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Electron Transfer Properties and Catalytic Competence of Cytochrome b_5 in the Fusion Protein Hmwb_5–EGFP in Reactions Catalyzed by Cytochrome P450 3A4

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Abstract—In the present paper we describe studies on molecular mechanisms of protein–protein interactions between cytochrome P450 3A4 (CYP3A4) and cytochrome b_5, the latter being incorporated into the artificial recombinant protein Hmwb_5–EGFP containing full-length cytochrome b_5 (functional module) and a mutant form of the green fluorescent protein EGFP (signal module) fused into a single polypeptide chain. It is shown that cytochrome b_5 within the fusion protein Hmwb_5–EGFP can be reduced by NADPH-cytochrome P450 reductase in the presence of NADPH, the rate of reduction being dependent on solution ionic strength, indicating that the signal module does not prevent the interaction of the flavo- and hemeproteins. Interaction of cytochrome P450 3A4 and Hmwb_5–EGFP was estimated based on spin equilibrium shift of cytochrome P450 3A4 to high-spin state in the presence of Hmwb_5–EGFP, as well as based on steady-state fluorescence anisotropy of the EGFP component of the fusion protein in the presence of CYP3A4. The engineering of chimeric protein Hmwb_5–EGFP gives an independent method to determine dissociation constant for the complex of cytochrome P450 and cytochrome b_5 that is less sensitive to environmental factors compared to spectrophotometric titration used before. Reconstitution of catalytic activity of cytochrome P450 3A4 in the reaction of testosterone 6β-hydroxylation in the presence of Hmwb_5–EGFP indicates that cytochrome b_5 in the fusion protein is able to stimulate the hydroxylation reaction. Using other fusion proteins containing either cytochrome b_5 or its hydrophilic domain to reconstitute catalytic activity of cytochrome P450 3A4 showed that the hydrophobic domain of cytochrome b_5 participates not only in hemeprotein interaction, but also in electron transfer from cytochrome b_5 to cytochrome P450.

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The role of cytochrome b_5, a small membrane-bound hemeprotein localized in endoplasmic reticulum membranes primarily in liver, in reactions catalyzed by cytochrome P450 is still unclear. There are at least four hypotheses explaining different mechanisms of the modulating effect of cytochrome b_5 on cytochrome P450 catalyzed reactions [1]:

– direct electron transfer from cytochrome b_5 to cytochrome P450, which is thought to be more effective then electron transfer from NADPH-cytochrome P450 reductase [1];

– decreasing of uncoupling of cytochrome P450-catalyzed reactions in the presence of cytochrome b_5 due to conformational changes in cytochrome P450 and consequent more rapid second electron transfer, as compared to NADPH-cytochrome P450 reductase, to the oxy-complex of substrate bound cytochrome P450 [1];

– formation of a binary complex of cytochrome b_5 and cytochrome P450 able to accept two electrons on interaction with NADPH-cytochrome P450 reductase, with subsequent sequential electron transfer from cytochrome b_5 to the oxy-complex of substrate bound cytochrome P450 [1];

– an effector (allosteric) role of cytochrome b_5, excluding its direct involvement in oxidation–reduction processes and electron transfer, appears to be due to changes in oxidation–reduction potential of cytochrome P450 that facilitates electron transfer from NADPH-cytochrome P450 reductase [2].

Cytochrome b_5 plays an important role in balancing the synthesis of sex hormones and glucocorticoids by regulating the activity of cytochrome P450c17. Cytochrome

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b_5 stimulates 17,20-lyase activity of cytochrome P450c17, being without effect on 17α-hydroxylase activity [3]. It is thought that uncoupling in regulation of the two activities of cytochrome P450c17 is realized using two different mechanisms: phosphorylation of a serine residue in cytochrome P450c17; changing the ratio of cytochrome P450c17 and NADPH-cytochrome P450 reductase in the presence of cytochrome b_5 [4].

It is difficult to explain the fact of activation by eukaryotic cytochrome b_5 of reactions catalyzed by prokaryotic cytochromes P450 since prokaryotes do not contain cytochrome b_5. Thus, cytochrome b_5 forms a specific complex with cytochrome P450cam from *Pseudomonas putida* that hydroxylates camphor [5]. It was recently shown that a soluble form of cytochrome b_5 from *Musca domestica* interacts with flavocytochrome $P450_{BM3}$ from *Bacillus subtilis*, demonstrating high affinity to cytochrome $P450_{BM3}$ and causing a small inhibitory effect on reactions of fatty acid hydroxylation catalyzed by this flavocytochrome [6]. The interaction of eucaryotic cytochrome b_5 with prokaryotic cytochrome P450 indicates the conservative structure of cytochrome P450 and confirms the important role of cytochrome b_5 functioning of cytochrome P450-dependent monooxygenase systems.

Until recently it was unclear if the effect of cytochrome b_5 *in vitro* has functional significance in the organism. Interesting results have been obtained in experiments on knockout of the cytochrome b_5 gene and in studies of pharmacokinetics of some drugs in liver of rats *in vivo* [7]. It was found that rats lacking the cytochrome b_5 gene demonstrate different pharmacokinetics for some chemicals metabolized by cytochrome P450. Direct participation of cytochrome b_5 in biosynthesis of sex hormones *in vivo* with participation of cytochrome P450c17 is confirmed by the fact that patients with inherited defects in the gene coding cytochrome b_5 suffer serious hormonal disturbance [8, 9]. These facts indicate that the effect of cytochrome b_5 on cytochrome P450-dependent reactions is not only found *in vitro* but also has serious functional significance *in vivo*.

In the present work, to study the role of cytochrome b_5 in reaction catalyzed by cytochrome P450 3A4, we used an earlier constructed fusion protein of cytochrome b_5 and green fluorescence protein [10]. This chimeric protein is able to shift spin equilibrium of cytochrome P450 3A4 and respond to inter-protein interactions by changing steady state fluorescence anisotropy. Reconstitution of testosterone 6β-hydroxylase activity of cytochrome P450 3A4 in the presence of NADPH-cytochrome P450 reductase and fusion protein Hmwb_5–EGFP, as well as other fusion proteins containing cytochrome b_5, shows the important role of the hydrophobic domain of cytochrome b_5 in stimulation of cytochrome P450 activity and direct participation of cytochrome b_5 in electron transfer.

MATERIALS AND METHODS

Instruments. In the present work we used a Specord M40 spectrophotometer (Carl Zeiss, Germany), CM2203 spectrofluorimeter (Solar, Belarus), HPLC 1090 LC (Hewlett Packard, USA) with Waters Bondapak C18 column (3.9 × 300 mm, particle size 15-20 μm), and LCQ Fleet mass spectrometric detector (Thermo Fisher Scientific, USA).

Preparation of recombinant proteins. Recombinant fusion protein Hmwb_5–EGFP was constructed and purified as previously described [10]. Recombinant rat liver cytochrome b_5 was expressed in *Escherichia coli* and purified using procedures described earlier [11]. Construction of a fusion protein consisting of hydrophilic domains of cytochrome b_5 and NADH-cytochrome b_5 reductase is described in the work [12]. Proteolytically stable NADPH-cytochrome P450 reductase was prepared as previously described [13]. Fusion proteins of cytochrome b_5 and NADPH-cytochrome P450 reductase were constructed and purified as described earlier [14]. Recombinant cytochrome P450 3A4 was purified according to the procedure described in [15] with minor modification using CHAPS as detergent for solubilization of membranes.

The concentration of oxidized cytochrome b_5 was determined using molar extinction coefficient ε_{413} = 117 $mM^{-1}\cdot cm^{-1}$ or from the reduced-minus-oxidized difference spectrum using molar extinction coefficient $\varepsilon_{424\text{-}409}$ = 185 $mM^{-1}\cdot cm^{-1}$ [16]. Concentration of cytochrome P450 3A4 was determined from difference spectra of carbonyl complex of sodium dithionite-reduced hemeprotein using molar extinction coefficient $\varepsilon_{450\text{-}490}$ = 91 $mM^{-1}\cdot cm^{-1}$ [17]. Concentration of NADPH-cytochrome P450 reductase was determined from its absorption spectrum using molar extinction coefficient ε_{456} = 21.4 $mM^{-1}\cdot cm^{-1}$ [18]. The purity of protein preparations was confirmed by SDS-PAGE [19].

Spectrophotometric titration of recombinant CYP3A4 with fusion protein Hmwb_5–EGFP. Spectrophotometric titration was done in tandem cells containing 0.5 μM solution of CYP3A4 in the experimental compartment and buffer solution in the reference compartment. The proteins were titrated in 50 mM Tris-HCl buffer containing 0.025% CHAPS, 150 μg/ml lecithin, and the necessary concentration of NaCl. After addition of 2 μl of 50 μM solution of Hmwb_5–EGFP into sample and reference compartments of the control cell, careful mixing, and incubation for 5 min, the difference spectra were recorded. The procedure was repeated until further addition of fusion protein did not result in spectral changes. From the spectra the fraction of the initial content of CYP3A4 complexed was calculated using the following equation:

$$f = \frac{\Delta A_{390-420}}{\Delta_{max} A_{390-420}}, \quad (1)$$

where f is fraction of complexed Hmwb_5–EGFP–CYP3A4, $\Delta A_{390\text{-}420}$ is difference in absorbance at wavelengths 390 and 420 nm, and $\Delta_{max}A_{390\text{-}420}$ is maximal difference in absorbance at wavelengths 390 and 420 nm.

Measurement of steady-state fluorescence anisotropy. Steady-state fluorescence anisotropy of EGFP in the Hmwb_5–EGFP fusion protein was calculated from fluorescence spectra (λ_{ex} = 480 nm, λ_{em} = 490-550 nm) obtained at four different positions of polarizers of excitation and emission using the equation:

$$r = \frac{I_{II} - kI_{\perp}}{I_{II} + 2kI_{\perp}}, \quad (2)$$

where r is fluorescence anisotropy, I_{II} is intensity of parallel polarized component of fluorescence under vertical polarization of exciting light, $I_{\perp}$ is intensity of perpendicular polarized component of fluorescence under vertical polarization of exciting light, and k is gauge factor.

All values were measured at 20°C.

Titration of chimeric protein Hmwb_5–EGFP by cytochrome P450 3A4 with registration by fluorescence anisotropy. When using fluorescence anisotropy, 0.5 μM solution of Hmwb_5–EGFP fusion protein was placed into the cell, and fluorescence emission spectra (λ_{ex} = 480 nm) were recorded at four different positions of polarizer of excitation and emission. Fluorescence anisotropy was calculated using Eq. (2). All values were measured at least 5 times. Then the necessary volume of 50 μM solution of CYP3A4 was added, and the fluorescence anisotropy was again measured. This manipulation was repeated until further addition of fusion protein did not result in spectral changes. From these measurements the amount of protein complex relative to initial content of Hmwb_5–EGFP was calculated using the following equation:

$$f = \frac{\Delta r}{\Delta_{max} r}, \quad (3)$$

where f is amount of Hmwb_5–EGFP–CYP3A4 complex, Δr is change in anisotropy of fluorescence of Hmwb_5–EGFP at a particular concentration of CYP3A4, and $\Delta_{max}r$ is maximal change of fluorescence anisotropy.

Determination of dissociation constants for complex between cytochrome P450 3A4 and Hmwb_5–EGFP. The dissociation constant of complex of cytochrome P450 3A4 and Hmwb_5–EGFP was determined by approximation of experimental results to the equation:

$$f = \frac{c}{K_d + c}, \quad (4)$$

where f is amount of Hmwb_5–EGFP–CYP3A4 complex, K_d is dissociation constant, and c is the concentration of Hmwb_5–EGFP used for spectrophotometric titration or CYP3A4 used for titration with registration of anisotropy.

Enzymatic reduction of cytochrome b_5 in Hmwb_5–EGFP. To measure enzymatic reduction of cytochrome b_5 in Hmwb_5–EGFP by NADPH-cytochrome P450 reductase, to 50 mM Tris-HCl buffer (pH 7.4, with specified concentration of sodium chloride) the necessary amount of protein was added, the sample was carefully mixed and incubated at the studied temperature for 15 min, and the sample was placed in the constant-temperature cell compartment. The necessary amount of freshly prepared 10 mM solution of NADPH in water was added, the mixture was immediately mixed, and changes of optical density at 424 nm were registered (ε_{red}-ε_{ox} = 123,000 $M^{-1}\cdot cm^{-1}$).

Reconstitution of hydroxylase activity of CYP3A4 *in vitro*. To a mixture of 15 μl lecithin vesicles (20 mg/ml) and 15 μl of CHAPS solution, definite amounts of highly purified CYP3A4, NADPH-cytochrome P450 reductase, and cytochrome b_5 (or chimeric proteins containing cytochrome b_5) were added. The final mixture was kept in a thermostat at 37°C for 10 min, and then to this mixture 50 mM Tris-HCl buffer (pH 7.4 at 37°C), containing 10 mM $MgCl_2$, 100 μM testosterone, and NADPH-regenerating system (sodium isocitrate and NADPH-isocitrate dehydrogenase) were added. The final mixture contained 50 mM Tris-HCl (pH 7.4 at 37°C), 10 mM $MgCl_2$, 100 μM testosterone, 0.5 μM cytochrome P450 3A4, 1 μM NADPH-cytochrome P450 reductase, 8 mM sodium isocitrate, and 0.1 IU/ml of NADPH-isocitrate dehydrogenase.

The concentration of cytochrome b_5 or its content in fusion proteins in different experiments consisted of 0.1, 0.25, 0.5, 0.75, and 1 μM.

The incubation mixture was kept in a thermostat for 5 min with vigorous mixing, and 0.5 ml was taken as starting point (zero point) and 1 mM NADPH (final concentration) was added. After 2, 5, and 10 min of incubation, samples of 0.5 ml were withdrawn and immediately mixed with 5 ml of dichloromethane, the mixture was vigorously mixed and centrifuged to separate phases, and the aqueous phase was discarded. The dichloromethane layer was evaporated under an argon stream, the dry residue was dissolved in 100 μl of methanol, and the products were analyzed by HPLC and mass spectrometry by applying 10 μl of methanol solution into a Waters Bondapak C18 reverse-phase column (3.9 × 300 mm, particle size 15-20 μm). The column was eluted at rate 1.2 ml/min at 30°C with methanol–water (60 : 40 v/v) mobile phase for 25 min. Mass spectra were obtained using the LCQ Fleet mass detector with APCI-interface ion source calibrated for positive ionization at vaporizer temperature of 350°C. The sample was analyzed in scanning mode in the range m/z 85-500. Quantitative calculations were performed using the area of chromatographic peaks.

Calculation of dipole moments and protein charges. Dipole moments of cytochrome b_5 and cytochrome P450 3A4 were calculated using the DS Viewer Pro pro-

gram and web-server Protein Dipole Moment Server (PDMS, http://bip.weizmann.ac.il/dipol) [20] using coordinates for cytochrome b_5 and CYP3A4 from PDB (pdb code 1AW3 and 1TQN, respectively). Protein charge at pH 7.0 was calculated using the Vector NTI 10.0.1 program [21].

Docking of proteins. Docking of proteins was performed using the GRAMM-X web-server http://vakser.bioinformatics.ku.edu/resources/gramm/grammx [22]. For docking, coordinates for three-dimensional structure of the hydrophilic domain of cytochrome b_5 (1AW3), hydrophilic domain of NADPH-cytochrome P450 reductase (1AMO), and cytochrome P450 3A4 (1TQN), were used.

Dissociation constants for Hmwb_5–EGFP–CYP3A4 complex determined by two independent methods at different solution ionic strength

Ionic strength, mM	K_d, M	
	spin state change	fluorescence anisotropy of EGFP
0	0.3 ± 0.03	0.4 ± 0.05
100	1.2 ± 0.02	1.3 ± 0.03
200	3 ± 0.3	2.9 ± 0.04

RESULTS AND DISCUSSION

The main procedure for determining the dissociation constant characterizing the interaction between cytochrome b_5 and cytochrome P450 until now has been spectrophotometric detection of spin equilibrium changes in cytochrome P450 in favor of high-spin state (type I spectral changes) induced by cytochrome b_5 during complex formation. Since spin equilibrium is subject to the effects of factors such as temperature, pH, and the presence of substrate or its analog, this method does not insure correct results. Besides, in some cases the interaction with cytochrome b_5 does not result in appearance of spectral changes, as in the case of interaction of cytochrome b_5 with cytochrome P450cam [23].

Fusion protein Hmwb_5–EGFP constructed by us earlier has the ability to induce type I spectral changes upon addition to cytochrome P450 3A4 (Fig. 1), indicating formation of a highly specific complex of the two hemeproteins. The dissociation constant for Hmwb_5–EGFP calculated based on spectrophotometric experiments (table) was very similar to that of for cytochrome b_5 found in the literature [24]. The absence of lecithin (or other phospholipids) in the solution results in anomalous spectral response (Fig. 1a), reflecting cytochrome P450 denaturation that is confirmed by registration of the carbonyl complex of sodium dithionite-reduced cytochrome P450 3A4 (transition of P450 to P420). Interaction of detergent-solubilized cytochromes P450 and b_5 results in denaturation of the former. The reason for this is not clear, although description of such phenomenon is known in literature for cytochrome b_5. This fact confirms the necessity for the hemeprotein to be in a phospholipid environment, like in a membrane, to realize its functional properties.

The interaction of CYP3A4 with fusion protein Hmwb_5–EGFP is confirmed not only by type I spectral changes (titration curve in Fig. 2a), but also changes in steady-state fluorescence anisotropy of Hmwb_5–EGFP (titration curve in Fig. 2b). The formation of a relatively tight complex results in change in time of rotational correlation of the fluorophore of EGFP and allows calculation of the portion of molecules of Hmwb_5–EGFP bound with CYP3A4. It is necessary to stress that the character of the titration in the two cases are different. In first case, the concentration of CYP3A4 is constant while the concentration of Hmwb_5–EGFP is changing, and the interaction is assessed based on changing cytochrome P450 spin state, while in the second case vice versa, the interaction is assessed based on changes in fluorescence of the reporter module of Hmwb_5–EGFP. The two methods of investigation of interaction of Hmwb_5–EGFP with CYP3A4 are independent, since for spectrophotometric detection the measurement is carried out for changes in spin state of CYP3A4, while during registration of fluorescence anisotropy the state of Hmwb_5–EGFP is determined. Dissociation constant values for the CYP3A4–Hmwb_5–EGFP complex determined by the two independent methods at different ionic strengths are presented in the table. As can be seen, the two methods give similar results under similar conditions. The importance of electrostatic interactions for complex formation is followed from the dependence of dissociation constant on ionic strength. Increase in ionic strength is followed by increase in dissociation constant. It is, however, unknown how the two proteins interact with each other, since information on localization of cytochrome P450 in the membrane is absent. It is thought that cytochrome P450 is partially imbedded into the membrane, but it is still not possible to localize and purify this hydrophobic domain as in the case of cytochrome b_5. As follows from the three-dimensional structure of CYP3A4, the heme is localized in the center of the molecule, close to the protein center of mass. The CYP3A4 molecule has significant dipole moment — 585 D (Fig. 3). The hydrophilic domain of cytochrome b_5 also has a large dipole moment (403 D); it forms an acute angle in the direction with the iron atom of heme of 133°. The charge of molecules at physiological pH (3.4 for CYP3A4, −10.68 for cytochrome b_5, and −15.88 for Hmwb_5–EGFP) [21] as well as significant val-

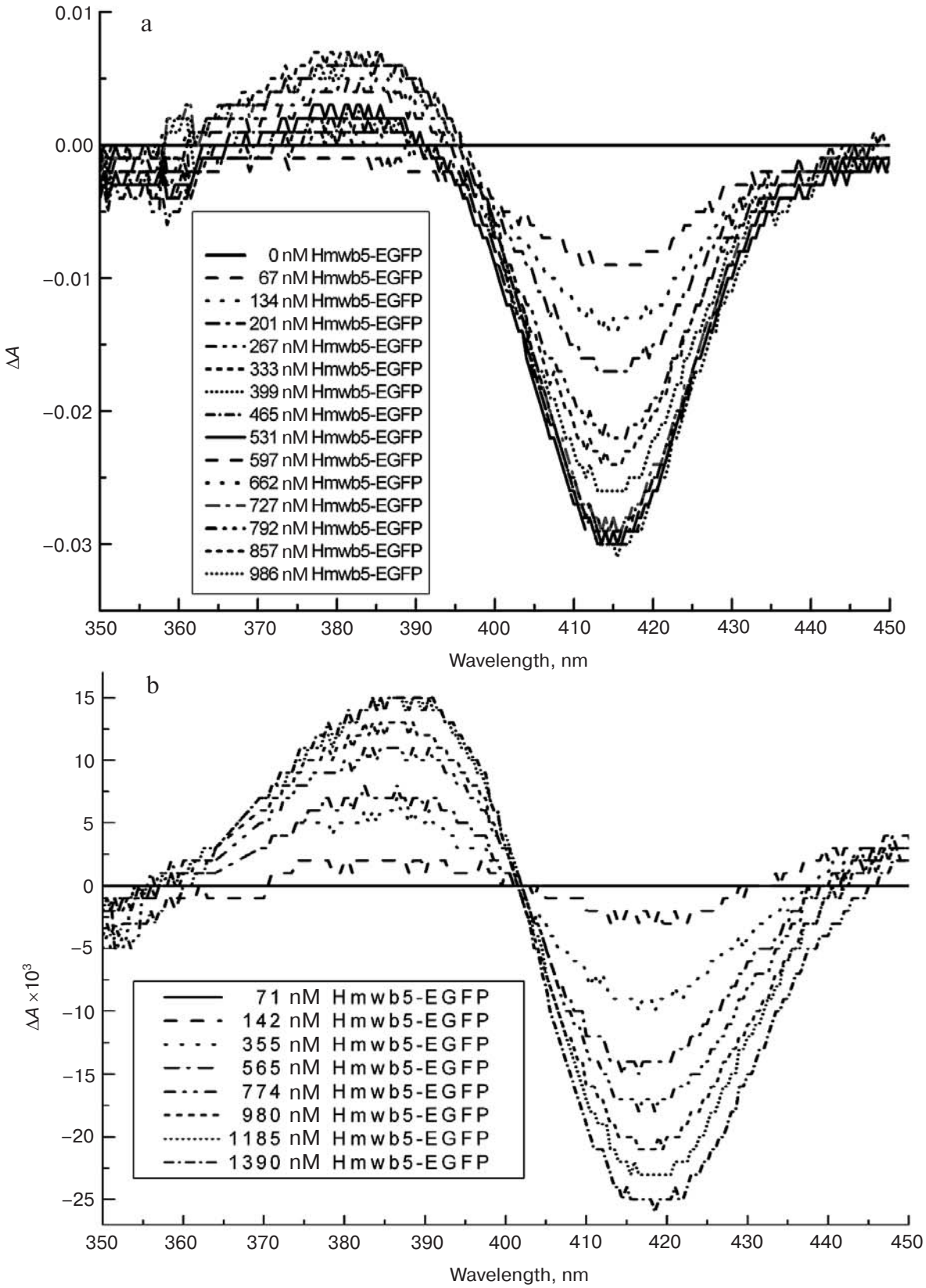

Fig. 1. Spectral titration of cytochrome P450 3A4 with Hmwb_5–EGFP in the absence (a) and presence (b) of lecithin.

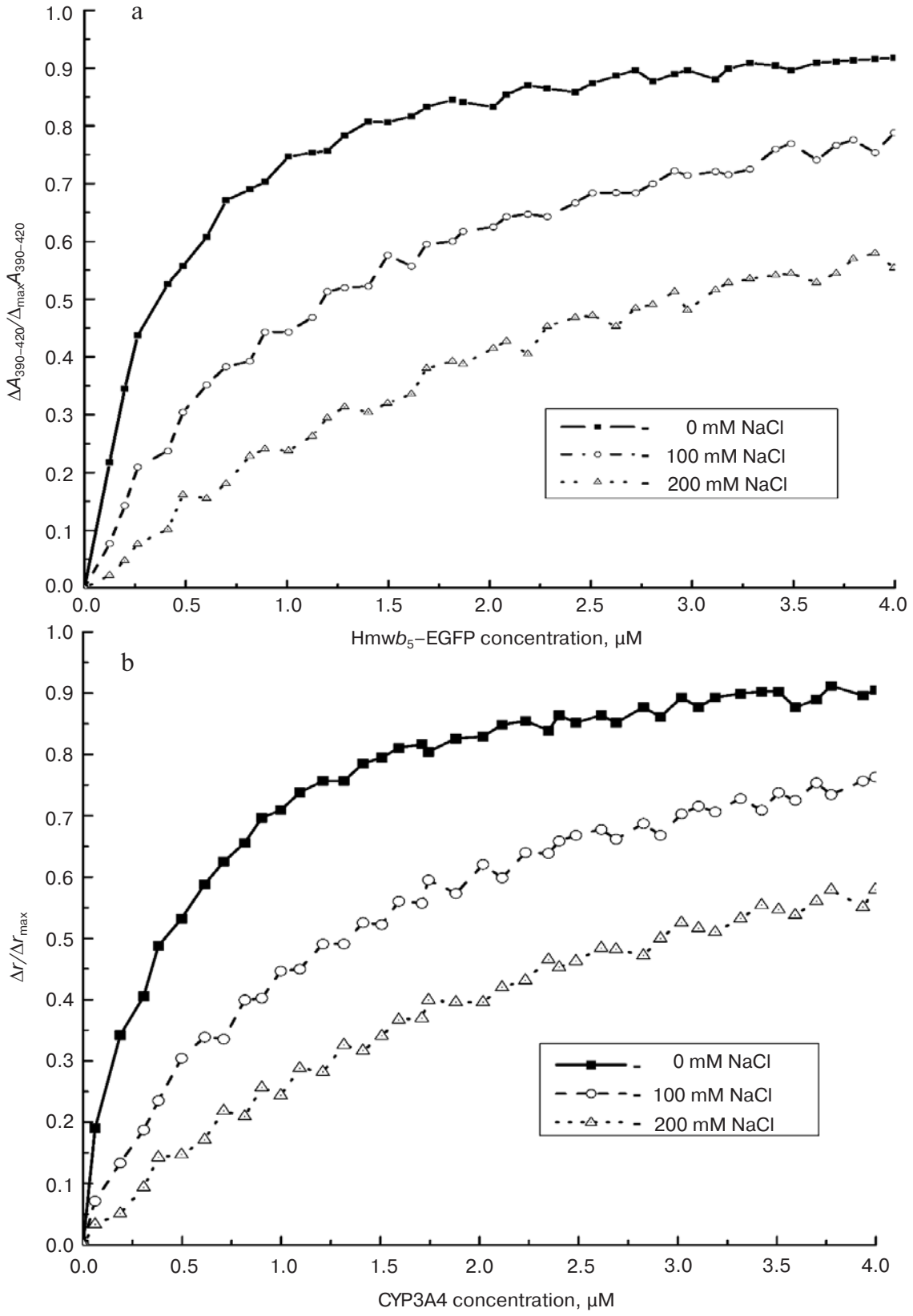

Fig. 2. Titration curves of Hmwb_5–EGFP with CYP3A4 obtained by spectrophotometric registration (a) and titration curves of CYP3A4 with Hmwb_5–EGFP obtained by registration of steady-state fluorescence anisotropy (b).

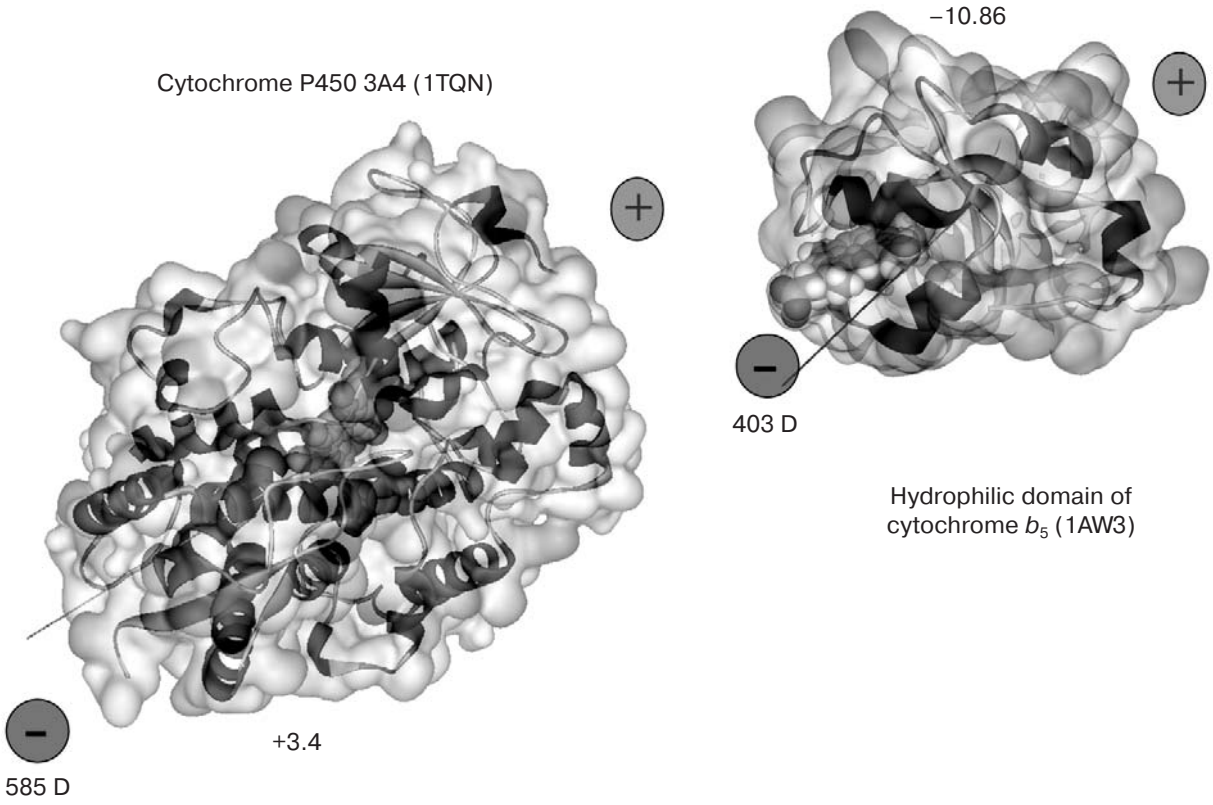

Fig. 3. Charge distribution on the surface of CYP3A4 and hydrophilic domain of cytochrome b_5.

ues of dipole moments indicate the importance of electrostatic interactions and allow identification of the surfaces that might be involved in interaction of the two proteins.

Cytochrome b_5 in the Hmwb_5–EGFP fusion protein does not lose its ability to be reduced by NADPH-cytochrome P450 reductase. The reduction of the hemeprotein is followed by characteristic changes in absorption spectrum (Fig. 4), the reaction rate being highly dependent on ionic strength. The increase in NaCl concentration increasing ionic strength of the solution from 0 to 150 mM increased the reaction rate (Fig. 4, inset). This phenomenon, as shown earlier, is connected with decrease in activation energy of the reduction reaction and its description for the example of the fusion protein of the hydrophilic domain of cytochrome b_5 with the hydrophilic domain of NADH-cytochrome b_5 reductase is presented in our previous work [12]. Using the steady state fluorescence anisotropy to follow complex formation between fusion protein Hmwb_5–EGFP and NADPH-cytochrome P450 reductase was not effective, probable due to the fact that electron transfer is occurs during random collisions of molecules.

Reconstitution of an active hydroxylating system with participation of CYP3A4, NADPH-cytochrome P450 reductase, and Hmwb_5–EGFP *in vitro* demonstrated a stimulating effect of the fusion protein on the reaction of testosterone 6β-hydroxylation. Mass spectra of testosterone (a) and the product of reaction catalyzed by CYP3A4, 6β-hydroxytestosterone (b), are shown in Fig. 5. The characteristic feature of the fusion protein in this system is the less stimulating potential (about 50%) as compared to the stimulating effect of cytochrome b_5 (Fig. 6). The reason for this might be decrease in efficiency of interaction of the fusion protein with CYP3A4 or some changes in the mechanism of the second electron transfer to the oxy-complex of substrate-bound cytochrome P450. The second suggestion is based a hypothesis presuming a functional role of cytochrome b_5 as donor of the second electron for cytochrome P450. Since dissociation constants for the fusion protein and cytochrome b_5 (literature data) are very similar, the second explanation is more likely. As known, the electron transfer in proteins for distances up to 20 Å is realized via tunneling with participation of aromatic amino acid residues [25-27]. The relatively large molecule of EGFP fused with cytochrome b_5 via its hydrophobic domain might result in some steric effects expressed in changes of localization of the hydrophobic domain. These changes as shown do not eliminate the interaction with cytochrome P450, but appear to result in a shift of some site of the molecule participating in electron transfer. This changes the character of tunneling and decreases the rate constant for the second electron transfer to the oxy-complex of the substrate-bound cytochromes P450.

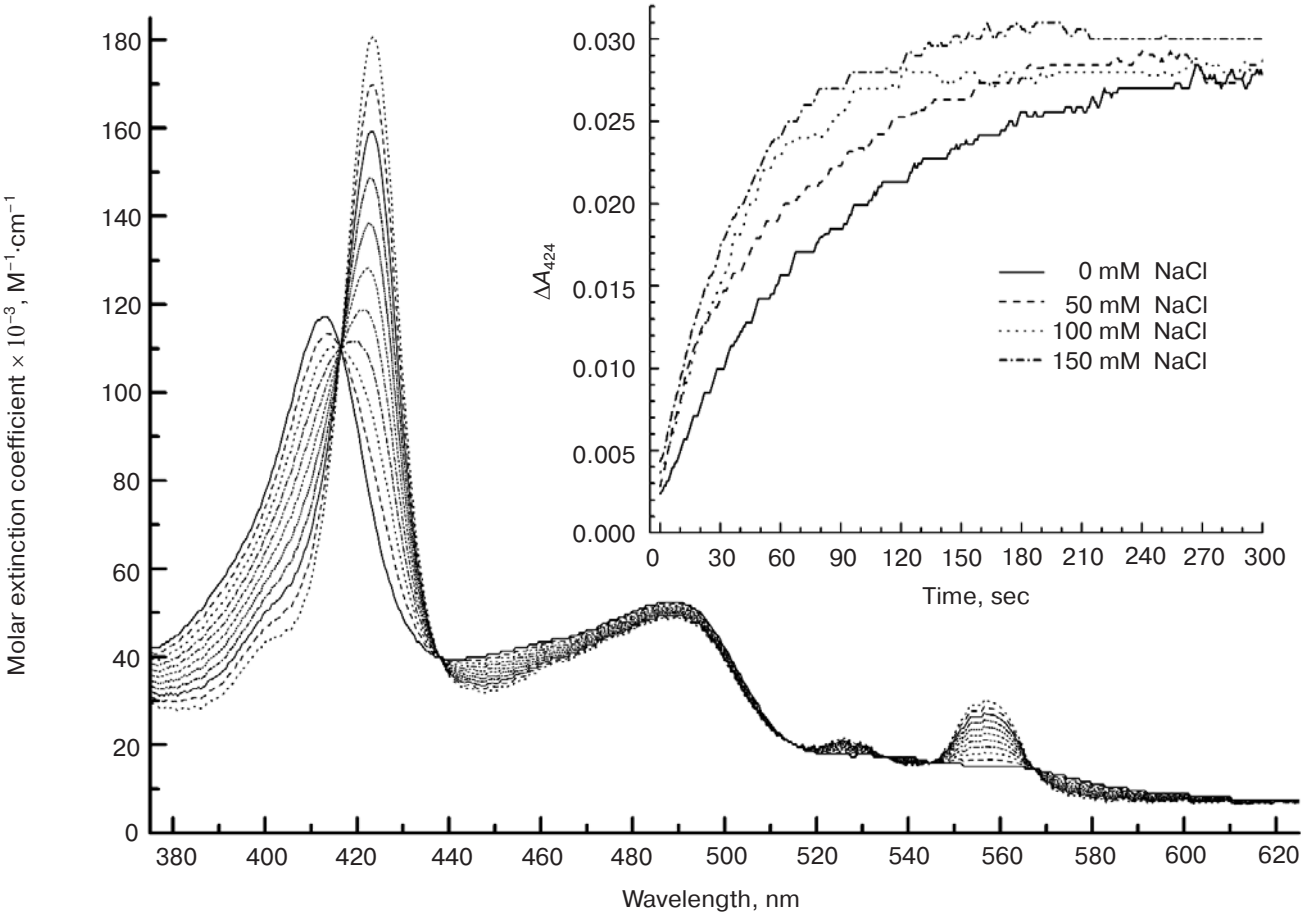

Fig. 4. Spectral changes on enzymatic reduction of Hmwb_5–EGFP by NADPH-cytochrome P450 reductase and kinetic curves of cytochrome b_5 reduction in solutions of different ionic strength (inset).

To compare the effects caused by different fusion proteins containing cytochrome b_5 or its hydrophilic domain on reactions catalyzed by cytochrome P450, we used for reconstitution of hydroxylase activity *in vitro* other earlier constructed fusion proteins: hydrophilic domain of cytochrome b_5–hydrophilic domain of NADH-cytochrome b_5 reductase, and fusion proteins of cytochrome b_5 and CPR with different combination of hydrophobic domains of these proteins (Hmwb_5–HmwCPR, full-length cytochrome b_5–full-length NADPH-cytochrome P450 reductase; Hmwb_5–LmwCPR, full-length cytochrome b_5–hydrophilic domain of NADPH-cytochrome P450 reductase; Lmwb_5–HmwCPR, hydrophilic domain of cytochrome b_5–full-length NADPH-cytochrome P450 reductase; Lmwb_5–LmwCPR, hydrophilic domain of cytochrome b_5–hydrophilic domain of NADPH-cytochrome P450 reductase).

The results of these experiments showed that maximal stimulating activity was achieved with full-length cytochrome b_5 and fusion protein Hmwb_5–HmwCPR. Fusion proteins Hmwb_5–EGFP, Hmwb_5–LmwCPR, and Lmwb_5–HmwCPR demonstrated similar stimulating effect, while fusion proteins not containing hydrophobic domains (Lmwb_5–Lmwb_5Red and Lmwb_5–LmwCPR) had practically no effect on the activity of cytochrome P450 (Fig. 7a).

The results obtained in experiments when the single donor of the first electron was provided by NADPH-cytochrome P450 reductase in a fusion protein were presented earlier [28]. In this case the activity observed using fusion protein Hmwb_5–HmwCPR almost two times exceeded the activity obtained when using the separate proteins. Activities for Hmwb_5–LmwCPR and Lmwb_5–HmwCPR were approximately the same.

It is necessary to stress the inability of the hydrophilic domain of cytochrome b_5 to stimulate the hydroxylation reaction and the absence of activity of cytochrome P450 when using as a donor of the first electron the hydrophilic domain of NADPH-cytochrome P450 reductase. It is also necessary to stress the inability of the hydrophilic domain of cytochrome b_5 to induce type I spectral changes, i.e. the inability of the hydrophilic domain of cytochrome b_5 to interact with

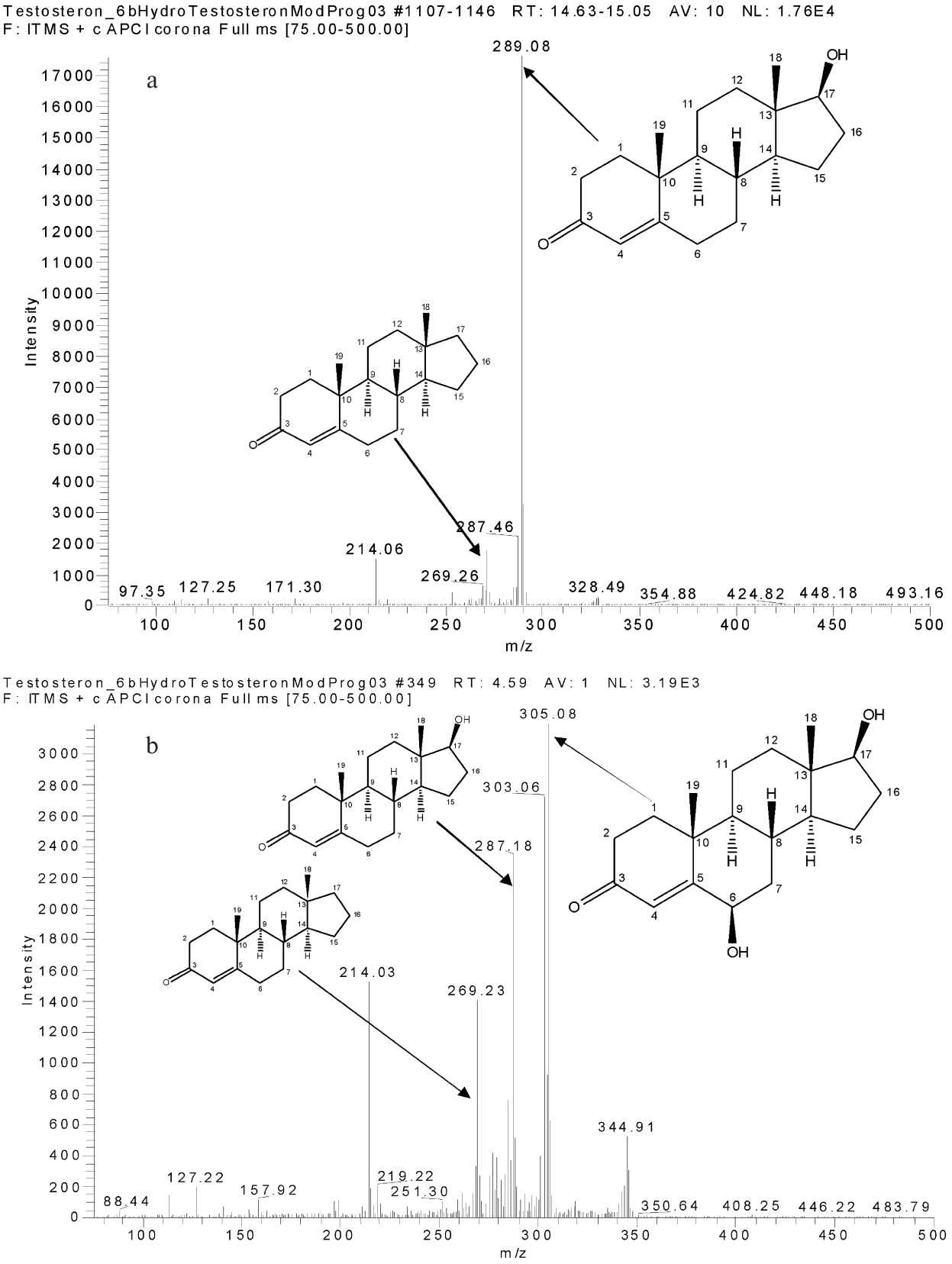

Fig. 5. Mass spectra of testosterone (a) and the reaction product of testosterone 6β-hydroxylation by CYP3A4—6β-hydroxytestosterone (b).

cytochrome P450. The absence of interaction between the hydrophilic domain of cytochrome b_5 and cytochrome P450 was shown earlier using affinity chromatography [15].

Additional evidence was obtained in experiments on construction of chimeric protein containing the hydrophobic domain of cytochrome b_5 (45 amino acid residues) instead of the hydrophobic domain of NADPH-cytochrome P450 reductase (56 amino acid residues). It was shown that such protein is able to support hydroxylation reactions catalyzed by cytochrome P450 [28]. The results indicate the necessity of protein fixation in phospholipid membrane to demonstrate maximal activity of cytochrome P450. Since alignments of sequences of hydrophobic domains of cytochrome b_5 and NADPH-cytochrome P450 reductase shows the absence of any similarity (Fig. 7c), the similar stimulating ability of Hmwb_5–LmwCPR and Lmwb_5–HmwCPR indicates that the hydrophobic domains regardless on sequence act as a membrane anchor.

The results are consistent with the assumption that the hydrophobic domain participates not only in protein orientation in the phospholipid membrane, but also participates in electron transfer. High stimulating activity of Hmwb_5–HmwCPR is explained by absence of steric effects changing electron tunneling. Similar stimulating efficiency of Hmwb_5–EGFP, Hmwb_5–LmwCPR, and Lmwb_5–HmwCPR is explained by both steric effects eliminating the tunneling effect for the first two proteins as well as complete substitution of amino acid residues participating in electron transfer.

The questions of what amino acid residues are involved in electron tunneling from cytochrome b_5 to the oxy-complex of the substrate-bound cytochrome P450 and what orientation is adopted by the hydrophobic domain of cytochrome b_5 in a phospholipid membrane are still opened. The experiments on insertion of cytochrome b_5 into a phospholipid membrane have shown that the C-terminal amino acid residue might be localized on the same side of the membrane where the hydrophilic domain is localized, as well as vice versa. The mode of localization of the hydrophobic domain of the hemeprotein in the membrane *in vivo* is unknown. The presence in the hydrophobic domain of three conservative residues of the relatively rare amino acid residue tryptophan (W109, W110, and W113, two being adjacent, while one is removed by two residues) suggests their possible participation in electron tunneling.

The maximal stimulating effect of cytochrome b_5 (or its fusion proteins) is reached at ratio of cytochrome b_5/cytochrome P450 concentration of 1 : 1. If this ratio becomes more than 1, the inhibiting effect of cytochrome b_5 on hydroxylation reaction might be seen. This phenomenon is explained by competition of cytochrome P450 and cytochrome b_5 for electrons supplied by NADPH-cytochrome P450 reductase. However, there

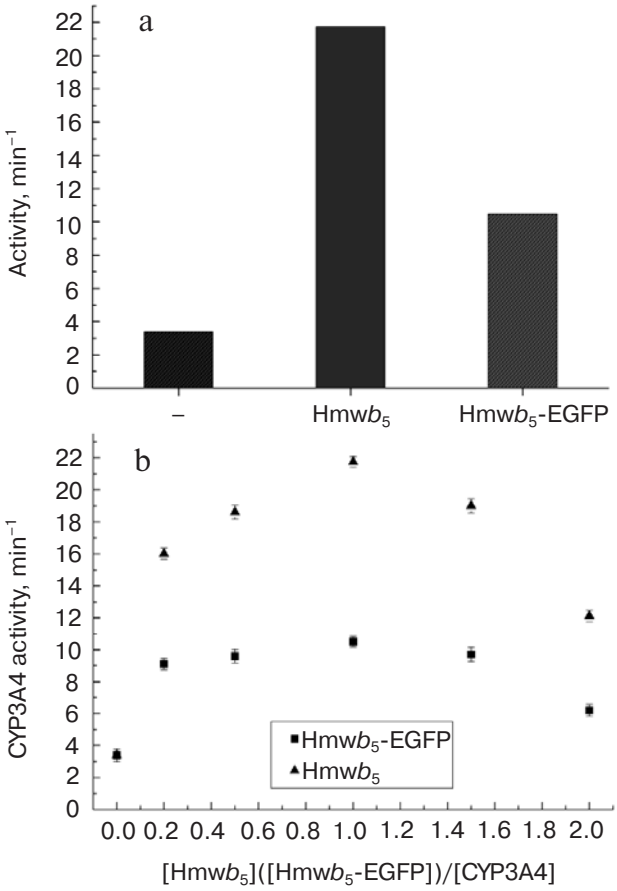

Fig. 6. Effect of cytochrome b_5 and Hmwb_5–EGFP on the reaction of testosterone 6β-hydroxylation catalyzed by CYP3A4.

are many unsolved questions in such explanation. Thus it is shown that maximal stimulating effect of cytochrome b_5 on the reaction of N-demethylation of benzphetamine catalyzed by cytochrome P450 is observed at low ratios of [CPR]/[P450], although the competitive model cannot explain this phenomenon [29].

Molecular docking of cytochrome b_5 and CYP3A4, as well as NADPH-cytochrome P450 reductase and CYP3A4, indicates the possibility of formation of the inter-protein complexes presented in Fig. 8. The dipole moment of the complex between cytochrome b_5 and CYP3A4 is 621 D, e.g. larger than for the individual proteins (403 and 585 D). For the complex of NADPH-cytochrome P450 reductase and CYP3A4, the dipole moment is 2847 D. It is necessary to stress that the structure of the complex of NADPH-cytochrome P450 reductase and CYP3A4, built using the GRAMM-X program, appears to be not optimal since CYP3A4 forms contacts with the surface of FAD-, but not the FMN-domain of NADPH-cytochrome P450 reductase, as follows from the model of intermolecular electron transfer between these proteins. The reason for this might be differences in three-dimensional structure of proteins in solution and in

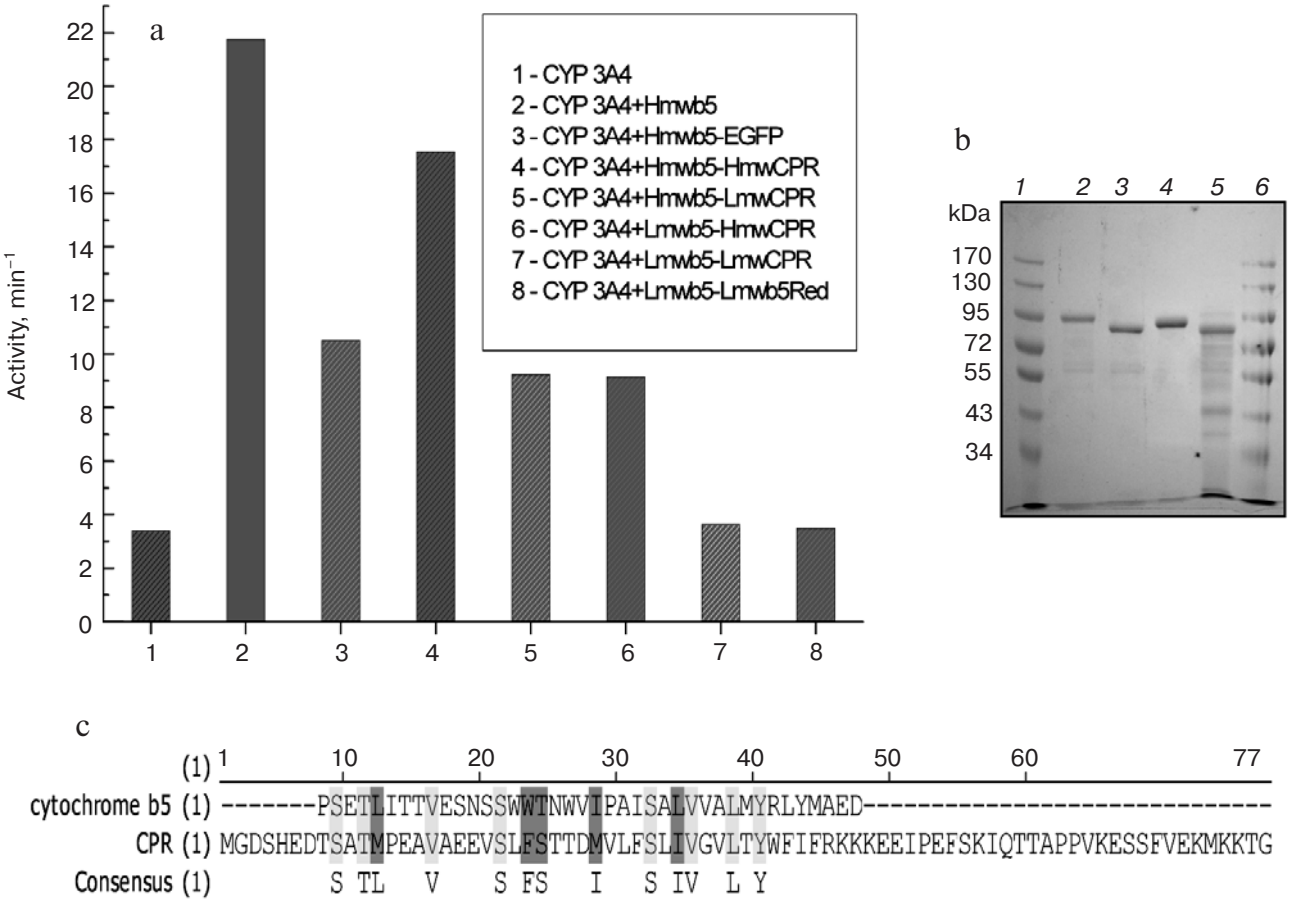

Fig. 7. Effect of different fusion proteins containing cytochrome b_5 on reaction of testosterone 6β-hydroxylation catalyzed by CYP3A4 (a); SDS-PAGE of fusion proteins between cytochrome b_5 and NADPH-cytochrome P450 reductase: *1*, *6*) molecular weight standard; *2*) Hmwb_5–HmwCPR; *3*) Hmwb_5–LmwCPR; *4*) Lmwb_5–HmwCPR; *5*) Lmwb_5–LmwCPR (b); and alignment of amino acid sequences of hydrophobic domains of cytochrome b_5 and NADPH-cytochrome P450 reductase (c).

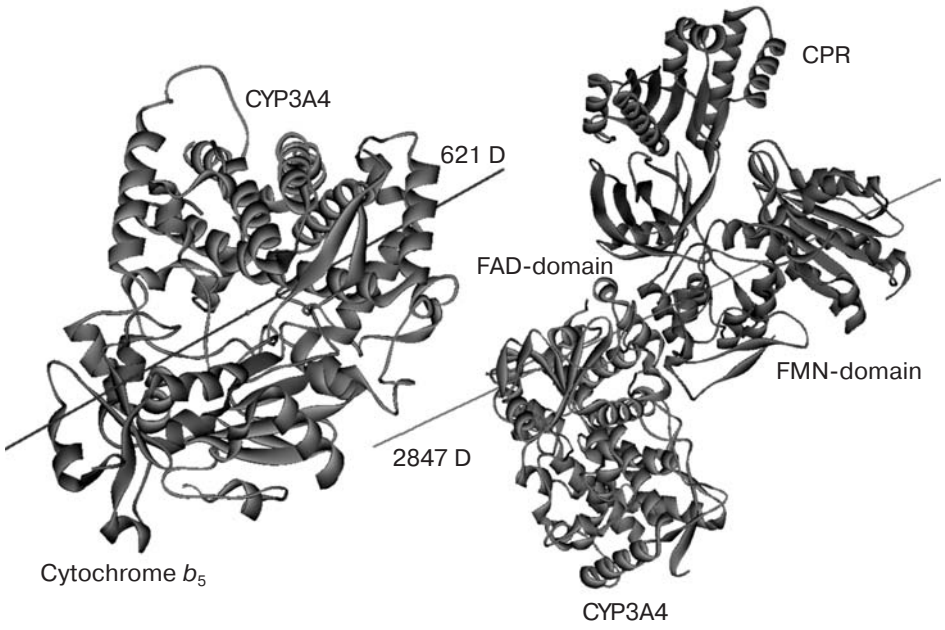

Fig. 8. Theoretical structures of complexes of cytochrome b_5–CYP3A4 and CPR–CYP3A4 obtained using the GRAMM-X program.

crystals as well as the absence of the hydrophobic domain of NADPH-cytochrome P450 reductase in the model used.

Thus, the presently available experimental data indicate a complicated and multiple role of cytochrome b_5 in the reaction catalyzed by cytochrome P450, which appears to consist of regulation of xenobiotic and steroid hormone metabolism and biosynthesis. In the present work, we studied the interaction between cytochrome P450 3A4 and constructed a fusion protein of cytochrome b_5 and green fluorescent protein. It was found that Hmwb_5–EGFP induced type I spectral changes and inter-protein interactions may be observed by registration of changes in steady-state fluorescence anisotropy of the fusion protein under complex formation. The dissociation constants for the complex determined by two independent methods were very similar. Comparison of stimulating activity of cytochrome b_5 in different fusion proteins suggests that the hydrophobic domain of cytochrome b_5 participates not only in the interaction between the two proteins, but also participates in electron transfer, and a cluster of tryptophan residues localized in the hydrophobic domain of cytochrome b_5 appears to be involved in electron transfer.

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