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Molecular organization (topography) of cytochrome $P\text{-}450_{11\beta}$ in mitochondrial membrane and phospholipid vesicles as studied by trypsinolysis

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Cytochrome $P\text{-}450_{11\beta}$ from adrenal cortex is an intrinsic membrane protein embedded in the inner mitochondrial membrane. Topography of the protein inside a phospholipid bilayer was examined using controlled proteolysis of purified cytochrome $P\text{-}450_{11\beta}$ following its integration into artificial liposomes. Inclusion of the protein into phospholipid vesicles led to a marked stabilization of the cytochrome activity. Trypsin treatment of the liposome-integrated cytochrome resulted in the rapid disappearance of the native protein moiety (47 kDa), while a major 34 kDa peptide component was formed. This peptide core retained the heme moiety and part of the cytochrome steroid-11β hydroxylase activity. Very similar observations were obtained when inside-out vesicles prepared from isolated adrenocortical mitoplasts were examined with the same approach. It is thus suggested that adrenocortical cytochrome $P\text{-}450_{11\beta}$ is embedded in the inner mitochondrial membrane as well as in artificial liposomes by a major hydrophobic domain associated with the heme moiety while a limited domain remains accessible on the matrix side of the membrane surface. The previous described phosphorylation of the cytochrome $P\text{-}450_{11\beta}$ on a serine residue, by the cAMP-dependent protein kinase is suggested to occur in the protein domain oriented toward the membrane surface, the phosphorylation site being lost under mild proteolytic digestion of the membrane-integrated protein.

Introduction

Adrenal cortex mitochondria contain specific cytochromes *P*-450 responsible for major steroidogenesis steps such as cholesterol side chain cleavage ($P\text{-}450_{scc}$) and 11β-hydroxylation ($P\text{-}450_{11\beta}$), which play a pivotal role in the biosynthetic pathways leading to active corticosteroid hormones [1].

Abbreviations: cAMP, adenosine 3′,5′-cyclic monophosphate; Tris, tris (hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; TDG buffer, 10 mM Tris-HCl buffer (pH 7.5), containing 1 mM dithiothreitol and 2% glycerol.

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The cytochromes $P\text{-}450_{scc}$ and $P\text{-}450_{11\beta}$ are integral proteins of the inner mitochondrial membrane. They function as terminal steps in oxydoreduction cascades using reducing equivalents coming from NADPH through a flavoprotein (adrenodoxin reductase) and an iron sulfur protein (adrenodoxin), both of which being soluble components of the mitochondrial matrix [2]. Regulation of these mitochondrial cytochrome *P*-450 activities is believed to present major targets in the control of adrenocortical steroidogenesis [3], whereas these proteins may also be considered as specific markers in hormonally controlled tissue differentiation [4].

We have previously shown that purified active

bovine adrenocortical cytochrome $P\text{-}450_{11\beta}$ can be incorporated into phospholipid vesicles [5]. On the other hand, purified $P\text{-}450_{11\beta}$ was shown to be phosphorylatable by purified, cyclic-AMP-dependent protein kinase with concomitant increase of its affinity for adrenodoxin in vitro [6]. The present work was undertaken with the aim of a better understanding of the organization of the cytochrome moiety into the mitochondrial membrane. Using a purified $P\text{-}450_{11\beta}$ integrated into artificial liposomes as a model, limited trypsinolysis showed that $P\text{-}450_{11\beta}$ is most likely embedded, in the liposomal membrane, by a polypeptide domain which bears the catalytic site of the enzyme. On the other hand, phosphorylation by cyclic-AMP dependent protein kinase occurs on a serine residue in a protein domain which remains accessible from outside the lipid vesicle.

Materials and Methods

Deoxycorticosterone and corticosterone were purchased from Fluka. NADPH, phosphatidylcholine (from egg yolk) and sodium cholate were provided by Sigma. Lyophilized trypsin (211 U/mg) and lima bean trypsin inhibitor were obtained from Millipore Corporation. [1,2-^{3}H]Deoxycorticosterone (47 Ci/mmol) was provided by the CEA (Saclay, France) and sodium [^{125}I]iodide by the Radiochemical Center (Amersham). Protein A, octyl-Sepharose, Sephadex G-50 and Sephacryl S-1000 were purchased from Pharmacia. ACA-44 and ACA-54 Ultragel from IBF (France). Phosphatidylethanolamine was prepared from egg yolk according to Hawthorne [7]. Protein was assayed either according to Lowry et al. [8], the biuret method [9] or following Bradford et al. [10], as indicated.

Preparation of mitochondria, mitoplasts and inside-out mitochondrial vesicles. Adrenocortical mitochondria were prepared from fresh bovine adrenal glands processed at 4°C. The cortical tissue was homogenized with a Teflon homogenizer (2000 rpm) in a 5 mM Tris-HCl buffer (pH 7.4), containing 275 mM sucrose. The supernatant resulting from a $800 \times g$ 15 min centrifugation was centrifuged at $10000 \times g$ for 15 min. The resulting mitochondrial pellet was washed twice with the same buffer. Mitoplasts were prepared by removing the outer membrane from intact mitochondria, following hypo-osmotic shock [11,36]. After swelling at 0°C for 20 min in 20 mM sodium phosphate buffer (pH 7.4), containing 0.02% (w/v) bovine serum albumin, the suspension was centrifuged at $17000 \times g$ for 20 min. The resulting pellet was resuspended (5 mg protein/ml) in the same buffer without bovine serum albumin and centrifuged at $5000 \times g$ for 15 min; the resulting pellet will be referred to as the mitoplast fraction (inner membrane plus matrix).

Mitoplasts (2 ml, 20 mg/ml protein) were disrupted by sonication for 2×45 s with a 1-min interval using a probe sonicator (Ultrasons, Annemasse), with a 60 V setting. Mitoplast vesicles were isolated by ultracentrifugation ($100000 \times g$, 20 min), and further purified using the cytochrome *C*-Sepharose column procedure according to Ref. 12.

Adrenodoxin and adrenodoxin reductase were prepared according to Refs. 13 and 14, except that final steps were a chromatography over ACA-54 and ACA-44, respectively. Cytochrome $P\text{-}450_{11\beta}$ was purified according to Refs. 15 and 16 with minor modifications: an octyl-Sepharose affinity column was used [5] instead of aniline-Sepharose and elution of the cytochrome was performed with a buffer containing 0.5 M KCl and sodium cholate (0.5%). After dialysis against a buffer containing 0.7% sodium cholate, the preparation was applied onto an hydroxyapatite column (HA-Ultrogel). The flow-through fraction contained cytochrome $P\text{-}450_{11\beta}$, whereas cytochrome $P\text{-}450_{scc}$ was retained on the column. Cytochrome *P*-450 concentration was determined using the CO-reduced difference spectrum, taking an absorption coefficient of 91 $mM^{-1} \cdot cm^{-1}$ for the absorbance difference between 450 and 490 nm [17]. Antiserum against cytochrome $P\text{-}450_{11\beta}$ was raised in rabbits and used to prepare the immunoglobulin (IgG) fraction by DEAE-trisacryl chromatography after desalting over Sephacryl GF 05 [18]. 11β-Hydroxylase activity was measured using deoxycorticosterone (DOC) as the substrate. The assay medium contained deoxy[^{3}H]corticosterone (25 nmol), adrenodoxin (7.5 nmol), adrenodoxin reductase (60 pmol), NADPH (600 nmol) and cytochrome $P\text{-}450_{11\beta}$, either solubilized homogeneous (15 pmol) or liposome-integrated (20 μg proteins)

in 0.25 ml of 50 mM sodium phosphate buffer (pH 7.0). The reduction was started upon addition of either cytochrome $P\text{-}450_{11\beta}$ or cytochrome-containing vesicles and run for 1 min at 37°C and stopped by addition of 0.8 ml of methanol. The extracted steroids were separated and analysed as in Ref. 16.

Liposomes containing cytochrome $P\text{-}450_{11\beta}$ were prepared from a mixture of phosphatidylcholine and phosphatidylethanolamine (1 : 1, w/w) [5]. The phospholipid solution in chloroform was taken to dryness under a nitrogen stream then gently stirred at room temperature until dispersion after addition of a 10 mM Tris-HCl buffer (pH 7.4), containing 300 μM EDTA, 0.1 M NaCl, 10 μM deoxycorticosterone and 16 mg/ml sodium cholate. Purified cytochrome $P\text{-}450_{11\beta}$ was added to the liposome suspension which was purified by filtration through Sephadex G-50, as described (5).

Trypsinolysis of cytochrome $P\text{-}450_{11\beta}$. Soluble cytochrome $P\text{-}450_{11\beta}$, liposome-integrated $P\text{-}450_{11\beta}$ (0.2 mg protein/ml) and mitoplast vesicles (1 mg protein/ml) were treated with trypsin (0.2 mg/ml) at 15°C for various periods of time. The reaction was stopped either with trypsin inhibitor (0.22 mg/ml) for enzymatic activity and heme content measurements, or with trichloroacetic acid for electrophoretic analysis by polyacrylamide (15%) gel electrophoresis in the presence of 0.1% SDS, according to Laemmli [20].

Blotting procedure and immuno-detection of cytochrome $P\text{-}450_{11\beta}$. Following electrophoresis, the proteins were transferred onto nitrocellulose sheets using a Bio-Rad electro-blot apparatus and a 25 mM Tris-192 mM glycine buffer in 20% (v/v) methanol at pH 8.3 [21,22]. The tracks were soaked in 10 mM Tris-HCl buffer (pH 7.5), containing 1 mM dithiothreitol, 2% glycerol, 9‰ NaCl and 3% bovine serum albumin during 2 h at 37°C, then washed three times with the same buffer devoid of albumin. Incubation with anti $P\text{-}450_{11\beta}$ IgG was carried out overnight, the tracks were then thoroughly washed at 37°C, with the above buffer. The sheets were then incubated (2 h) with ^{125}I-labelled protein A ($5 \cdot 10^6$ cpm), previously prepared according to Ref. 19. After thorough washing with TDG buffer containing 1 M NaCl, 0.05% Nonidet P-40 and 0.25% gelatine then with water, the blots were dried and exposed to Kodak X-O mat film.

cAMP-dependent protein kinase catalytic subunit was prepared from bovine heart tissue, using DEAE-cellulose chromatography, ammonium sulfate precipitation, CM-Sephadex adsorption in the presence of cAMP, and filtration through ACA-44, according to Ref. 23.

Phosphorylation of soluble and liposomal cytochrome $P\text{-}450_{11\beta}$ was performed with cAMP-dependent protein kinase (catalytic subunit) as described [6].

Cholesterol assay. Protein fractions (1 ml) were extracted three times with diethyl ether (5 ml); the extracts were pooled, washed with 2 ml of 1 M NaOH and taken to dryness. Trimethylsilyl ether derivatives preparation and gas chromatography analysis was performed as previously described using cholesterol butyrate as an internal standard [24].

Results

1. Trypsin treatment of solubilized and liposome-integrated cytochrome $P\text{-}450_{11\beta}$

Purified cytochrome $P\text{-}450_{11\beta}$, either in solubilized form or previously integrated into phospholipid vesicles was submitted to trypsin digestion under identical controlled conditions at 15°C. The resulting peptide mixtures were analysed by SDS-polyacrylamide gel electrophoresis in time-course experiments. The corresponding electrophoretic patterns are illustrated in Fig. 1. The cytochrome-derived peptides were characterized by their apparent M_r with regard to a set of standard molecular weight protein markers, as indicated, and their respective amounts calculated following quantitative scanning of the gels. The corresponding data were plotted as illustrated in Fig. 2. As seen in Figs. 1 and 2, trypsin treatment resulted in the progressive disappearance of the native $P\text{-}450_{11\beta}$ moiety (47 kDa) which reached barely detectable levels after 90 min of incubation regardless of the type of cytochrome preparation. However, the rate of proteolysis was clearly decreased when the cytochrome was integrated into a liposomal structure. In this case, a half-life of about 12 min could be calculated, while it was roughly 5 min for the solubilized

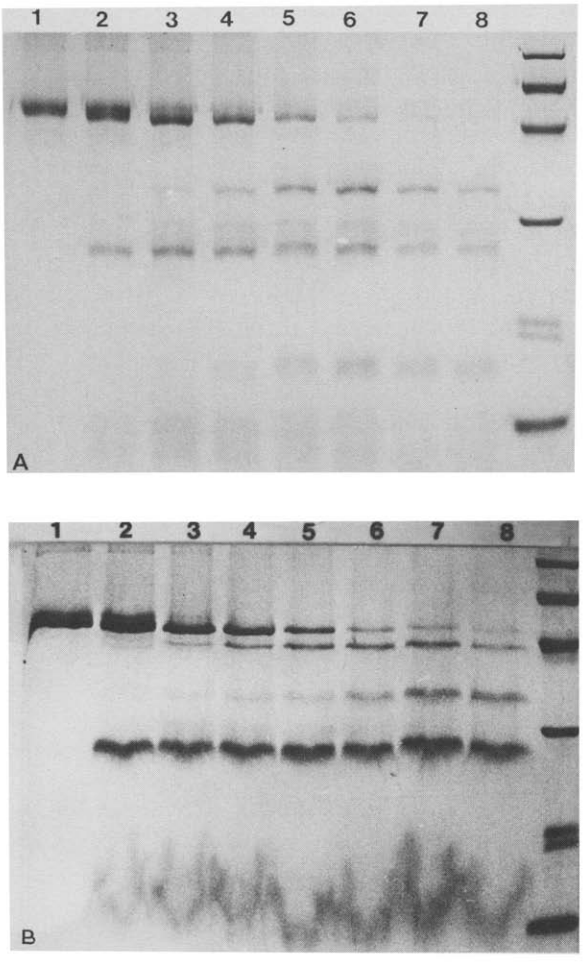

Fig. 1. Electrophoretic analysis of peptides resulting from trypsin treatment of adrenocortical cytochrome $P\text{-}450_{11\beta}$ with trypsin. Cytochrome $P\text{-}450_{11\beta}$ either solubilized (A) or liposomal (B) was incubated with trypsin (0.2 mg/ml). After incubation at 15°C for various periods of time, the reaction was stopped by trichloroacetic acid precipitation. The pellet was dissolved into SDS-buffer and subjected to SDS-gel electrophoresis followed by Coomassie blue staining. Lane 1, control (before addition of trypsin); lanes 2–8, after incubation for 0, 5, 10, 15, 30, 60 and 90 min, respectively. Molecular weight markers: 94 kDa, 67 kDa, 43 kDa, 30 kDa, 20 kDa and 14 kDa.

preparation. This indicates that the trypsin access to the cytochrome was impaired when the protein was included into a phopsholipid bilayer. Striking differences also occurred when the patterns of the peptides generated upon trypsinolysis of the two cytochrome *P*-450 preparations were compared. The solubilized protein yielded transciently major peptides of 34, 30 and 17 kDa which thereafter disappeared with time to become barely detectable after 2 h of incubation. By contrast, proteolysis of the liposome-integrated protein yielded a major 34 kDa peptide which represented about 80% of the products after one hour and was not further degraded thereafter. Both $P\text{-}450_{11\beta}$ preparations yielded within the first 10 min of trypsin attack a minor 45 kDa component which remained stable within the next 2 h of treatment. These observations strongly suggested that the 34 kDa fragment of the cytochrome may represent a lipophilic domain of the protein which was embedded in the liposomal structure and therefore refractory to further proteolysis. However, if the liposome-encapsulated cytochrome preparation resulted in a random distribution of the protein into the lipid bilayer, one might have expected an equal distribution between inner-oriented and outer-oriented forms with regard to the lipidic vesicle wall. In this situation, half of the total liposomal cytochrome should be expected to be refractory to the action of trypsin which was added to the medium bathing the vesicles. However, the above data disclosed that this was not the case: the native 47 kDa liposomal cytochrome was digested as if it was mostly (if not totally) accessible to trypsin attack. This would suggest an asymetrical distribution of the protein between the two vesicle phospholipid layers, with most of the protein integrated into the outer leaflet, with a hydrophilic domain emerging from the outer surface of the liposomes. Further experiments were thus designed to establish whether the cytochrome-containing phospholipid vesicles were either destroyed by trypsin treatment or possibly permeable to the protease. It was found that $P\text{-}450_{11\beta}$-containing liposomes behave as impermeable barriers to water-soluble components: ferricyanide included into the inner liposomal space during the vesicle preparation [31] was not released during 2-h incubations in the conditions used for trypsinolysis. The chromatographic distribution of the liposome population upon gel filtration chromatography over Sephacryl S-1000 was not modified following a 2-h incubation with trypsin, showing that the vesicles were not destroyed under these conditions (not shown). In addition, when ^{125}I-labeled tryp-

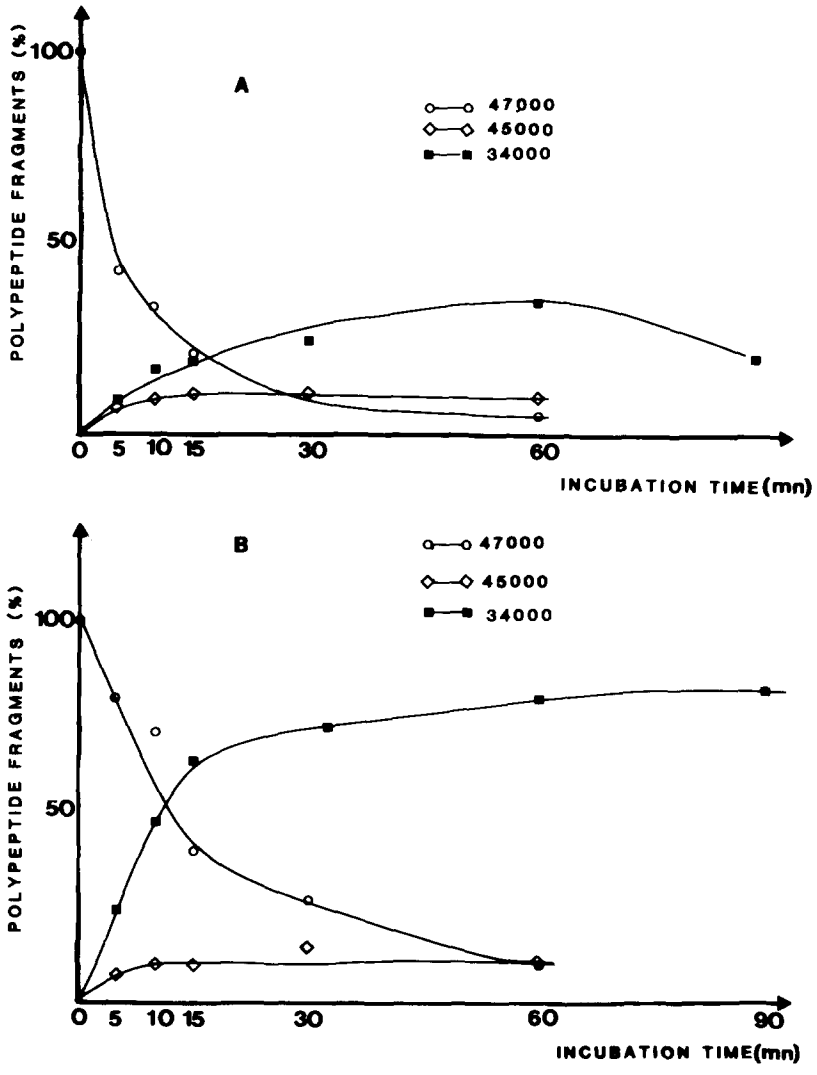

Fig. 2. Quantitative analysis of the peptide electrophoretic patterns as shown in Fig. 1. The variation of the relative amount of peptides visualized in Fig. 1 as a function of time was obtained following scanning of the Coomassie blue-stained gels.

sin was prepared and incubated with cytochrome $P\text{-}450_{11\beta}$-containing liposomes under conditions used for trypsinolysis experiments, subsequent isolation by ultracentrifugation disclosed that they were devoid of radiolabel. These observations indicated that trypsin did not become associated with the liposomes, thus ruling out a permeation of the protease into the vesicles.

2. Trypsinolysis of cytochrome $P\text{-}450_{11\beta}$ in mitochondrial, mitoplast and mitochondrial vesicle preparations

The adrenocortical mitoplast preparations were mostly free of outer mitochondrial components, as shown by a high content in cytochrome *P*-450 (relative content 1.35) as well as in cytochrome-*C* oxydase [26] and malate dehydrogenase activities [27] (relative specific activities 1.25 and 1.5, respectively), whereas their monoamine-oxidase [28,29] and their adenylate kinase [30] activities were very low (relative specific activities 0.25 and 0.15, respectively) as compared to those of intact mitochondria. The mitoplast preparations were subjected to sonication to yield inside-out inner

membrane mitochondrial vesicles [11]. Electron microscopy observation showed that the resulting preparation was mostly made of vesicles of an average diameter of 2000 Å with a virtual absence of any intact mitochondria (not shown). When the mitoplast preparations were passed through a cytochrome *c*-Sepharose column [12], two populations of vesicles could be isolated; about 80 to 90% of the preparation was recovered in the flow-through volume, as expected for mitochondrial inner membrane inside-out vesicles [12], whereas the remainder was eluted with 1 M KCl concentration. This was in agreement with the fact that full 11β-hydroxylase activity was expressed in these preparations, when adrenodoxin and adrenodoxin reductase were provided, suggesting that cytochrome $P\text{-}450_{11\beta}$ was accessible to these proteins added to the medium (Table I). The purified vesicles obtained by this procedure retained a high content in cholesterol (13.3 μg/mg protein) as compared to intact mitochondria (19.2 μg/mg protein) and mitoplasts (14.8 μg/mg protein). On the other hand, mitoplast preparations resulting from treatment of mitochondria by digitonin exhibited a severe loss of their cholesterol content (5.8 μg/mg protein) and were discarded from this study.

The various mitochondrial preparations were subjected to trypsin treatment under the aforementioned conditions and cytochrome $P\text{-}450_{11\beta}$-derived peptides were detected following polyacrylamide gel electrophoresis and immunoblotting. This detection method was first validated by analysing the proteolysis product generated upon treatment of the solubilized and the liposome-integrated cytochrome, as described above. In addition to the native cytochrome (M_r 47 kDa), the immunodetection procedure revealed the major $P\text{-}450_{11\beta}$-derived peptides previously described (i.e. of M_r 45, 34, 30 and 17 kDa) in the case of the solubilized cytochrome (not shown). When applied to the detection of liposome-integrated cytochrome proteolytic products, the major 34 kDa $P\text{-}450_{11\beta}$-derived peptide was clearly detected (Fig. 3). When whole mitochondria and mitoplast preparation were treated by trypsin, the immunoblot

TABLE I

EFFECT OF TRYPSIN TREATMENT ON STEROID 11β-HYDROXYLASE ACTIVITY OF SOLUBILIZED AND LIPOSOME-INTEGRATED CYTOCHROME $P\text{-}450_{11\beta}$ AND MITOCHONDRIAL VESICLES

Mitochondrial vesicles (1 mg/ml protein) or cytochrome $P\text{-}450_{11\beta}$ (0.1 μM, solubilized or liposomal) was incubated at 15 °C with or without trypsin (0.2 mg/ml). At various times of incubation, trypsin inhibitor (0.2 mg/ml) and in half of the assays trypsin inhibitor and sodium cholate (1% final concentration) were added. 11β-Hydroxylase activity (nmol corticosterone/min per mg protein) was assayed using an assay mixture containing deoxy[^{3}H]corticosterone (25 nmol, 0.05 μCi), adrenodoxin (7.5 nmol), adrenodoxin reductase (60 pmol), NADPH (60 nmol) and $P\text{-}450_{11\beta}$ (15 pmol) or vesicles (20 μg of proteins) in 0.25 ml of potassium phosphate buffer (50 mM, pH 7.0). The reaction was started upon addition of the cytochrome preparation, run at 37 °C for 1 min and stopped by addition of methanol.

Incubation time		0	1 h	2 h
Solubilized cytochrome *P*-450				
Control		910 ± 70	860 ± 70	690 ± 65
Trypsin			410 ± 38	80 ± 5
Liposomal cytochrome *P*-450				
Control, Cholate	(−)	349 ± 50	321 ± 45	311 ± 43
	(+)	905 ± 80	879 ± 80	874 ± 78
Trypsin, Cholate	(−)	349 ± 50	234 ± 45	200 ± 45
	(+)	905 ± 80	544 ± 60	531 ± 60
Vesicles				
Control, Cholate	(−)	43 ± 4.5	43 ± 4.5	43 ± 4
	(+)	45 ± 5	43 ± 4	
Trypsin, Cholate	(−)	43 ± 4.5	30 ± 3	28 ± 3
	(+)	48 ± 6	32 ± 3.5	

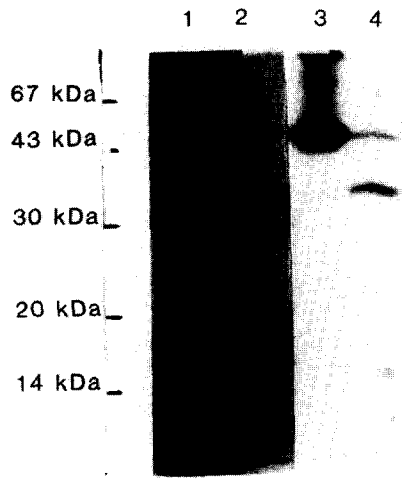

Fig. 3. Detection of immunoreactive cytochrome $P\text{-}450_{11\beta}$-related peptides by electrophoretic blotting of mitochondrial vesicle proteins following trypsin treatment. After electrophoresis, the proteins separated on the gel were transferred onto nitrocellulose. The nitrocellulose sheet was incubated with an anti-cytochrome $P\text{-}450_{11\beta}$ immunoglobulin preparation, then with ^{125}I-labeled protein A and subjected to autoradiography. Lanes 1 and 2, electrophoresis and blotting of liposomal cytochrome $P\text{-}450_{11\beta}$ before (lane 1) and after trypsinolysis (lane 2); lanes 3 and 4, electrophoresis and blotting of mitochondrial vesicles before (lane 3) and after action of trypsin (lane 4). Mitochondrial vesicles (1 mg/ml) or liposomal cytochrome $P\text{-}450_{11\beta}$ were incubated for 1 h with (or without) trypsin (0.2 mg/ml), aliquots (100 μl) were precipitated with trichloroacetic acid, centrifuged, the pellet dissolved in SDS buffer and analysed by SDS-polyacrylamide (10%) gel electrophoresis and blotting.

showed no immuno-reactive peptide other than the M_r 47 kDa native $P\text{-}450_{11\beta}$ (fig. 3). This indicates that access to the cytochrome embedded in the inner mitochondrial membrane was not possible for trypsin from the outside of either intact mitochondria or mitoplasts. By contrast, trypsin treatment of inside-out mitochondrial vesicles resulted in the occurrence of the cytochrome-derived 34 kDa peptide. No other immunoreactive $P\text{-}450_{11\beta}$-related peptide was detected (Fig. 3). This pattern was thus very similar to that obtained in the case of liposome-integrated cytochrome. This suggests that the protein was seen by the protease in a similar fashion either when it was integrated in an artificial liposomal membrane or when it was embedded in its natural inner mitochondrial membrane environment, provided this natural membrane has been previously inside-out oriented.

3. 11β-Hydroxylase activity and heme content of trypsin-treated cytochrome $P\text{-}450_{11\beta}$

The aforementioned experiments disclosed that membrane-integrated cytochrome $P\text{-}450_{11\beta}$ was proteolysed in a limited fashion, yielding a major 34 kDa fragment. We then addressed the question as to whether this 34 kDa domain of the cytochrome contains the active site of the enzyme and whether it retains catalytic activity of the intact protein. The 11β-hydroxylase activity as well as the heme content of the cytochrome (either solubilized, liposome-integrated or in inside-out mitoplast vesicles) were assayed at various times during trypsin treatment.

As can be seen in Table I, purified cytochrome $P\text{-}450_{11\beta}$ in a solubilized form exhibited a marked instability with time: about 15% of its activity was lost within 2 h, although no change in heme content was detected (Table II). Trypsin-treatment of this preparation resulted in a virtual total loss of activity within 2 h (Table I) while a parallel drop in heme content was observed (Table II). When the cytochrome was integrated into liposomes, its activity was stabilized, although its specific activity was reduced as compared to its solubilized counterpart (Table I). However, it was possible to recover a maximally active cytochrome by solubilizing the enzyme by liposome treatment with sodium cholate (Table I). Liposomal cytochrome 11β-hydroxylase activity was assayed during trypsin treatment both in intact liposomes and in cholate-solubilized preparations. As shown in Table II, trypsin treatment resulted in a loss of about 45% of the liposomal 11β-hydroxylase activity within 2 h (Table I); however, the heme content remained unchanged under these conditions (Table II).

Although the 11β-hydroxylase specific activity was lower in inside-out mitochondrial vesicle, as compared to that of the liposomal cytochrome preparation, its behavior toward trypsin treatment was qualitatively very similar. Whereas the activity remained highly stable in the vesicles during the 2-h incubation in the absence of protease, about half of the activity was lost after 2 h of trypsin treatment (Table I). At the same time, no detecta-

TABLE II

EFFECT OF TRYPSIN TREATMENT ON *P*-450 HEME CONTENT (NMOLES) OF SOLUBILIZED OR LIPOSOMAL CYTOCHROME $P\text{-}450_{11\beta}$ PREPARATION AND MITOCHONDRIAL VESICLES

After trypsin treatment for the indicated times, the incubation was stopped as in Table I and heme content was determined. Sodium cholate (1.2% final concentration) was added before measurement. Sodium dithionite was then added to the mixture, and the reduced versus reduced and carbon monoxide-bound difference spectra between 500 and 400 nm were recorded and used to calculate *P*-450 concentration according to Ref. 17.

Incubation time	*P*-450 heme content (nmol)					
	Vesicles		Liposomal cytochrome $P\text{-}450_{11\beta}$		Soluble cytochrome $P\text{-}450_{11\beta}$	
	Control	Trypsin	Control	Trypsin	Control	Trypsin
0	1.5	1.5	1.1	1.1	0.9	0.9
30 min					0.9	0.6
1 h	1.5	1.35	1	1.1	0.9	0.25
2 h	1.35	1.35	1.1	1	0.9	0.15

ble loss in heme content was observed (Table II). It may be noticed that, by contrast to the cytochrome included into artificial phospholipid liposomes, $P\text{-}450_{11\beta}$ in mitochondrial vesicles expressed its full activity, as shown by the absence of increase in its activity after solubilization following cholate treatment (Table I). It may thus be concluded that cytochrome $P\text{-}450_{11\beta}$ in solubilized form is freely accessible and looses its heme and its biological activity under trypsin attack. By contrast, when integrated into a membrane structure (either artificial liposomes or inside-out mitoplasts), the cytochrome is only partly accessible to trypsin. In this case, the initial proteolytic process remains limited and does not reach the 34 kDa cytochrome domain which retains the heme moiety. Loss of a cytochrome fragment oriented toward the surface of the membrane structure, and therefore most likely involved in the interaction of the enzyme with adrenodoxin would be in agreement with the simultaneous partial loss of 11β-hydroxylase activity observed.

4. Phosphorylation and trypsinolysis of cytochrome $P\text{-}450_{11\beta}$

Cytochrome $P\text{-}450_{11\beta}$ in the soluble form was phosphorylated by incubation with the catalytic subunit of cAMP-dependent protein kinase and [γ-^{32}P]ATP; 1 mole of ^{32}P per mole of protein was incorporated on a serine residue [4]. When liposomal cytochrome was incubated in the same conditions, the protein was also phosphorylated

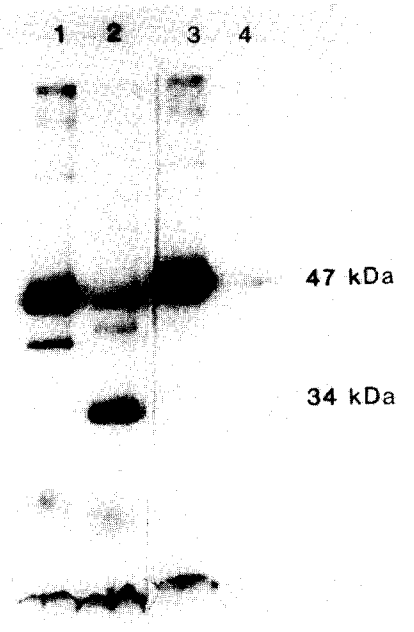

Fig. 4. Phosphorylation and trypsinolysis of liposome-integrated adrenocortical cytochrome $P\text{-}450_{11\beta}$. Liposome-integrated $P\text{-}450_{11\beta}$ (1.3 μM) was incubated in a 200 μl mixture containing 0.1 μM ATP, 0.8 μCi [^{32}P]ATP, 5 mM $MgCl_2$, 0.2 mg/ml of purified cAMP-dependent protein kinase catalytic subunit in 10 mM Tris-HCl buffer (pH 7.4), during 30 min at 15°C. Then, trypsin (0.2 mg/ml) was added, followed by a 30 min incubation at 15°C (same incubation for the control). The reaction was stopped by trichloroacetic acid precipitation; the pellet was dissolved in SDS buffer and submitted to SDS-polyacrylamide gel electrophoresis. Lane 1, control: phosphorylated liposome-integrated cytochrome $P\text{-}450_{11\beta}$ (Coomassie blue staining); lane 2, trypsinolysis of phosphorylated liposomal cytochrome $P\text{-}450_{11\beta}$ (Coomassie blue staining); lanes 3 and 4, autoradiography of lanes 1 and 2, respectively.

(Fig. 4) and 0.8 mole of ^{32}P per mole was incorporated into a serine residue. After trypsinolysis of soluble ^{32}P-labelled cytochrome $P\text{-}450_{11\beta}$ under afore mentioned conditions, no ^{32}P label could be detected in the resulting peptide products. When liposome integrated ^{32}P-labelled cytochrome $P\text{-}450_{11\beta}$ was submitted to trypsin digestion, no label was found associated with the major 34 kDa peptide fragment (Fig. 4). This suggests that the phosphorylatable site of the cytochrome was part of the protein domain oriented toward the outside surface of the membrane, which was removed and possibly further degraded and lost during the proteolytic process.

Discussion

Cytochrome $P\text{-}450_{11\beta}$ is an integral inner mitochondrial membrane protein in adrenocortical cell and understanding of its activity should take into account this particular situation. The major aim of this work was to examine some aspects of the organization of the cytochrome $P\text{-}450_{11\beta}$ molecule embedded in artificial liposomal vesicles as well as in its natural mitochondrial membrane by using as a probe its susceptibility to trypsin attack. This firstly required to define controlled proteolytic conditions since low trypsin concentration (i.e. 2 μg/ml) has previously been shown unable to attack liposome-associated cytochrome $P\text{-}450_{11\beta}$ [5]. On the other hand, one may question the limitations of the cytochrome-derived peptides detection using a polyclonal antiserum. However, it was considered to meet the main objective of the study since it allows the detection of the major peptides characterized by Coomassie blue staining. The major findings of this study disclosed that (i) purified cytochrome $P\text{-}450_{11\beta}$ under solubilized form appeared freely accessible to proteolytic digestion. (ii) When integrated either in an artificial or a natural membrane structure, cytochrome $P\text{-}450_{11\beta}$ was largely protected to trypsin; in both situations proteolysis was limited and a similar 34 kDa peptide fragment was produced, which remained associated with the membrane structure and retained the heme moiety. However, in this situation, a partial loss of 11β-hydroxylase activity was observed. These data are in agreement with a model visualizing the cytochrome as embedded in its membrane support by an hydrophobic 34 kDa peptide domain associated with the heme nucleus. The protein may emerge at the surface of the membrane by a domain which is accessible to trypsin and which is likely to be involved in the interaction of the cytochrome with adrenodoxin, required to support 11β-hydroxylase activity. This model is supported by the very similar behavior of the cytochrome included either into liposomal bilayers or embedded in the inner mitochondrial membrane, provided the latter were oriented inside-out following sonication. This situation in vivo would be in keeping with the known location of adrenodoxin and adrenodoxin reductase in the mitochondrial matrix compartment.

However, some of the observations made with liposome-integrated cytochrome $P\text{-}450_{11\beta}$ deserve comments. First, the specific activity of the solubilized enzyme was reduced by about half following liposome inclusion. Since full activity could be recovered by solubilizing again the liposomal protein, a first explanation of these observations might have been an even distribution of the cytochrome between an inside and an outside-oriented forms in the lipidic layer. In this situation, only half of the cytochrome would be accessible to the 11β-hydroxylase cofactors (adrenodoxin, adrenodoxin reductase) added to the medium. However, this was apparently not the case since (i) trypsin treatment of the liposomal cytochrome led to the virtual total disappearance of the native 47 kDa protein indicating that the bulk of the protein was accessible to trypsin from the outer surface of the vesicles. (ii) Study of the liposomal cytochrome P-450 spectrum in the presence of added adrenodoxin, adrenodoxin reductase and NADPH showed that more than 80% of the cytochrome could be reduced under these conditions. Similar observations have already been reported in the case of cytochrome $P\text{-}450_{scc}$ incorporated into phosphatidylcholine vesicles [32]. All together, these observations may be best explained by an asymetrical distribution of the cytochrome in the phospholipid vesicle wall i.e. most of the protein being oriented toward the outer leaflet of the membrane, with its hydrophilic domain turned toward the outer surface of the vesicles and therefore accessible to trypsin. This distribution would

be favored by the constraint in the liposomal wall, which would tend to exclude embedded proteins toward the outer leaflet of the vesicle. This situation did not noticeably change the gross conformation of the protein, as judged by comparing its circular dichroism spectrum to that of its solubilized counterpart. Both spectra (210 nm, $\theta = 5 \cdot 10^3$) appeared identical (not shown). This asymetrical distribution of cytochrome $P\text{-}450_{11\beta}$ in the liposomes was also confirmed by the phosphorylation experiments. Incorporation of one mole of phosphate on a serine residue was observed when liposome-integrated cytochrome $P\text{-}450_{11\beta}$ was phosphorylated by the cAMP-dependent protein kinase subunit. This phosphate was lost after trypsinolysis and should thus be located outside the phospholipid bilayer in the protein domain which is accessible to trypsin. Phosphorylation of the cytochrome was shown to increase its binding to adrenodoxin [6]; this is in line with a localization of the phosphorylatable site on the protein domain exposed at the membrane surface and most likely involved in the interaction with adrenodoxin [34].

The reversible loss of cytochrome specific activity following its incorporation into liposomes may be explained by the nature of its lipidic environment in the vesicle wall. It has been shown that cytochrome *P*-450 activity could be greatly influenced by the nature of the phospholipids used in such reconstitution experiments. For example adrenocortical $P\text{-}450_{scc}$ integrated in artificial vesicles has been found to exhibit a higher activity when cardiolipins were included in the phospholipid mixture [32,37]. This suggests that the PE-PC liposomal vesicles used in this work might not optimally support cytochrome $P\text{-}450_{11\beta}$ activity and may be modified in order to mimic all the functional properties of the lipidic environment of the cytochrome in the intact cell membranes.

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