2$, 10 mM for NaCl and KCl. □ , before desorption; ▨, after desorption

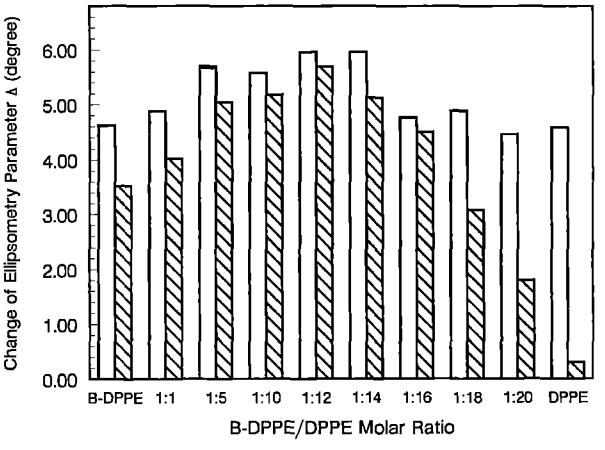

Fig. 5. Comparison of the amounts of avidin binding onto supported planar monolayers was studied with ellipsometry at 20 °C. The supported planar monolayers were prepared with different molar ratios of B-DPPE/DPPE using the LB technique. For adsorption assay, avidin (1.0 mg/ml in water, pH 6.5) was added in the cuvette to the final concentration of 30 μg/ml. To detach the non-specific adsorption, a 5 mM $CaCl_2$ solution was flowed through the chamber for about 60 min. □, before desorption; ▨, after desorption

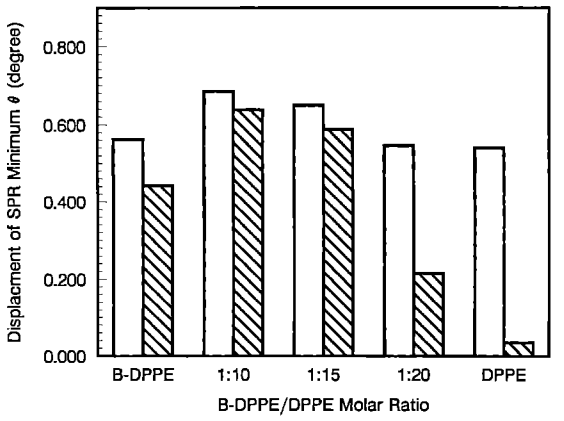

Fig. 6. Comparison of the amounts of avidin binding onto supported planar monolayers containing various molar fractions of B-DPPE lipids was studied with surface plasmon resonance at 20 °C. □, before desorption; ▨, after desorption

2. Influence of membrane fluidity on avidin binding

Monolayers and liposomes (curved bilayer) are useful models of biological membranes. As thoroughly discussed in a review article (Gabrielli 1991), the possible choice of model membranes both in mono- and bi-layers yielded complementary and comparable information. Therefore we used the two types of model systems to study the influence of membrane fluidity on avidin binding as follows. *Monolayer (at air/water interface) study.* In recent years several laboratories have studied the pattern formation of pure and mixed lipid monolayers at an air/water interface with the microfluorescence film balance (Loesche et al. 1983; Gaub et al. 1986). The analyzing process of the pattern formation was in most cases according to the phase separation in the liquid-solid co-existence region. Following this approach we further studied the effects of the phase behavior of model receptor containing monolayers on specific binding.

After spreading a lipid monolayer of B-DPPE (containing 2% RDB-DOPE, EX = 540 nm, EM = 610 nm) on the air/water interface, the fluorescent molecules were well distributed at low surface pressure. With compression of area, we observed the appearance of regions of intense fluorescence and regions of dark dots, showing the coexistence of liquid and solid states of the monolayer (Fig. 7 a). Further compression would enlarge the dark areas.

In contrast to the lipid pattern, we generated a two-dimensional pattern of avidin. A 2% DOPE containing B-DPPE (without stained lipid) monolayer was formed at an air/water interface. Around liquid-solid coexisting surface pressure, FITC-avidin (EX = 495 nm, EM = 535 nm) was injected into the subphase. 40 min later, the protein pattern was observed (Fig. 7 b). At this stage, the patterns of lipid (shown in Fig. 7 a) and protein (Fig. 7 b) looked similar, but they arose from different causes. For Fig. 7 a, the dark domains were the areas where lipids were in the solid state; and for Fig. 7 b, these were the regions where fewer proteins were adsorbed.

Similar results were also obtained by a double labeling system, RDB-DOPE containing B-DPPE at the air/water interface and FITC-avidin in the subphase. Because of the energy-transfer between FITC and the RDB group, when the exciting FITC collided with the filter block of 420 ~ 490 nm, a red figure (emitted by RDB group of lipid) was seen, suggesting a FITC-avidin/biotin-lipid binding. The lipid monolayer and the receptor bound FITC-avidin patterns were then surveyed separately (Fig. 7 c). With green excitation (filter block: 510 ~ 560 nm), an image was taken of the lipid layer (on the left side of Fig. 7 c). For the same condition after irradiating with green light for several minutes to quench RDB, the pattern of the protein layer (in green) was observed with blue excitation (on the right side of Fig. 7 c). The bright regions of these two patterns looked nearly the same, but they came from different sources. One was from fluid lipid regions, and the other was from receptor bound avidin domains. This observations was in agreement with the results of Fig. 7 a and Fig. 7 b.

An interesting phenomenon was observed one or more hours later. The fluorescence intensity of the dark domains of the protein pattern gradually increased, and eventually obscured the original pattern. This suggested that the receptor bound FITC-avidin even occurred in the solid state regions of the B-DPPE layer. During a further observation, we noticed that the figure of the lipid layer remained constant while the FITC-avidin pattern lost contrast, indicating that the RDB-DOPE molecules did not exchange with the solid-phase lipids. The observed blurring of the avidin pattern may be caused by two molecular mechanisms. One is that the B-DPPE molecules in the solid phase may still exchange with the FITC-avidin bound B-DPPE complexes in the liquid phase. The other is that the avidins from subphase bind directly to the monolayer surface to form new complexes in the dark domains. To interpret this phenomenon, we think that the intermolecular interaction between membrane attached FITC-avidin must be considered. The fluorescence micrographs of Fig. 7 b and Fig. 7 c (right side) show that FITC-avidins attached to the membrane assemble into two-dimensional aggregates. This observation provided direct evidence that a relatively strong protein-protein interaction exists, which induced avidins to be aggregated rather than to be homogeneously distributed. Therefore, we interpret the observation as the result of direct protein binding from the subphase rather than lateral exchange in the monolayer.

Liposome study. Four types of liposome samples were prepared. The difference between the sample types was in the chemical composition: B-DPPE/DPPC = 1:3.4 and 1:1.7, and B-DMPE/DPPC = 1:4.0 and 1:2.0. Calorimetric studies showed that the melting points for the two mixtures of B-DPPE/DPPC were around 41 °C, which is approximately the same as for the pure DPPC liposome. However, in the case of the mixtures of B-DMPE/DPPC, the transition broadened dramatically to a temperature range of 30 °C ~ 40 °C. This was probably due to the shorter carbon-hydrogen chains of B-DMPE reducing the strength of interchain interaction. For the present binding studies, we were only interested in knowing the temperatures of the

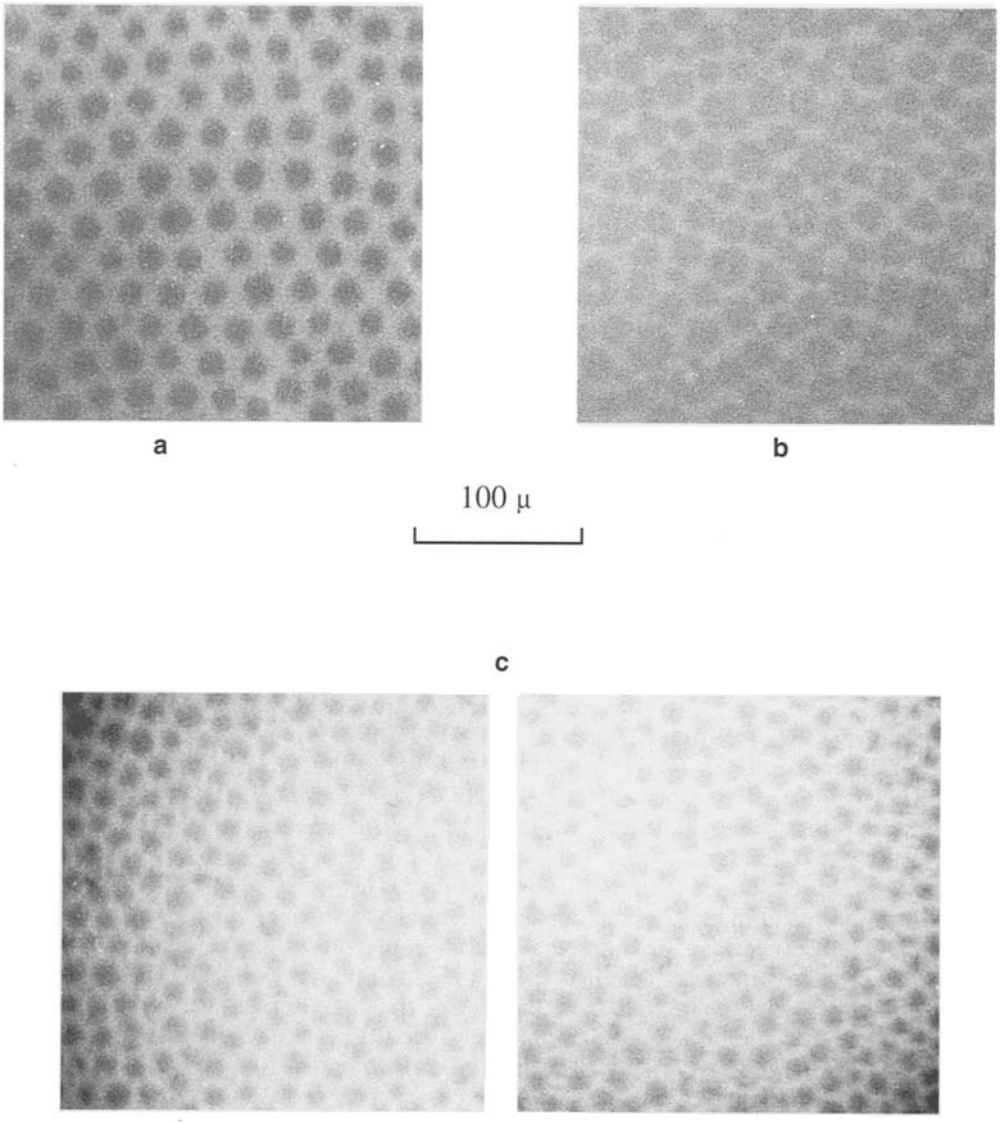

Fig. 7 a–c. Fluorescence micrographs of monolayers at the air/water interface were observed directly with a long working distance objective by focusing on the air/water interface at 25 °C. **a**, the observed monolayer was pure B-DPPE containing 2% RDB-DOPE (EX = 540 nm, EM = 610 nm). No FITC-avidin was added into subphase at this stage. The bright region of **a** indicated fluid dyed-DOPE-containing B-DPPE. In the case of **b**, the monolayer was 2% DOPE containing B-DPPE (without stained lipid), and in the subphase FITC-avidin was injected. Therefore, the brightness of the regions of **b** came from the receptor bound FITC-avidin (EX = 495 nm, EM = 535 nm). Two micrographs are shown in **c**. In both cases, a B-DPPE monolayer (containing 2% RDB-DOPE) at the air/water interface and avidin (labeled with FITC) in the subphase were stained with dye. Two images were taken from the same film and conditions. Exciting with green light the bright region (in red) of the image (on the left side) was observed. Under the same conditions, after irradiating with green light for several minutes to quench RDB, the bright region (in green) of the image (on the right side) was excited with blue light. The pattern on the left side of **c** thus displayed the liquid (in bright) and solid (in dark) lipid regions respectively. The picture on the right side of **c** distinctively exhibited the protein-rich region (in bright) and the pure lipid domains (in dark)

liposome in the gel or fluid state, and thus the phase behavior of the lipid mixtures did not need to be analyzed in detail. Since all four sample types of liposomes were in the gel state at 25 °C and in the liquid state at 55 °C, we studied the property of the specific binding of avidin to membrane bound model receptors at these two temperatures.

In order to obtain quantitative information on avidin binding to biotin containing liposomes, a specific approach using a fluorometric assay was established according to the method of Lin and Kirsch (1977). This was based on the quenching of the tryptophan fluorescence of avidin by biotin. Since the fluorescence quenching was only dependent on avidin/biotin specific binding, the influence of the nonspecific adsorption was absolutely removed. For each sample type, the avidin fluroescence intensity was measured as a function of the volume of the added liposome suspension. For example, the insert of Fig. 8 shows the decrease of the fluorescence intensity of avidin following titration with a biotin containing liposome, B-DPPE/DPPC (1:1.7) in the liquid state (55 °C) and in the gel state (25 °C). Figure 8 shows that more liposomes must be added at 25 °C than at 55 °C, in order for the four types of biotin containing liposomes to interact completely with the same amount of avidin solution. These results suggested that avidin interacted more weakly with biotin-lipid at 25 °C than at 55 °C. Therefore, at 25 °C, more biotin containing liposomes were needed to quench the same amount of avidin. In a parallel measurement study, no difference was detected for free biotin's interaction with avidin at these two temperatures.

Discussion

There are some significant differences between the molecules of avidin and streptavidin. Each of the four avidin subunits has a carbohydrate side chain that reportedly contains four to five residues of mannose and three residues of N-acetylglucosamine. This oligosaccharide chain on

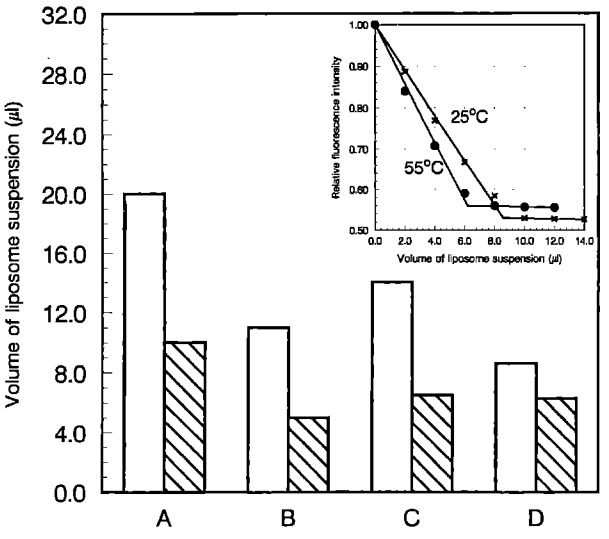

Fig. 8. Volumes of liposome suspension needed to interact completely with the same amount of avidin were determinated with a sensitive fluorometric assay. 100 μg avidin dissolved in 1.0 ml 0.2 M sodium phosphate buffer (pH 6.8) was titrated with liposome suspension. Fluorescence was measured at 350 nm with excitation at 290 nm using a Fluorescence Spectrophotometer 850. (*A*) liposome of a 1:4.0 B-DMPE/DPPC mixture; (*B*) 1:2.0 B-DMPE/DPPC mixture; (*C*) liposome of a 1:3.4 B-DPPE/DPPC mixture; and (*D*) 1:1.7 B-DPPE/DPPC mixture. The temperatures were at 25 °C (□) and 55 °C (▨) respectively. *Insert*: Fluorescence titratin curves with addition of biotin containing liposome suspension to quench the fluorescence intensity of avidin. Liposome: B-DPPE/DPPC, molar ratio 1:1.7; ×, measured at 25 °C; ●, at 55 °C

avidin is N-linked to asparagine residue 17, and exhibits a high degree of heterogeneity (Bruch and White 1982). Avidin is a positively charged protein (pI = 10.5), while streptavidin is a neutral protein. We compared the amine acid sequences of the subunits of avidin with those of streptavidin. This demonstrated that the avidin subunit contains more arginine and lysine residues than the streptavidin subunit (Argarana et al. 1986). We believe that this difference probably contributes to the different actions of avidin and streptavidin on charged membranes.

Ringsdorf and coworkers have reported their work on streptavidin (Blankenburg et al. 1989). They chemically synthesized many kinds of biotinyl-lipids with different lengths of the hydrophobic tails. A study on the interaction between streptavidin and various biotinyl-lipid monolayers at the air/water interface showed that a certain length of swinging arm was necessary for the streptavidin/biotinyl-lipid combination. Their results suggested that the binding site of biotin stretches into the protein molecule. This is one kind of steric hindrance, which may be designated using the name of lengthwise steric hindrance. Since the size of avidin is much larger than that of the biotin group, the lateral space in another requirement restricting protein binding. With the LB film technique the space between biotin groups could be precisely controlled by varying the molecular ratio of B-DPPE and DPPE. The results shown in Fig. 5 and Fig. 6 obviously show that this kind of steric hindrance exists and we call this lateral steric hindrance.

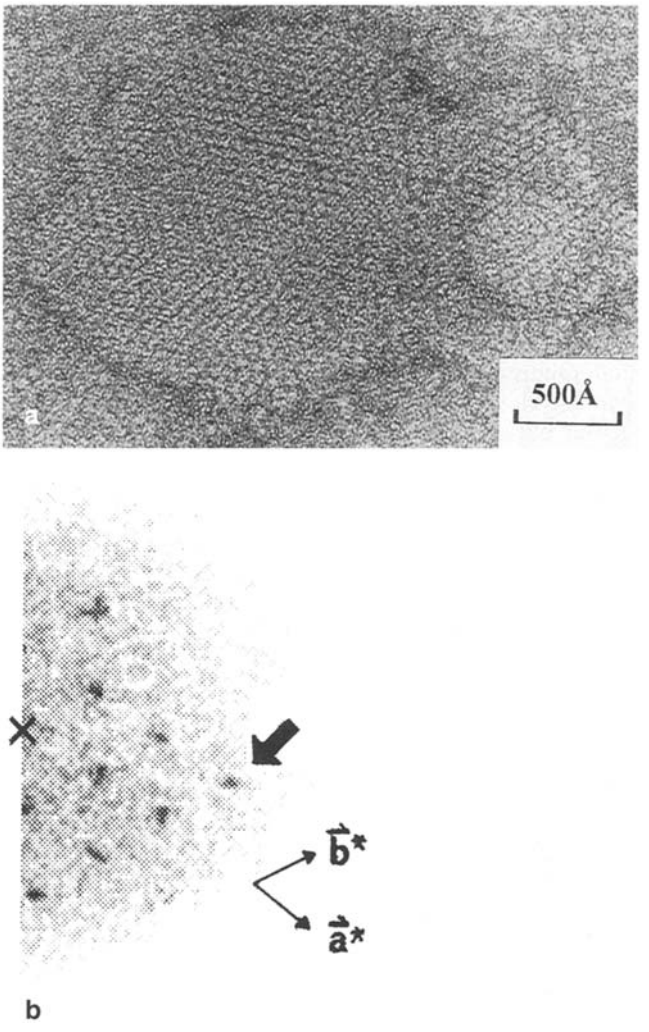

Fig. 9 a, b. Electron micrograph of ordered arrays of avidin on a lipid monolayer. Lipid solution, 1.2 μl of chloroform and methane (1:1 by volume) containing B-DPPE at 0.12 mg/ml and DOPC at 0.4 mg/ml, was applied to the surface of 18 μl of protein solution (avidin at 200 μg/ml in 50 mM Tris-HCl, pH 7.0, and 150 mM NaCl) in a teflon well (4 mm in diameter and 1 mm in depth). After incubation at room temperature or at 4 °C for at least 12 hours, lipid films were picked up on carbon coated grids which had been deposited for 5 minutes on top of the incubating solution, carbon side facing the lipid film. The grids were washed with one drop of distilled water and negatively stained with 1% uranyl acetate (UA), pH 4.0 for 30 to 60 s. **a** Electron micrograph of a negatively stained two-dimensional crystal of avidin on B-DPPE/DOPC monolayer. The accelerator voltage is 100 KV. **b** The computed diffraction pattern of the obtained avidin two-dimensional crystal showed that the crystal had an average unit cell parameters $a = 6.6 \pm 0.2$ nm, $b = 6.8 \pm 0.1$ nm, $\gamma = 121 \pm 4°$. The (2,1) reflection, at 1/2.7 nm is indicated by an arrow. The cross symbol is the position of the origin peak

Techniques for the two-dimensional crystallization of proteins have been developed (Kornberg and Darst 1991; Uzgiris and Kornberg 1983). We have obtained two-dimensional (2D) crystals of avidin on a lipid layer (shown in Fig. 9). Since the work on the two-dimensional crystallization of avidin is beyond the topic of the present article, we present only one electron micrograph for the purpose of interpreting our experimental results about lateral steric hindrance. Image analysis of electron micrographs

of the crystals reveals p2 symmetry with the unit cell parameters $a=6.6\pm0.2$ nm, $b=6.8\pm0.1$ nm, $\gamma=121\pm4°$. The effective area of the avidin molecule is calculated to be about 2000 $Å^2$. The cross-section area of one PE molecule in the rigid state is about 40 $Å^2$ (Silver 1985), and in the fluid state about 60 $Å^2$ (Fettiplace et al. 1971; Levine and Wilkins 1971; Haydon and Hladky 1972). Therefore, an estimated cross-section of 50 $Å^2$ of lipid molecules in the supported monolayer was used. Assuming there are two effective binding sites on one protein bound to a planar lipid monolayer, the optimum ratio of BPE/PE for the specific avidin/biotinyl-lipid binding was around 1:20. Under these ideal conditions, the biotin groups distributed themselves in a perfect matchable arrangement. The molecular package was an ordered formation and the total amount of the bound protein reached its highest value. While the proteins self-assembled into 2D crystals, the BPE molecules underwent a redistribution in the monolayer, requiring a certain mobility of lipid molecules. As to the solid supported monolayer, however, the lipid had little mobility, thus the lipid molecules could not make a perfect matchable arrangement. Therefore, the optimum mixing ratio of 1:12 (B-DPPE/DPPE) derived from our experiments was a little higher than the ideal ratio, which was understandable.

In addition to the lateral steric hindrance, the membrane fluidity is another important factor in increasing the ligand binding, which we concluded according to the evidence of the monolayer study and the liposome study (curved bilayer). In the liquid state of the lipid monolayer, the model receptors have more lateral and rotational mobility, and this promotes the molecular process of ligand binding. The dependence of receptor function on the state of the lipid bilayers was discussed by Shinitzky (1987), but our present study emphasized the effect of the physical state of the membrane on ligand-receptor binding.

In conclusion our results that the steric hindrance and the fluidity of membrane bound model receptors are the two factors influencing the molecular process of avidin binding. The effect of lateral steric hindrance was confirmed in the present research work. Concerning the nonspecific adsorption of avidin, our study showed that it is dominated by electrostatic forces and thus may be eliminated by a suitable amount of bivalent cations.

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