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IN VITRO SYNTHESIS OF MITOCHONDRIAL CYTOCHROMES P-450(scc) AND P-450(11-β) AND MICROSOMAL CYTOCHROME P-450(C-21) BY BOTH FREE AND BOUND POLYSOMES ISOLATED FROM BOVINE ADRENAL CORTEX

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SUMMARY: In vitro synthesis of mitochondrial cytochromes P-450(scc) and P-450(11-β), and microsomal cytochrome P-450(C-21) programmed by bovine adrenal cortex polysomes was carried out using rat liver cell sap and wheat germ lysate systems. Synthesis of P-450 proteins in the cell-free systems was determined by immunoprecipitation and immunoadsorption using monospecific antibodies to each species of P-450, and the sizes of the in vitro products were analyzed by SDS-polyacrylamide gel electrophoresis. Both free and bound polysomes synthesized these three species of P-450 in the cell-free systems. P-450(scc) and P-450(C-21) were synthesized apparently as the mature size products, whereas P-450(11-β) was synthesized as a putative precursor approximately 5,000 daltons larger than the mature form. Mitochondrial and microsomal P-450 proteins seem to share common sites of synthesis in the cytoplasm of adrenal cortex cells.

Several species of cytochrome P-450 are present in the membranes of endoplasmic reticulum and mitochondria in adrenal cortex cells, and they catalyze hydroxylation reactions of steroids in the biosynthesis of adrenocortical hormones. Two of them, P-450(scc) and P-450(11-β), are located in the inner membrane of mitochondria, and another one, P-450(C-21), is located in the membrane of endoplasmic reticulum. These three species of cytochrome P-450 have been purified to homogeneity and well characterized (1-10). Moreover, they are immunologically distinct from one another (6,10-13). Existence of these multiple species of Cytochrome P-450 on different organelles in adrenal cortex cells provides us with a unique system to study the mechamism of intracellular distribution of newly synthesized hydrophobic membrane proteins to various cell organelles.

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We studied (11-13) the cytoplasmic sites of synthesis of these three P-450 species by examining the presence of their nascent peptides on free and bound ribosomes isolated from bovine adrenal cortex, and we found that the nascent peptides of all three P-450 species are associated with both free and bound ribosomes. In this communication, we report the in vitro synthesis of P-450(scc), P-450(11-β), and P-450(C-21) by free and bound polysomes isolated from bovine adrenal cortex in rat liver cell sap and wheat germ lysate systems.

MATERIALS AND METHODS

Preparation of free and bound polysomes from bovine adrenal cortex, and in vitro translation of isolated polysomes in rat liver cell sap and wheat germ lysate systems were carried out as described in the preceding paper (14). Monospecific rabbit antibodies against each one of P-450(scc), P-450(11-β), and P-450(C-21) were prepared and characterized as described previously (11-13). These antibodies were used in isolating in vitro synthesized P-450 peptides by direct immunoprecipitation or by immunoadsorption using Protein A-Sepharose as described in the preceding paper (14). In vitro synthesized P-450 peptides isolated by immunoadsorption were analyzed by SDS-polyacrylamide gel electrophoresis by the method of Laemmli (15) with some modifications using 10 % separation gel. After the electrophoresis, the gel was cut into 2 mm slices, and the slices were dissolved in NCS solubilizer, and the radioactivity was counted in toluene-Triton X-100-PPO scintillant.

RESULTS

The in vitro synthesis of P-450(scc), P-450(11-β), and P-450(C-21) was determined by immunoprecipitation of P-450 peptides from the translation products programmed by isolated polysomes in rat liver cell sap system. Table I shows that P-450(scc) antibodies immunoprecipitated 0.8 % and 1.0 % of the total translation products programmed by free and bound polysomes, respectively. Similarly, P-450(11-β) antibodies immunoprecipitated 0.9 % and 1.3 % of the total translation products programmed by free and bound polysomes, respectively. Table II shows the results of another experiment, in which P-450(C-21) antibodies immunoprecipitated about 0.1 % of the total translation products programmed by free and bound polysomes. These results demonstrated that both free and bound polysomes synthesized P-450(scc), P-450(11-β), and P-450(C-21) in the in vitro translation systems.

Table I. In Vitro Synthesis of P-450(scc) and P-450(11-β) by Free and Bound Polysomes in Rat Liver Cell Sap System.

In vitro translation programmed by bovine adrenal cortex free and bound polysomes was performed in rat liver cell sap system, and P-450(scc) and P-450(11-β) peptides were isolated from the total translation products by direct immunoprecipitation. The immunoprecipitates were washed several times, and the radioactivity was counted. Immunoprecipitates of control immunoglobulin(IG) with anti-rabbit goat IG were prepared as the control.

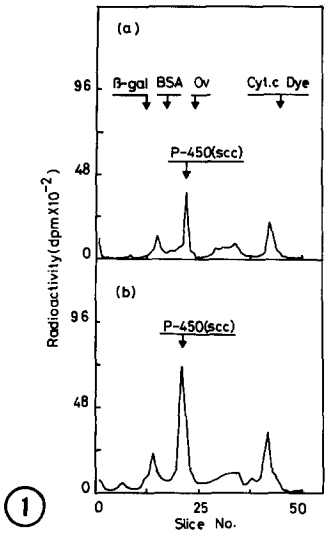
Polysomes	Antibodies	Radioactivity			
		Total	Immunoprecipitates		
		cpm x 10^{-3}	cpm	% of total	%(-control)
free	anti-P450(scc)	144.8	1210	0.83	(0.8)
"	anti-P450(11-β)	144.8	1350	0.93	(0.9)
"	control IG	144.8	50	0.03	-
bound	anti-P450(scc)	48.9	540	1.09	(1.0)
"	anti-P450(11-β)	48.9	670	1.37	(1.3)
"	control IG	48.9	40	0.07	-

To determine the sizes of cell-free synthesized P-450 peptides, the cell-free translation products immunoadsorbed to Protein A-Sepharose were analyzed by SDS-polyacrylamide gel electrophoresis. Fig. 1 shows that a main radioactivity peak corresponding to the mature size of P-450(scc) appeared in the products immunoadsorbed to P-450(scc) antibodies from the total translation products synthesized by either free or bound polysomes, suggesting that P-450(scc) was synthesized apparently as the mature size.

Table II. In Vitro Synthesis of P-450(C-21) by Free and Bound Polysomes in Rat Liver Cell Sap System.

Experimental conditions were the same as described in the legend to Table I. Ovalbumin immunoprecipitates were prepared as the control.

Polysomes	Antibodies	Radioactivity			
		Total	Immunoprecipitates		
		cpm x 10^{-3}	cpm	% of total	%(-control)
free	anti-P450(C-21)	205.6	4160	0.20	(0.10)
"	anti-Ovalbumin	205.6	2220	0.10	-
bound	anti-P450(C-21)	220.7	3510	0.15	(0.09)
"	anti-Ovalbumin	220.7	1350	0.06	-



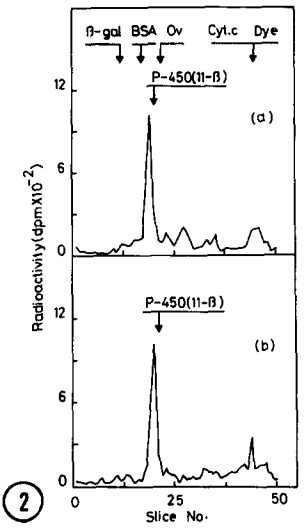


Fig. 1, SDS-Polyacrylamide Gel Electrophoretic Analysis of P-450(scc) Synthesized by Free (a) and Bound (b) Polysomes in Rat Liver Cell Sap System.
P-450(scc) peptides were isolated from total translation products by immunoadsorption using Protein A-Sepharose, and analyzed by SDS-polyacrylamide gel electrophoresis using 10 % separation gel. Molecular weight markers were β-galactosidase (β-gal, MW 130,000), bovine serum albumin (BSA, MW 67,000), ovalbumin (Ov, MW 45,000), and cytochrome c (Cyt. C, MW 12,000). The position of authenic P-450(scc) (MW 50,000) is also shown.

Fig. 2, SDS-Polyacrylamide Gel Electrophoretic Analysis of P-450(11-β) Syntheiszed by Free (a) and Bound (b) Polysomes in Rat Liver Cell Sap System.
P-450(11-β) peptides were isolated from total translation products by immunoadsorption using Protein A-Sepharose, and analyzed by SDS-polyacrylamide gel electrophoresis using 10 % separation gel. Molecular weight markers used were the same as described in the legend to Fig. 1. The position of authentic P-450(11-β) (MW 45,000) is also shown.

A small radioactivity peak at a large molecular weight position possibly represents the dimer of P-450(scc), since the position corresponds with the molecular weight (100,000 daltons) of the dimer.

Fig. 2 shows that a radioactivity peak corresponding to a molecular weight larger than that of mature P-450(11-β) by about 5,000 daltons appeared in the products immunoadsorbed to P-450(11-β) antibodies from the total translation products synthesized by either free or bound polysomes in rat liver cell sap system. The same large molecular weight products were immunoadsorbed to P-450(11-β) antibodies from the total translation products programmed by total polysomes in wheat germ lysate system (Fig. 3). The

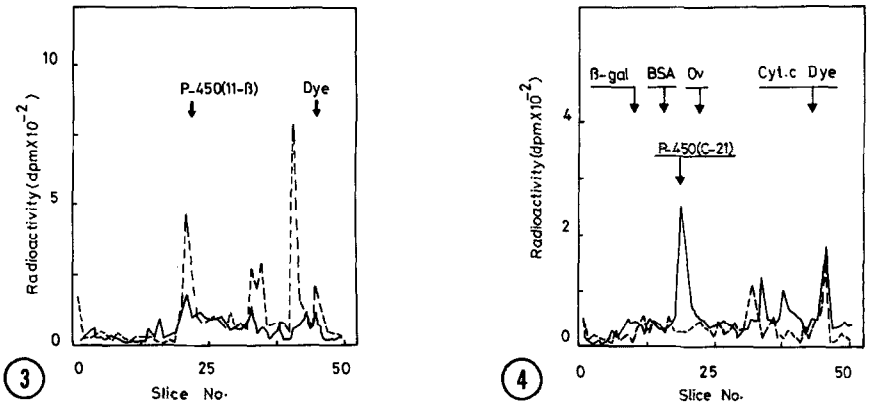


Fig. 3, Competition between In Vitro Synthesized P-450(11-β) and Authentic P-450(11-β) for Immunoreaction with Antibodies.
In vitro synthesized P-450(11-β) peptides were isolated by immunoadsorption from the total translation products programmed by total polysoems in wheat germ lysate ssytem in the absevce (-----) and presence (———) of authentic P-450(11-β) whose amount was three times excess than the equivalent amount of the antibody used. The analysis by SDS-polyacrylamide gel electrophoresis was carried out as described in the legend to Fig. 1.

Fig. 4, SDS-Polyacrylamide Gel Electrophoretic Analysis of P-450(C-21) Synthesized by Free (———) and Bound (-----) Polysomes in Rat Liver Cell Sap System.
P-450(C-21) peptides were isolated from the total translation products by immunoadsorption using Protein A-Sepharose, and analyzed by SDS-Polyacrylamide gel electrophoresis using 10 % separation gel. Molecular weight markers used were the same as described in the legend to Fig. 1. The position of authentic P-450(C-21) (MW 47,000) is also shown.

radioactivity peak was diminished when competing authentic P-450(11-β) was added to the translation products before addition of the antibodies (Fig. 3), suggesting that the peak represented a large precursor of P-450(11-β).

Fig. 4 shows that a radioactivity peak corresponding to the mature size of P-450(C-21) appeared in the products immunoadsorbed to P-450(C-21) antibodies from the total translation products synthesized by free polysomes in wheat germ lysate system, whereas no such peak appeared at the corresponding position in the case of bound polysomes. Other smaller radioactivity peaks possibly represent proteolytic fragments of P-450(C-21) peptides. The absence of mature peptides of P-450(C-21) in the products of bound polysomes could be attributable to a protease activity associated with bound polysomes. Higher proportions of smaller peptides appeared in the products

immunoadsorbed to P-450(C-21) antibodies when the *in vitro* translation of either free or bound polysomes was carried out in rat liver cell sap system (data not shown).

DISCUSSION

This study demonstrated that both free and bound polysomes isolated from bovine adrenal cortex synthesize three species of cytochrome P-450, P-450(scc), P-450(11-β), and P-450(C-21), in cell-free systems. Synthesis of these P-450 species by both free and bound polysomes could not be attributed to the cross-contamination between these two types of polysomes, since exclusive synthesis of adrenodoxin reductase by free polysomes (14) indicated a good separation between them.

P-450(scc) and P-450(C-21) were synthesized apparently as the mature size products in the cell-free systems used. However, we can not rule out the existence of large precursor forms of these P-450 species, since a small difference in the molecular size between cell-free products and mature forms may not be detectable by the technique used in this study. Further investigations are needed in order to ascertain the existence of large precursor forms of P-450(scc) and P-450(C-21). On the other hand, cell-free synthesized P-450(11-β) was about 5,000 daltons larger than the mature form, and this large cell-free product competed with authentic P-450(11-β) for immunoreaction with the antibodies. We suggest that P-450(11-β) was synthesized as a large precursor form in both rat liver cell sap and wheat germ lysate systems. The *in vitro* synthesis of liver microsomal cytochrome P-450 has recently been reported (16-20), and a 3-methylcholanthrene-inducible form, which is often referred to as cytochrome P-448, is reported to be synthesized as a large precursor form (20).

The synthesis of typical integral membrane proteins like P-450(scc), P-450(11-β), and P-450(C-21) by both free and bound polysomes is noteworthy. Two species of liver microsomal cytochrome P-450 have recently been found to be synthesized exclusively on membrane-bound polysomes (16-21). Although

primary sequences of the three cytochrome P-450s examined in this study are not yet known, synthesis of each of these cytochrome P-450 species could continue up to near completion on free polysomes if the membrane-binding hydrophobic segment of each P-450 peptide is located at the C-terminal end. Recently, two membrane proteins of endoplasmic reticulum have also been reported to be synthesized mainly on free polysomes (22-24). Our observations suggest that mitochondrial P-450(scc) and P-450(11-β), and microsomal P-450(C-21) share common sites of synthesis in the cytoplasm of adrenal cortex cells, and their intracellular distribution occurs by some post-translational mechanism.

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