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Cytochrome P-450$_{LM2}$ oligomers in proteoliposomes

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The effects of bifunctional cross-linking reagents on the purified hexameric cytochrome P-450$_{LM2}$ in an aqueous medium and on the proteoliposomal cytochrome P-450$_{LM2}$ have been compared. In both cases, cross-linking is shown to result in the appearance of a range of additional protein bands in SDS electrophoretograms. The number and the positions of these bands seem to be similar in the solubilized and in the proteoliposomal cytochromes. No additional bands appear when the purified cytochrome P-450 was pretreated with 0.2% Emulgen 913, which decomposes cytochrome P-450$_{LM2}$ hexamers. The results indicate that the membrane-bound cytochrome P-450 can exist in the oligomeric (presumably hexameric) form.

Cytochrome P-450; Hexamer; Proteoliposome; Cross-linking

1. INTRODUCTION

Some indications of the existence of cytochrome P-450 oligomers in membranes of the liver endoplasmic reticulum have been reported in the past 10 years [1–4]. In our group, the hexameric organization of cytochrome P-450$_{LM2}$ has been shown in studies on the purified hemoprotein in aqueous solution [5,6] and on the immobilized hemoprotein [7–9].

We have investigated the oligomer organization of cytochrome P-450$_{LM2}$ in the proteoliposomal membrane. The purified hexameric cytochrome P-450$_{LM2}$ in solution and cytochrome P-450$_{LM2}$ in the proteoliposomes were treated with bifunctional cross-linking reagents. Since the major part of the cytochrome P-450 molecule protrudes from the membrane [10], we may assume that accessibility of the cytochrome to cross-linking reagents in solution and in the membrane should be rather similar. As the experiments showed, the SDS electrophoretic patterns of the cross-linked proteoliposomal cytochrome P-450 resemble those of the solubilized cytochrome. This fact indicates that cytochrome P-450$_{LM2}$ can form oligomers not only in the solubilized state but also in the membrane.

2. MATERIALS AND METHODS

Cytochrome P-450$_{LM2}$ was isolated from rabbit liver microsomes as described elsewhere [5,6]. The final preparation contained solely cytochrome P-450 hexamers. The SDS electrophoresis of this preparation [6] revealed a single band corresponding to monomer of the LM2 isoform. The A_{418}/A_{280} ratio was found to be 1.95, i.e. close to the maximal value, which shows that the obtained cytochrome was free of the detergent and other contaminations.

The concentration of the solubilized cytochrome P-450 was estimated by the carbon monoxide difference spectrum method [11] or by absorption of the oxidized protein at 418 nm (ε_{418} = 107 $\mu M^{-1} \cdot cm^{-1}$). The former method was used for determining the amount of cytochrome P-450 in proteoliposomes.

As the lipid component for proteoliposome reconstitution, we used synthetic dimyristoyl phosphatidyl choline, microsomal phospholipids or a mixture containing egg phosphatidylcholine, bovine brain phosphatidylethanolamine (or synthetic distearoyl phosphatidylethanolamine), and phosphatidic acid in the proportion 10:1:1.

To form proteoliposomes, the cholate dialysis technique or its gel-filtration modification were employed. In addition, incorporation of cytochrome P-450 into the lipid membrane was carried out by incubating the solubilized cytochrome with sonicated liposomes or with liposomes prepared by the reverse phase method ([12,13] see also [14] for details). The incubation was carried out at 37°C (which is above the phase transition temperature of the majority of the phospholipids used) or, when indicated, at a lower temperature. The formed proteoliposomes were separated from liposomes and from an excess of the protein by passing through a Sephacryl S-1000 column (0.5 × 15 cm). To prevent proteoliposome sorption on Sephacryl, the liposome suspension was passed through the column before the proteoliposome separation.

When proteoliposomes were prepared with the cholate-dialysis or the cholate-gel filtration method, a mixture containing 20 mg of phospholipid, 150 μl of buffer (25 mM HEPES, 0.1 M NaCl, 0.1 mM EDTA and 2.5% glycerol, pH 7.5), 50 μl of 20% sodium cholate and 0.3 ml cytochrome P-450$_{LM2}$ (about 10 nmol) was incubated for 1 h at 4°C. The cholate was removed by dialysis against the buffer or by using a Sephadex G-25 column washed with a phospholipid and cholate mixture and equilibrted with the buffer. Fractions containing proteoliposomes were layered onto a 0–20% Ficol-400 gradient and centrifuged at 280 000 × *g* for 10–12 h, 6°C, in an SW-40 rotor of a Beckman centrifuge. Proteoliposomes were concentrated as a thin layer in the upper quoter of the centrifuge tube (buoyant density 1.04–1.06 $g \cdot cm^{-3}$). In some experiments 0.1 M potassium phosphate, pH 7.5, was used instead of HEPES buffer.

The enzymatic activity of cytochrome P-450 was assayed as the rate of benzphetamine *N*-demethylation in the presence of NADPH-cytochrome P-450 reductase or of cumene hydroperoxide [7].

As bifunctional cross-linking reagents, we used DMS, DTBP,

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Abbreviations: DFDNB, 1,5-difluoro,2,4-dinitrobenzene; DFNPS, di(4-fluoro-3-nitrophenyl)-sulfon; DMS, dimethylsuberimidate; DTBP, dimethyl-3,3-dithiobispropionimidate; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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DFDNB and DFNPS (effective length of the molecule, 1.08, 1.19, 0.45 and 0.85 nm, respectively). The cross-linking was performed at 20°C and 37°C. SDS electrophoresis of the cross-linked proteins was carried out in a 5–15% polyacrylamide gel gradient.

Bacteriorhodopsin from *Halobacterium halobium* was a generous gift from Dr A.D. Kaulen.

3. RESULTS AND DISCUSSION

It is well known that partial cross-linking of promoters in water-soluble oligomeric proteins results in the formation of dimers, trimers etc., which can be detected by SDS electrophoresis, the number of zones on the electrophoretogram being equal to that of promoters in the native oligomer.

As one can see on the SDS electrophoretograms in Fig. 1, the cross-linking of the solubilized cytochrome $P\text{-}450_{LM2}$ hexamer results in the appearance of additional bands of higher molecular masses than the monomer. An increase in the concentration of the cross-linking reagents or in the treatment time leads to an increase in the number of bands and in their densities. Such an effect is observed not only with DMS (the length of the molecule 1.08 nm) but also with DFDNB (0.45 nm). This fact indicates the tight contact of subunits in the hexamer.

The major band of the cross-linked cytochrome P-450, (about 45 kDa) corresponding to the cytochrome P-450 protomer, was found to be of somewhat higher mobility than that of the native cytochrome. In the region of 90–110 kDa, corresponding to the dimer, two bands are visible, a phenomenon that can be explained by assuming that the dimer-forming protomers are cross-linked in two different fashions. This may well be due to the fact that the hexamer-forming protomers are organized in two layers [5,6].

One more diffuse band (probably a trimer) is detected near 150 kDa. In the 200–300 kDa region, several bands are observed. Molecular masses above 100 kDa can be estimated only approximately because of the possible intramolecular cross-linking which may prevent complete denaturation of the protein globule.

The SDS-electrophoretic patterns in the cases of DMC (Fig. 1A), DFDNB (Fig. 1B), as well as DFNPS and DTBP proved to be similar as a characteristic feature of the cytochrome $P\text{-}450_{LM2}$ hexamer.

At the same time, the treatment of the solubilized cytochrome P-450 by such detergents as Emulgen 913 which was found to induce dissociation of hexamers [15], decreased the cross-linking significantly (Fig. 1). A similar effect was observed when octylglucoside, CHAPS and cetyltrimethylammonium bromide were used instead of Emulgen 913 (not shown). These data are in agreement with our previous study on detergent-induced cytochrome P-450 hexamer decomposition [8,9].

In experiments with DFDNB, it has been demonstrated that the Emulgen 913-induced decrease in the cross-linking was not due to the competition between DFDNB and Emulgen 913 for interaction with the protein. Modification of cytochrome P-450 by DFDNB was followed by a characteristic shift of the DFDNB spectrum (not shown).

In the next series of experiments, the cross-linking of cytochrome P-450, incorporated into proteoliposomes, was studied. Several methods of incorporation and various phospholipid mixtures were tested.

When the non-detergent procedure of the proteoliposome formation was used, cytochrome P-450 was incubated with liposomes obtained by sonication or by the reverse phase method. In the latter case, the diameter of the formed proteoliposomes was rather large, being of the same order of magnitude as that of

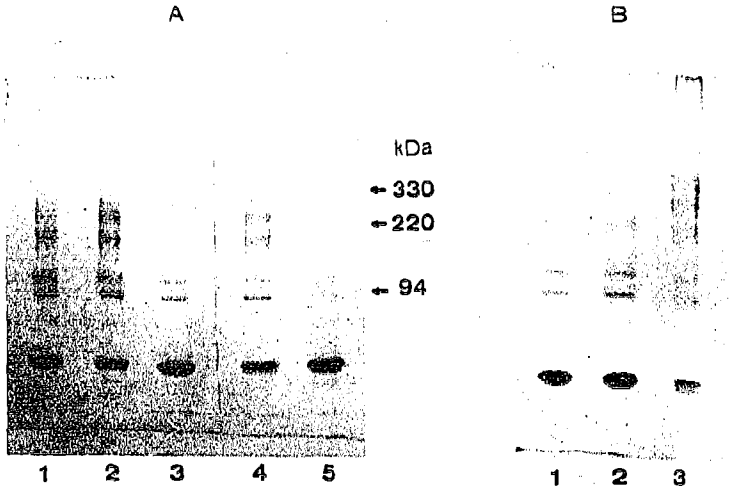

Fig. 1. SDS electrophoresis of the cross-linked purified hexameric cytochrome $P\text{-}450_{LM2}$ in polyacryl amide gel. (A) cross-linking by DMS. (1) 1 min treatment with 10 mM DMS; (2) 10 mM DMS, 5 min; (3) 2 mM DMS, 1 min; (4) 2 mM DMS, 30 min; (5) 10 mM DMS, 1 min in the presence of 0.2% Emulgen 913. For reference, the positions of myosin (330 kDa), ferritin (220 kDa) and glycogen phosphorylase (94 kDa) are indicated by arrows. (B) cross-linking with DFDNB (30 min). (1), (2) and (3), 0.05, 0.1 and 1 mM DFDNB, respectively.

microsomes. However, liposomes were mainly multimembranous so that cytochrome P-450 incorporation proved to be small (25 pmol protein × mg phospholipid $^{-1}$).

20-fold higher incorporation values were obtained when cytochrome P-450 was inlaid into bacteriorhodopsin proteoliposomes. The latter were prepared from liposomes reconstituted by the reverse phase method. Such a favourable effect of bacteriorhodopsin agrees with the previous observation that the incorporation of other hydrophobic proteins into bacteriorhodopsin proteoliposomes proceeds much better than into liposomes [16]. This may be due to the fact that bacteriorhodopsin stimulates the formation of monomembrane (rather than multimembrane) vesicles [14, 16] and causes a lateral heterogeneity of the membrane [16]. The enzymatic activity of cytochrome P-450 in bacteriorhodopsin-containing proteoliposomes proved to be somewhat higher than in proteoliposomes without bacteriorhodopsin. Bacteriorhodopsin was found to be without effect on the cytochrome P-450 spectrum.

The cross-linking of cytochrome P-450 embedded in the proteoliposome membrane, like that of the solubilized cytochrome P-450, resulted in the appearance of additional bands in the SDS polyacrylamide gel. In the solubilized and the proteoliposomal cytochrome, the number and position of the bands were shown to be similar (Fig. 2).

It seems important that the cross-linking of cytochrome P-450 protomers took place irrespective of the method of proteoliposome formation, including cholate-dialysis techniques. As it was previously found in our group [8], the treatment of the immobilized, [^{14}C]iodoacetamide-modified cytochrome P-450 hexamers with a cholate solution of phospholipids resulted in elution of some radioactivity. Cholate and phospholipids, taken separately, were ineffective. Apparently the removal of cholate by dialysis gives rise to reassociation of hexamers from products of their dissociation in the proteoliposome membrane.

Experiments with the bacteriorhodopsin-cytochrome P-450 proteoliposomes showed that the cross-linking of these two proteins did not take place. In the electrophoretogram (Fig. 3), one can see (i) a bacteriorhodopsin band (about 26 kDa) and (ii) bands of the cytochrome P-450 protomer and oligomers in the same positions as in the cytochrome proteoliposomes without bacteriorhodopsin. The cross-linking of bacteriorhodopsin results in the formation of a dimer band of about 52 kDa.

Variations in the structure of the bifunctional reagents (DMS, DTBP, DFDNB and DFNPS were used) and in the phospholipid component of proteoliposomes were without any detectable effect upon the proteoliposomal cytochrome P-450 cross-linking. In this study, individual synthetic phospholipids, phospholipid mixtures and microsomal phospholipids were used. In the case of proteoliposomes, composed of bacteriorhodopsin and cytochrome P-450, no indications of bacteriorhodopsin-cytochrome P-450 cross-linking were obtained, even when bacteriorhodopsin was in a 20-fold excess over cytochrome P-450.

Thus, the above results indicate that the hexameric structure of cytochrome P-450$_{LM2}$ discovered in experiments on the solubilized protein [5,6] seems to be preserved in proteoliposomes. This fact is consistent with our previous observation that the immobilized cytochrome P-450 hexamers cannot be dissociated by the phospholipid treatment [8,9], as well as with data on rotational diffusion of cytochrome P-450 [3] and with other evidence indicating the existence of cytochrome

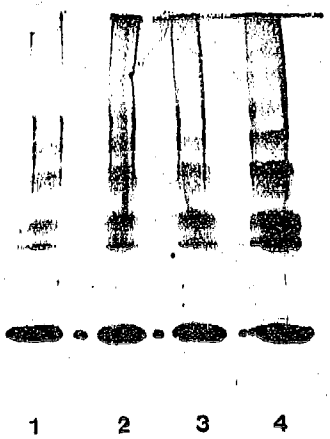

Fig. 2. SDS electrophoresis of cytochrome P-450 cross-linked in solution (4) and in proteoliposomes prepared by the cholate-gel filtration procedure (1–3). Egg phosphadityl choline was used as the lipid component. (1) and (3), 2 mM DMS; (2), 4 mM DMS, 30 min treatment; (4), 2 mM DMS, 10 min.

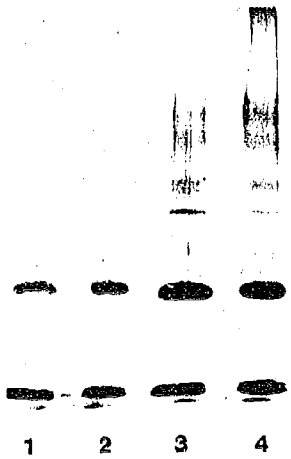

Fig. 3. SDS electrophoresis of cytohrome P-450 and bacteriorhodopsin cross-linked in proteoliposomes. Lipid component, microsomal phospholipids. (3) and (4), proteoliposomes with cytochrome P-450 and bacteriorhodopsin. (1) and (2), bacteriorhodopsin. 10 mM DMS in all the cases. (1), 5 min treatment; (2) and (4), 30 min treatment; (3), 1 min treatment.

P-450 oligomers in the membrane [4]. One can mention in this context that cytochrome H-450, the water-soluble cytochrome P-450 analog from cytosol, was isolated as a 400 kDa oligomer composed of 64 kDa protomers [17]. The hexamer structure of a monoxygenase from a methanogenic bacterium was recently observed in an electron microscopy study [18].

The hexameric organization of cytochrome $P\text{-}450_{LM2}$ may be important for its stabilization. We found that the transition of the immobilized cytochrome P-450 to the inactive P-420 form occurs much faster in dimers than in hexamers. It seems also possible that the hexamer arrangement results in the formation of an internal 'reaction chamber' differing in dielectric constant from the water solution or the membrane core, which may be favourable for a mono-oxygenation reaction catalyzed by the cytochrome. Yet, another possibility might be that the hexameric structure of cytochrome P-450 is needed for the regulation of its activity.

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