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Conformational dynamics and molecular interaction reactions of recombinant cytochrome P450scc (CYP11A1) detected by fluorescence energy transfer

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Abstract

Bovine adrenocortical cytochrome P450scc (P450scc) was expressed in *Escherichia coli* and purified as the substrate bound, high-spin complex (16.7 nmol of heme per mg of protein, expression level in *E. coli* about 400–700 nmol/l). The recombinant protein was characterized by comparison with native P450scc purified from adrenal cortex mitochondria. To study the interaction of the electron transfer proteins during the functioning of the heme protein, recombinant P450scc was selectively modified with fluorescein isothiocyanate (FITC). The present paper shows that modified P450scc, purified by affinity chromatography using adrenodoxin–Sepharose to remove non-covalently bound FITC, retains the functional activity of the unmodified enzyme, including its ability to bind adrenodoxin. Based on the efficiency of resonance fluorescence energy transfer in the donor–acceptor pair, FITC–heme, we calculated the distance between Lys^{338}, selectively labeled with the dye, and the heme of P450scc. The intensity of fluorescence from the label dramatically changes during: (a) denaturation of P450scc; (b) changing the spin state or redox potential of the heme protein; (c) formation of the carbon monoxide complex of reduced P450scc; (d) as well as during reactions of intermolecular interactions, such as changes of the state of aggregation, complex formation with the substrate, binding to the electron transfer partner adrenodoxin, or insertion of the protein into an artificial phospholipid membrane. Selective chemical modification of P450scc with FITC proved to be a very useful method to study the dynamics of conformational changes of the recombinant heme protein. The data obtained indicate that functionally important conformational changes of P450scc are large-scale ones, i.e. they are not limited only to changes in the dynamics of the protein active center. The results of the present study also indicate that chemical modification of Lys^{338} of bovine adrenocortical P450scc does not dramatically alter the activity of the heme protein, but does result in a decrease of protein stability. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Heterologous expression; Cytochrome P450scc; Selective chemical modification; Fluorescence energy transfer; Conformational dynamics; Molecular interaction reaction

1. Introduction

Cytochrome P450scc (CYP11A1)(P450scc) catalyzes the cholesterol side chain cleavage reaction, the initial and key reaction in the regulation of steroid hormone biosynthesis. P450scc is a monooxygenase located on the inner mitochondrial membrane

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of most steroidogenic tissues. In the process of catalysis, oxidized (ferri-) P450scc forms a complex with the substrate (cholesterol) which results in a change of the spin state of the heme iron from the low to the high-spin state. This complex of ferric P450scc and substrate then accepts an electron transferred from NADP(H^+) via the electron transfer partners, adrenodoxin reductase and adrenodoxin, and the heme iron of P450scc is reduced to its ferro form. Reduced P450scc, in a complex with cholesterol, binds molecular oxygen. It then accepts a second electron from another molecule of reduced adrenodoxin, and activates inert molecular oxygen so that one atom of oxygen is inserted into the substrate and the other atom of oxygen is reduced to water. In most P450 catalyzed reactions, the hydroxylated product is released from the active site of the P450 and the oxidized heme protein returns to its initial ferric low-spin state [1]. However, to convert cholesterol to pregnenolone, P450scc needs at least six electrons supplied by its electron transfer partners, i.e. three cycles of the P450. This requires the operation of multiple association and dissociation reactions of P450scc and reduced adrenodoxin during a catalytic cycle. Thus, the extremely complicated mechanism of the reaction whereby cholesterol is converted to pregnenolone requires the coordinated functioning of several catalytically important sites in the heme protein molecule, such as operation of the substrate entrance channel for cholesterol binding, accessibility and preparation of the site responsible for the interaction(s) with reduced adrenodoxin, positioning the sites involved in internal electron transfer, and operation of the intrinsic site responsible for the reaction where 'activated oxygen' interacts with cholesterol to carry out the chemistry of cholesterol side chain cleavage [2,3].

Coordinating the functions of these different sites may be achieved via a type of signal transfer between these sites. This transfer of information may be carried out by means of changes in the conformation of polypeptide chains. In recent times, more and more attention is being paid to the role of conformational dynamics in understanding the function of protein molecules [4–7]. There is more and more evidence pointing to the importance of transitions between different conformational states of proteins [9–12]. However, until now, the question of the magnitude of these changes taking place in a protein molecule, as well as the size of polypeptide chain fragments involved in such conformational changes, remains unanswered.

The aim of the present work is to use a selective chemical modification of recombinant cytochrome P450scc with the fluorescent dye, FITC, to determine changes in heme protein conformation. Measurements of the efficiency of fluorescence energy transfer in the donor–acceptor pair, FITC–heme, permits the monitoring of changes in intramolecular distances between the fluorescent labeled amino acid residues and heme. This method also permits one to follow changes of this distance under different conditions presumed to result in changes of heme protein conformation.

2. Materials and methods

2.1. Chemicals

In the present study, we used isopropyl-1-thio-β-D-galactopyranozide (IPTG) (Gibco-BRL, USA), yeast extract, bactopepton, trypton (Difco, USA), cholesterol, deoxy-corticosterone, sodium cholate, Tween-20, Coomassie G-250, glucoso-6-phosphate, glucoso-6-phosphate dehydrogenase, polyethyleneglycol 6000 (Serva, Germany), Sepharose 4B, Sephadex G-50, Sephacryl S300 (Pharmacia, Sweden), δ-aminolevulinic acid, cholesterol oxidase, lysozyme, phenylmethyl-sulfonylfluoride (PMSF), NADPH, fluorescein isothiocyanate (Sigma, USA), guanidine chloride (Merck, Germany).

Dioleoylphosphatidylcholine was kindly provided by Dr. M.A. Kissel (Institute of Bioorganic Chemistry) and was judged to be pure by thin layer chromatography in a system containing methanol/chloroform/water (6.5:2.4:0.1). The dioleoylphosphatidylcholine sample did not contain any lysoforms.

2.2. Isolation and purification of the proteins

Cytochrome P450scc, adrenodoxin and adrenodoxin-reductase were purified from bovine adrenocortical mitochondria using affinity chromatography [13,14]. The purity of the preparations was monitored by SDS-PAGE according to Laemmli [15].

The concentrations of adrenodoxin reductase and adrenodoxin were determined spectrophotometrically using molar extinction coefficients 11 mM^{-1} cm^{-1} at 450 nm and 10 mM^{-1} cm^{-1} at 414 nm, respectively [16]. The concentration of active P450scc and its inactivated form (cytochrome P420) was determined from the carbon monoxide difference spectrum of the reduced heme proteins (CO spectrum), using molar extinction coefficients 91 mM^{-1} cm^{-1} at 450 nm and 114 mM^{-1} cm^{-1} at 420 nm, respectively [17]. Spectrophotometric studies were carried out using a Shimadzu UV-3000 (Japan) or Specord M40 (Carl-Zeiss, Germany) spectrophotometer.

The plasmid containing the hybrid trp/lac (trc) promoter and the cDNA coding the amino acid sequence of the mature bovine adrenal mitochondrial P450scc (pTrc99A), was kindly provided by Prof. Michael R. Waterman (Vanderbilt University, Nashville, TN). Construction of the plasmid is described in the paper [18].

2.3. Heterologous expression of P450scc

Heterologous expression of P450scc in *Escherichia coli* (JM109) cells was carried out according to [19] with some modifications. The final ampicillin concentration was 200 mg/l. After IPTG addition the *E. coli* cells were incubated for 72 h at 22°C with shaking at 140 rpm. Using these conditions, the expression level of active recombinant P450scc averaged 500 nmol per liter of the culture medium.

2.4. Analysis of the protein composition of the bacterial cells

Analysis of the protein composition of the bacterial cells, after expression of P450scc, was performed by SDS-PAGE. Immunochemical analysis of the recombinant P450scc was carried out by immunoblotting in the presence of specific antibodies [20].

The level of heterologous expression of the functionally active P450scc in the *E. coli* cells was measured spectrophotometrically by determining the heme protein content using the difference spectra of the carbon monoxide complex of reduced P450scc. For that purpose, a 2-ml aliquot of an overnight cell culture was centrifuged for 5 min at 5000 rpm. The cell pellet was suspended in 2 ml of 50 mM potassium-phosphate buffer, pH 7.4 and sodium dithionite was added. The sample was divided into two spectrophotometer cuvettes and carbon monoxide was bubbled into the sample cell. The maximal difference in optical density at 450 nm was reached in 40–50 min. The content of recombinant P450scc was measured spectrophotometrically in every sample at different steps of the purification procedure.

2.5. Purification of recombinant P450scc

The *E. coli* cells obtained from 1 l of culture medium were collected by centrifugation at 5000 rpm and suspended in 20 mM potassium-phosphate buffer, pH 7.4, containing 20% glycerol, 1 mM EDTA and 1 mM dithiotreitol (buffer A) in the ratio 1:3 (wet weight/volume). To disrupt the bacterial cell wall, lysozyme was added to the cell suspension to a final concentration of 100 μg/ml. The mixture was incubated for 30 min at room temperature and frozen after the addition of PMSF (0.4 mM). The thawed, lysed cells were homogenized and disrupted by ultrasonic treatment (UZDN-1, current 20 A) on ice: 10 times for 10 s with 1-min intervals. After sonic treatment the cell debris were removed by centrifugation at 5000 rpm during 30 min. Emulgen 913 was added to the supernatant at final concentration 1% (v/v) and the mixture was incubated 1 h on ice. The non-solubilized membrane residues were removed by centrifugation for 1 h at 18 000 rpm.

Twenty ml of 50 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol (buffer B), containing 50% polyethyleneglycol 6000, was added to each 30 ml of supernatant obtained after solubilization of the membranes. The mixture was stirred for 30 min at room temperature and centrifuged for 1 h at 25 000 rpm. The pellet was dissolved in one-fifth the initial volume of supernatant using buffer A containing 0.1 μM cholesterol and the mixture was incubated for 40 min at room temperature. It was then diluted 5 times with buffer B and applied to an adrenodoxin-Sepharose 4B column (1×2 cm). The column was washed with buffer B until the absorbance of Emulgen 913 (265–290 nm) disappeared. P450scc that was tightly bound to the column was eluted with buffer B containing 1 M sodium chloride and 0.3% sodium cholate (buffer C).

The content of total protein in the preparations of

recombinant P450scc during the different steps of purification was determined by the Lowry method [21].

2.6. Enzymatic reduction of P450scc

The rate of enzymatic reduction of P450scc was determined spectrophotometrically using a reconstituted system containing the mitochondrial electron transfer proteins adrenodoxin reductase and adrenodoxin at a ratio of adrenodoxin reductase/adrenodoxin/P450scc of 0.5:2:1. The rate was calculated based on the formation of the carbon monoxide complex of reduced P450scc where the P450 is reduced either chemically or enzymatically [23].

2.7. Determination of cholesterol side chain cleavage activity

Cholesterol side chain cleavage activity of recombinant P450scc was determined by a HPLC method according to the procedure described in [22]. The amount of progesterone formed from pregnenolone by cholesterol oxidase was monitored at 240 nm by normal-phase HPLC using a column (Zorbax-Sil, 4.6×250 mm) and an isocratic solvent system of *n*-hexane/isopropanol, as previously described for bovine adrenocortical P450scc. Deoxycorticosterone was used as an internal standard.

2.8. Studies of the interaction of P450scc with cholesterol

Cholesterol-induced spectral changes were recorded during titration of the Tween-20 induced low-spin form of natural and recombinant P450scc (final concentration 0.2 μM in buffer A containing 0.01% Tween-20). Cholesterol in ethanol (1 mM solution) was added and the amplitude of the type I spectral response in the difference spectrum recorded 1 h after substrate addition. An equivalent volume of ethanol was added to the control cell. The reduced carbon monoxide spectra were recorded before and after the titration to prove the absence of denaturation of the P450scc during recording of the spectra.

2.9. Studies of the interaction of P450scc with adrenodoxin

The spectral changes induced by addition of adrenodoxin to the native and recombinant P450scc were recorded by difference spectrophotometry using P450scc in buffer A, containing 0.025% Tween-20. Two spectrophotometric cells, divided into two compartments (split cell technique), were used. P450scc (0.5 μM) was placed in one compartment of each cell and buffer A was placed in the second compartment of the cell. Different amounts of adrenodoxin were added to the P450scc containing compartment in the sample cell and to the buffer solution compartment in the reference cuvette and the cells were incubated for 15 min at room temperature prior to recording the difference spectrum. The absence of denaturation during the incubation was proved by recording the carbon monoxide difference spectra of reduced P450scc.

2.10. Chemical modification of P450scc with fluorescein isothiocyanate

Modification of the lysine residue Lys^{338} of P450scc with fluorescein isothiocyanate was carried out according to [23]. P450scc (final concentration 0.1 mM) in buffer C was incubated with a six-molar excess of the reagent for 45 min at room temperature. The excess reagent was removed by gel-filtration on Sephadex G-50 (fine) column (1×25 cm) using buffer A, containing 0.3% sodium cholate. The degree of modification of P450scc was determined from the absolute absorption spectrum using a molar extinction coefficient of 74 mM^{-1} cm^{-1} at 496 nm for FITC and subtracting the heme absorbance (equal 1/10 A_{393}) from the optical density at 496 nm: $A_{FITC} = A_{496} - 1/10\ A_{393}$.

Selectively modified P450scc was additionally purified by affinity chromatography on adrenodoxin–Sepharose 4B. P450scc was applied to the column in buffer A, washed with buffer A containing 0.2 M sodium chloride and eluted with buffer C. The effectiveness of this procedure in removal of non-covalently bound FITC was checked by precipitation of the purified, modified P450scc with acidic acetone

[24]. The pellet was dissolved in buffer A, containing 6 M guanidine chloride and the amount of covalently bound FITC was determined from the absorbance at A_{496}. Apo-cytochrome P450scc was prepared by a similar method [25].

2.11. Limited proteolysis of P450scc with trypsin

Limited proteolysis with trypsin of the native or recombinant FITC labeled P450scc was carried out at a P450scc concentration of 10 μM and a molar ratio of trypsin/cytochrome P450scc of 1:10 [26]. The products of proteolytic digestion of P450scc were analyzed by immunoblotting in the presence of antibodies against the F1 and F2 fragments of the bovine adrenocortical P450scc.

2.12. Preparation of inactivated form of P450scc

Denaturation of FITC labeled P450scc to form cytochrome P420 was carried out by thermal treatment (45°C, 2 h). The content of cytochrome P420 was determined spectrophotometrically from the carbon monoxide difference spectra of the reduced heme protein.

2.13. Studies of aggregation state of FITC-labeled P450scc

Studies of the aggregation state of FITC-labeled P450scc and its dependence on buffer composition were carried out using gel-filtration on Sephacryl S-300 column (1.5×70 cm). Human spleen ferritin (450 kDa), rabbit immunoglobulin G (150 kDa) and horseradish peroxidase (40 kDa) were used as protein standards.

2.14. Enzymatic preparation of the low-spin form of P450scc

To remove endogenous cholesterol from the active site of native P450scc, 1 ml of the FITC-labeled P450scc (30 μM) was incubated with adrenodoxin (1 nmol), adrenodoxin-reductase (0.5 nmol) as well as regenerating system containing $NADP^+$ (100 nmol), glucose-6-phosphate (500 nmol), and glucose-6-phosphate dehydrogenase (0.5 IU). After incubation for 30 min at room temperature, the low-spin form of P450scc was separated from the added proteins by affinity chromatography using an adrenodoxin–Sepharose column as described above.

2.15. Preparation of proteoliposomes

Proteoliposomes containing P450scc were prepared by a gel-filtration method according to [27] using synthetic phospholipid–dioleoylphosphatidylcholine at a weight ratio of lipid/protein = 1:2.8.

2.16. Measurement of fluorescence spectra

The fluorescence spectra of FITC labeled P450scc were recorded using a fluorimeter SOLAR SFL (Belarus) at an excitation wavelength setting of 470 nm and an excitation and emission slit setting of 5 nm. The final concentration of fluorescent label was 1 μM with the exception of the experiments where P450scc was titrated with cholesterol where a concentration (0.5 μM) of the label was used. Measurements were carried out at 4°C except for studies where the stability of FITC-labeled P450scc was determined.

The distance (R) between donor and acceptor of fluorescence energy was determined based on the equation:

$$R = R_0(1/E-1)^{1/6},$$

where the efficiency of the fluorescence energy transfer is equaled to $E = 1-Q/Q_0$, Q/Q_0 is the ratio of quantum yields of the label in the presence and absence of acceptor, respectively [28]. As the critical Forster radius (R_0) for the donor–acceptor pair (FITC–heme) for the low-spin form of P450scc we used the distance 3.98 nm [29]. Since the absorbance spectrum of P450scc changes during high- to low-spin transition, CO complex formation or denaturation to form cytochrome P420, we estimated the changes of the R_0 for these states based on the integral of overlapping between donor (FITC) emission and acceptor (Q-bonds of heme) absorbance spectra [28]. The integral of overlapping was calculated using the original computer program HAND kindly provided by Dr. S.M. Bachilo (Institute of Molecular and Atomic Physics, National Academy of Sciences of Belarus, Minsk, Belarus).

3. Results and discussion

3.1. Optimization of conditions for the heterologous expression of P450scc

The expression conditions described in Section 2 allows one to attain a high level of heterologous expression in *E. coli* of a functionally active form of recombinant P450scc, i.e. equal to 500 nmol/l of the culture (or 30 nmol/g of the dry cell weight). The significant increase of the expression level of P450scc obtained in the present study was the result of optimization of the temperature conditions used for the growth of the bacterial cells. It was noted that there is an increase in the growth of the *E. coli* when the temperature is increased to 28–30°C and this results in an increase in the content of a protein with the same electrophoretic mobility as P450scc and having common antigenic determinants, but the amount of spectrally detected cytochrome P450scc decreases. It appears that accelerated protein synthesis causes dramatic changes in the folding of the protein (or insertion of the heme) leading to a denaturation that results in the formation of inclusion bodies since the yield of the solubilized P450scc protein dramatically decreases.

3.2. Purification of recombinant P450scc from E. coli

To avoid the loss of large amount of recombinant P450scc during its purification we excluded the commonly used stage of the cell fractionation to obtain membrane fraction [18,30] as described in the paper [19]. In contrast to this work, the ultrasonic treatment of lysed *E. coli* cells was carried out before detergent addition. In this case, the decrease of the amount of spectrally detected P450scc did not exceed 10% (Table 1).

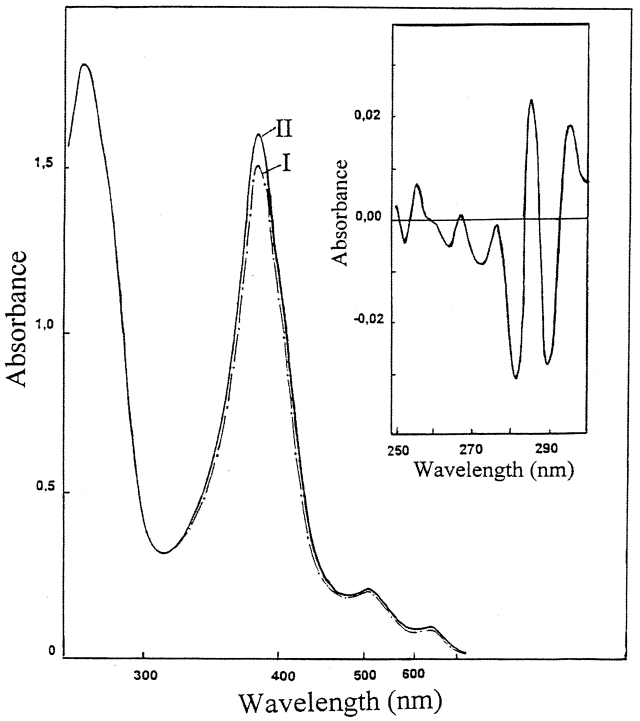

Fig. 1. Absolute absorption spectra of bovine adrenocortical (I) and recombinant (II) P450scc in 50 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol. Inset: second derivative of UV spectra.

The choice of Emulgen 913 as a detergent to solubilize P450scc is connected with the fact that this detergent allows one to get the maximal yield of cytochrome P450scc in solution. Moreover, the storage at −20 degrees for at least 1 month of the supernatant containing solubilized P450scc obtained using this detergent does not result in a significant spectral change. At the same time, storage of the cytochrome P450scc solubilized with sodium cholate (0.6%) results in a dramatic increase in the amount of denatured heme protein. Treatment of the solubilized P450scc with polyethyleneglycol results in the precip-

Table 1
Isolation and purification of recombinant P450scc expressed in *E. coli* cells

Purification step	Quantity of spectrally detected P450scc (nmol)	Yield (%)	P450 content (nmol/mg protein)	Purification degree	Yield (%)
Bacterial culture	500	100	0.146	1	100
Sonicated lysed cells	450	90	0.106	0.72	90
Solubilisate	325	72	0.124	0.85	68
PEG precipitate	210	65	0.230	1.58	42
Adrenodoxin–Sepharose	190	90	16.7	114	38

itation 65% from spectrally detected heme protein. This precipitated P450scc remains in a functionally active form and contains only a small amount of the detergent. The yield of P450scc at the step of affinity chromatography was 90%.

3.3. Spectral properties of recombinant P450scc

Highly purified recombinant P450scc contained 16.7 nmol of heme per mg of protein. Recombinant P450scc predominantly was in the high-spin state ($\Delta A_{390-470}/\Delta A_{416-470} = 2.0$ [31]) and was characterized by a spectrophotometric index (A_{416}/A_{280}) equal to 0.88 (Fig. 1) indicating that the purified P450scc did not contain any impurities of the denatured form of the heme protein (i.e. P420). This is the first time that recombinant P450scc has been purified in the stable high-spin state.

Removal of Emulgen 913 from the final preparations of highly purified recombinant P450scc permits the recording of the absolute absorbance spectra including the ultraviolet region (Fig. 1) as well as the second derivative of the ultraviolet spectrum. The latter gives information on the micro-environment of the aromatic amino acid residues in the heme protein and indicates that purified recombinant P450scc is very similar to native cytochrome P450scc.

The similarities of the spectral properties as well as functional parameters of recombinant and adrenocortical bovine P450scc (Table 2) supported the conclusion that equivalent processes of protein folding for the P450scc occur in bacterial and in mammalian cells. This allows one to use recombinant P450scc as a model for the natural P450scc in experiments.

3.4. Chemical modification of recombinant P450scc with FITC

As has been shown previously [23], chemical modification of P450scc with FITC results in the selective reaction of the fluorescent dye with the ε-amino group of Lys^{338}. This, however, is followed by non-specific absorption of the dye (about 30% of the total amount of the bound dye) on the surface of the protein. The non-specifically absorbed dye rather strongly interacts with hydrophobic sites of P450scc and is not removed by gel-filtration even in the presence of detergents.

Since the intrinsic fluorescence of the non-specifically bound dye might cause an interference in the determination of the distance in the donor–acceptor

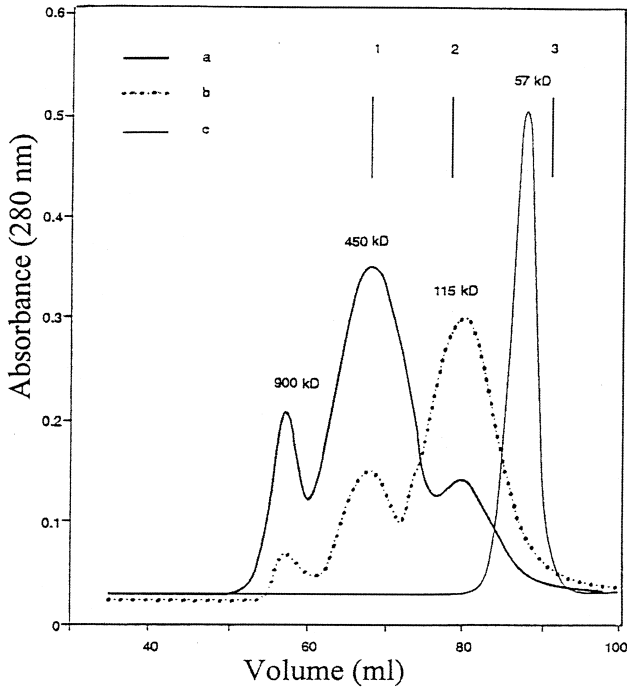

Fig. 2. Gel-filtration of recombinant P450scc on Sephacryl S-300 column equilibrated with: (a) 50 mM potassium-phosphate buffer, pH 7.4; (b) 50 mM potassium-phosphate buffer, pH 7.4, containing 0.2 M NaCl; and (c) 50 Mm potassium-phosphate buffer containing 1.0 M NaCl and 0.3% sodium cholate. 1, 2 and 3, elution volumes of standard proteins: ferritin, immunoglubulin G and horseradish peroxidase, respectively.

Table 2
Functional characteristics of adrenocortical and recombinant bovine P450scc

P450scc	Heme content (%)	$\Delta A_{390-470}/\Delta A_{420-470}$	Cholesterol side chain cleavage activity, (nmol/nmol P450/min)	Efficiency of enzymatic reduction (%)	Interaction with adrenodoxin		Interaction with cholesterol	
					K_d (μM)	ΔA_{max}	K_d (μM)	ΔA_{max}
Natural	75	1.6	3.6	89	0.36	0.042	4.00	0.030
Recombinant	94.5	2.0	3.7	91	0.36	0.046	3.90	0.029

Table 3
Purification of FITC-labeled P450scc

Protein sample	*F/P	F/P	Content of non-specifically bound label (%)	Yield (%)	Efficiency of enzymatic reduction (%)	Cholesterol side chain cleavage activity (nmol/nmol P450/min)
P450scc	0	0	–	–	91	3.7
FITC-labeled P450scc before chromatography on adrenodoxin–Sepharose	1.28	0.9	28	100	58	2.1
Non-bound fraction	2.3	1.17	49	19	22	Nd
Washing with buffer A	2.0	0.92	54	8	29	nd
Washing with 0.2 M NaCl	1.35	0.89	34	34	33	nd
FITC-labeled P450scc after adrenodoxin–Sepharose chromatography	0.87	0.87	0	38	92	3.6

F/P, degree of modification; **F/P*, spectrally detected modification degree.

pair, as measured by resonance fluorescence energy transfer, we used a different method to remove the non-specifically bound FITC from the P450scc protein labeled at Lys^{338}.

Best results were achieved by using affinity chromatography of the FITC labeled P450scc on adrenodoxin–Sepharose 4B. The precipitation of the affinity purified P450scc with acidic acetone proved that 100% of the FITC is covalently bound to the heme protein. SDS-PAGE of the products of limited proteolysis by trypsin of the P450scc labeled with FITC showed that all the label is located in the fragment F2. Since there are indications that modification of P450scc Lys^{338} results in the loss of adrenodoxin-binding ability [32,33], we checked the interaction of P450scc and adrenodoxin (Table 3). The data presented in the table indicate that the adrenodoxin-binding ability of the purified labeled P450scc is retained while the P450scc which was damaged during the modification with FITC, as well as non-covalently bound reagent, are removed. Thus, the amino group of Lys^{338} is not critical for direct physical interaction of P450scc with adrenodoxin. The differences in the effect of modification of this amino acid on the interaction of P450scc with adrenodoxin reported in this and previous studies may be the result of the different conditions used for the modification reaction, but most probably with the mode and degree of purification of the labeled protein.

3.5. Studies of the molecular dynamics of P450scc as measured by fluorescence energy transfer

The retention of enzymatic activity by P450scc selectively modified with FITC indicates that the modified amino acid residue is not involved directly and is not essential for P450scc activity. This observation provides the unique possibility to use this modification: (a) to detect large-scale conformational motions in the P450scc molecule; and (b) to assess the role of large-scale conformational changes on the conformational dynamics of P450scc.

The large-scale conformational dynamics of P450scc that occur as a result of changes in the content of α-helix and polarity of the tyrosine microenvironment, for example by changes of pH and temperature, was measured by circular dichroism spectroscopy [34]. In the present work, we measured changes in the intensity of FITC fluorescence in the labeled P450scc and calculated the distance between the Lys^{338} containing the label and heme, based on the efficiency of Forster energy transfer in the donor–acceptor pair, FITC–heme. Then, based on changes of fluorescence intensity of the label, we determined changes in the intramolecular distance in P450scc under the following conditions:

1. denaturing caused by guanidine hydrochloride (total unfolding of the protein globule);

2. the effect of temperature on the conversion of P450scc to cytochrome P420;
3. the transition to the low-spin form of P450scc;
4. the transition from the oligomeric to the monomeric form resulting from changes in the composition of the buffer (Fig. 2);
5. enzymatic reduction in a reconstituted system and formation of the carbon monoxide complex of reduced P450scc.

Table 4 presents the results of measurements of FITC fluorescence intensity using P450scc (natural and recombinant) under the conditions described above. This table also presents changes in the calculated distances between Lys^{338} and heme. The differences in values obtained when using natural or recombinant P450scc result from differences in the content of heme in the preparations of labeled heme protein. An increase of heme content results in an increase in the efficiency of fluorescence energy transfer and consequently a decrease in the FITC fluorescence intensity.

Fig. 3 shows the dependence of FITC fluorescence intensity on P450scc concentration and the heme content of the P450scc. Since the purified bovine adrenocortical P450scc contains only 75% heme, while recombinant P450scc contains 95%, the data obtained for recombinant P450scc seems to be more accurate. However, we were interested primarily not only in the value of the distance that was determined but also in the possibility that one could measure changes of this distance and thereby assess the scale of these changes reflecting the conformational dynamics of P450scc.

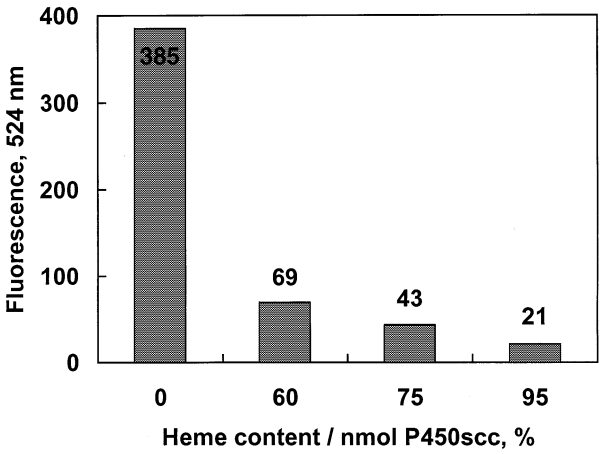

Fig. 3. The dependence of the intensity of FITC (10^{-6} M) fluorescence in labeled P450scc on the heme content.

As shown by the data presented in Table 4, the direction of the changes in fluorescence intensity, both in natural and recombinant P50scc, is the same, although they are greater in magnitude with the recombinant heme protein. This may reflect the molar differences in the ratio of heme to protein in the two preparations.

It is known that reduced P450scc is less stable than the oxidized heme protein and that the low-spin form

Table 4
Intensity of FITC fluorescence and calculated distances between Lys^{338} and heme in bovine adrenocortical and recombinant P450scc

P450scc	Fluorescence intensity (relative units)		Distance (nm)				
	Natural	Recombinant	R_o	Natural		Recombinant	
				nm	%	nm	%
FITC-apo-P450scc	388	385	–	–	–	–	–
FITC-cytochrome P420							
Guanidine hydrochloride	306	298	4.06	5.08	181	4.99	203
Thermoinactivation	165	124	4.06	3.86	137	3.58	146
FITC-labeled P450scc (oxidized)							
High-spin oligomer	43	20.5	3.98	2.81	100	2.46	100
Low-spin oligomer	59	34.0	3.98	2.98	106	2.70	110
High-spin monomer	62	38.5	3.98	3.02	107	2.76	112
FITC-labeled P450scc (reduced)							
Enzymatic reduction	65	42	3.98	3.06	109	2.80	114
CO-complex	74	55	3.96	3.11	111	2.93	119

R_0, the critical Forster radius calculated for different spectral states of FITC-cytochrome P450scc based on the integral of overlapping between donor (FITC) emission and acceptor (Q-bonds of heme) absorbance spectra using the computer program HAND.

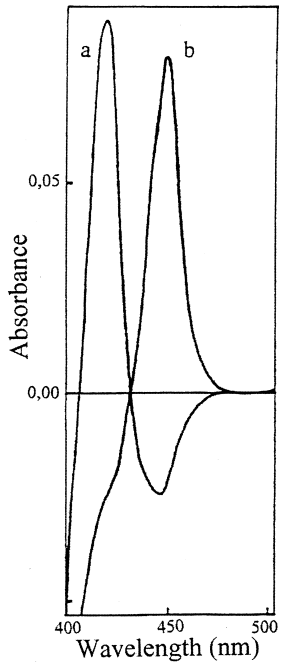

Fig. 4. Carbon monoxide difference spectra of reduced P450scc in its monomeric (a) and oligomeric (b) forms after 2 h storage at 20°C.

of oxidized P450scc is less stable than the high-spin form of the heme protein [1,3]. Our experiments show that monomeric P450scc in solution is completely denatured when stored for more than 2 h at room temperature, while oligomeric P450scc, treated in the same way, retains 90% of its P450 form (Fig. 4). Thus, the above-mentioned conformational changes are the result of destabilizing effects on the P450scc molecule and when one measures the efficiency of fluorescence energy transfer these conformational changes are expressed as an increase of the intramolecular distance between Lys^{338} and the heme group of P450scc.

Table 5
Effect of stabilizing agents on the changes of FITC fluorescence intensity (ΔF) and distance R between FITC-Lys^{338} and heme in recombinant P450scc molecule

FITC-labeled P450scc	ΔF (%)	R (nm)	R^* (nm)
Complex with adrenodoxin	42	2.24	2.46
Complex with cholesterol	35	2.49	2.70
Membrane-bound P450scc	44	2.42	2.70

R^*, distance between Lys^{338} and heme in control sample of FITC-labeled P450scc.

Participation of P450scc in intermolecular interactions (complex formation with cholesterol [35], insertion into a phospholipid membrane [1]) stabilizes the heme protein molecule. The measurement of the efficiency of resonance fluorescent energy transfer in the molecule of FITC-labeled P450scc stabilized by intermolecular interactions indicates a decrease of the distance between Lys^{338} and heme of P450scc (Table 5). The interaction of low-spin P450scc (prepared by enzymatic conversion of the bound endogenous substrate) with cholesterol results in a 35% decrease of FITC fluorescence intensity. The insertion of P450scc into an artificial phospholipid membrane, followed by conversion of the P450scc to its low-spin form, results in a decrease of FITC fluorescence intensity by 44%, compared to the low-spin form of the heme protein in solution. The formation of the complex of P450scc with adrenodoxin results in a 2-fold decrease of FITC fluorescence.

The data obtained indicate that measurement of resonance fluorescence energy transfer in the molecule selectively labeled at Lys^{338} may be used for

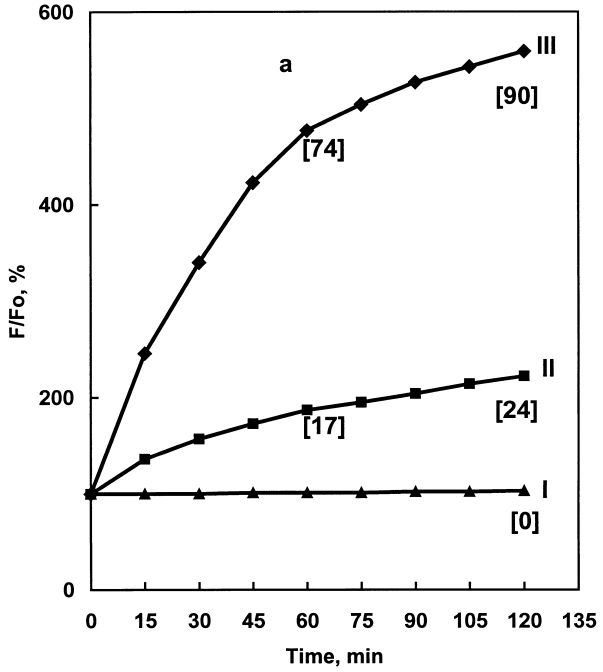

Fig. 5. Effect of temperature on the intensity of FITC fluorescence (524 nm) in P450scc: I, 4°C; II, 20°C; and III, 40°C. The data in the square brackets show the percent content of cytochrome P420 in the samples. Cytochrome P450scc concentration was 1 μM.

monitoring the conformational mobility of P450scc and allows one to detect the participation of P450scc in intermolecular interactions. These data also indicate that many of the biological functions of P450scc are carried out via the coordinated dynamics of its large polypeptide fragments and that these molecular changes are not restricted only by the conformational mobility of the active site.

Partially disturbing the conformational mobility of P450scc by inserting a large hydrophobic reagent molecule, i.e. chemical modification of the ε-amino group of Lys^{338} with FITC, without affecting directly the catalytic properties of P450scc, resulted in a decrease of the P450scc's stability. Fig. 5 shows the kinetics of fluorescence increase of FITC as influenced by incubation at different temperatures, which correlates with the increase of cytochrome P420 content in the sample of the labeled heme protein. At 4°C, the intensity of FITC fluorescence in labeled P450scc is practically not changed with time of incubation, while an increase of temperature results in an increase of FITC fluorescence. The rate of the increase of FITC fluorescence is highly dependent on the temperature at which P450scc is incubated. The carbon monoxide difference spectra of reduced P450scc confirms the appearance of cytochrome P420 in these samples (Fig. 5, data in square brackets). In contrast to FITC-labeled recombinant P450scc, the same concentration of native P450scc does not contain cytochrome P420 after incubation for 2 h at room temperature. The content of cytochrome P420 after incubation at 40°C does not exceed 40% (data not shown).

The decreased stability of FITC-labeled P450scc appears to result from a change of position or the degrees of freedom of movement for Lys^{338}. As a result of the inclusion of the fluorescent label, such changes may lead to a further unfolding of the polypeptide globule due to its inability to return to the initial conformation with minimal free energy [12] corresponding to the specific folding of the polypeptide chain in the region of Lys^{338}.

From that point of view, the dependence of the rate of denaturation on temperature for the FITC-labeled P450scc is best explained by the fact that an increase of temperature results in an increase of conformational mobility of the protein. The increase of conformational mobility of P450scc facilitates mutual transitions between different conformational states, thereby accelerating the relaxation processes that tend to move the system into its equilibrium state [8]. In our case, it appears that the relaxation process facilitates the tendency to unfold due to the change of the energy landscape (total energy) of P450scc caused by inclusion of the fluorophor molecule.

These results suggest that it may be possible to use site-directed mutagenesis to change the energetic landscape of the protein molecule [36]. It has been found that the conformational mobility of recombinant lysozyme increases after substitution of valine at position 6 for isoleucine [37]. Although this mutation does not change dramatically the catalytic properties of the enzyme, the authors suggest that there may be changes of protein function during evolution due to changes in the flexibility of the protein molecule after point mutations. It is also suggested that conformational changes in the P450 molecule may be due to mutations that alter or are responsible for a loss of catalytic activity [38]. Based on these facts it is suggested that a disturbance of the interaction between adrenodoxin and a molecule of the mutant recombinant P450scc [39] may be also connected

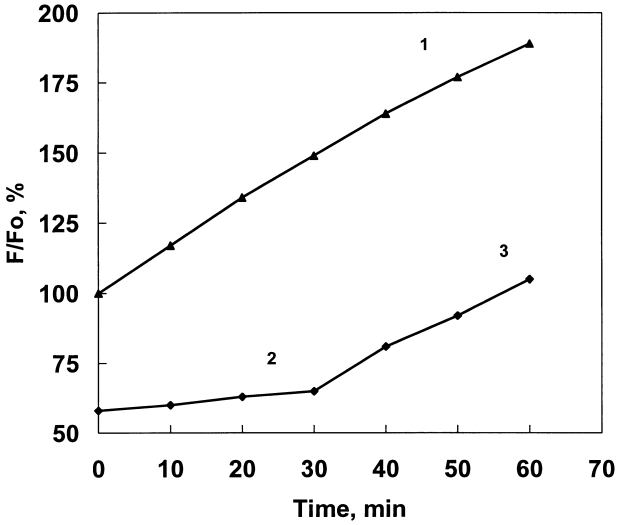

Fig. 6. Kinetics of the changes in intensity of fluorescence of FITC labeled P450scc (524 nm). 1, In the absence of adrenodoxin; 2, in the presence of 20-fold molar excess of adrenodoxin; 3, in the presence of 20-fold molar excess of adrenodoxin after addition of adrenodoxin reductase (10 μM). Temperature of measurement was 20°C. Cytochrome P450scc concentration was 1 μM.

with changes in the conformational mobility of the heme protein molecule.

The interaction of adrenodoxin with FITC-labeled P450scc decreases the rate of increase of FITC fluorescence intensity (Fig. 6). The amount of cytochrome P420 formed is also decreased (up to 2% per h). An addition of adrenodoxin reductase to the complex of P450scc and adrenodoxin restores the inactivation rate of the FITC-labeled heme protein. This result indicates that the flavoprotein causes a dissociation of the complex. A decrease in the rate of inactivation is also observed after substrate addition to FITC-labeled P450scc when compared to the rate of inactivation seen with only the low-spin form of P450scc. Membrane-bound FITC-labeled P450scc is also much more stable than the low-spin form of the heme protein.

The distance between Lys^{338} and heme in the molecule of FITC-labeled P450scc increases in the presence of destabilizing factors, while the opposite (i.e. a decrease in distance) is seen in the presence of stabilizing factors. Perhaps, stabilizing factors, such as an interaction of P450scc with adrenodoxin, or the formation of an enzyme–substrate complex with cholesterol, as well as the binding of the protein to a membrane, restrict the mobility of the P450scc molecule and fix it in a definite conformational state with a minimal energy corresponding to a more tight folding of the protein molecule.

The same interpretation can be made to explain the results of P450cam–substrate interactions by analyzing the temperature dependence of the CO band using IR spectra of the reduced carbon monoxide complex [9]. There is an indication of a decreased mobility of P450cam after its interaction with substrate [35]. This led to the conclusion that an increase of P450cam thermostability during the formation of the substrate–enzyme complex results in a decrease of the mobility of the heme microenvironment (heme pocket) where the thermoinactivation process of P450cam starts.

By analogy, we can explain the change of P450scc conformation after interaction with adrenodoxin [40]. This is shown by our experiments where we see a decrease of the distance between Lys^{338} and heme group and a decrease in the rate of P450scc inactivation to form cytochrome P420.

In summary, the results described in the present work allow one to make the conclusions listed below.

(1) We have shown that recombinant and native P450scc both have very similar structural, functional and immunochemical properties and thus recombinant P450scc may by used in model experiments instead of the native heme protein.

(2) Chemical modification of Lys^{338} of P450scc with FITC does not dramatically affect the interaction of P450scc with adrenodoxin. Some differences are noted between our experimental data presented here and previously published results. We explain these differences by: (a) the fact that no one previously has used immobilized adrenodoxin to purify FITC-labeled P450scc; and (b) there are some differences in modification conditions used.

(3) Selective chemical modification of P450scc Lys^{338} with FITC proved to be an efficient approach to follow conformational changes as well as reactions of intermolecular recognition by using resonance fluorescent energy transfer in the donor acceptor pair FITC–heme. Conformational changes taking place in P450scc are not only connected with the active site, but also look like large-scale conformational changes involving large fragments of a P450scc polypeptide chain.

(4) Chemical modification of P450scc Lys^{338} with FITC does not dramatically affect the functional properties of the heme protein, but disturbs its stability. This likely results from changing the conformational mobility of the heme protein molecule at the site of modification.

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