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PREPARATION OF A HIGH-ACTIVE MICROSOMAL MONOOXYGENASE SYSTEM RECONSTITUTED BY MEANS OF SELF-ASSEMBLY

ILYA B. TSYRLOV, VLADIMIR M. MISHIN, OLGA A. GROMOVA, NATALIYA E. ZAKHAROVA and VYACHESLAV V. LYAKHOVICH

Department of Biochemistry, Institute of Clinical and Experimental Medicine, Academy of Medical Sciences of the U.S.S.R., Siberian Branch, Novosibirsk 630091, U.S.S.R.

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Abstract—The present work is devoted to study of the role of glycerol in preservation of the lipoprotein nature of cytochrome P-450 during the stages of sodium cholate-caused solubilization of microsomal fraction and subsequent self-assembly of a monooxygenase system after prolonged dialysis. The system thus reconstituted is characterized by the highest catalytic activity of cytochrome P-450 compared to other reconstituted liver microsomal hydroxylating systems presently described in literature.

Investigation of the mechanism of function and principles underlying the organization of multicomponent membrane-bound enzyme systems through solubilization, fractionation into individual components and subsequent reconstitution currently occupies a prominent place in the works devoted to the biochemistry of mitochondria [1] and microsomes [2, 3]. Thus, it has been found that solubilized fractions of cytochrome P-450 and NADPH-cytochrome P-450 reductase, and phosphatidylcholine are those components whose recombination ensures partial restoration of catalytic monooxygenase activity of the artificial system [2]. On the other hand, this approach is largely associated with the identification and more detailed study of the properties of components which are solubilized by sodium cholate and subsequently purified. In general, it helps to identify only enough components and conditions under which model monooxygenase systems would function.

In this sense, the approach of Archakov *et al.* [4] seems to be more promising. Here, sodium cholate is removed by prolonged dialysis from a solubilized fraction containing all the functional and structural components of the smooth endoplasmic membranes, thus creating the conditions for self-assembly of the microsomal monooxygenase system. This makes it possible in principle to evaluate not only the contribution of each component to the overall hydroxylation activity of the reconstituted system but also the character of interactions between these components and with the units of the membranous structure, that is, to bring out all the essential factors in the organization and function of the monooxygenase systems.

However, the absence of any protecting agent (excluding dithiothreitol) during solubilization of the liver microsomal fraction with sodium cholate reduced this part of the work [4] to an attempt at reconversion of hemoprotein P-420 to P-450 by using dialysis against a solution not containing a detergent. This procedure confirmed the data described earlier by Ishikawa and Yamano [5] concerning the appearence of a negligible amount of cytochrome P-450 spectra in the ultraprecipitate of the solubilized fraction after dialysis, and the system as a whole was marked by weak functional activity [4].

The present work is devoted to a study of the role for glycerol during the stages of solubilization and dialysis in the process of reconstitution by means of self-assembly of microsomal monooxygenase system and to the assessment of the effectiveness of this method as compared to those described in literature [2–4, 6, 7].

MATERIAL AND METHODS

Male Wistar rats (180–200 g) used in the experiments, were killed by decapitation. Livers were perfused *in situ* with 1.15% KCl solution containing 0.2 mM dithiothreitol. Isolation of microsomal fraction (fraction I) was done as described previously [8] in a medium including 20 mM Tris–HCl buffer, 1.15% KCl and 0.2 mM dithiothreitol, pH 7.4. Preparation of an agranular endoplasmic membrane (so called microsome "ghosts" fraction) was conducted as in the work [4] except that EDTA was not included in the medium containing 100 mM Tris–HCl buffer and 1.0 mM dithiothreitol (TD buffer). The obtained "ghosts" fraction was diluted by a TD buffer containing 20% glycerol (TDG buffer) until the protein content reached 15 mg/ml. The "ghosts" fraction was treated with sodium cholate (final concentration 2.6%, w/v). Then the clear yellow solution was centrifuged at 150,000 *g* for 120 min at 4° on ultracentrifuge VAC-601.

The supernatant obtained (fraction II) was divided into two equal parts. The solubilized fraction from the first part was dialyzed against a solution (1:70, v/v) containing 20 mM Tris–HCl buffer, 0.2 mM dithiothreitol, 0.1% (w/v) sodium cholate, and 10% glycerol for 20 hr at 4°; the other part was dialyzed against the same solution but without glycerol. Then the dialysis was continued against a solution (1:250, v/v) without detergent for 22 hr at 4°. The contents of the dialysis sacks (N 250-9, Sigma) were transferred

to centrifugal tubes and centrifuged at 150,000 *g* for 90 min. The pellets obtained were suspended in a TD buffer, pH 7.4. The precipitate obtained by ultracentrifugation of the diffusate after dialysis against a solution with the omission of glycerol was designated as fraction III, and the precipitate after dialysis against a solution, including 10% glycerol as fraction IV.

In all the fractions the microsomal protein content, the amount of cytochromes P-450 and P-420, the rate of NADPH-cytochrome P-450 reductase, and also the activity of aminopyrine *N*-demethylation and aniline *p*-hydroxylation were determined as described by us previously [8]. All the spectral assays were conducted in a Hitachi model 356 two-wavelength double-beam spectrophotometer.

In addition, reconstitution by self-assembly of the microsomal hydroxylation system in the presence of EDTA and the absence of glycerol in the solubilization and dialysis steps had been obtained in accordance with the method of Archakov *et al.*, [4].

The data presented are the average of 3–4 preparations; each of which included fractions isolated from liver microsomes of twenty rats.

RESULTS AND DISCUSSION

As can be seen from Table 1, the solubilized fraction II contained cytochrome P-450 in concentration of 0.64 nmoles/mg protein, which is 71 per cent of P-450 level in the intact microsomal fraction I; no contamination whatsoever by cytochrome P-420 had been observed in the preparation. The subsequent dialysis was not accompanied by the appearance of any conversion of P-450 to P-420, whether or not the dialysis solution contained glycerol (fractions III and IV). Thus the introduction of glycerol during solubilization step makes it possible to fully retain hemoprotein P-450 in native form at subsequent self-assembly. In the experiments of Archakov *et al.* [4] solubilization of the microsome "ghosts" fraction in the absence of glycerol caused the conversion of more than half of cytochrome P-450 and the appearance of considerable amounts of P-420 in the reconstituted vesicular microsome-like structures.

The mechanism of preventing the conversion of P-450 to P-420, with the aid of polyols, during the treatment of microsomal fraction with high concentrations of sodium cholate, has not been sufficiently elucidated as yet [5]. In this sense, it is possible to assume that polyols hinder either the extraction of the phospholipids [9] surrounding P-450 and coupling with this hemoprotein [10] or prevent the binding of cholate to the hydrophobic membrane proteins [11] and subsequential conformational changes in the cytochrome P-450 structure. Indeed, the treatment with sodium dodecyl sulfate, specifically binding to the hydrophobic proteins with subsequent formation of dodecyl sulfate-protein complex [12] or with trypsin, causing hydrolysis of hydrophilic proteins both leads to pronounced conversion of P-450 to P-420, whether the incubation medium contains glycerol or not.

The solubilized system (fraction II) in our work displays some drug-hydroxylating activity with regard to aminopyrine and aniline (Table 1). This is confirmed by the results of Lu *et al.* [13] demonstrating that in a reconstituted microsomal monooxygenase system, sodium cholate, at a concentration of 1 mg/ml, stimulates *N*-demethylation activity to 48 per cent of the control. In fraction II the amount of sodium cholate is 0.9 mg/ml, which, evidently, explains the observed aniline and aminopyrine metabolism.

It should be noted that this reconstituted system, electron-microscopically presented as closed vesicular structures (not shown), possessed pronounced NADPH-cytochrome P-450 reductase activity, mak-

Table 1. Cytochrome P-450 content, activity of NADPH-cytochrome P-450 reductase and substrate metabolism in the microsomal fractions of presented work (fractions I–IV) and the same fractions (A–C) in the work of Archakov *et al.* [4]

Fractions	Cytochromes		Activity of NADPH-dependent cytochrome P-450 reductase (nmole acceptor reduced · min^{-1} · mg $protein^{-1}$)	*p*-Hydroxylation aniline (nmole *p*-aminophenol formed · min^{-1} · mg $protein^{-1}$)	*N*-demethylation	
	P-450 (nmole · mg $protein^{-1}$)	P-420			Aminopyrine (nmole HCHO formed · min^{-1} · mg $protein^{-1}$)	Dimethylaniline
Fraction I (100%)	0.9	0	2.2	0.58	1.46	n.d.
Fraction II (%)	0.64 (71)	0	0	0.04 (7)	0.13 (9)	n.d.
Fraction III (%)	0.59 (66)	0	0.44 (20)	0.19 (33)	0.43 (30)	n.d.
Fraction IV (%)	0.69 (77)	0	0.62 (28)	0.18 (31)	0.19 (13)	n.d.
Fraction A (100%)	0.57	0	2.6*	0.64	n.d.	11.0
Fraction B (%)	0.25 (44)	0.59	0*	0	n.d.	0
Fraction C (%)	0.42 (74)	0.42	0*	0.07 (11)	n.d.	2.1 (20)

* Activity of the reductase was determined by us. Fractions A–C were isolated in full conformity with the method described by Archakov *et al.* [4]. n.d.—not detected.

Table 2. Molecular activity of cytochrome P-450 in different reconstituted microsomal systems

Type of reaction and substrates / No fractions	*p*-Hydroxylation aniline (nmole *p*-aminophenol formed·min^{-1}·nmole P-450^{-1})	*N*-demethylation		
		Aminopyrine (nmole HCHO formed·min^{-1}·nmole P-450^{-1})	Dimethylaniline (nmole HCHO formed·min^{-1}·nmole P-450^{-1})	Benzphetamine (nmole HCHO formed·min^{-1}·nmole P-450^{-1})
1. Fraction I	0.64	1.62		
2. Fraction III	0.32	0.73		
2./1. (%)	(50)	(45)		
3. Fraction A	1.12		20	
4. Fraction C	0.17		5	
4./3. (%)	(15)		(25)	
5. Microsomes*	0.62			2.67
6. Reconstituted system†	0.22			0.78
6./5. (%)	(35.5)			(29)

* Most typical data from literature presented.

† Data on metabolism of aniline taken from work [6]; data on metabolism of benzphetamine taken from work [7].

ing it detectable by a commonly-used method [14]. This indicates the preservation of the hydrophobic character of the interactions between the protomers of the reconstituted microsomal electron-transport chain, namely, cytochrome P-450 and its reductase [10, 15].

In evaluating the drug-metabolizing activity of this reconstituted system, it may be concluded that the rate of *p*-hydroxylation reaction is 33 per cent and the rate of *N*-demethylation reaction is 30 per cent of the level of fraction I; these values exceed those obtained in a system where glycerol had not been used [4] by 3 and 1.5 times, respectively (Table 1).

The data presented in Table 2 seem to be more comparable, since they sum up the results of several laboratories regarding the reactivation of such a specific characteristic as the molecular activity of the enzyme (cytochrome P-450) when using various substrates subjected to *p*-hydroxylation and *N*-demethylation. For convenience, a comparision is drawn between the molecular activity of cytochrome P-450 from different reconstituted systems with the corresponding molecular activity of P-450 from intact microsomal fractions from which these systems (or components of the systems) have been obtained.

Table 2 shows that when using aniline as a substrate, cytochrome P-450 in the reconstituted microsomal system of Archakov *et al.* [4] displayed the least molecular activity; then comes the reconstituted system of Lu *et al.* [6] and that of Fujita and Mannering [3], whose results are similar to the former. Finally, the highest mooxygenase molecular activity, 50 per cent of the level of intact microsomes, was obtained when using the method described in this work. The same was observed when comparing the molecular activity of *N*-demethylases of different reconstituted systems (Table 2).

Thus, comparison of the methods used for reconstituting microsomal monooxygenases from the solubilized structural and(or) functional components, offered by literature, shows that the most effective reactivation of catalytic properties of a reconstituted enzyme system is observed when the lipoprotein nature of cytochrome P-450 [16] is preserved at the stages of detergent-caused solubilization with the aid of glycerol and after subsequent removal of this polyol during dialysis and self-assembly.

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