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Selective chemical modification of Cys^{264} with diiodofluorescein iodacetamide as a tool to study the membrane topology of cytochrome P450scc (CYP11A1)

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Abstract

Cys^{264} of cytochrome P450scc (CYP11A1) was selectively labelled with diiodofluorescein iodacetamide in solution and in proteoliposomes. The labelling affected the interaction of P450scc with adrenodoxin and significantly inhibited the side-chain cleavage activity of the soluble and membrane-bound hemeprotein in the reconstituted system. In proteoliposomes both the labelled and unlabelled hemepoteins were susceptible to trypsin and split into F1 and F2, two fragments corresponding to the two main domains of P450scc. These results suggest that the hinge connecting the two domains in the region Arg^{250}–Asn^{257} is exposed to the surface of the membrane and involved in the interaction of P450scc with adrenodoxin.

Key words: Cytochrome P450scc (CYP11A1); Domain; Membrane topology; P450 liposomes; Chemical modification; Bovine adrenal cortex

1. Introduction

The transformation of cholesterol (CHL) to pregnenolone (PG), the first step of adrenocortical steroidogenesis, is catalyzed by cytochrome P450scc (CYP11A1) localized in the inner mitochondrial membrane. For catalysis P450scc requires two electron transfer components, i.e. NADPH-adrenodoxin reductase (AR) and adrenodoxin (AD), the latter interacting directly with the hemeprotein [1,2]. The P450scc molecule contains two cysteine residues, i.e. Cys^{264} (Cys^{303} for the signal peptide-containing precursor) and Cys^{422} (Cys^{461}). The latter acts as the fifth ligand of the heme [3], whereas the function of the former is unknown.

We previously used antibodies against intact P450scc molecule and its two tryptic fragments, F1 (Ile^{1}–Arg^{250}) and F2 (Asn^{257}–Ala^{481}), to study the topology of the hemeprotein in the inner mitochondrial membrane [4,5]. In this paper we studied the functional importance of the central region of the P450scc sequence, which includes the trypsin-sensitive, interdomain hinge, and the topology of the region in proteoliposomes by using diiodofluorescein iodoacetamide (DIFIA)-labelled Cys^{264} as a reference marker.

2. Materials and methods

2.1. Chemicals and proteins

Sodium cholate, CHL, PG, cholestenon, progesterone, NADPH, Tween 20, trypsin, soybean trypsin inhibitor, protease from *S. aureus* V8, choleterol oxidase from *Nocardia erythropolis*, molecular weight standards for electrophoresis, and CL were all from Serva (Germany). Other lipids were from Lipid Products (UK). Guanidinium chloride, maleic acid anhydride, iodacetamide, DTT, solvents for HPLC were from Merck (Germany). Sepharose 4B, Sephadex G-25M, Sephacryl S-1000 were from Pharmacia (Sweden). Diiodofluorescein iodoacetamide (DIFIA) was synthesized according to [6] and kindly provided by Prof. A. Stier (Max Planck Institute for Biophysical Chemistry, Göttingen, Germany). P450scc, AD and AR were purified from bovine adrenocortical mitochondria to electrophoretic homogeneity using specific affinity adsorbents [7]. Antibodies against P450scc and its peptide fragments F1 and F2 were raised in rabbits and purified according to the procedure described earlier [4].

2.2. Labelling procedures

Labelling of P450scc was performed in 50 mM potassium phosphate buffer, pH 7.2, containing 0.15% sodium cholate, 0.5 M NaCl, 0.5 mM EDTA, 10% glycerol and 0.2% methanol (labelling buffer). P450scc (200 nmol) and DIFIA (2 μmol) were mixed in a final volume of 5 ml. Incubation was carried out at 4°C for 10 h and then at 23°C for 1 h. After incubation, the mixture was applied to a Sephadex G-25M column (2.5 × 12 cm) equilibrated with 50 mM sodium phosphate buffer (PB), pH 7.2, containing 20% glycerol (storage buffer) to remove the free label. The amount of noncovalently bound label in the final preparation was estimated by SDS-PAGE followed by scanning of the fluorescent bands by a Shimadzu Gel scanner GS-9000 at 517 nm.

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Abbreviations: PC, phosphatydyl choline; PE, phosphatydyl ethanolamine; CL, cardiolipin; DTT, 1,4,-dithiotreitol.

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P450scc incorporated into liposomes was labelled in 50 mM PB, pH 7.4, containing 0.1 M NaCl, 0.5 mM EDTA, 0.1 mM DTT and 20% glycerol (standard buffer). For labelling, 16 μl of the stock solution of DIFIA in the same buffer (DIFIA concentration of 2.353 mM) was added to 100 μl of a suspension of P450scc-containing liposomes (P450scc concentration, 37 μM). The mixture was incubated at 4°C for 8 h and then at 23°C for 30 min. Subsequently the sample was applied to a Sephadex G-25M column (0.4 × 2.5 cm) equilibrated with the same buffer. Protein-free liposomes were treated with DIFIA under the same conditions to assess possible nonspecific incorporation or adsorption of the label to the membrane.

2.3. Incorporation of P450scc into liposomes

P450scc and DIFIA–450scc were incorporated into unilamellar liposomes (PC/PE/CL/CHL, 2:2:1:0.6 by weight) by means of octylglucoside dialysis. A lipid mixture containing egg PC (3.5 mg), egg PE (3.5 mg), bovine heart CL (1.8 mg) and CHL (1.1 mg) was dried and then dissolved in 9 ml (final volume) of 50 mM potassium phosphate buffer, pH 7.4, containing 100 mM KCl, 0.5 mM EDTA, 0.1 mM DTT, 20% glycerol, 4 μM P450scc (or DIFIA–P450scc) and 0.43% octylglycoside (lipid/protein ratio, 5:1 by weight). The mixture was dialysed at 4°C for 48 h against standard buffer in the presence of the Bio-Beads SM-2 (Bio-Rad, USA). The dialysed mixture was then gel-filtrated and fractionated on Sephacryl S-1000 and fractions of largest vesicles were collected. Finally, the suspension of P450scc-liposomes was concentrated using a Centricon-100 microconcentrator (Amicon, USA) to a P450scc concentration of 16–30 μM.

2.4. Interaction with adrenodoxin and enzymatic activity of DIFIA–P450scc

Differential spectral titration of P450scc with AD was performed according to [8] in 50 mM PB, pH 7.4 containing 25 μM CHL and 0.03% Tween 20 at 23°C. Defined amounts of AD were added from a stock solution to avoid final dilutions of the reaction mixtures greater than 4% [9].

CHL side chain cleavage activity of P450scc was determined in the reconstituted system as described earlier [4] followed by treatment of the reaction mixture with CHL oxidase to convert CHL and PG into the 3-one-4-en steroids, i.e. cholestenon and progesterone, respectively [10]. The conversion reaction was initiated by addition of 50 μl of CHL oxidase (0.4 U) in 20 mM potassium phosphate buffer, pH 7.4, containing 1% sodium cholate, to 1 ml of the reaction mixture. After incubation at 37°C for 10 min, the steroids were extracted with dichloromethane. The extract was dried under a stream of nitrogen and analysed by reverse-phase HPLC on a Nucleosil-gel $5C_{18}$ column (4 × 100 mm; Macherey-Nagel, Germany) with a gradient solvent system of 70–100% of methanol.

2.5. Proteolysis of soluble and membrane-bound DIFIA–P450scc with trypsin

DIFIA–P450scc and DIFIA–P450scc-containing liposomes were diluted with standard buffer up to a P450scc concentration of 3 μM. Trypsin was added to the suspension up to the molar ratio P450scc/trypsin of 50:1 or 20:1. The suspension was incubated for 30–60 min at 23°C and then soybean trypsin inhibitor added up to a ratio inhibitor/trypsin of 4:1. The samples were incubated 60 min at 4°C. 2-Mercaptoethanol and SDS were added to the sample up to 5% and 2%, respectively. The sample was boiled for 3 min and then analysed by SDS-PAGE followed by immunoblotting in the presence of antibodies against P450scc and its fragments F1 and F2.

2.6. Analytical methods

SDS-PAGE was carried out according to Laemmli [11] in a Bio-Rad Mini-Protean II Cell. To detect DIFIA–P450scc and its fragments after proteolysis, gels were scanned and processed by an imagination system consisting of Cromato-VUE Transilluminator TM-36 (UVP, USA) as

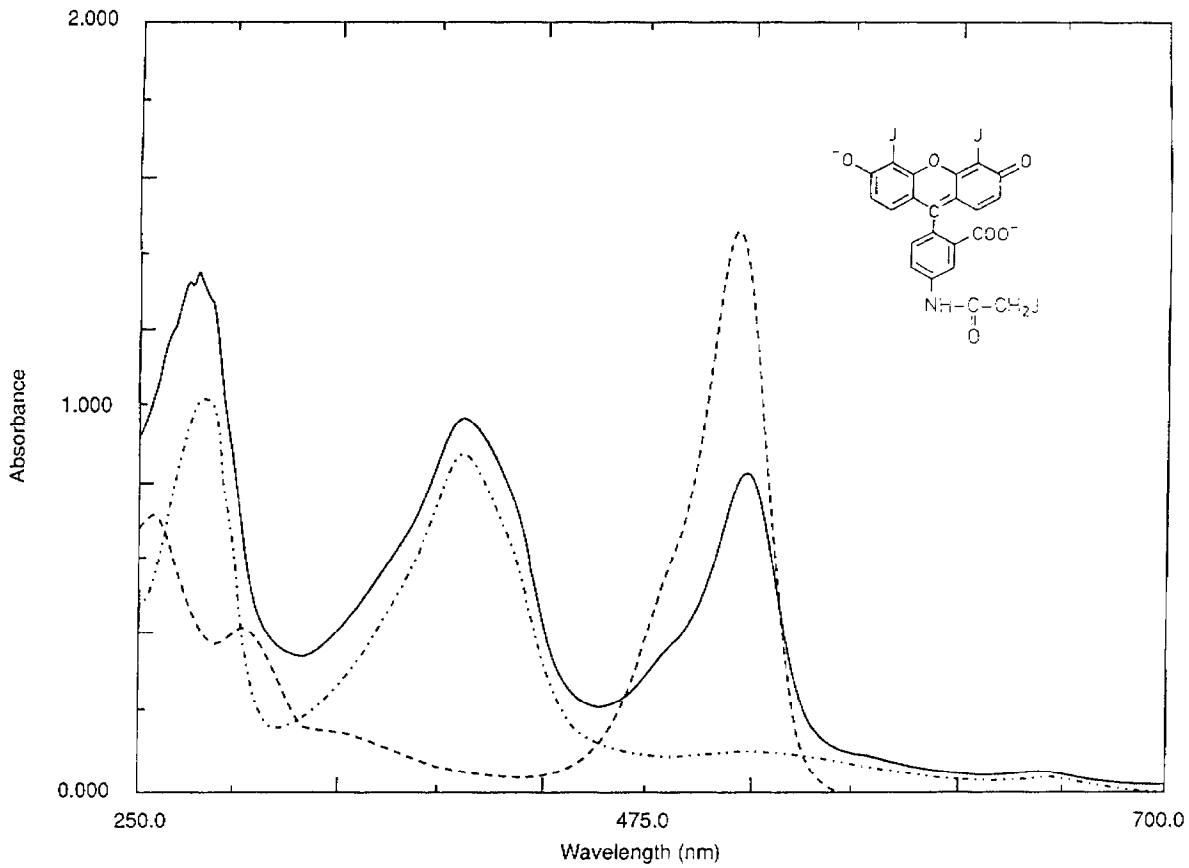

Fig. 1. Labelling of P450scc in solution with DIFIA. (– ·· – ·· –) intact P450scc in labelling buffer, (- - - -) DIFIA in labelling buffer; (——) DIFIA–P450scc in storage buffer. Inset: chemical structure of DIFIA.

an UV-light source and Mitsubishi video copy processor (Mitsubishi, Japan). After scanning the gel was stained with Coomassie BB R-250. Immunoblotting was performed in Bio-Rad Mini-Transblot apparatus according to the procedure described previously [4].

P450scc concentrations were determined from the absolute spectra or from CO-difference spectra of dithionite-reduced preparations of liposomes using an extinction coefficient of 91 $mM^{-1} \cdot cm^{-1}$ at 393 or 450 nm, respectively [12].

The amount of DIFIA bound to P450scc was determined from its characteristic absorbance at 517 nm using an extinction coefficient of 75 $mM^{-1} \cdot cm^{-1}$.

Proteolysis of DIFIA–P450scc with *S. aureus* V8 protease was performed in 0.1 M ammonium bicarbonate buffer, pH 8.3, according to [13]. Separation of the peptide products labelled with DIFIA was carried out by reverse-phase HPLC as described [14]. Structure analysis of the peptide fractions was carried out using a Gas-phase Sequenator 477A from Applied Biosystem (USA) followed by on-line detection of the PTH-amino acids by HPLC according to standard programs.

3. Results

3.1. Labelling of P450scc with DIFIA

Labelling of P450scc with DIFIA, an SH-specific fluorescent reagent, caused a shift of the absorption maximum of the fluorophore from 512 nm to 516–518 nm (Fig. 1). The use of 50 mM potassium phosphate buffer containing 0.5 M NaCl and 0.15% sodium cholate prevented the inactivation of P450scc and its conversion into the P420 state (confirmed by the reduced CO difference spectrum) during the labelling procedure. After gel filtration of the labelled hemeprotein on Sephadex G-25M, the amount of noncovalently bound DIFIA decreased to 7–9.5% as determined by SDS-PAGE followed by absorbance scanning at 517 nm. On the other hand, in P450scc labelled with diiodofluorescein isothiocyanate, a lysine-specific, hydrophobic reagent, about 60% of the label was noncovalently bound even after gel filtration [15]. The DIFIA–P450scc prepared by the method described in section 2.2 contained about 1 mole of DIFIA per mole of the hemeprotein. When the labelled protein was digested with *S. aureus* V8 protease and the peptide fragments were separated by HPLC, only one major labelled peptide (absorbing at 517 nm) was obtained and its sequence was determined to be Phe-Arg-Asn-Tyr-Pro-Gly-Ile-Leu-Tyr-Cys(*)-Leu-Leu-Lys-Ser-Glu, where * indicates the position of the label. This peptide corresponds to the P450scc sequence Phe^{255}–Glu^{269} [16,17]. No other labelled peptides were found. It was therefore concluded that DIFIA specifically modifies Cys^{264} leaving the second cysteine residue Cys^{422} unlabelled and that the central region of P450scc including Cys^{264} is accessible for the chemical modification under the conditions employed. Treatment of liposomal bound P450scc with DIFIA also resulted in specific labelling of the hemeprotein (data not shown). However, the final DIFIA/P450scc molar ratio was only 0.5–0.6. Neither an increase in the incubation time nor the use of a large excess of DIFIA enhanced the amount of bound DIFIA.

3.2. Interaction of DIFIA–P450scc with AD

Although DIFIA labelling of P450scc did not change the structure around the heme significantly (monitored by EPR spectroscopy of the heme iron, not shown) and did not convert the hemeprotein into the P420 state, the labelling affected the interaction of P450scc with AD. Fig. 2A shows the results of spectral titration of P450scc with AD, indicating that AD binds to the hemeprotein with a spectral dissociation constant (K_s) of 3.8 μM.

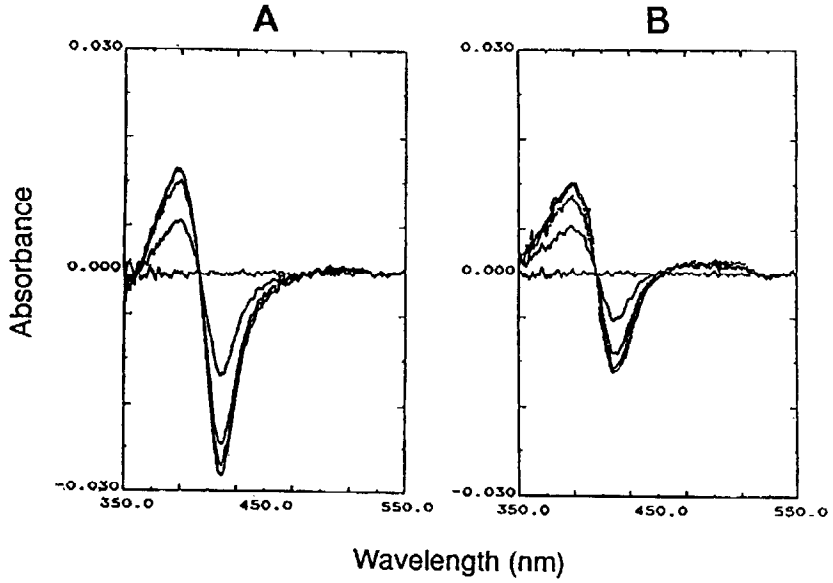

Fig. 2. Spectrophotometric titration of intact P450scc (A) and DIFIA–P450scc (molar ratio 1:1) (B) The concentration of P450scc was 2.3 μM. AD was sequentially added up to the final concentrations 3.4, 10.4, 17.3 and 24.2 μM.

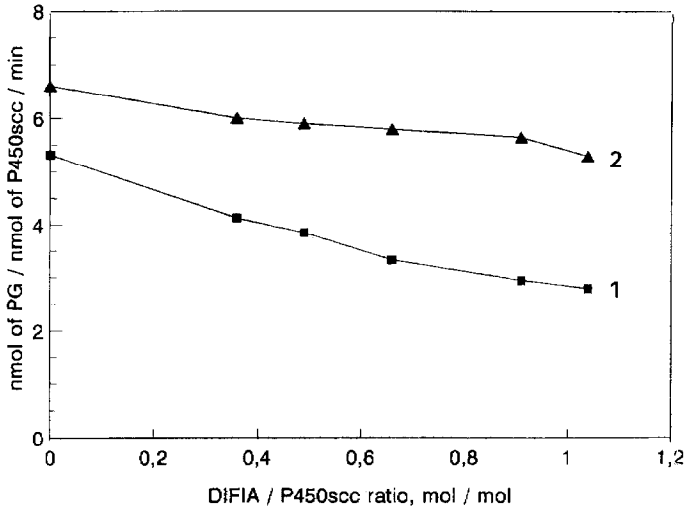

Fig. 3. Enzymatic activity of DIFIA–P450scc preparations with different molar ratio of labelling. The reconstituted system contained in a final volume of 1 ml 0.5 μM P450scc or DIFIA–P450scc, 0.4 μM of AR, 100 μM CHL, 360 μM NADPH and 2.5 μM (line 1) or 5 μM (line 2) AD in 50 mM PB, pH 7.4, containing 0.2% Tween 20. The activity of intact P450scc is 5.3 nmol of PG/nmol of P450scc/min for the molar ratio AD/P450scc of 5:1 (line 1) and 6.6 nmol of PG/nmol of P450scc/min for the rato 10:1 (line 2).

When DIFIA–P450scc was used, the spectral change induced by AD binding was significantly diminished with a K_s value of 6.0 μM (Fig. 2B). The maximal spectral change was also decreased 1.6-fold by the labelling. It was thus evident that DIFIA labelling significantly reduces the capacity of P450scc to interact with AD.

3.3. *Effect of DIFIA labelling on the reconstituted enzyme activity*

Since DIFIA labelling of P450scc decreases its ability to bind AD, it is not surprising that the labelling also affected its CHL side-chain cleavage activity in the reconstituted system. The inhibitory effect depends on both the amount of DIFIA bound to P450scc and the AD/P450scc molar ratio in the reaction mixture (Fig. 3). A 10-fold excess of AD almost prevents the inhibition, but at a ratio AD/P450scc of 5 the activity of DIFIA–P450scc (1:1 molar ratio) was only 53% of that of the intact hemeprotein. We next measured the activities of variously composed reconstituted systems consisting of NADPH, AR, AD and (1) intact P450scc, (2) DIFIA–P450scc (1:1), (3) DIFIA–P450scc-containing liposomes, (4) P450scc-containing liposomes, or (5) P450scc-containing liposomes treated with DIFIA after incorporation of the hemeprotein into the vesicles (final DIFIA/P450scc ratio = 0.51). As shown in Table 1, liposomal bound intact P450scc can catalyze the conversion of CHL to PG more effectively (81% stimulation) than in solution. Treatment of liposomal bound P450scc with DIFIA inhibited the activity by 38%. On the other hand, liposomes containing P450scc that had been labelled (DIFIA/P450scc molar ratio = 1) before incorporation into vesicles retained only 32% of the activity as compared to liposomes containing intact P450scc.

3.4. *Tryptic digestion of soluble and liposomal bound DIFIA–P450scc*

Limited tryptic digestion of DIFIA–P450scc (molar ratio 1:1) led to the formation of two main peptide fragments having apparent molecular masses between 26,000 and 29,000 Da as estimated by SDS-PAGE (Fig. 4B, lanes 6 and 7). Only one of these fragments having lowest molecular mass was fluorescent (Fig. 4A, lanes 6 and 7). These molecular masses correspond to those of the fragments F1 (Ile^{1}–Arg^{250}) and F2 (Asn^{257}–Ala^{481}) (Fig. 4B, lanes 1 and 2), which were previously detected after trypsinolysis of unlabelled P450scc [18]. Soybean trypsin inhibitor inhibited the formation of F1 and F2 from intact P450scc (Fig. 4B, lanes 3 and 4). Separation of the trypsin digest of DIFIA–P450scc by SDS-PAGE followed by immunoblotting with antibodies raised against F1 and F2 actually showed that the fragments were reactive with the antibodies (not shown). Limited trypsinolysis of liposomal bound intact P450scc followed by SDS-PAGE revealed that the hemeprotein was digested to F1 and F2 to a comparable extent as unbound P450scc (not shown). Labelling of liposomal bound P450scc with DIFIA and subsequent trypsin digestion (not shown) as well as trypsinolysis of liposomal bound DIFIA–P450scc (1:1) (Fig. 4B, lanes 8 and 9) also gave similar eletrophoretic patterns on SDS-PAGE. In all these cases, immunoblotting using anti-F1 and anti-F2 antibodies showed that the label was detectable only in F2. These results indicate that the hinge region (Arg^{250}–Asn^{257}) of intact and DIFIA-modified P450scc is accessible to trypsin both in solution and in proteoliposomes. It can also be concluded that labelling of P450scc with DIFIA does not influence the intramolecular location of this hinge region.

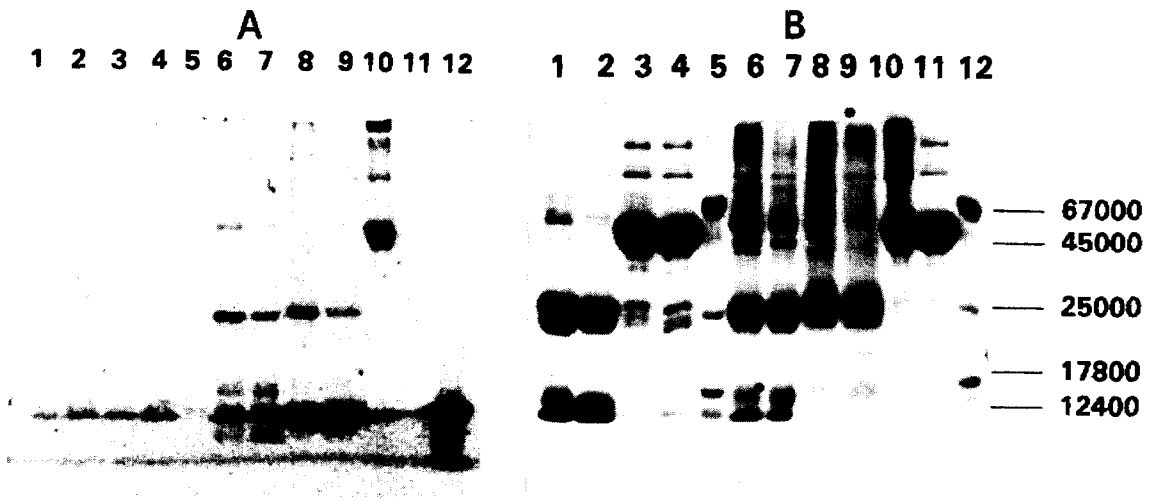

Fig. 4. Limited proteolysis of soluble and membrane-bound DIFIA–P450scc with trypsin. (A) unstained 12% PAGE SDS-electrophoresis slab photographed under UV light. (B) the slab stained with Coomassie BB R-250. Samples contained equal amounts of: 1, soluble P450scc treated with trypsin at molar ratio P450scc/trypsin of 50:1 for 30 min; 2, as 1 (the ratio 20:1, 60 min); 3 and 4, P450scc preincubated with soybean trypsin inhibitor and after that treated with trypsin under the conditions of the samples 1 and 2, respectively; 5, molecular weight standards (shown in Da); 6 and 7, DIFIA–P450scc with molar ratio of the labelling 1:1 treated with trypsin under the conditions of the samples 1 and 2, respectively; 8 and 9, DIFIA–P450scc proteoliposomes treated with trypsin under the same conditions; 10, DIFIA–P450scc; 11, soluble P–450scc; 12, molecular weight standards as in 5+ pure DIFIA. The samples containing P450scc and DIFIA–P450scc were taken in highest excess to be analysed by UV scanning.

4. Discussion

Despite numerous studies on membrane-bound P450-dependent oxygenase systems (for review see [1,2,19]), little is known of the mechanism of inner mitochondria membrane-associated electron transfer from AR to P450scc via AD and the topology of the hemeprotein in the membrane. Based on studies on limited proteolysis of the inner mitochondrial membrane and immunochemical assay of P450scc, we have recently suggested that P450scc penetrates the membrane and the trypsin-sensitive hinge region (Arg^{250}–Asn^{257}) is exposed to the mitochondrial matrix space [5,7].

Table 1
Enzymatic activity of membrane-bound P450scc and DIFIA–P450scc*

Preparation	Activity (nmol of PG/nmol of P450scc/min)	%
P450scc-liposomes	4.73	181.0
P450scc-liposomes labelled with DIFIA (1:0.51)	2.94	112.6
DIFIA-P450scc (1:1) liposomes	1.53	58.6
DIFIA-P450scc (1:1) in solution	1.23	47.1
P450scc (intact) in solution	2.61	100.0

*P450scc and DIFIA–P450scc were incorporated into liposomes consisting of PC/PE/CL/CHL with a weight ratio 2:2:1:0.6. The reconstituted system contained in a final volume of 1 ml: 0.5 μM P450scc, 4 μM AD, 0.28 μM AR, 100 μM CHL and 360 μM of NADPH in 50 mM PB, pH 7.4 with 0.01% Tween 20. To prevent the solubilization of liposomes, the concentration of Tween 20 was decreased in comparison with the standard activity experiments described above for DIFIA–P450scc in aqueous solution (Fig. 3).

The results obtained in this work suggest that the interdomain hinge in the P450scc sequence including Cys^{264} is directly involved in the interaction of hemeprotein with AD on the surface of the liposomal membrane. It is also likely that this interaction occurs outside the phospholipid membrane between the hinge region, which is protruding to the outside, and externally added AD. However, it cannot as yet be decided whether Cys^{264} is directly involved in the interaction with AD or the inhibition of AD binding is due to a steric hindrance effect of the relatively bulky DIFIA molecule. Taken together, the results described above provide evidence that (1) Cys^{264} of P450scc can be specifically labelled with DIFIA without changing the basic structure of the hemeprotein, (2) Cys^{264} is located at or near the AD binding area, and (3) this area is exposed to the outer surface of the liposomal membrane and accessible to AD added externally. It should be noted that the central region of the bovine P450scc sequence contains two lysine residues, i.e. Lys^{267} and Lys^{272}, which have been identified by chemical modification with succinic anhydride as being involved in AD binding through charge ion pairing [14]. It has also been found that the P450scc sequence Leu^{266}–Leu^{272} is one of the antigenic determinants of the P450scc molecule [20] and antibodies against P450scc and F2 fragment have been shown to strongly inhibit the CHL side-chain cleavage activity of P450scc [4].

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