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Immobilized cytochrome P-450$_{LM2}$

Dissociation and reassociation of oligomers

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Subunit interactions in the purified hexameric cytochrome P-450$_{LM2}$ have been studied using covalent binding of one of the 6 protomers to an insoluble matrix. High ionic strength, large-scale pH changes, guanidine chloride and sodium cholate taken at membrane-solubilizing concentrations, had no effect on the aggregation state of the immobilized hemoprotein. SDS caused a 6-fold decrease in the amount of the bound cytochrome. Non-ionic detergents (Emulgen 913, octylglucoside, Tritons) induced hexamer dissociation. In the presence of Emulgen 913 (>0.2%), monomers and immobilized dimers were obtained as cytochrome P-450 was studied in an aqueous medium and in the immobilized state, respectively. Immobilized dimers could be reconstituted to hexamers by treatment with an excess of solubilized monomers after removal of the detergent. In the presence of various phospholipids, which increased the immobilized cytochrome P-450$_{LM2}$ demethylase activity and induced characteristic spectral changes, no hexamer dissociation was shown. The data obtained are thus in agreement with the suggestion that hexameric arrangement is inherent in the cytochrome P-450 when it is bound to the native membranes.

Cytochrome P-450; Hexamer; Immobilization; Detergent; Phospholipid

1. INTRODUCTION

SDS-electrophoresis of purified microsomal cytochromes P-450 reveals a single 50-kDa band which corresponds to the monomeric form of the enzyme [1]. At the same time, some cytochrome P-450$_{LM2}$ oligomers of various molecular masses (270–700 kDa) were described when the native enzyme was studied [2,3]. In our previous studies, a monodisperse 330 kDa cytochrome P-450$_{LM2}$ preparation was obtained. Electron microscopy showed its hexameric organization [4,5]. It was also found that the cytochrome can be immobilized by means of covalent binding of one of the 6 protomers to an insoluble matrix [6]. In the present paper we have studied dissociation and reassociation of the immobilized cytochrome P-450$_{LM2}$ hexamers.

2. MATERIALS AND METHODS

Cytochrome P-450$_{LM2}$ was isolated from the rabbit liver as was described previously [5].

Immobilization of cytochrome on the bromocyane-activated Sepharose 4B was performed according to the procedure developed in this group [6]. The excess of the activated groups at the Sepharose surface, which did not combine with the protein, was titrated by glycine (in some cases, by [^{14}C]glycine). The non-bound cytochrome, as well as the detergents, were removed by washing with 20% glycerol and 100 mM KH_2PO_4 (or 25 mM Hepes/100 mM NaCl), pH 7.5. The cytochrome P-450 concentration in solution was measured spectrophotometrically [7] using a Hitachi-557 spectrophotometer. To measure the concentration of the immobilized cytochrome P-450, an Aminco DW2a spectrophotometer was employed. To the same purpose Coomassie G-250 staining and in some cases the CTAB-induced spectral shift were used [6].

Analytic centrifugation was carried out in a Spinco ϵ ultracentrifuge (60,000 rpm), equipped with a scanning system, measuring the optical density at 418 nm. The samples contained 100 mM KH_2PO_4, pH 7.5, 10% glycerol and detergent as indicated in the Figure captions.

The demethylase activity of the immobilized cytochrome P-450 was measured in a reconstituted system containing NADPH-cytochrome P-450 reductase or cumene hydroperoxide [6].

To modify cytochrome P-450 by iodoacetamide, a solution of 100 mM KH_2PO_4, pH 7.5, containing a 6-fold excess of [^{14}C]iodoacetamide was mixed with the solution of cytochrome P-450 (50–60 nmol/ml and incubated for 3 h, 4°C. Then the excess of [^{14}C]iodoacetamide was removed using a Sephadex G-25 column. Under such conditions, about 5% of the cytochrome P-450 molecules proved to be modified so that no measurable formation of inactive cytochrome P-420 took place. The radioactivity of the modified cytochrome P-450 was measured according to the standard procedure [8].

3. RESULTS AND DISCUSSION

Immobilization of cytochrome P-450$_{LM2}$ was performed under conditions when only one monomer of each hexamer was covalently bound to the bromocyane-activated Sepharose [6] so that dissociation of the immobilized cytochrome P-450 by 0.1% SDS resulted in a

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Abbreviations: SDS, sodium dodecyl sulfate; CTAB, cetyltrimethylammonium bromide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine

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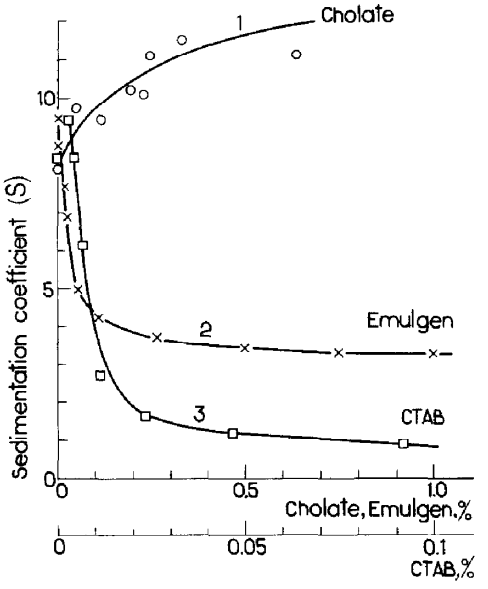

Fig. 1. Effect of detergent upon the sedimentation coefficient of cytochrome P-450 in the aqueous medium. The cytochrome P-450 concentration was 4×10^{-6} M, $T = 20°C$.

6-fold decrease in the amount of the bound cytochrome (Table I; see also Figs. 3 and 4).

At the same time, cholate concentrations which are known to solubilize biomembranes, failed to dissociate the immobilized hexamers (Table I). This agrees with the fact that the cholate concentrations did not decompose the hexamers in solution as was shown in the ultracentrifugation experiment (Fig. 1, curve 1). Some increase in the sedimentation coefficient was due to the cholate binding by cytochrome P-450 [9]. In this connection it should be mentioned that much lower cholate concentration (0.1–0.2%) were found to effectively dissociate complexes of cytochrome P-450 with various proteins [10].

Non-ionic detergents such as Emulgen 913 (polyoxyethylene nonylphenol ether, 800 kDa), octylglucoside

Table I

Effect of various treatments upon the cytochrome $P\text{-}450_{LM2}$ immobilized on Sepharose 4B

Treatment	Amount of the protein retained on Sepharose 4B (%)
-	100
0.8% Sodium cholate	100
0.1% SDS	16
0.2% Emulgen 913	33
1% Emulgen 913	33
0.8% Octylglucoside	33
Liposomes[a]	100
2 M NaCl	100
2 M KCl	100
5 M Guanidine chloride	> 90

[a]Egg PC, the mixture of egg PC, bovine brain PE and phosphatidic acid (10:1:1), or synthetic dilauroylphosphatidylcholine were used.

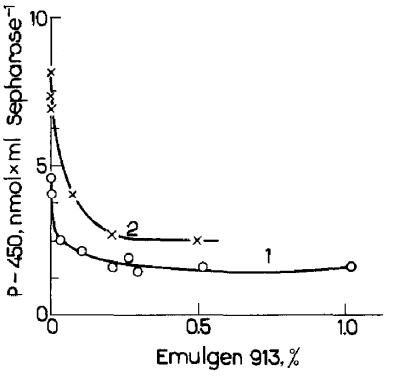

Fig. 2. Emulgen 913-induced dissociation of immobilized cytochrome P-450.

and Tritons were shown to dissociate the cytochrome P-450 hexamers. As can be seen in Fig. 1 (curve 2), one amount of Emulgen 913, higher than the concentration of micelle formation, decreased the cytochrome P-450 sedimentation coefficient. An increase in the Emulgen 913 level from 0.2 to 1% did not cause any further decrease in the sedimentation coefficient. As was recently found in our group [11] by means of the sedimentation equilibrium measurements in D_2O solutions of different density [11], molecular mass of product of the cytochrome $P\text{-}450_{LM2}$ hexamer dissociation in 0.2% Emulgen 913 is equal to 50 ± 8 kDa. In calculations, contribution of the bound detergent was taken into account. In the presence of 0.2% Emulgen 913, no demethylation activity of cytochrome P-450 was observed.

When the immobilized cytochrome P-450 was treated with 0.2% Emulgen 913, the amount of the Sepharose-bound cytochrome was found to decrease by factor 3 independently of the initial quantity of the bound cytochrome pool (Table I and Fig. 2). One percent of Emulgen 913 failed to induce further dissociation of the immobilized cytochrome P-450. This could be achieved by 0.1% SDS which caused a two-fold decrease in the amount of the bound cytochrome treated with Emulgen 913.

A similar study was carried out using [^{14}C]iodoacetamide-modified immobilized cytochrome P-450 (Fig. 3). The Emulgen 913 treatment caused elution of about 4-fold larger portion of the [^{14}C]protein than subsequent SDS treatment. So, one can conclude that Emulgen 913 dissociates the immobilized hexamer to one immobilized dimer and *four* solubilized monomers and the SDS treatment results in dissociation of the immobilized dimer to one immobilized monomer and *one* solubilized monomer.

The immobilized cytochrome P-450 monomers, obtained by means of SDS treatment, proved to be inactive. As to the 0.2% Emulgen 913 treatment, the immobilized dimers still catalyzed *N*-demethylation of dimethylamine provided that the detergent was washed

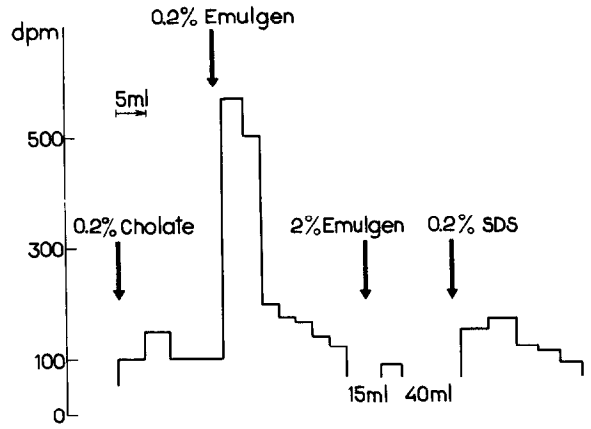

Fig. 3. The detergent-induced elution of [1-^{14}C]iodoacetamide-modified cytochrome P-450 immobilized on Sepharose 4B.

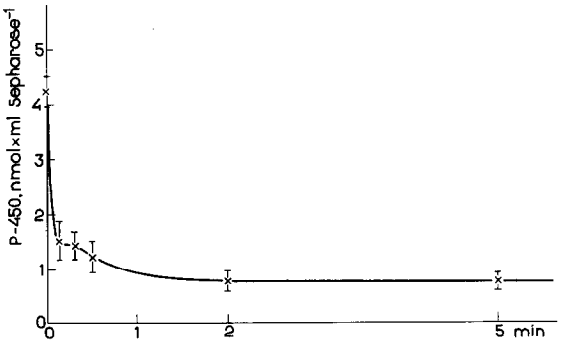

Fig. 4. The SDS-induced dissociation of cytochrome P-450 immobilized on Sepharose 4B, SDS concentration, 0.1%.

out (not shown). It was also found that immobilized hexamers could be reconstituted from: (1) immobilized dimers; and (2) solubilized monomers obtained by means of Emulgen 913 treatment. Reconstitution was shown to occur when the detergent was removed by Amberlite XAD-2 (Table II). The reconstituted cytochrome exhibited the same spectral and catalytic properties as the immobilized hexamers [6].

Formation of immobilized dimers from hexamers was shown to occur when octylglycoside was used instead of Emulgen 913 (Table I). In solution, monomerization and inactivation of cytochrome P-450 by octylglycoside was described [12]. In our experiments, a cationic detergent CTAB (0.3%) as well as 0.5% Triton X-305 and 1% Triton WR-1339 caused some decrease (up to 50%) in the amount of the immobilized cytochrome P-450. Noticeable lowering of the cytochrome P-450 sedimentation constant by CTAB (Fig. 1, curve 3) seems to indicate that this detergent is more effective in dissociating the soluble cytochrome than immobilizing one and thus resembles in this respect the action of 0.2% Emulgen 913.

The immobilized cytochrome P-450 was found to preserve the hexameric organization when: (1) stored at 4°C in the solutions of potassium phosphate or Hepes, pH 7.5, supplemented with 20% glycerol; (2) washed with a 500-fold volume of these buffers; (3) treated with 2 M NaCl or KCl, or (4) treated with 5 M guanidine chloride. The same stability was inherent in the cytochrome P-450 dimers obtained by Emulgen 913 treatment.

Two-layer structure of hexamer [5] suggests existence of two types of intersubunit contacts. Resistance of the immobilized dimers to some detergents is most probably due to higher stability of one of these contact types when covalently-bound monomer is involved.

In this context, one should point to the two-phase SDS dissociation of the cytochrome P-450 hexamer (Fig. 4). Such relationships are in agreement with the general principles of organization of quasispherical hexamer proteins [13].

Significantly, various phospholipids did not dissociate the immobilized cytochrome P-450 hexamers (Table I). At the same time, phospholipids induced some activation of the immobilized enzyme and a characteristic change in its spectrum [6] described by Schenkman et al. [14]. The magnitude of the effect indicated that almost 50% of the Sepharose-bound cytochrome P-450 could contact the added phospholipids. Incubation at 37°C and repeated treatment of the immobilized cytochrome P-450 with phospholipids did not decrease the concentration of the enzyme bound to Sepharose (Table I) and did not induce transfer of the [^{14}C]iodoacetamide-modified cytochrome to the phospholipid phase. That transfer was absent even when the mixture of immobilized cytochrome P-450 and phospholipids was sonicated, the procedure resulting in the decomposition of the Sepharose granules.

In some experiments, Sephadex G-25 was used as a matrix instead of Sepharose 4B. In this case, all of the

Table II

Reconstitution of cytochrome P-450 hexamers from immobilized dimers and solubilized monomers

No.	Preparation	Amount of bound cytochrome (nmol/ml wet Sepharose 4B)
1	Immobilized hexamers	4.2
2	Immobilized dimers obtained due to the 0.2% Emulgen 913 treatment of preparation 1	1.4
3	Preparation 2 after incubation with solubilized hexamers and washing with the buffer solution	3.4
4	Preparation 2 after incubation with an excess of solubilized monomers in the Emulgen 913 solution and amberlite XAD-2 treatment	4.3

Incubation in cases 2–4: wet Sepharose with the immobilized cytochrome (1 ml) was added to 5 mM cytochrome solution (5 ml) containing detergent when indicated. The mixture was kept at 4°C for 20 h.

immobilized cytochrome P-450 was accessible to the added phospholipids, because the bound cytochrome molecules were localized only on the surface of the granules since their pores were too small for the protein to penetrate the granular core. Also in this system, the protein could not be transferred to the phospholipid phase. Thus we may conclude that cytochrome P-450 preserves its hexameric organization in the presence of phospholipids. This fact is consistent with our assumption that the hexameric organization can be inherent in the membrane-bound cytochrome P-450 [5,6,10,11].

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