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CYTOCHROME P450scc SPIN STATE TRANSITIONS IN THE THIN SOLID FILMS

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Summary

Langmuir-Blodgett films of cytochrome P450scc were prepared on the solid supports and their spectral properties were investigated. Being immobilized, hemoprotein changes its spin state from initially high to low spin. This transition is reversible since after the solubilization of hemoprotein, the spin state equilibrium is shifted towards high-spin state. Anaerobic reduction of film incorporated cytochrome P450scc by electron transfer chain (NADPH→adrenodoxin reductase→adrenodoxin) revealed the low rate of the reaction that coincides well with the content of the hemoprotein low-spin form. We suggest that particularly regular orientation of solid cytochrome P450scc are of crucial importance for this phenomenon.

Key words: Cytochrome P450scc (CYP11A1); Langmuir-Blodgett films; Spin-state equilibrium.

1. Introduction

In mitochondria of adrenal cortex, cytochrome P450scc (P450scc; CYP11A1) catalyses the key steroidogenic reaction of cholesterol side chain cleavage to form pregnenolone. The enzyme is active in the presence of two electron transfer proteins, NADPH-adrenodoxin reductase (ADR) and adrenodoxin (AD). Recently the first attempt was undertaken to prepare Langmuir-Blodgett (LB) films of P450scc [1]. At present, Langmuir films of proteins are of a particular interest as an approach to get reproducible, oriented, and well-controlled layers of immobilized macromolecules with well defined properties. In spite of the first positive results in preparation of P450scc LB films, we, however, still have no so much knowledge about structural and functional properties of P450scc in the thin solid films.

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It has been known that physico-chemical and enzymatic properties of cytochrome P450s connected with their spin state. For instance, the rate of P450scc reduction in high-spin state is much faster than in low-spin state [2]. Both high-spin (S=5/2) as well as low-spin (S=1/2) states are two distinct configurations of five 3d electrons in the heme iron. Low to high spin conversion results in heme iron liberation from sixth ligand and it displacement out of heme plane [3]. Spectrally low to high and high to low spin conversions of cytochrome P450 are well determined as changes in position of Soret band which is located at 393 nm (for high-spin state) or at 416 nm (for low-spin state). Among other cytochrome P450 species, P450scc is a unique subject for the investigation of spin states. Spin-state equilibrium of this hemoprotein is influenced by the temperature [4, 5, 6], pH [7, 8] and depends on binding with substrates [9] and AD [10].

Here we present results of our investigations of LB films containing P450scc. We have found that P450scc, which was in a high-spin form before the deposition, in the LB film exists only in a low-spin form. It can be inferred that in monolayers, self organised at the air-water interface, hemoprotein molecules are kept under the control of strong intermolecular protein-protein interactions. The strength of these interactions is quite sufficient to fix definite conformational and spin states in P450scc molecule. The structure of the organised P450scc layers is too rigid that neither cholesterol nor AD are capable to influence on the hemoprotein spin state. However, this phenomenon is reversible since after solubilization of films, P450scc changes its spin state again to the high spin. Moreover, we studied electron transfer properties of P450scc in LB films and found their dependence on the low-spin state of the hemoprotein.

2. Materials and methods

2.1. Chemicals and proteins. Sodium cholate, cholesterol, D-glucose, sodium dithionite, NADPH, methyl viologen, Tween 20, dimethyl-dichlorosilane were from Sigma (USA). Glucose oxidase from *Aspergillus niger* and catalase from bovine liver were from Serva (Germany). P450scc, ADR and AD were purified from beef adrenal cortex according to the procedures described earlier [11, 12].

2.2 Deposition of cytochrome P450scc thin films.

2.2.1 Deposition of Langmuir-Blodgett films. Monolayers of P450scc were formed in a Langmuir trough (MM-MDT Corp., Russia) [13] with a surface area 240 cm^2 at surface pressure 25 $mN \cdot m^{-1}$. As the subphase, a 5 mM sodium phosphate buffer, pH 7.4, was used. Solution of 80 μM P450scc in Na-phosphate buffer was spread over the subphase. The transfer of LB films of P450scc from the subphase surface on the supports was performed by touching the support in parallel to the subphase surface (the Langmuir-Schaefer method) [14]. Quartz plates 0.6 x 3.5 cm were used as supports. For hydrophobization, plates were treated with dimethyl-dichlorosilane. After the film had been deposited, the samples were dried in a nitrogen flux. This procedure was repeated 20-50 times for multilayer deposition. Being prepared, films were washed in 50 mM Na-phosphate buffer, pH 7.4.

2.2.2 Deposition of films with random orientation of cytochrome P450scc molecules. Thin films with random orientation of hemoprotein molecules on the surface of support were deposited as follows: 0.1 ml of 80 μM solution of P450scc in 50 mM Na-phosphate buffer, pH 7.4, was distributed uniformly on the surface of hydrophobized quartz plate. Preparation was placed near the anhydrous silica gel. The drying procedure was continued overnight at 4°C.

2.3 Solubilization of cytochrome P450scc LB films. For the solubilization of P450scc LB films, four plates with 45 layers of hemoprotein were prepared on hydrophobic supports. Solubilization procedure was done in 2 ml of the solution of 0.3 % sodium cholate, 1 M NaCl, 50 mM Na-phosphate, pH 7.4, during 1 h with the slow constant stirring.

2.4 Analytical methods. Quartz plates with LB films of P450scc were analysed spectrophotometrically in 50 mM Na-phosphate buffer, pH 7.4, in the cells with 1 cm optical path length. Thin films with random orientation of P450scc molecules were analysed under dry conditions.

Cytochromes P450 and P420 content was determined from CO-difference spectra of dithionite-reduced preparations using an extinction coefficients of 91 $mM^{-1}cm^{-1}$ [15] and 110 $mM^{-1}cm^{-1}$ [16], respectively. In some cases, methyl viologen (2 μM) was added to promote the reduction of low-spin form of P450scc. No spectral disturbance by methyl viologen was observed.

Spin-state equilibrium of P450scc was characterised by the index $(A_{393}-A_{470})/(A_{416}-A_{470})$ [17]. Tween 20-induced low-spin P450scc was used as the reference.

NADPH-dependent P450scc reduction in LB film was measured under anaerobic conditions as follows: the reaction mixture contained 4 μM AD, 2 μM ADR, 40 mM glucose, 0.1 mg/ml glucose oxidase, 0.1 mg/ml catalase, and 300 μM NADPH. The solution was bubbled with CO and the reaction was started by immersion of the plate with the P450scc LB film into the reaction mixture.

All experiments were carried out at room temperature. Absorbance spectra were recorded using a Jasco 7800 UV/VIS spectrophotometer.

3. Results

In accordance with the conventional purification procedure, isolated P450scc still contains tightly bound molecules of endogenous cholesterol [18]. At the neutral pH and room temperature about 70 % of the P450scc is in a high-spin state [19]. Typical absorbance spectrum of such kind of preparation which we used in our experiments is shown in Fig. 1.

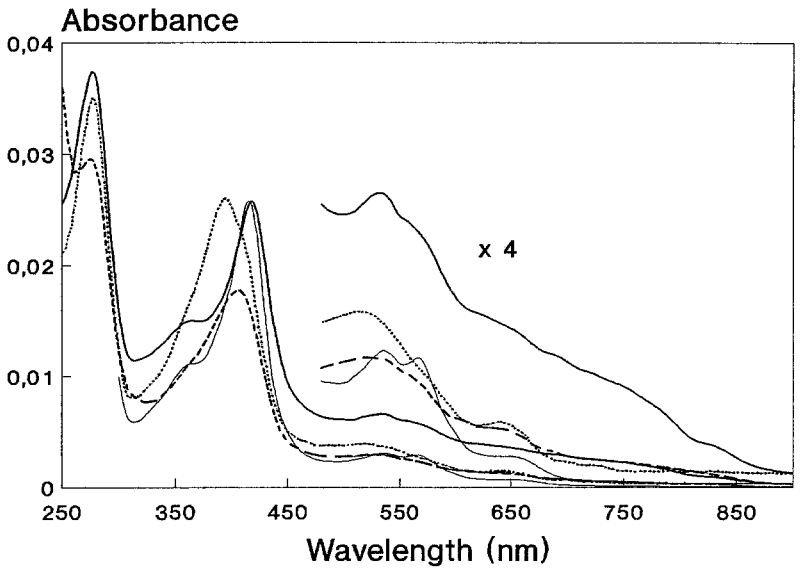

Fig. 1. Absorption spectra of cytochrome P450scc: (···) initial high-spin hemoprotein in 50 mM Na-phosphate buffer; (—) 50 layers of cytochrome P450scc on hydrophobized quartz plate; (---) hemoprotein in 50 mM Na-phosphate, 0.3 % sodium cholate and 1 M NaCl after solubilization from LB films; (—) Tween 20-induced low-spin cytochrome P450scc.

This spectrum has maxima at 393, 526 and 645 nm that are characteristic for oxidised substrate-bound high-spin P450scc. After deposition of P450scc LB films both on quartz and hydrophobic quartz, absorbance spectra of hemoprotein were changed. They had peaks at 416, 535, 570 nm and a shoulder around 360 nm (Fig. 1). There was no more peak at 645 nm. This spectrum is typical for low-spin substrate-free hemoprotein. Moreover, cholesterol when added at 50 μM final concentration and 4 μM AD did not cause any visible changes in the position of the Soret band of the hemoprotein.

We tried to desorb the hemoprotein from the supports to understand the influence of LB films preparation procedure on the stability of P450scc: is this low-spin form of cytochrome P450 or this is a mixture of cytochrome P450 and its inactive form, cytochrome P420? LB films of the hemoprotein stick rather strong to the supports. As follows from the absorption spectra of LB films, after 1 hour solubilization treatment in 0.3 % of sodium

cholate and 1 M NaCl, only approximately 25 % of immobilized protein was eluted from the films. The Soret band of the solubilized P450scc has the position at 406 nm that is intermediate for low- and high-spin states (Fig. 1). Indexes of spin-state equilibrium of initial, LB film deposited and solubilized preparations of P450scc are summarised in Table 1. P450scc in LB films has spin-state equilibrium index equal to that of low-spin Tween 20-induced preparation and after the solubilization from the films spin-state equilibrium index is intermediate between low- and high-spin states of hemoprotein.

After deposition-solubilization treatment, cytochrome P450 content was measured by reduced CO-difference spectrum (Fig. 2). Cytochrome P450 composed about 93 % of the whole hemoprotein content and was almost equal to cytochrome P450 content (96 %) in the initial preparation. Hence, P450scc is rather stable during the experiment, although reduced CO-difference spectrum in LB films indicates the presence of both cytochromes P450 and P420 (Fig. 2).

As a functional test for P450scc LB films activity, we studied the reduction of the CO-bound hemoprotein by the electron transport chain NADPH→ADR→AD→P450scc. However, we failed to detect the reduction of P450scc in aerobic conditions. The reduction was only seen in the presence of glucose oxidase-catalase deoxygenative system (Fig. 3). Consequently, the rate of P450scc reduction is comparatively slow because in aerobic conditions soluble molecular oxygen is able to oxidize hemoprotein with the rate which

Table 1. Indexes of spin-state equilibrium of P450scc.

Preparation	$(A_{393}-A_{470})/(A_{416}-A_{470})$
P450scc initial	1.4 ± 0.05
P450scc in LB film	0.55 ± 0.01
P450scc after desorbtion from LB films	1.03 ± 0.01
P450scc low-spin (Tween 20-induced form)	0.56 ± 0.01

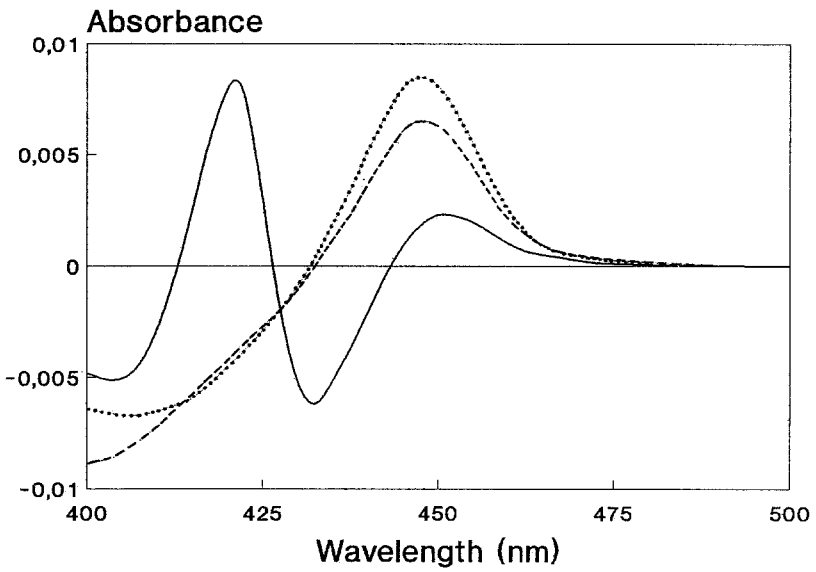

Fig. 2. CO-difference spectra of the dithionite-reduced preparations of cytochrome P450scc: (···) hemoprotein before the deposition into LB films; (—) 50 layers of cytochrome P450scc on hydrophobized quartz plate; (---) hemoprotein in 50 mM Na-phosphate, 0.3 % sodium cholate and 1 M NaCl after solubilization from LB films.

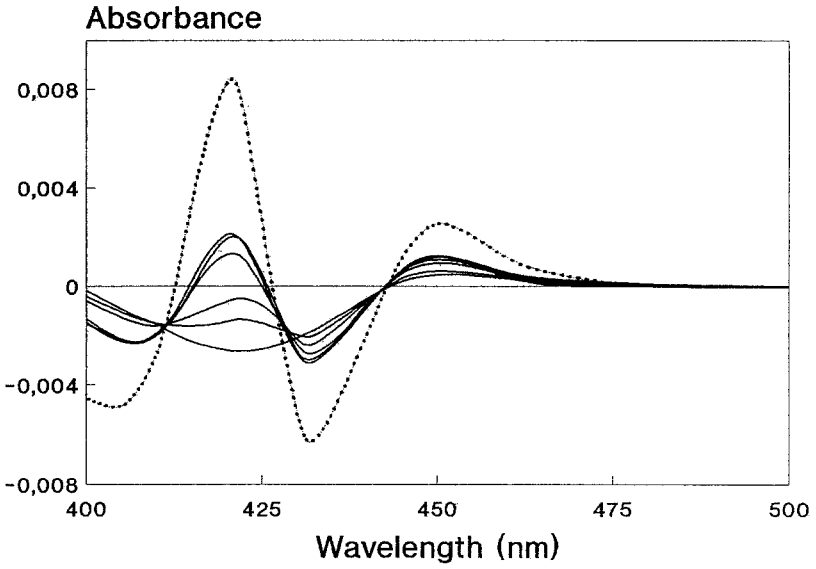

Fig. 3. Kinetics of the anaerobic reduction of CO-bound cytochrome P450scc in the LB film by the dissolved electron transport chain NADPH→ADR→AD (—) and by the sodium dithionite (···).

exceeds the rate of P450scc reduction. These results indicate also that electrons can be donated to LB film incorporated P450scc from the soluble AD and that there is no any limitations in mutual orientation of reacting molecules.

Floating of proteins at the air-water interface and surface tension are able to influence on the conformation of protein molecules and their functions [14]. To check the influence of these factors on spin-state equilibrium of P450scc, we prepared solid films with random orientation of the hemoprotein on the support. As in the case of P450scc LB films, the spectrum of the hemoprotein after evaporation procedure differs from that of the initial preparation in the solution (Fig. 4). It has two overlapped peaks at 393 nm and at 416 nm indicating that spin-state equilibrium was shifted to the low-spin state. Film with random orientation of hemoprotein was weakly sticked to the supports. Immediately after immersion into the 50 mM Na-phosphate buffer it was solved rapidly and completely.

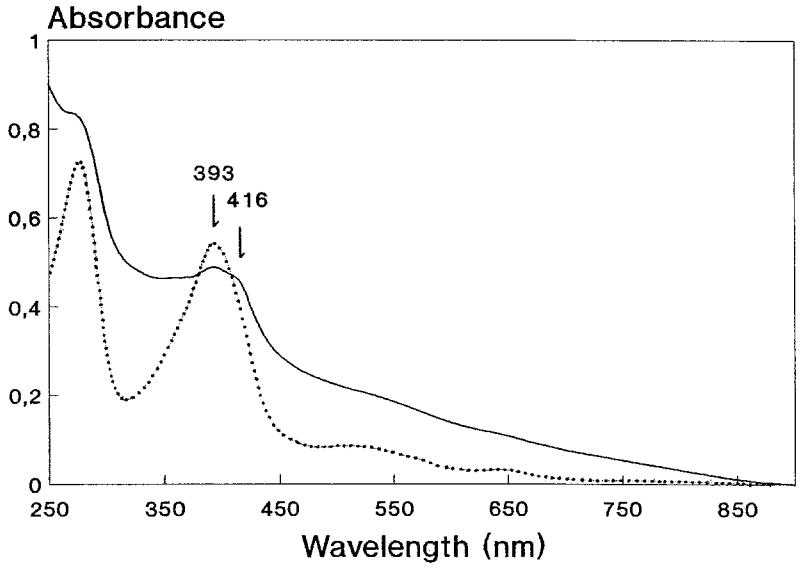

Fig. 4. Absorption spectra of cytochrome P450scc: (—) the film with random orientation of hemoprotein molecules on the surface of quartz support; (···) hemoprotein in 50 mM Na-phosphate after solubilization from the film with random orientation of hemoprotein molecules.

Moreover, solubilized P450scc preparation restored the high-spin state (Fig. 4). Deposition-solubilization treatment did not affect the stability of P450scc and CO-difference spectrum of the hemoprotein after the solving procedure was equal to that of the initial preparation (data not shown).

To check the influence of surface tension on spin-state equilibrium of P450scc we prepared also LB films either at 15 $mN \cdot m^{-1}$ and at 40 $mN \cdot m^{-1}$. The absorption spectra of these films were identical to the spectrum of the LB film deposited at 25 $mN \cdot m^{-1}$ (data not shown).

4. Discussion

There are at least two factors, temperature and pH, which affect reversibly on the P450scc spin-state equilibrium and do not cause the removal of cholesterol from the substrate-binding centre of the enzyme. P450scc spin state shifts from high to low spin when the temperature is increased [5, 6]. Change in pH from 6.5 to 8.0 leads to the high to low spin transition [20]. As in the case of P450scc LB films, substrate addition at high temperature does not convert the enzyme back to the high-spin state. However, a temperature decrease reverses the spectral changes completely and a change in pH from 8.0 to 6.5 increases the high-spin content, although no substrate is added [19]. Conformational transitions of P450scc molecules upon temperature and pH treatment were reported [19]. Our results indicate that during thin films preparation, P450scc molecules can undergo changes of their conformation too. In the process of organisation in a monolayer at the air-water interface they appear to form new intermolecular bonds. Our data also indicate that protein-protein interactions are much more stronger in ordered layers than in the films with the random orientation of hemoprotein molecules. Conformational changes cause a movement of cholesterol molecule or at least a movement of its side-chain group in the vicinity of heme group [21]. As the result of this movement, heme iron changes its spin-state from high to low. It is possible that this process is influenced by the lack of water molecules

on the protein surface and really high to low spin transition is affected by the combination of two factors: protein-protein interactions in particular mutual orientation and partial dehydration of protein globule.

In addition to the question of spin transitions of P450scc in the films, there is a question of pH-maintenance at the air-water interface. Carbon dioxide from the air may be dissolved in the upper layers of subphase resulting in decrease the pH. As it was stressed above, the pH lowering results in increase in the content of hemoprotein high-spin form. Nevertheless, after deposition in the LB film, P450scc exists only in a low-spin form, that indicates the absence of dramatic changes in pH of solution during preparation of LB film.

The most striking effects of P450scc low-spin conversion is drastic decrease in its redox potential and its ability to interact with AD [22, 23]. Therefore electron transfer from reduced AD to low-spin oxidised P450scc is less favourable. One can see that our observation concerning low-spin state of P450scc in LB films is in line with its low reduction rate in the native electron transfer chain. It should be pointed out that at the air-water interface, AD-binding site of the hemoprotein is turned to the water phase. Nevertheless, due to its size and charge, AD interacts mainly with P450scc from upper layer (or layers) of LB film and does not penetrate into the inner sublayers.

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