Atomic Force Microscopy Studies of Langmuir−Blodgett Films of Cytochrome P450scc: Hemeprotein Aggregation States and Interaction with Lipids

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Tapping mode atomic force microscopy was applied for a study of the molecular organization and aggregation states of purified bovine adrenocortical cytochrome P450scc present in thin solid films. The films of pure cytochrome P450scc or cytochrome P450scc mixed with lipids were transferred onto substrates of highly oriented pyrolytic graphite or mica. Images of the cytochrome P450scc films show that the topology of the proteins is drastically dependent on surface pressure. An effect of hemeprotein on the structural organization of lipid was observed. Images of the hemeprotein molecules and/or their aggregates provide a new insight for the further understanding of the nature of hemeprotein-hemeprotein and hemeprotein−lipid interactions as well as the membrane topology of cytochrome P450scc.

Introduction

Atomic force microscopy (AFM)—a rather new and fast developing high-resolution technique—is a powerful instrument for studies of surfaces at molecular and atomic resolution.[1] It has recently proved useful for imaging biological specimens.[2−10] The important advantage of this method, as compared to conventional techniques, is the ability to establish conditions close to physiologically relevant ones. During the past decade AFM has been widely applied to investigations of the spatial organization of biological macromolecules, biomembranes, lipids, or protein−lipid membranes. In addition to providing new information on molecular structure, the method permits an evaluation of the state of aggregation of protein molecules and the orientation of proteins in lipid structures. For example, the structure of cholera toxin oligomers,[7,11] pertussis toxine oligomers,[5] hydrated bacteria surface protein,[8] extracellular gap junction surface,[6] photosynthetic membranes,[3,4] bacteriorodopsin lattice,[2] bacteriorodopsin photoreaction unit,[10] heat shock protein oligomers,[12] gramicidin A aggregates,[13] etc., have been observed using AFM. Moreover, AFM is a unique tool for the characterization of proteins dispersed as Langmuir−Blodgett (LB) films. These techniques make it possible to visualize 2D and 3D structures of proteins or/and lipid thin solid films formed using LB technology.

In the present work we report on the application of the AFM technique for the study of bovine adrenocortical cytochrome P450scc distributed on LB films. Cytochrome P450scc (CYP11A1) is hemeprotein and a member of a large group of mixed function monooxygenase enzymes. It is an integral mitochondrial membrane-bound enzyme which catalyzes the first reaction of the steroidogenic pathway, i.e., the conversion of cholesterol to pregnenolone.[14] This reaction requires the input of six electrons that are transferred to the hemeprotein from NADPH via the flavoprotein, NADPH-adrenodoxin reductase, and the iron−sulfur protein [2Fe-2S], adrenodoxin.

Thin solid films of cytochrome P450scc can be deposited on solid supports by Langmuir−Schaefer techniques using either hydrophobic or hydrophilic adsorption. Moreover, the hemeprotein does not denature upon spreading on the water surface after being transferred from the interface film to the solid support, because it still exhibits specific electron-transfer activity.[15] The monolayer film consisting of cytochrome P450scc molecules exhibits a well-defined surface pressure dependence. This is reflected by a change of the molecular orientation of the proteins at the surface. It has been shown that monolayers of P450scc can be transferred from the air/water interface onto a solid substrate and covalently immobilized without damage to

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the hemeprotein structure. On the basis of these results a model of the molecular orientation of cytochrome P450scc molecules at the air−water interface has been proposed.[16]

The aim of the present work is to apply AFM as well as the Langmuir−Schaefer technique to the study of the aggregation states and molecular organization of bovine adrenocortical cytochrome P450scc present in thin solid films. This report extends our understanding of the nature of protein−protein interactions, protein−lipid interactions, as well as the possible topology of this unique hemeprotein on the mitochondrial membrane.

Materials and Methods

Abbreviations. Abbreviations used are as follows: P450scc, cytochrome P450scc, CYP11A; AFM, atomic force microscopy; LB film, Langmuir−Blodgett film; HOPG, highly oriented pyrolytic graphite; PC, phosphatidylcholine.

Preparation of P450scc. Cytochrome P450scc was purified from bovine adrenocortical mitochondria by affinity chromatography as described.[17] Mitochondria were isolated from adrenal cortex tissue according to Schnaitmann and Greenawalt.[18] To remove a significant portion of the outer membrane components, the mitochondria were subjected to a swell−shrink−sonicate procedure followed by centrifugation in a three-step discontinious sucrose gradient according to Hovius et al.[19] The mitochondrial lipid fraction was prepared by chloroform extraction of bovine adrenal mitochondrial membranes according to ref 20. Final concentration of the lipids in the extraction was determined as described by Vaskovsky et al.[21] Egg yolk phosphatidylcholine was purchased from the Sigma Chemical Co. All other reagents were used as received. Freshly cleaved mica (muscovite) or HOPG were used as the substrates.

Deposition of the Films. Monolayers of cytochrome P450scc were formed in a Langmuir trough (MM-MDT Inc., Russia) with dimensions of 550 × 200 × 7 mm and a volume of 95 mL. Protein monolayer formation as well as transfer of the monolayer to the substrate was performed from the surface of the 25 mM K-phosphate buffer, pH 7.4 solution. A 10 μM solution of cytochrome P450scc was spread on the subphase using a Hamilton rheodyne syringe. After equilibration for 5 min, the monolayers were compressed at a rate of 2 mm/s. When cytochrome P450scc/lipid monolayers were formed, PC or lipids extracted from mitochondrial membranes were first dissolved in chloroform and spread on the surface of the subphase. The concentration of the lipids in the chloroform solution before spreading on the air−liquid interface was 0.48 mg/mL. Usually not more than 50 μg of the lipids was used for obtaining "pure-lipid" LB films. To obtain "mixed" films containing both lipids and P450scc, the lipids (20 μg) were spread on the surface of the support and incubated for 15 min to allow the chloroform to completely evaporate. P450scc was then added (concentration of 0.03−0.06 μg/mL in 10 mM K-phosphate buffer) and deposited over a noncompressed lipid monolayer so that the initial lipid/protein ratio was 0.8/1.0 (w/w). After 15 min of incubation, the area was compressed and the LB films were deposited at various surface pressure values, however, never exceeding 35 mN/m. Transfer of the films from the subphase surface onto the substrates was performed by "touching" the support, in a parallel mode, to the subphase surface (analogous to the Langmuir−Schaefer method). After deposition of the film, the samples were dried under nitrogen gas, washed with 25 mM K-phosphate buffer, pH 7.4, and finally dried with a stream of nitrogen gas.

Atomic Force Microscopy Imaging. AFM experiments were carried out using an AFM Nanoscope (IIIa Digital Instruments, Santa Barbara, CA) in the tapping mode. This method proved to be the most useful for studies of such soft and flexible specimens as lipids and proteins. Commercially available cantilevers Nanoprobe for the tapping mode (Digital Instruments, Santa Barbara, CA) were used. The length of the cantilevers was 125 μm and the resonant frequency 308−340 kHz. The cantilever root mean square amplitude prior to scanning (A_0) was chosen experimentally in order to obtain higher image quality in the interval 50−150 nm. The setpoint (root mean square cantilever amplitude while scanning A_s) was chosen automatically by the AFM-operating program, the setpoint ratio A_s/A_0 being 0.91. We applied the user-friendly software FemtoScan 001 (Advanced Technologies Center, Moscow, Russia)[22] for image processing.

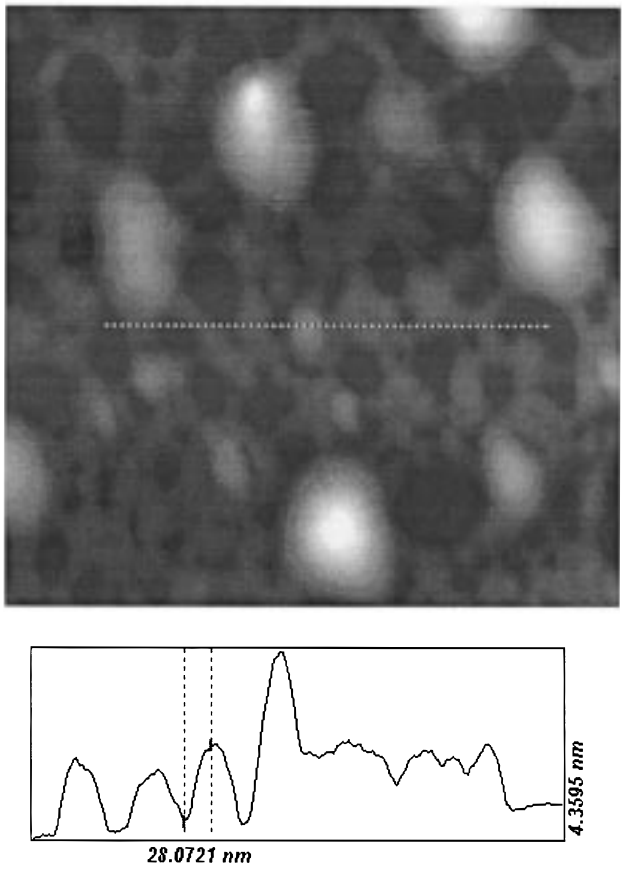

Figure 1. LB film of cytochrome P450scc on HOPG surface. Image size 680 × 680 nm^2. Surface pressure 10 mN/m. Cross section shows the film thickness.

Results

Films Prepared from Highly Purified Bovine Adrenocortical Cytochrome P450scc. In the first set of experiments we studied LB films containing only cytochrome P450scc mounted on HOPG and mica substrates. To optimize the procedure used for preparation of the films, we tested the deposition of films at various values of surface pressure. At a pressure of 10 mN/m the structure of the film resembled a network with variable thickness (Figure 1). Areas of substrates were distinguishable, so it was possible to measure the thickness of the film. The thickness of the network was determined to be 4.4 nm (see the legend of Figure 1). In addition we observed bubbles in the network of 40 nm diameter and 10 nm height. In some cases bigger bubbles of variable sizes were observed (some with a diameter as wide as 90 nm and as high as 15 nm). At a pressure of 25 mN/m the structure was quasiperiodical; i.e., the film was uniform

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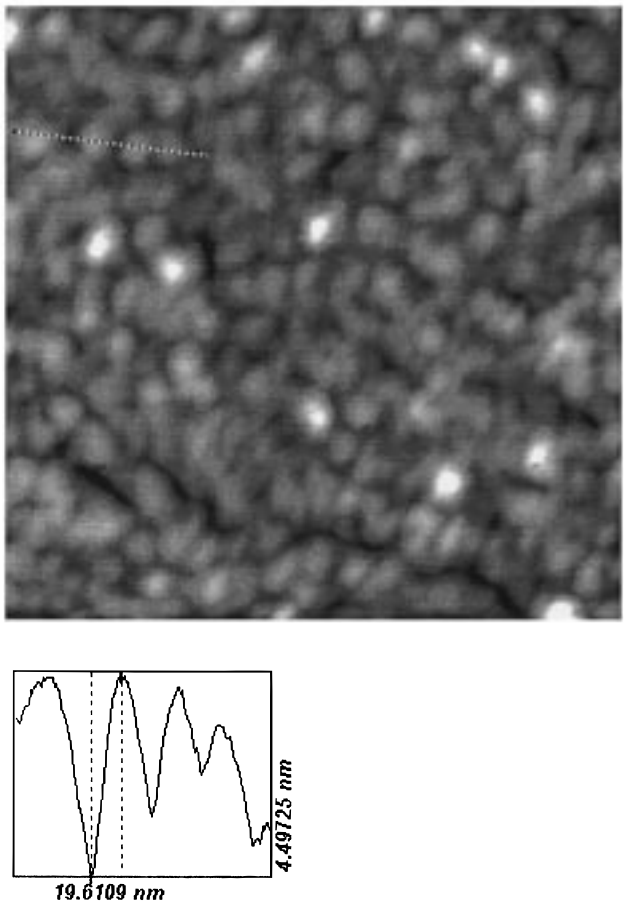

Figure 2. LB film of cytochrome P450scc on a mica surface. Image size 520 × 520 nm^2. Surface pressure 25 mN/m. Cross section gives the idea of corrugation amplitude and cluster size.

and practically covered all the substrates surface (Figure 2). In this case it was impossible to measure the thickness of the film. LB films of the reaction centers from *Rhodobacter sphaeroides* were previously studied by scanning tunneling microscopy, and their periodic structure was determined.[23] One can speculate that more order should have been observed when the films of P450scc were observed at higher pressure. But at a pressure of 35 mN/m the films looked almost the same as those obtained at a pressure of 25 mN/m (data not shown).

Films Prepared with Cytochrome P450scc Using PC. In this set of experiments we studied LB films of cytochrome P450scc with PC deposited on HOPG and mica. The samples were prepared as described above. The surface pressure was 25 mN/m. As a control experiment, we used LB films prepared with pure PC, i.e., without adding the protein (see Figure 3). In this case the film is not continuous and forms islands. In the picture shown in Figure 3 one can see that the film consists of domains composed of parallel bands. Similar structures were previously observed in films prepared from lipids and other surfactants.[24−26] This is the ripple phase of the lipid mono- and bilayer.[24] The thickness of each region of the film is constant and lies in the region between 1.2 and 3.2 nm. This indicates that each band represents a horizontal cylinder or semicylinder.[26] The boundry between regions

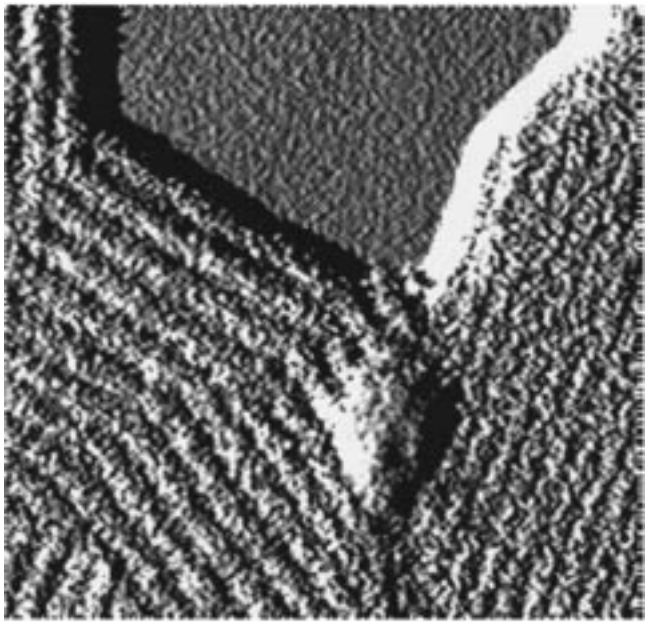

Figure 3. LB film of PC on HOPG surface. Image size 120 × 120 nm^2. Lighting is applied.

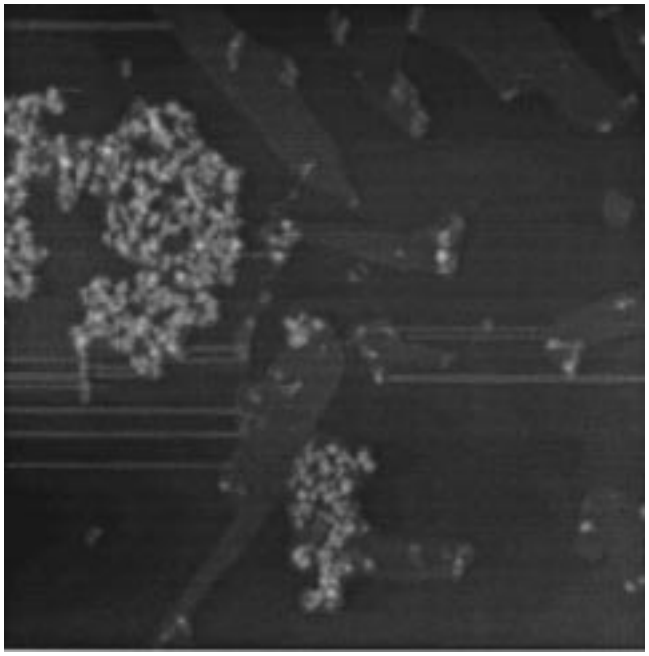

Figure 4. LB film of cytochrome P450scc with PC on HOPG surface. Image size 2000 × 2000 nm^2.

of the parallel bands is vague, and no identifiable separations are observed. The width of the bands is approximately constant within a single domain but varies from one domain to another in the range of 4.8−7.5 nm. The corrugation height is 0.2−0.5 nm. Small protrusions are observed on the film, and we believe these are due to the presence of a contamination which influences the structure of the film. The protrusions are less than 1 nm high and rare. They are easily distinguished from protein molecules (see below). In Figure 3 we applied lighting (FemtoScan 001 software) in order to show bands more distinctly.

The typical appearance of the film of cytochrome P450scc with PC is shown in Figure 4. The distribution of the protein in the film is not uniform. Regions of the film that do not contain protein are in the ripple phase, and their thickness varies from 1.5 to 3 nm. It is worth noting that

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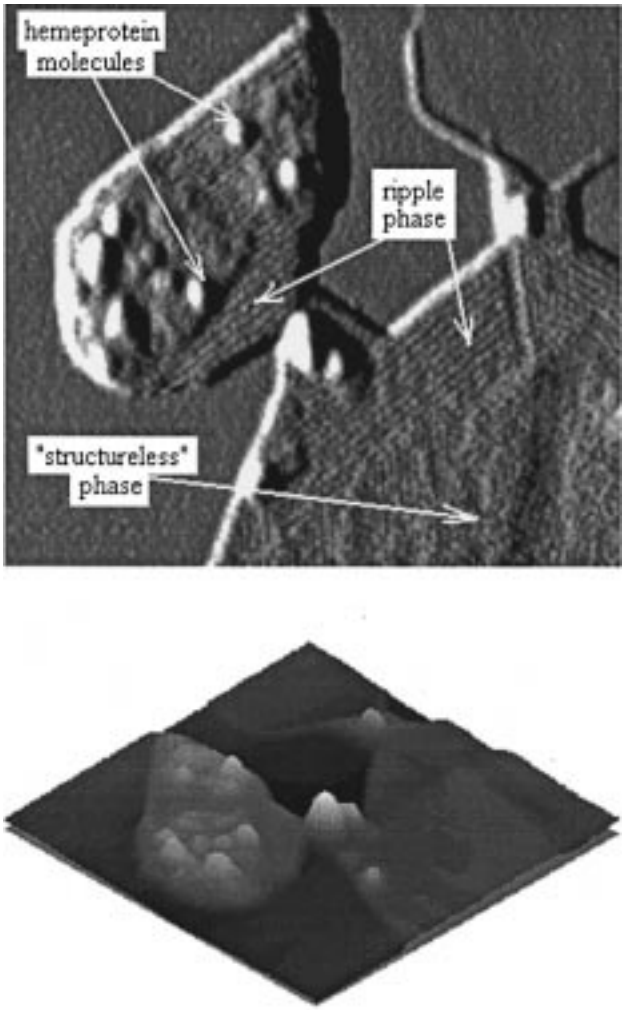

Figure 5. LB film of cytochrome P450scc with PC on HOPG surface. Image size 460 × 460 nm^2. Lighting is applied. 3D view is given below.

films prepared using pure PC, and also those prepared with cytochrome P450scc, all demonstrate angles of the film bound and directions between bands as a multiple of $\pi/3$. Since the lattice of HOPG is a hexagon, this observation confirms the role of a lipid/substrate interaction in the formation of ripple structures as proposed in ref 25.

Figure 5 gives a more detailed view of this film. Here we can see that the ripple phase is not the only one present. Another phase, whose structure is not resolved, coexists. The thickness of the regions of this "structureless" phase is from 2 to 5 nm. This "structureless phase" probably represents bi- or multilayers of lipid. In these "structureless" regions we observed numerous protrusions that are 4−5 nm higher than the surface of the film. Such protrusions are absent in control experiments, so they may be related to protein molecules. It is important to note that such protrusions were never observed in the regions composed of bands. The lateral size of the observed molecules, as measured at half-height of the extending part, is 15 to 25 nm.

For comparison we deposited the same film onto mica. In this case the film proved to be flat, and neither ripple structures nor incorporated protein molecules were observed (see Figure 6). The film thickness was 1.5 nm, which corresponds to the thickness of a PC monolayer. The origin of the big bubbles seen in Figure 6 is not yet clear.

Films Prepared Using P450scc with Mitochondrial Lipids. In the next set of experiments we substituted

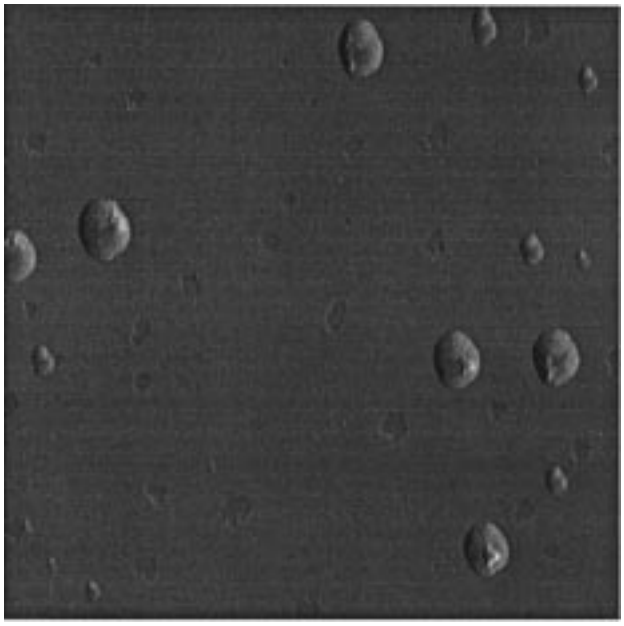

Figure 6. LB film of cytochrome P450scc with PC on the mica surface: film thickness, 1.5 nm; image size, 700 × 700 nm^2.

PC with lipids extracted from mitochondria prepared from bovine adrenal cortex (mitochondria lipids). It is known that the membrane fraction of bovine adrenal mitochondria contains cholesterol, PC, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, and cardiolipin as the main lipid components.[27] Thus, LB films prepared using lipids extracted from such mitochondrial membranes may be more representative of the natural environment of P450scc. As in the case where we examined LB films prepared using PC with P450scc, we carried out a series of control experiments where we studied LB films prepared using only mitochondrial lipids (without the protein). Again, we observed band structures similar to those seen with LB films containing only PC, i.e., a ripple phase of lipid layer (Figure 7). In this case the film thickness was 1−3 nm, the bandwidth varied in the same interval between 4.5 and 7.5 nm, and a corrugation height of 0.2−0.5 nm was observed. The main difference between LB films prepared using mitochondrial lipids and PC was in the shape of the bound film: it is a curve and not a broken line (compare Figures 3 and 7). In this case the thickness of the LB film prepared using mitochondrial lipids was not constant even within the domains where the band direction was the same. It varied from 1.2 to 3 nm.

Figure 8 illustrates the coexistence of the two phases, ripple and "sructureless", in the unresolved structure segment of the film prepared using P450scc and mitochondrial lipids. Similar to the case described above, molecules of P450scc get incorporated only into the "structureless" phase. This means that molecules of P450scc do not get incorporated into the ripple phase of LB films prepared using mitochondrial lipids. The height and diameter measured at half-height of the protrusions calculated from the "structureless" phase surface are in the range of 2.5−5 nm and 20−60 nm, respectively. As expected, higher protrusions have larger diameters.

An important aspect of these studies is the process of self-organization in the lipid/protein film as seen at the subphase/air interface in the LB trough. We deposited the film onto the substrates after 15, 30, and 60 min of incubation with the protein (Figure 9). Qualitative dif-

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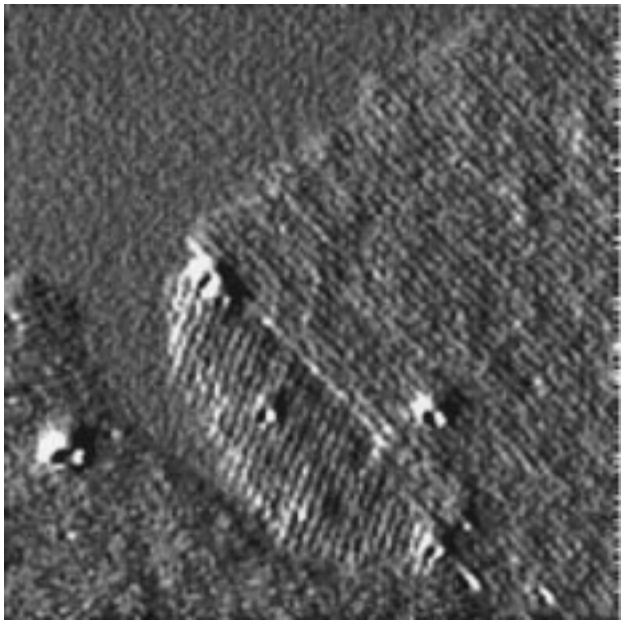

Figure 7. LB film using lipids extracted from adrenal cortex mitochondria on the HOPG surface. Image size, 310 × 310 nm^2. Lighting is applied.

ferences in the appearance of the film were evident at each time stage. However, it is not easy to interpret these pictures. In Figure 9 a one can see hollows—this is the surface of HOPG which means that this film contains holes. There are some flat regions, which are very similar to our "structureless" lipid phase but look a little bit thicker. Close to the holes are balls which look like aggregated protein. Figure 9a shows a double-tip image; i.e., this is the sum of two equivalent slightly shifted images. The edge of the holes looks doubled, and the string in the middle looks duplicated, too. Despite this fact the structure remains clear. The value of the shift is negligible compared to the diameter of holes the size of big bubbles. The thickness of the film is not changed either. In Figure 9b one can see granular structures, which we propose correspond to P450scc molecules as hemeprotein oligomers. After 60 min of hemeprotein incorporation into the lipid monolayer these particles were also observed (Figure 9c), but their dimensions are considerably in excess of the particles seen after 30 min of incubation. Possibly, these observations indicate a higher extent of time-dependent protein oligomerization.

Discussion

In the present work we used AFM to study the molecular organization of purified bovine adrenocortical cytochrome P450scc placed on thin solid LB films using HOPG or mica as substrates. The application of AFM makes it possible to visualize the molecular size of objects when dispersed on smooth solid surfaces and to control the influence of processes which take place at the air/water interface during the formation of thin films.

We obtained AFM images in air. Such an approach simplifies the experimental procedure and what is even more important makes investigated soft biological objects more stable which sometimes allows for better resolution. Probably, the drying might alter the structure of a protein molecule but does not affect the aggregation and distribution of hemeprotein molecules in the film. When the monolayer is transferred onto the solid substrate, molecules are fixed and their distribution is not changed. In addition, it is known[28] that at air humidity 45% and higher the surface of a phospholipid (DPPC or DPPE) film with charged heads oriented to the air is covered by the layer of water. Samples were imaged in air in ambient conditions, relative humidity being 60−70%. Thus, we suppose that in our case the phospholipid film and hemeprotein molecules remained hydrated.

Using this approach we confirmed recently reported data[16] describing immunochemical studies of P450scc present in thin solid LB films. In this study[16] it was shown that an increase of surface pressure results in a more tight packing of hemeprotein molecules in the film (compare Figures 1 and 2). At a comparatively low surface

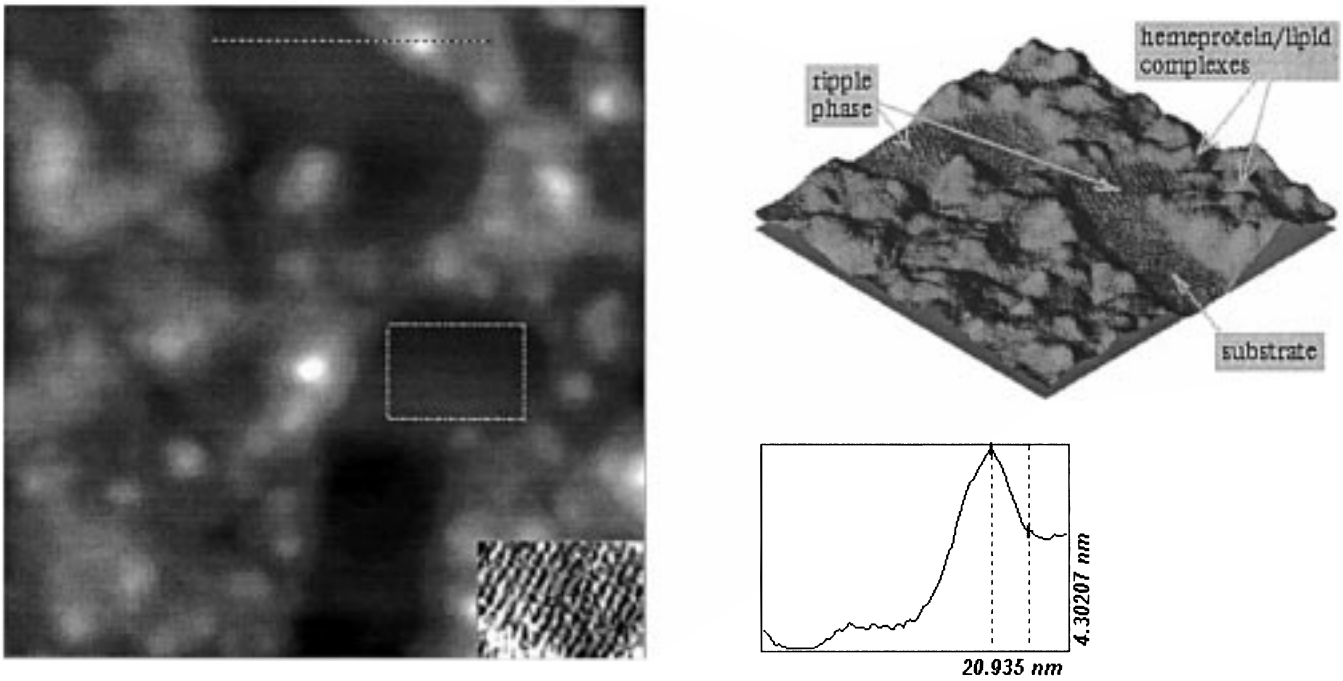

Figure 8. LB film of cytochrome P450scc with mitochondria lipids on the HOPG surface. Image size 330 × 370 nm^2. 3D view (lighting is applied) and cross section are given on the right-hand side.

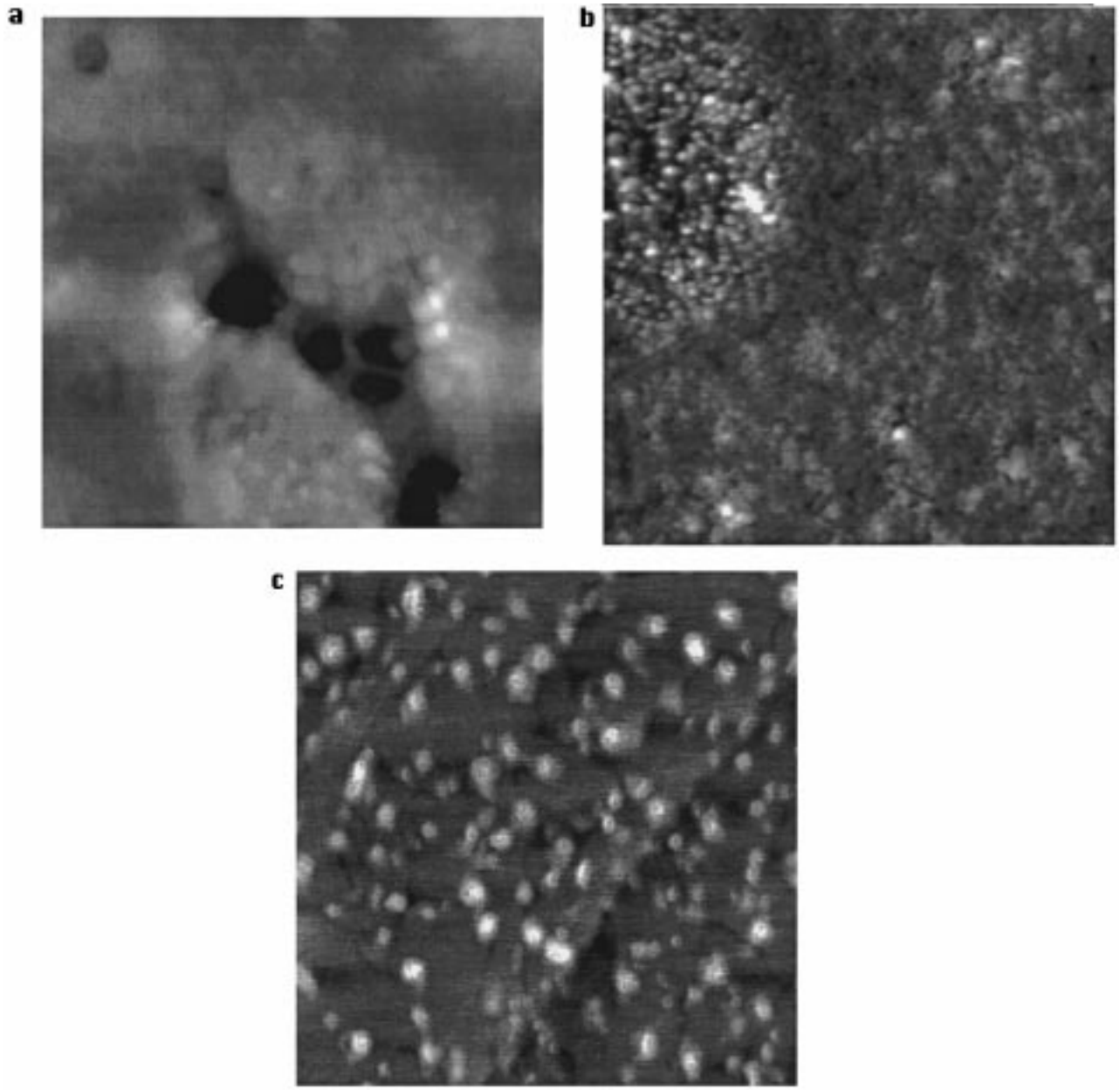

Figure 9. LB film of cytochrome P450scc with mitochondria lipids on the HOPG surface deposited after different time of incubation with hemeprotein: a, 15 min; b, 30 min; c, 60 min. Image size 1.2 × 1.2 mm^2.

pressure (10 mN/m) the surface density of the hemeprotein is not high and the film thickness is irregular with holes in the film structure. At a surface pressure of 25 mN/m molecules form a rather dense monolayer with a quasi-periodical structure. The structural similarity of the films deposited at 25 and 35 mN/m can be explained if we accept the fact that the P450scc molecule is not a ball, it is asymmetric. During the compression of the monolayer, the orientation of the hemeprotein molecules is altered[16] resulting in no observable difference in packing of the films deposited at 25 and 35 mN/m.

One purpose in using AFM for the study of thin LB films containing P450scc was to investigate hemeprotein−lipid and hemeprotein−hemeprotein interactions. Earlier studies using limited proteolysis indicated that this hemeprotein (P450scc) is localized on the matrix side of the inner mitochondrial membrane.[29] Moreover, it was shown that P450scc has a transmembrane organization. Although the intrinsic molecular organization of P450scc in the bovine adrenal mitochondrial membrane remains unknown, highly purified P450scc exists as a monomer in the presence of 1 M sodium chloride and 0.3% sodium cholate. However, P450scc does form oligomers in the absence of detergent and sodium chloride. With gel filtration and chemical cross-linking methods, it was shown that P450scc is present as an octamer of the heterologous type with a molecular weight of about 400 000.[30]

The present study allows us to conclude that HOPG is a more appropriate substrate for hemeprotein−lipid film deposition than mica. The structure of protein−lipid films on the HOPG is variable in composition since the distribution of the hemeprotein in the films is not

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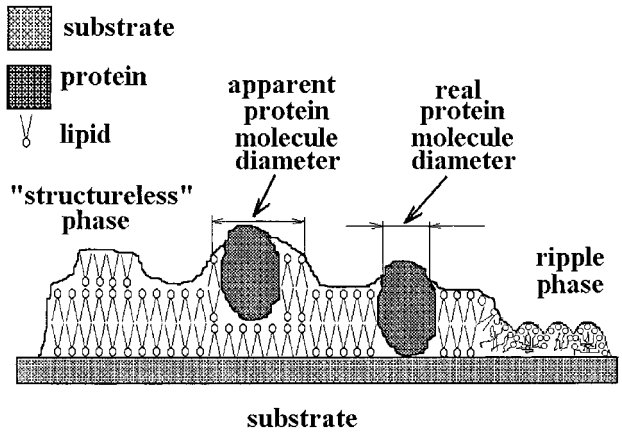

Figure 10. Proposed simplified models of the membrane topology explaining the difference in the apparent dimensions of the cytochrome P450scc molecules.

homogeneous. Regions of the film that do not contain protein are in the "ripple phase". The image of P450scc in the LB film, as shown in Figure 5, indicates that the ripple phase is not the only one present—there is another phase in the structure which is not completely resolved and we call this phase the "structureless" phase. The thickness of the regions corresponding to this "structureless" phase is about 2−5 nm suggesting the presence of bi- or multilayers. In the "structureless" regions of the film we observed numerous protrusions which are 4−5 nm higher than the film surface. Such protrusions are absent in control experiments. On the basis of these results we suggest that these protrusions are related to P450scc molecules in the film. It is important to note that such protrusions are never observed in the regions composed of bands. The estimated lateral size of the P450scc molecules measured at the half-height of the protruding structures is about 15−25 nm. Recently,[31] we obtained images of cytochrome P4502B4 monomers, and the diameter of these monomer hemeprotein moleculaes proved to be about 4−5 nm. This result indicates that the diameter of protrusions formed with P450scc are four to five times larger than that seen with P450 2B4, confirming the conclusion that P450scc is present as aggregates in the thin solid LB films studied here.

To further explain the observed differences in the size of the protrusions, we propose two noncontradicting models. According to the first model the bigger protrusion may represent more than one unresolved hemeprotein molecule. The second model proposes that the bigger protrusions may represent a P450scc molecule surrounded by the lipids which then self-organize to form a protein−lipid complex. To illustrate these different hypotheses we show in Figure 10 a schematic drawing of the two possible models as they might appear in the film. These proposed models are simplified presentations. In reality, more complicated constructions possibly occur, including several monolayers, steps, various orientation of lipid molecule's tails and heads, etc. Again, we named the phase "structureless" because its structure is not resolved. The real orientation of the lipid molecules is not known. The fact that the P450scc did not get incorporated into lipid films in the ripple phase seems to us to be natural since the transmembranous P450scc molecule needs both sheets of the membrane in order to get incorporated. P450scc molecules incorporated in the LB film are observed surrounded by the "structureless" phase.

All these results lead us to conclude that the formation of the "structureless" phase formation is due to P450scc present at the subphase/air interface in the Langmuir trough. Thus, cytochrome P450scc molecules play the role of self-organization centers in the film by adsorbing and orienting lipid molecules.

On the basis of the results obtained here we estimate the size of the P450scc molecule, in the direction perpendicular to the membrane plane, as 5 nm. At present there are no exact data on the dimensions of a cytochrome P450scc molecule. The cytochrome P450s for which 3D sructures are known from X-ray crystallographic studies each have a triangular prism shape about 6.5 nm on each side and 3.5 nm thick.[32] However, the dimensions of the cytochrome P450 molecules associated with membranes remain less clear and molecules may exist according to our proposed membrane topology model.

It is known that in AFM imaging lateral dimensions are generally always overestimated due to a broadening effect created by the probe.[33] Since one is working in an air environment, this effect is almost inevitable, even in the gentle tapping mode. In the present studies the observed diameter measured at the half-height of the smallest protrusions is about 14 nm. This figure is probably exaggerated due to the effect of tip broadening. In the approximation model[33] of the structure of P450scc we estimate the real diameter of a molecule to be 3−5 nm. The images obtained using P450scc molecules absorbed onto the substrates and a solution where lipids are absent revealed that the sizes of the particles lie in a very wide range of 25−60 nm. The only possible explanation for this difference in size is to propose that P450scc exists in the buffer solution in a dynamic equilibrium between monomer and oligomer states resulting in various degrees of oligomerization.

Conclusions

In this study, we have shown that atomic force microscopy can be successfully applied for the investigation of cytochrome P450-containing LB films. The structure of the thin film containing the hemeprotein depends on surface pressure and the type of substrates used. These AFM measurements revealed different hemeprotein deposition morphologies for LB films of pure P450scc and for hemeprotein−lipid films. Cytochrome P450scc molecules appear to play a role as self-organization centers in the LB film by adsorbing and orienting lipid molecules. The estimation of size of molecules from the images of hemeprotein and hemeprotein−lipid films permit us to hypothesize the existence of protein oligomers both in the subphase buffer solution and in thin solid films.

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