Pergamon

0960-0760(95)00199-9

J. Steroid Biochem. Molec. Biol. Vol. 55, No. 5/6, pp. 507–514, 1995

0960-0760/95 $9.50 + 0.00

Organization of 3β-Hydroxysteroid Dehydrogenase/Isomerase and Cytochrome $P450_{scc}$ into a Catalytically Active Molecular Complex in Bovine Adrenocortical Mitochondria

N. Cherradi, E. M. Chambaz and G. Defaye*

CEA, Biochimie des Régulations Cellulaires Endocrines, INSERM Unité 244, Département de Biologie Moléculaire et Structurale, CEN.G, 17 rue des Martyrs, F-38054 Grenoble Cedex 9, France

We have previously reported the co-localization [Cherradi *et al.*, *Endocrinology* 134 (1994) 1358–1364] of 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD) and cytochrome $P450_{scc}$ (cyt. $P450_{scc}$) in the inner membrane and in the intermembrane contact sites of adrenocortical mitochondria. This observation raises the question of a possible functional association between the two proteins. Isolated bovine adrenocortical mitochondria are able to convert cholesterol to progesterone without the need of exogenous cofactors. An association of 3β-HSD and cyt. $P450_{scc}$ is observed during the purification of 3β-HSD from mitochondria. The behaviour of 3β-HSD on a column of Heparin–Sepharose is modified by the presence of cyt. $P450_{scc}$. Immunoprecipitations from mitochondria with either anti-cyt. $P450_{scc}$ or anti 3β-HSD antibodies result in a co-precipitation of the two proteins. Both proteins engaged in these immunocomplexes are catalytically active. The interaction was further demonstrated by the surface plasmon resonance method using purified components. An affinity constant of 0.12 μM between 3β-HSD and $P450_{scc}$ was obtained. These observations suggest that $P450_{scc}$ and 3β-HSD may associate into a molecular complex in the mitochondrial compartment and may constitute a functional steroidogenic unit, thus opening new possibilities in the regulation of the production of progesterone and its flow in the adrenocortical cell.

J. Steroid Biochem. Molec. Biol., Vol. 55, No. 5/6, pp. 507–514, 1995

INTRODUCTION

The biosynthesis of hormonal steroids from cholesterol involves several hydroxylations catalysed by enzymatic complexes of which the terminal enzyme is a specific cytochrome *P*450 (cyt. *P*450) [1, 2]. These complexes are localized either in mitochondria or in endoplasmic reticulum. In mitochondria, the side chain of cholesterol is cleaved by cyt. $P450_{scc}$ giving pregnenolone and in the same organelle are also carried out the last steps of corticosterone, cortisol and aldosterone formation, i.e. 11β- and 18-hydroxylations. In the endoplasmic reticulum are found 17α and 21-hydroxylases.

In the steroidogenic pathway, the enzyme 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD) catalyses the oxydative conversion of 3β-hydroxy-Δ^5-steroids, such as pregnenolone, to 3-keto-Δ^4-steroids, such as progesterone [3]. Therefore, 3β-HSD plays a crucial role in the biosynthesis of all classes of active hormonal steroids, progesterone, mineralocorticoids and glucocorticoids as well as androgens and oestrogens.

Steroidogenesis is thus characterized by an important flow of substrates from mitochondria to endoplasmic reticulum and back to mitochondria. The mechanism regulating this intracellular traffic is not well understood.

The enzyme 3β-HSD is found both in endoplasmic reticulum and in mitochondria [4, 5] and it utilizes NAD^+ as the only coenzyme factor. In mitochondria, it functions by using endogenous NAD^+. 3β-HSD and

Proceedings of the Workshop on the Molecular and Cell Biology of Hydroxysteroid Dehydrogenases, Hannover, Germany, 19–22 April 1995.

*Correspondence to G. Defaye.

cyt. $P450_{scc}$ are localized in the same mitochondrial compartments, i.e. in the inner membrane and in the intermembrane contact sites [5]. It has been postulated that these contact sites are involved in the increased transport of cholesterol to the inner mitochondrial membrane that occurs under acute action of ACTH [6]. Sauer *et al.* [4], as well as Cherradi *et al.* [7], have suggested that the mitochondrial isoform of the 3β-HSD is located with its active site facing the matrix space. Since the cyt. $P450_{scc}$ also has its active site facing the matrix [6], the direct transformation of cholesterol to progesterone would be possible due to the presence of cyt. $P450_{scc}$ and 3β-HSD in the same mitochondrial compartments.

In the present study, we show that isolated mitochondria are able to produce progesterone from cholesterol when substrates of the tricarboxylic acid cycle are provided. This report also provides evidence that cyt. $P450_{scc}$ is tightly associated with 3β-HSD. The two proteins copurify throughout sequential steps of chromatographic separation. *In vitro* studies using either purified enzymes or isolated mitochondria show that the two proteins retain their catalytic activities in the molecular complex. This interaction has been further confirmed by surface plasmon resonance measurements [8, 9].

Such a molecular organization would introduce a novel concept in the functional regulation of adrenocortical steroidogenic functions.

MATERIALS AND METHODS

Specific reagents

Synthetic 1-24 ACTH (Synacten) and metyrapone were provided by Ciba (Basel, Switzerland). [^{3}H]Pregnenolone was purchased from the Radiochemical Center (Amersham, England). Anti-progesterone antibodies were obtained from Biosys (France). Specific rabbit anti-pregnenolone, anti-$P450_{scc}$ and anti-3β-HSD polyclonal antibodies were raised in our laboratory. Aminogluthetimide was from Sigma and trislostane was a generous gift from Wintrop (France).

Preparation of bovine adrenocortical cells (BAC cells) and subcellular fractions

BAC cells were prepared by successive digestion of fresh adrenal glands (fasciculata-reticularis zone) according to Duperray and Chambaz [10]. For the preparation of subcellular fractions, the cell layer was scraped off and homogenized in a Tris (5 mM, pH 7.4), saccharose (250 mM) buffer. After centrifugation (600 *g*, 10 min), the supernatant was centrifuged at 10,000 *g* for 10 min. The pellet corresponds to the mitochondrial fraction. The supernatant was further centrifuged at 100,000 *g* for 30 min to yield the microsomal fraction.

Pregnenolone and progesterone production by adrenocortical mitochondria

After 5 days of culture, BAC cells were incubated without (control) or with ACTH (3×10^{-7} M) for 2 h, in Ham's F12 medium containing aminogluthetimide (0.5 mM). Isolated mitochondria (150 μg of protein) were incubated for 10 min at 37°C without or with trilostane (10 μM) in Tris–HCl (10 mM, pH 7.4) buffer containing $MgCl_2$ (5 mM), KCl (10 mM), saccharose (250 mM) and metyrapone (an inhibitor of cyt. $P450_{11\beta}$) (5 μM) to a final volume of 500 μl. The reaction was started by addition of a mixture of malate and isocitrate (10 mM final concentration of each) then stopped by addition of dichloromethane. Pregnenolone and progesterone, extracted by the organic solvent, were quantified by radioimmunoassay.

Purification of mitochondrial 3β-HSD and cyt. $P450_{scc}$

Cyt. $P450_{scc}$ was partially purified from bovine adrenocortical mitochondria following the procedure of Suhara *et al.* [11]. Mitochondria were sonicated and the membrane proteins extracted by cholate (1%, w/v). Solubilized proteins were recovered in the supernatant of a 100,000 *g* centrifugation (45 min). Ammonium sulfate (32–45% of saturation) precipitation was followed by a chromatographic purification step using aniline–Sepharose. The cytochrome $P450$ concentration was assayed using the CO-induced difference spectrum as described by Omura and Sato [12].

The purification of mitochondrial 3β-HSD was assayed as previously described for the microsomal enzyme [13]. Briefly, mitochondrial proteins (10 mg/ml) were solubilized by cholate (0.6%, w/v) in the presence of NAD^+ (0.01 mM) then loaded on a DEAE–cellulose column. The eluted fractions having a high 3β-HSD specific activity were pooled. This pool was loaded on a Heparin–cellulose column. The mitochondrial enzyme was eluted with the equilibration buffer containing 1 M KCl.

N-terminal amino acid sequencing and tryptic cleavage of mitochondrial 3β-HSD

These experiments were carried out by J. Garin (CEN-G, Grenoble) by automated Edman degradation using an Applied Biosystem gas phase sequencer. After SDS–PAGE and transfer on a Problott membrane, the band corresponding to 3β-HSD was cut and Edman degradation was performed.

Tryptic digestion was carried out on 3β-HSD eluted from the SDS–PAGE gel. The digestion was performed with TPCK-treated trypsin in an enzyme/substrate ratio of 1:20 (mol/mol).

Immunoprecipitation of 3β-HSD and cyt. $P450_{scc}$ from mitochondria

At day 4 of culture, BAC cells were washed with DMEM medium without methionine containing 5%

(v/v) of Ham's F-12 then incubated in the same medium in the presence of foetal calf serum (10%, v/v) and [^{35}S]methionine (50 μCi/ml) during 15 h.

Subcellular fractionation was performed. Mitochondrial and microsomal membrane proteins were extracted with cholate (0.6%, w/v). After centrifugation (100,000 *g*, 20 min), the supernatant was diluted (0.1 mg/ml) in RIPA buffer and incubated with anti-3β-HSD or anti-$P450_{scc}$ antibodies (dilution 1/100) for 2 h at 4°C, followed by incubation with Protein-A Sepharose for 30 min. Immunoprecipitates were collected and washed 4 times with the immunoprecipitation buffer. The final immunocomplexes were either analysed by SDS–PAGE [14] followed by autoradiography or assayed for 3β-HSD and cyt. $P450_{scc}$ enzymatic activities.

3β-HSD activity

3β-HSD activity was monitored by using [^{3}H]pregnenolone (20 μM) as substrate and NAD^+ (200 μM) in 50 mM (K)phosphate, pH 7.4 containing 0.1 mM EDTA. Incubation time was 5 min at 37°C. The steroids were separated by silica gel TLC with chloroform/acetone (90/10 v/v) as solvent using commercial samples of pregnenolone and progesterone as standards.

Side chain cleavage activity

Side chain cleavage activity was assayed by monitoring the formation of [^{3}H]pregnenolone from [^{3}H]cholesterol as described by Katagiri *et al.* [15]. The reaction mixture (300 μl) contained cyt. $P450_{scc}$ (20–50 pmol), adrenodoxin (1.2 nmol), adrenodoxin reductase (0.1 nmol), NADPH (120 nmol) and [^{3}H]cholesterol (60 nmol, 0.02 μCi) in phosphate buffer (30 mM, pH 7.4) containing Tween 20 (0.3%, w/v). The reaction was run for 3 min at 37°C and the steroids separated by TLC in cyclohexane/ethyl acetate (6/4 v/v).

Protein complex formation

Purified microsomal 3β-HSD (1 nmol) and cyt. $P450_{scc}$ (5 nmol) were incubated for 15 min at room temperature in buffer B (0.5 ml). The mixture was either loaded on to a Heparin–Sepharose column equilibrated as for 3β-HSD purification, or immunoprecipitated with anti-$P450_{scc}$ or anti-3β-HSD antibodies.

Surface plasmon resonance analysis of 3β-HSD and $P450_{scc}$ interaction

The interaction between 3β-HSD and cyt. $P450_{scc}$ was investigated with a new Biosensor technology called real time biospecific interaction analysis (RT BIA), using plasmon resonance (BIACORE, Pharmacia). Purified microsomal 3β-HSD was immobilized on to the hydrogel matrix on a CM5 sensorchip. After activation of the matrix with a mixture of *N*-hydroxysuccinimide and *N*-ethyl-*N*′-[3-(diethylamino) propyl]carbodiimide (EDC) to form reactive esters, 3β-HSD was diluted (1/10) in 10 mM acetate buffer (pH 5.2) and injected in the activated sensorchip. Unreacted sites on the matrix were blocked with 1 M ethanolamine hydrochloride (pH 8.5). The immobilized amount obtained was about 8000 RU corresponding to about 8 ng of 3β-HSD/mm^2. Different concentrations of cyt. $P450_{scc}$ (3000, 1500, 750 and 375 nM) were then injected on to the immobilized 3β-HSD. The sensorgrams obtained from the interaction of 3β-HSD and cyt. $P450_{scc}$ provide the basis for calculation of association and dissociation rate constants and under saturating conditions also the stoichiometry of the interaction.

RESULTS

Cytochrome $P450_{scc}$ and 3β-HSD activities in bovine adrenocortical mitochondria

The formation of pregnenolone and progesterone by isolated mitochondria was studied in the absence of exogenous electron donors (NADPH and NAD^+). Mitochondria from ACTH-stimulated and untreated cells were used. By treating the cells with ACTH and aminogluthetimide, an inhibitor of cyt. $P450_{scc}$, the inner mitochondrial membrane accumulates endogenous cholesterol. Mitochondria were then incubated with malate and isocitrate both being components of the tricarboxilic cycle. Figure 1 shows the conversion of cholesterol to pregnenolone (in the presence of

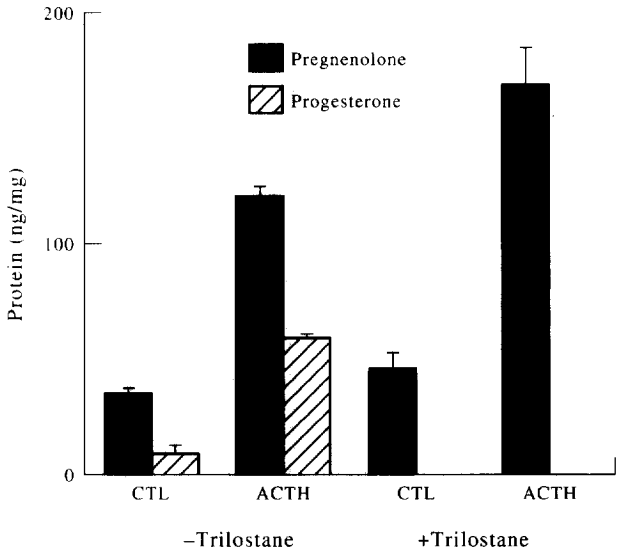

Fig. 1. Endogenous production of progesterone by isolated mitochondria from BAC cells. Isolated mitochondria from BAC cells treated without or with ACTH (10^{-8} M), in the presence of aminogluthetimide (0.5 mM), were incubated during 10 min with a mixture of malate/isocitrate (1/1, v/v, final concentration 10 mM of each). Pregnenolone and progesterone were extracted and quantified by a radioimmunoassay. Results are the mean ± SD of three independent experiments.

trilostane, an inhibitor of 3β-HSD activity) and of pregnenolone to progesterone (in the absence of trilostane), by bovine adrenal mitochondria. As expected [6], the higher cholesterol content in the mitochondria derived from ACTH-treated cells led to a 4 times greater production of pregnenolone than in mitochondria derived from untreated cells. In the absence of trilostane, pregnenolone is transformed into progesterone. These results show that mitochondrial 3β-HSD can metabolize pregnenolone, produced by the side chain cleavage of endogenous cholesterol to progesterone, using endogenous NAD^+ available in the matrix space.

Copurification of 3β-HSD and $P450_{scc}$ from adrenocortical mitochondria

The presence of active 3β-HSD in the mitochondria raises the question of its identity with the microsomal enzyme in terms of catalytic properties and amino acid sequence. The purification of mitochondrial 3β-HSD was attempted following the procedure previously described for the microsomal enzyme [13] using two chromatographic steps. The mitochondrial enzyme was eluted from a DEAE–cellulose column with an increased concentration of detergent as the microsomal enzyme. The 3β-HSD containing fractions were loaded onto a Heparin–Sepharose column and enzymatic activity determined in the emerging fractions. As illustrated in Fig. 2(A), only a weak 3β-HSD activity was found in the filtrate of the Heparin–Sepharose (about 10% of the total activity loaded). In contrast, the microsomal 3β-HSD was recovered in the filtrate of the Heparin–Sepharose column (95% of the total activity loaded). SDS–PAGE analysis of the fractions, corresponding to the loaded material (lane L), the filtrate (lane F) and the elution with KCl (1 M) (lane E) are shown in Fig. 2(B). Small amounts of 3β-HSD (43 kDa) and cyt. $P450_{scc}$ (50 kDa) were recovered in the filtrate. The isomerase was eluted together with cyt. $P450_{scc}$ and cyt. $P450_{11\beta}$. The figure also illustrates the SDS–PAGE analysis of the microsomal 3β-HSD. In this case, the enzyme was not retained on the column and was homogeneous after this chromatographic step. Therefore, the Heparin–Sepharose column is not a purification step for the mitochondrial enzyme as it was for the microsomal one.

Other chromatographic steps, such as aniline–Sepharose, octyl-Sepharose, ammonium sulfate precipitation, also led to a copurification of 3β-HSD together with cyt. $P450_{scc}$ (data not shown). These results indicate that the two proteins, cyt. $P450_{scc}$ and 3β-HSD copurify throughout different sequential chromatographic steps.

To ensure that the presence of cyt. $P450_{scc}$ is responsible for the behaviour of 3β-HSD on the Heparin–Sepharose column, the purified microsomal enzyme and a partially purified cyt. $P450_{scc}$ fraction were

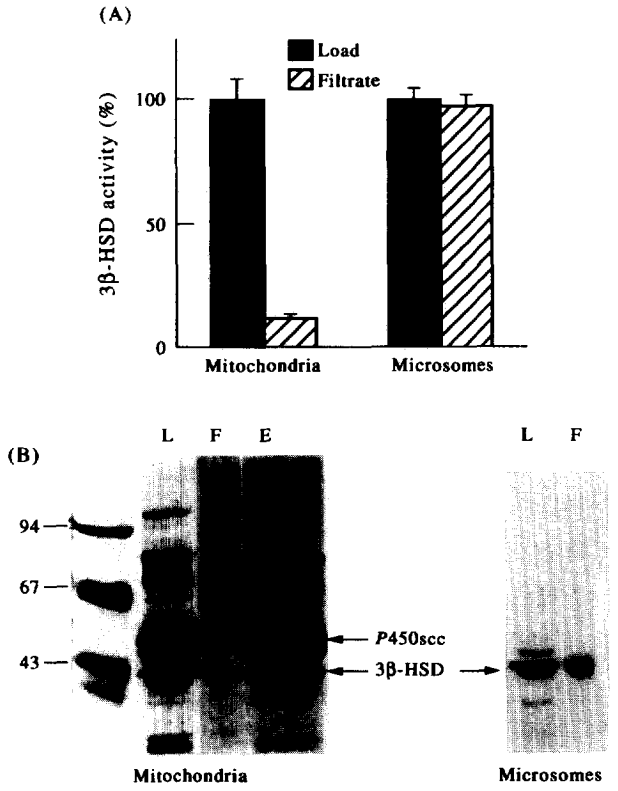

Fig. 2. Purification of 3β-HSD and cyt. $P450_{scc}$ on a Heparin–Sepharose column. (A) 3β-HSD activities of the fractions (eluates from DEAE–cellulose from mitochondrial or microsomal 3β-HSD purification) before loading on the Heparin–Sepharose column (load) and recovered in the filtrate of the column. (B) SDS–PAGE analysis of the corresponding fractions from mitochondria (load (L); filtrate (F) and eluate with buffer containing 1 M KCl (E)) and microsomes (load (L); filtrate (F)). The results are representative of five independent experiments.

coincubated. The mixture was then loaded onto a Heparin–Sepharose column. As shown in Fig. 3(A), the percentage of 3β-HSD activity recovered in the filtrate (≅10% of the loaded activity) was identical to that obtained when mitochondrial proteins were loaded on the column. By contrast, most of the purified microsomal enzyme activity (90%) loaded was recovered in the filtrate. SDS–PAGE analysis of the different fractions shows that most of the 3β-HSD protein was recovered in the eluate (1 M KCl) of the column together with $P450_{scc}$ whereas small amounts of 3β-HSD and $P450_{scc}$ were detected in the filtrate [Fig. 3(B)].

N-terminal amino acid sequence and tryptic peptides analysis of mitochondrial 3β-HSD

After SDS–PAGE analysis of the eluate from the DEAE–cellulose column, the band corresponding to 3β-HSD was extracted. N-terminal sequence analysis was performed. The first 21 amino acids obtained are underlined in Fig. 4. Tryptic digestion of mitochondrial 3β-HSD yielded six peptides which were sequenced (Fig. 4). These peptides are identical to those

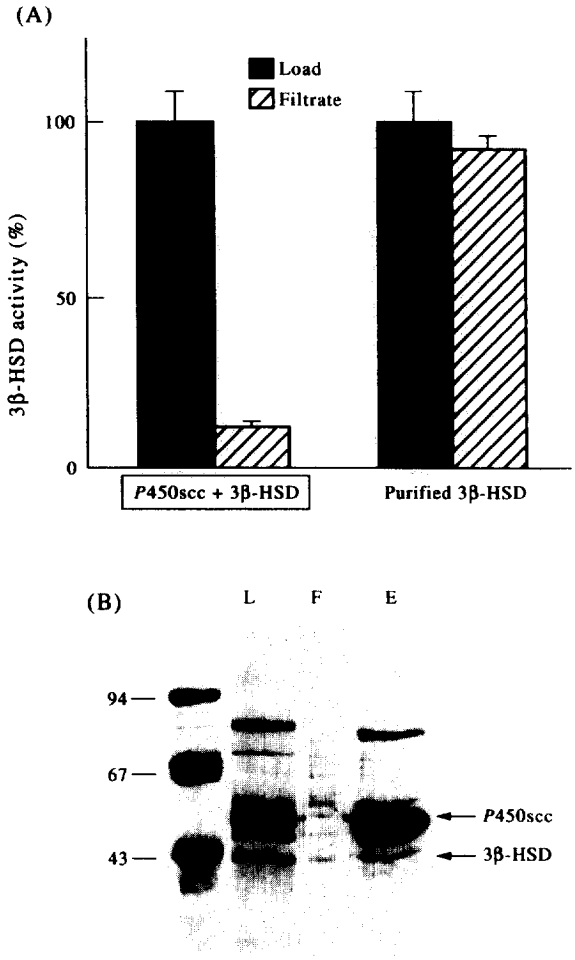

Fig. 3. Coelution of 3β-HSD and cyt. $P450_{scc}$ from Heparin–Sepharose. (A) Microsomal 3β-HSD (1 nmol) and cyt. $P450_{scc}$ (5 nmol) were incubated together (15 min) then loaded on a Heparin–Sepharose column. The 3β-HSD activities loaded on the column (load) or in the filtrate were measured. 3β-HSD was also loaded alone on a Heparin–Sepharose. (B) SDS–PAGE analysis of the corresponding fractions of the co-incubation of 3β-HSD and cyt. $P450_{scc}$.

previously described in 3β-HSD from bovine ovary [16] or adrenal cortex [17].

Mitochondrial 3β-HSD and $P450_{scc}$ are associated in a molecular complex

The previous results led us to investigate a possible interaction between $P450_{scc}$ and 3β-HSD in the mitochondria. BAC cells were labelled with [^{35}S]methionine and immunoisolation of 3β-HSD or cyt. $P450_{scc}$ was carried out using the mitochondrial protein extract and the corresponding antibodies. SDS–PAGE analysis of the immunoprecipitates is shown in Fig. 5. Lane 1 corresponds to the immunoprecipitation of 3β-HSD from the microsomal protein extract. Both cyt. $P450_{scc}$ and 3β-HSD are detected in the immunocomplexes obtained with 3β-HSD-antibody (lane 2), as well as in the immunoprecipitates obtained with anti-$P450_{scc}$ (lane 3).

Furthermore, measurement of 3β-HSD activity in the supernatants after immunoprecipitation shows that about 90 and 60% of the 3β-HSD activity were immunoprecipitated with 3β-HSD and cyt. $P450_{scc}$ antibodies, respectively. These observations prompted us to examine in more detail the 3β-HSD and $P450_{scc}$ molecular interaction *in vitro* using purified components.

3β-HSD and $P450_{scc}$ form a stable complex in vitro

To determine whether a direct interaction can take place between the two proteins, partially purified $P450_{scc}$ was incubated with purified microsomal 3β-HSD. At the end of the incubation, immunoprecipitations were carried out with antisera against either 3β-HSD or cyt. $P450_{scc}$. Figure 6 illustrates the side chain cleavage and the 3β-HSD activities of the immunocomplexes. After immunoprecipitation with 3β-HSD antiserum, about 90% of the 3β-HSD and 50% of the side chain cleavage activities were recovered. Similar results were obtained with anti-cyt. $P450_{scc}$, 50% of the side chain cleavage and 60% of the 3β-HSD activities were recovered in the immunoprecipitates.

Surface plasmon resonance analysis of 3β-HSD and cyt. $P450_{scc}$ interaction

In order to obtain kinetic parameters of the 3β-HSD–$P450_{scc}$ interaction, we used a surface plasmon resonance approach [8, 9].

The 3β-HSD was immobilized on the dextran surface. The variation of the resonance angle recorded was 8000 RU (RU: resonance units) corresponding to 8 ng/mm^2. Association of cyt. $P450_{scc}$ with the immobilized 3β-HSD was followed for about 3 min

1	**MAGWSCLVTG**	**GGGFLGQRII**	**C**LLVEEKDLQ	EIRVLD**KVFR**	**PEVR**EEFSKL
51	QSKIKLTLLE	GDILDEQCLK	GACQGTSVVI	HTASVIDVRN	AVPR**ETIMNV**
101	**NVK**GTQLLLE	ACVQASVPVF	IHTSTIEVAG	PNSYREIIQD	GR**EEEHHESA**
151	**W**SSPYPYSKK	LAEKAVLGAN	GWALKNGGTL	YTCALRPMYI	YGEGSPFLSA
201	YMHGALNNNG	ILTNHCKFSR	**VNPVYVGNA**	**WAHILALR**AL	RDPKKVPNIQ
251	GQFYYISDDT	PHQSYDDLNY	TLSKEWGFCL	DSRMSLPISL	QYWLAFLLEI
301	VSFLLSPIYK	YNPCFNRHLV	TLSNSVFTFS	YKKAQR**DLGY**	**EPLYTWEEAK**
351	QKTK**EWIGSL**	**VK**QHKETLKT	KIH		

Fig. 4. N-terminal amino acid and tryptic peptides sequence of mitochondrial 3β-HSD. The N-terminal and the six peptides sequences obtained are underlined on the described sequence from bovine ovary [16].

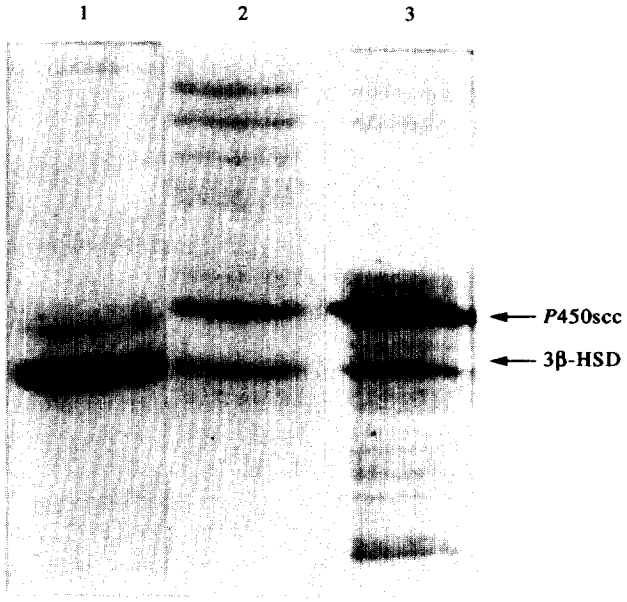

Fig. 5. Immunoprecipitation of 3β-HSD and cyt. $P450_{scc}$. BAC cells were labelled with [^{35}S]methionine and immunoprecipitations were performed with anti-3β-HSD or anti-$P450_{scc}$ antibodies as described in Materials and Methods. SDS–PAGE analysis of the different immunocomplexes was followed by autoradiography. Lane 1, immunoprecipitation of 3β-HSD from microsomes; lanes 2 and 3, immunoprecipitation from mitochondria of 3β-HSD and cyt. $P450_{scc}$, respectively.

[Fig. 7(A)]. The dissociation was monitored in buffer flow. Figure 7(B) shows the real time measurements of the association and dissociation phases of cyt. $P450_{scc}$ with immobilized 3β-HSD, performed at several concentrations of cyt. $P450_{scc}$ [Fig. 7(B)].

To determine whether the binding of 3β-HSD to cyt. $P450_{scc}$ was specific, the interactions between 3β-HSD and $P450_{11\beta}$ as well as the interaction between cyt. $P450_{scc}$ and the dextran surface were analysed. As seen on the sensorgrams, no significant interactions were detected in either case [Fig. 7(A)].

An analysis of the curves of the dissociation phase gave an average of the constant K_d for four different concentrations. The constant K_a was also determined and the ratio K_d/K_a yielded an apparent affinity constant K_D. The values of these constants are reported in Table 1. The binding ratio is 1/1.

DISCUSSION

In previous studies, we have shown that 3β-HSD activity is present in both mitochondria and microsomes isolated from bovine adrenal cortex homogenates [4]. In the mitochondria, the enzyme has been found in the inner membrane as well as in the intermembranes contact sites. Immunodetection of 3β-HSD and cyt. $P450_{scc}$ in these two submitochondrial compartments revealed a co-localization of the two proteins. Furthermore, the best substrate of 3β-HSD in the mitochondria is pregnenolone. Taken together, these observations raise the question of the functional significance of such an organization in the mitochondrial membrane.

The data presented in this paper clearly show that isolated adrenocortical mitochondria are able to produce sequentially pregnenolone (from cholesterol, by the side chain cleavage activity of cyt. $P450_{scc}$) then progesterone (3β-HSD activity) when substrates of the tricarboxylic acid cycle are added. This transformation from cholesterol to progesterone suggests that 3β-HSD and cyt. $P450_{scc}$ may constitute a functional unit in the mitochondrial membrane.

A molecular association was suggested first by the observation that, during purification of 3β-HSD from mitochondria, the enzyme remains associated with cyt. $P450_{scc}$. Therefore, the purification procedure giving pure 3β-HSD from microsomes failed to yield a pure enzyme from mitochondria. The presence of cyt. $P450_{scc}$ is responsible of this behaviour. Indeed, when purified microsomal 3β-HSD and cyt. $P450_{scc}$ were coincubated before being loaded on a Heparin–Sepharose column, 3β-HSD was retained whereas the isolated iomerase passed through the gel and was recovered in the filtrate of the column.

Attempts to sequence the mitochondrial 3β-HSD revealed that its N-terminal sequence which contains the NAD binding site is identical to that of the microsomal enzyme [13]. Six peptides obtained by tryptic digestion of the enzyme show no differences with the published sequence of the microsomal enzyme from adrenal cortex [17] or ovaries [16].

Cyt. $P450_{scc}$ and 3β-HSD form a molecular complex which remains stable after immunoisolation from mitochondria. Immunoprecipitations with anti-cyt. $P450_{scc}$

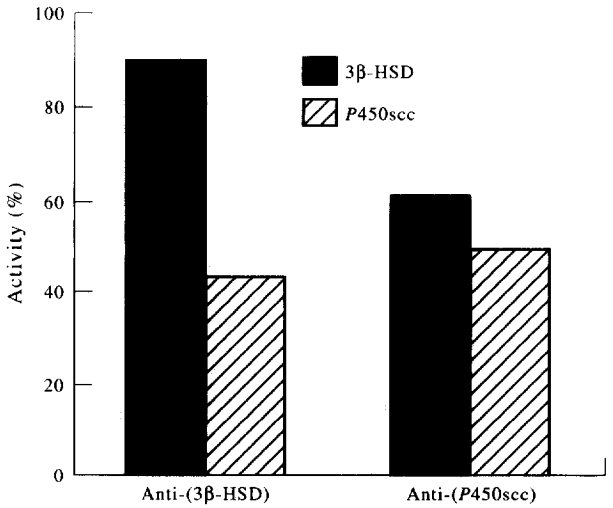

Fig. 6. Enzymatic activities of the immunocomplexes. 3β-HSD (1 nmol) and cyt. $P450_{scc}$ (5 nmol) were incubated (10 min) together before immunoprecipitation with either anti-cyt. $P450_{scc}$ or anti-3β-HSD antibodies. The 3β-HSD or side chain cleavage activities were measured in each immunocomplex. 100% represents the total activity before immunoprecipitation.

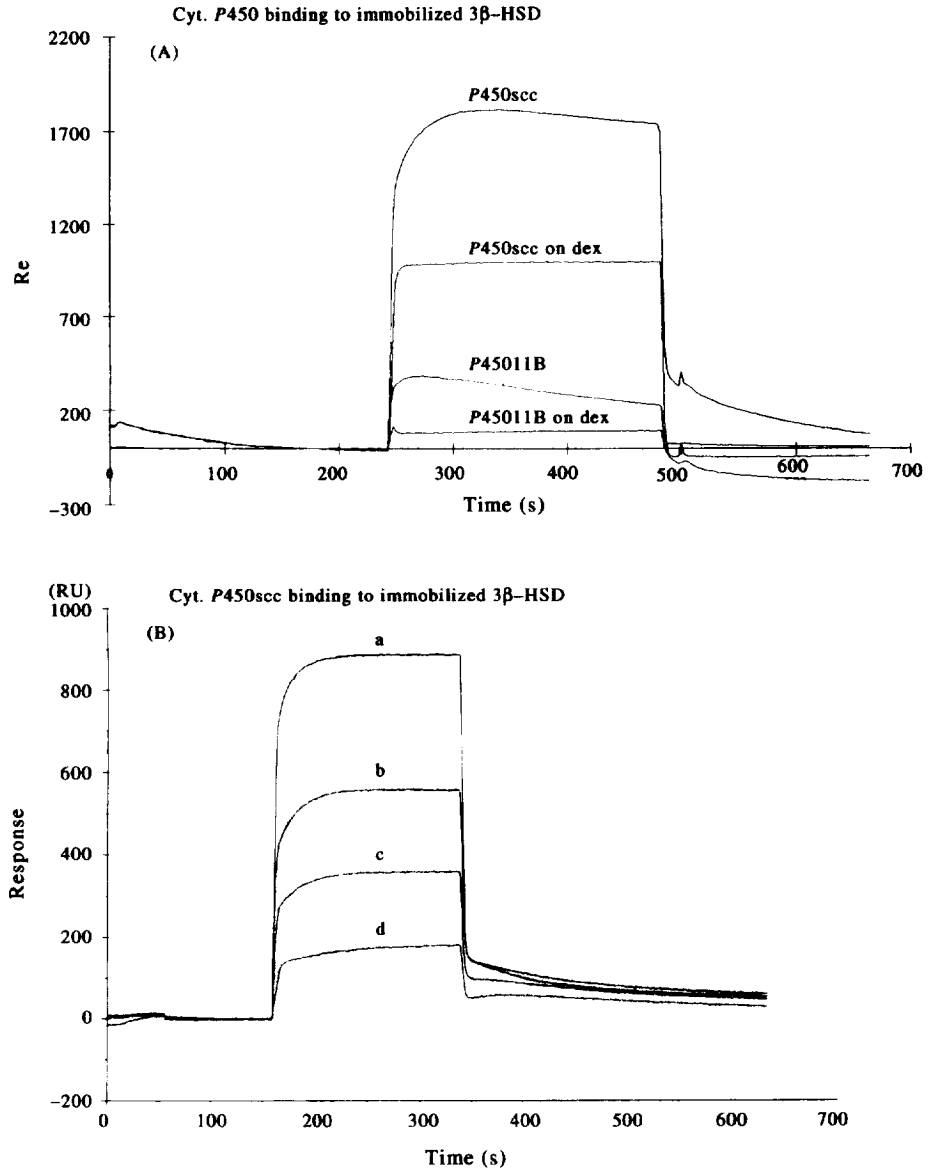

Fig. 7. Binding of cyt. $P450_{scc}$ to immobilized 3β-HSD studied by surface plasmon resonance. 3β-HSD was immobilized on the dextran surface (8 ng/mm^2). Plotted is the resonance signal (RU) as a function of time. Association and dissociation are observed. The results correspond to four independent experiments. (A) Cyt. $P450_{scc}$ was injected at a concentration of 5 μM on immobilized 3β-HSD ($P450_{scc}$). The same concentration of $P450_{scc}$ was injected on the dextran matrix ($P450_{scc}$ on dex). The same injections of cyt. $P450_{11\beta}$ are reported on immobilized 3β-HSD ($P450_{11\beta}$) and on the dextran matrix ($P450_{11\beta}$ on dex). (B) Several concentrations of cyt. $P450_{scc}$ injected into the system (3, a; 1.5, b; 0.75, c; and 0.375 μM, d).

or anti-3β-HSD antibodies show that a stable association takes place between the two proteins and that these immunocomplexes possess two enzymatic activities. This observation is consistent with the result showing the copurification of cyt. $P450_{scc}$ and 3β-HSD from mitochondrial extracts.

Table 1. Kinetic constants for the binding of cyt. $P450_{scc}$ to immobilized 3β-HSD

K_D	3.3×10^4 M^{-1} S^{-1}
K_a	3.3×10^4 M^{-1} S^{-1}
K_d	4.1×10^{-3} S^{-1}
K_D	0.12 μM

Kinetic constants were obtained from the results reported in Fig. 7.

The kinetic constants of the cytochrome–dehydrogenase association were characterized for the first time using the surface plasmon resonance method, yielding an association constant of 0.12 μM. This interaction is specific and other mitochondrial cytochromes such as cyt. $P450_{11\beta}$ do not associate with 3β-HSD.

The present observations suggest that the co-localization of cyt. $P450_{scc}$ and 3β-HSD in mitochondrial contact sites may lead to a molecular complex which will favour a functional cooperation between the two

enzymes. In such a complex, pregnenolone produced by the cleavage of cholesterol by the cytochrome will be directly provided to the 3β-HSD to yield progesterone. However, the site of interaction and the possible regulation of the association between the two proteins remain to be defined.

Adrenal steroid biosynthesis is characterized by intricate inter-organelles transport of substrates between mitochondria and endoplasmic reticulum. The present finding that cyt. $P450_{scc}$ associates to 3β-HSD in an active molecular complex suggests that at least part of the pregnenolone formed does not leave mitochondria before being transformed to progesterone. It will be of interest to determine whether this pathway favoured by the interaction cyt. $P450_{scc}$ and 3β-HSD is regulated by intracellular factors, possibly under extracellular hormonal signals.

Acknowledgements—This work was supported by the INSERM (Unité 244), the CEA (DSV/DBMS/BRCE), the Association pour la Recherche contre le Cancer, the Fédération Nationale des Centres de Lutte contre le Cancer, the Fondation pour la Recherche Médicale and the Ligue Nationale Française contre le Cancer. We would like to thank Helena Nilshans (Pharmacia Biosensor, Uppsala, Sweden) for all the experiments concerning surface plasmon resonance. We are indebted to C. Blanc-Brude for her assistance in primary culture preparations. We thank S. Lidy for her skilful secretarial work.

REFERENCES

1. Schulster D., Burstein S. and Cooke B. A.: *Molecular Endocrinology of the Steroid Hormones*. Wiley, New York (1976).
2. Hanukoglu I.: Steroidogenic enzymes: structure, function and role in regulation of steroid hormone biosynthesis. *J. Steroid Biochem. Molec. Biol.* **43** (1992) 779–804.
3. Lorence M. C., Murry B. A., Trant J. M. and Mason J. I.: Human 3β-hydroxysteroid dehydrogenase/$\Delta^{4,5}$ isomerase from placenta: expression in nonsteroidogenic cells of a protein that catalyzes the dehydrogenation/isomerization of C21 and C19 steroid. *Endocrinology* **126** (1990) 2493–2498.
4. Sauer L. A., Chapman J. C. and Dauchy R. T.: Topology of 3β-hydroxy-5-ene-steroid dehydrogenase/Δ^5-Δ^4-isomerase in adrenal cortex mitochondria and microsomes. *Endocrinology* **134** (1994) 751–759.
5. Cherradi N., Defaye G. and Chambaz E. M.: Characterization of the 3β-hydroxysteroid dehydrogenase activity associated with bovine adrenocortical mitochondria. *Endocrinology* **134** (1994) 1358–1364.
6. Jefcoate C. R., McNamara B. C., Artemenko I. and Yamazaki T.: Regulation of cholesterol movement to mitochondrial cytochrome $P\text{-}450_{scc}$ in steroid hormone synthesis. *J. Steroid Biochem. Molec. Biol.* **43** (1992) 751–767.
7. Cherradi N., Defaye G. and Chambaz E. M.: Dual subcellular localization of the 3β-hydroxysteroid dehydrogenase/isomerase. Characterization of the mitochondrial enzyme in the bovine adrenal cortex. *J. Steroid Biochem. Molec. Biol.* **46** (1993) 773–779.
8. Malmqvist M.: Biospecific interaction analysis using biosensor technology. *Nature* **361** (1993) 186–187.
9. Zhou M., Felder S., Rubinstein M., Hurwitz D. R., Ullrich A., Lax I. and Schlessinger J.: Real time measurements of kinetics of EGF binding to soluble EGF receptor monomers and dimers support the dimerization model for receptor activation *Biochemistry* **32** (1993) 8193–8198.
10. Duperray A. and Chambaz E. M.: Effect of prostaglandin E1 and ACTH on proliferation and steroidogenic activity of bovine adrenocortical cells in primary culture. *J. Steroid Biochem.* **13** (1980) 1359–1364.
11. Suhara K., Gomi T., Sato H., Itagaki E., Takemori S. and Katagiri M.: Purification and immunochemical characterization of the two adrenal cortex mitochondrial cytochrome P-450 proteins. *Arch. Biochem. Biophys.* **190** (1978) 290–299.
12. Omura T. and Sato R.: The carbon monoxide-binding pigment of liver microsomes. *J. Biol. Chem.* **239** (1964) 2370–2388.
13. Cherradi N., Guidicelli C., Defaye G. and Chambaz E. M.: Purification and characterization of 3β-hydroxysteroid-dehydrogenase-isomerase from bovine adrenal cortex. *J. Steroid Biochem. Molec. Biol.* **41** (1992) 831–836.
14. Laemli J. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **27** (1970) 680–685.
15. Katagiri M., Takemori S., Itagaki E. and Suhara K.: Purification of adrenodoxin and cytochrome P-450 (cholesterol desmolase and steroid 11β- and 18-hydroxylases). In *Methods in Enzymology* (Edited by S. Fleischer and E. Parker). Academic Press, New York (1978) Vol. LII, pp. 124–132.
16. Zhao H. F., Simard J., Labrie C., Breton N., Rhéaume E., Luu-The V. and Labrie F.: Molecular cloning, cDNA structure and predicted amino acid sequence of bovine 3β-hydroxy-5-ene steroid dehydrogenase/Δ^5-Δ^4 isomerase. *FEBS Lett.* **259** (1989) 153–157.
17. Rutherfurd K. J., Chen S. and Shively J. E.: Isolation and amino acid sequence analysis of bovine adrenal 3β-hydroxysteroid/steroid Isomerase. *Biochemistry* **30** (1991) 8108–8116.