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THE ROLES OF SOLUBLE FACTORS IN SQUALENE EPOXIDATION BY RAT LIVER MICROSOMES

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SUMMARY: Squalene epoxidation by rat liver microsomes requires a supernatant protein factor and an acidic phospholipid in addition to NADPH and molecular oxygen. This study has shown that both the protein factor and the phospholipid are necessary for externally added squalene to bind to the catalytic site on microsomal membranes. The epoxidation of squalene thus bound or biosynthesized *in situ* from mevalonic acid proceeds effectively if the protein factor is present. Thus, the supernatant protein factor seems to play a dual function in both the binding and epoxidation of squalene in the *in vitro* assay system. The phospholipid is not required for the epoxidation of bound squalene.

Cholesterol biosynthesis involves a step in which squalene is converted to 2,3-oxidosqualene (1). This epoxidation catalyzed by rat liver microsomes requires the hepatic soluble fraction in addition to NADPH and molecular oxygen (2,3). So far, three different proteins, *i.e.* "squalene and sterol carrier protein" (4,5), "sterol carrier protein$_1$" (6,7), and "supernatant protein factor" (SPF) (8,9), have been purified from the soluble fraction of rat liver and shown to stimulate the microsomal epoxidase activity. Bloch and coworkers (8,9) have further shown that a combination of purified SPF, an acidic phospholipid and FAD can fully substitute the crude soluble fraction in eliciting the microsomal epoxidase activity; both SPF and the phospholipid are required obligatorily, but the requirement for FAD is not absolute. Nothing is, however, known of the roles of these soluble factors in the epoxidation reaction. By using phosphatidylinositol (PI) as an acidic phospholipid, we have now obtained evidence that both SPF and the phospholipid are necessary for exogenous squalene (added in the form of a mixed micelle

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Abbreviations used are: SPF, supernatant protein factor required for microsomal epoxidation of squalene; PI, phosphatidylinositol.

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with Tween 80) to bind to the proper catalytic site on microsomal membranes and that SPF, but not the phospholipid, is also required for the epoxidation per se of bound squalene.

MATERIALS AND METHODS

Soybean PI and FAD were purchased from Sigma, NADPH from Oriental Yeast Co., Tween 80 from Nakarai Chemical Co., and precoated thin-layer plates (silica gel 60) from Merck. D,L-[2-^{14}C]mevalonolactone (27.3 mCi/mmol) was obtained from New England Nuclear and converted to mevalonic acid before use. [^{14}C]Squalene was prepared enzymatically from [^{14}C]mevalonic acid as described by Popjåk (10). Its radiochemical purity was found to be 98 % by thin-layer chromatography.

Male, Sprague-Dawley rats (100-150 g) were killed by exsanguination. The livers were perfused with 1.15 % KCl solution and homogenized with 5 vol. of the KCl solution in a Potter homogenizer. The homogenate was centrifuged at 10,000 x g for 20 min, and microsomes were sedimented from the supernatant fraction by centrifugation at 78,000 x g for 90 min. The microsomes were washed at least twice with 0.1 M Tris-HCl buffer (pH 7.5) and suspended in the same buffer.

For purification of SPF, the 78,000 x g supernatant fraction obtained above was re-centrifuged at 78,000 x g for 90 min. The clear supernatant fraction was subjected to ammonium sulfate fractionation in the presence of 0.5 mM dithiothreitol. The precipitate formed between 40 and 70 % saturation was dissolved in 50 mM Tris-HCl buffer (pH 7.5) containing 0.5 mM dithiothreitol, and desalted by passing a Sephadex G-25 column. SPF was purified from the desalted solution by the method of Ferguson and Bloch (9), except that the final isoelectric focusing step was omitted. The SPF preparation thus obtained was estimated to be 30-40 % pure.

Liver microsomes containing [^{14}C]squalene biosynthesized in situ from D,L-[2-^{14}C]mevalonic acid were prepared essentially as described by Popjåk (10). In brief, a 10,000 x g supernatant fraction of rat liver was incubated anaerobically at 37°C for 2 h with [^{14}C]mevalonic acid and other ingredients, and then the mixture was centrifuged at 78,000 x g for 90 min. The microsomes thus precipitated were washed with 50 mM Tris-HCl buffer (pH 7.5). About 90 % of the radioactivity associated with the washed microsomes was shown to be due to squalene by thin-layer chromatography.

The standard reaction mixture for epoxidation assay contained 0.1 M Tris-HCl buffer (pH 7.5), 1.5 mM NADPH, 1 mM EDTA, 10 μM FAD, 40 μmol of [^{14}C] squalene (about 20,000 cpm) dispersed with Tween 80 (final concentration, 0.03 %, w/v) (11), microsomes (2 mg protein), purified SPF (20 μg), and 0.1 mg of PI in a final vol. of 1.0 ml. An aqueous suspension of PI (0.1 %, w/v) was sonicated in a Branson Sonifier for a total period of 10 min (with 9 interruptions each for 1 min) at the output setting of #7. The sonicated suspension was centrifuged at 105,000 x g for 90 min and the supernatant fraction was used as the phospholipid supplement. The epoxidation reaction was started by addition of NADPH and run aerobically (with shaking) at 37°C. After incubation the mixture was saponified, the unsaponified materials were extracted with petroleum ether, and subjected to thin-layer chromatography as described by Yamamoto and Bloch (12). The spots of squalene, 2,3-oxidosqualene and sterols were scraped off from the plate and their radioactivities were determined in a Beckman LS-250 liquid scintillation spectrometer. The epoxidase activity was calculated from the determined radioactivities and expressed as the sum of 2,3-oxidosqualene and sterol products formed.

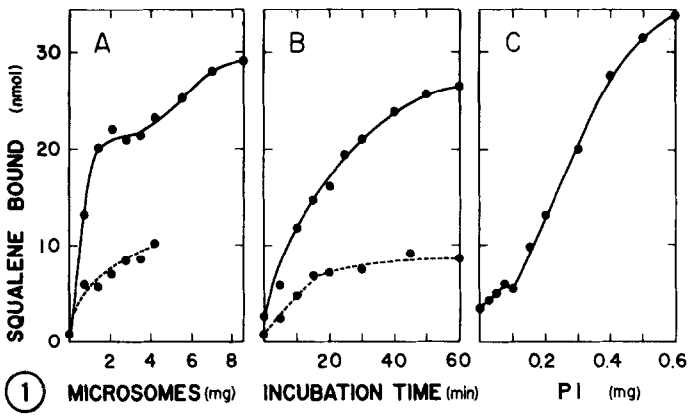
Binding of [^{14}C]squalene to microsomes was measured in the same reaction mixture from which NADPH (and other components, if necessary) was omitted. After incubation at 37°C for a desired period of time, the microsomes were recovered by centrifugation at 78,000 x g for 90 min and washed with 50 mM

Tris-HCl buffer (pH 7.5). The amount of squalene bound was estimated by measuring the radioactivity of the washed microsomes. The epoxidation of the bound squalene was determined as described above, except that [^{14}C]squalene (and other components, if necessary) was omitted from the reaction mixture.

Protein was determined by the method of Lowry et al. (13).

RESULTS AND DISCUSSION

When the standard reaction mixture for epoxidation assay (cf. "Materials and Methods") from which NADPH and SPF were omitted was incubated at 37°C, considerable binding of [^{14}C]squalene to microsomes occurred. This binding was dependent on the concentration of microsomes (Fig. 1A) and on the incubation time (Fig. 1B). Only a very low level of binding was detected after incubation at 0°C. The binding was also dependent on the concentration of PI (Fig. 1C); only insignificant binding was observed on incubation in the absence of the phospholipid. Addition of purified SPF to this system caused further increase in the binding and the increase was dependent on the concentration of SPF (Fig. 2). However, it should be noted that practically no



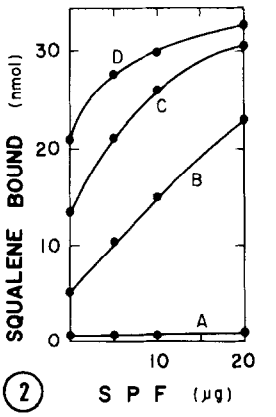


Fig. 1. Effects of the concentration of microsomal protein (A), incubation time (B), and the concentration of PI (C) on squalene binding to rat liver microsomes in the presence of PI. Binding experiments were carried out as described under "Materials and Methods" in the absence of NADPH and SPF. The concentration of microsomal protein was 2.1 mg per tube except for A, where it was varied as indicated. Incubation time was 30 min except for B, where it was varied as indicated. The concentration of PI was 0.3 mg (——) or 0.1 mg (-----) per tube except for C, where it was varied as indicated.

Fig. 2. Squalene binding to microsomes in the presence of both PI and SPF. The experimental condition were the same as Fig. 1, except that indicated amount of SPF (per tube) was included in the incubation mixture and 0 (A), 0.1 (B), 0.2 (C) and 0.3 mg (D) of PI was added.

Table I. Epoxidation of squalene previously bound to microsomes in the presence of PI alone (Expt. A) and both PI and SPF (Expt. B)

Addition	Products formed (nmol)	
	Expt. A	Expt. B
None	0.73	0.71
NADPH	0.98	3.52
NADPH, PI	1.00	3.08
NADPH, SPF	0.62	10.12
NADPH, PI, SPF	0.57	8.41

Squalene was bound to microsomes as described under "Materials and Methods", except that the mixture contained 0.3 mg of PI but no SPF (Expt. A) and 0.1 mg of PI and 20 μg of SPF (Expt. B). 2.0 mg protein of the microsomes (containing 28 and 26 nmol of bound squalene in Expts. A and B, respectively) were suspended in the fresh reaction mixture from which NADPH, PI and SPF had been omitted. The epoxidation reaction was then started by adding 1.5 mM NADPH, 0.1 mg of PI, and/or 20 μg of SPF as indicated, and incubation was carried out aerobically at 37°C for 30 min.

binding took place when the incubation was performed in the presence of SPF alone. It seemed that SPF facilitated the transfer of squalene from the Tween-squalene micelle to microsomes only in collaboration with the acidic phospholipid. Egg phosphatidylcholine, a neutral phospholipid, was much less effective in causing squalene binding than PI (data not shown). It is of interest to note in this connection that only acidic phospholipids have been shown to activate the microsomal epoxidase activity (8).

Having observed the binding of squalene to microsomes in the presence of PI and SPF, it was of interest to examine whether or not the squalene thus bound could be epoxidated effectively. For this purpose, microsomes to which [^{14}C]squalene had been incorporated in the presence of either PI alone or PI plus SPF were washed and resuspended in the fresh reaction mixture lacking NADPH, [^{14}C]squalene, PI and SPF. Then, the reaction was started by addition of NADPH and the extent of epoxidation was determined. It was thus found that virtually no epoxidation took place with the microsomes to which squalene had been bound in the presence of PI alone (Table I, Experiment A). Fortification of the reaction mixture with SPF or SPF plus PI caused no stimulation. With the microsomes to which squalene had been incorporated in the presence of both SPF and PI, on the other hand, epoxidation of the bound squalene proceeded at

markedly high rates if the reaction mixture contained either SPF or SPF plus PI (Table I, Experiment B). In the absence of supplementary additions, however, the epoxidation activity was significantly lower and the addition of the phospholipid alone did not stimulate the activity.

These results suggest that squalene binding in the presence of PI alone is mostly nonspecific and the squalene thus bound can undergo epoxidation only very poorly and that coexistence of SPF and PI during the binding process is necessary for squalene to bind to the "right" position, most probably the catalytic site of the epoxidase system. Nothing is, however, known of the mechanism of this interplay of SPF and PI. One possibility is that SPF in collaboration with PI acts as a carrier transporting the substrate to the catalytic site. However, gel filtration and sucrose density gradient centrifugations studies showed that binding of [^{14}C]squalene to SPF did not occur even in the presence of PI (data not shown). Another conclusion salient from Table I is that SPF, but not the phospholipid, is also required for epoxidation of squalene that has been bound to the "right" position, although it is again unclear whether this role of SPF is catalytic or not. It is to be noted that the requirement of SPF for epoxidation of bound squalene does not seem to be absolute; even in the absence of SPF significant epoxidation was observed, though clearly less than in the presence of SPF (Table I, Experiment B).

It is further suggested from the results described above that the binding of squalene to the "right" (catalytic) site on microsomes is essential for the epoxidation reaction to take place and the binding is a time-dependent process. This could be supported by the time-course experiments shown in Fig. 3. When the reaction was started by addition of NADPH immediately after mixing microsomes with SPF, PI [^{14}C]squalene and the other components of the standard assay mixture, the time course of epoxidation showed a definite lag period. The lag phase, however, disappeared completely when the mixture was preincubated for more than 20 min before the addition of NADPH (Fig. 3A). These time courses can be readily explained by assuming that the lag phase

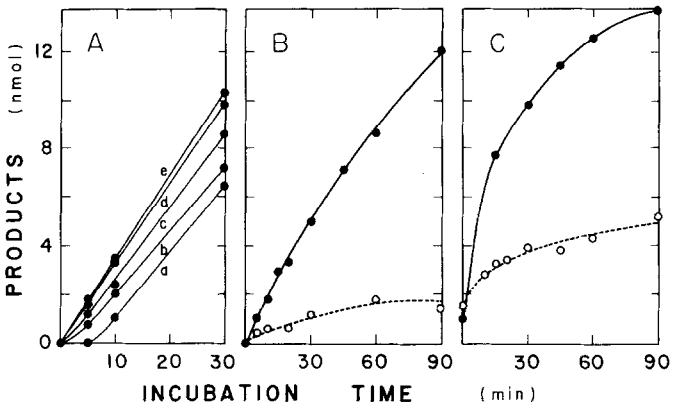


Fig. 3. Time courses of squalene epoxidation. A, Effect of preincubation. The standard reaction mixture (microsomes, 2.1 mg protein/tube) was used. Before starting the reaction by adding NADPH, the mixture was preincubated for 0 (a), 5 (b), 10 (c), 20 (d), and 60 min (e). B, Epoxidation of squalene previously bound to microsomes in the presence of both PI and SPF. Squalene binding to microsomes was carried out as in Table I, Expt. A. 2.0 mg protein of the microsomes thus prepared (containing 27 nmol of bound squalene) and 0 (--O--) or 20 µg (—●—) of SPF were mixed with the other components of the standard reaction mixture and the reaction was started by adding NADPH. C, Epoxidation of squalene biosynthesized *in situ* from [^{14}C]mevalonic acid. Microsomes containing biosynthesized squalene were prepared as described under "Materials and Methods". 2.0 mg protein of the microsomes thus prepared (containing 18 nmol of biosynthesized squalene) and 0 (--O--) or 20 µg (—●—) of SPF were mixed with the other components of the standard reaction mixture and the reaction was started by adding NADPH.

represents the time during which a sufficient amount of exogenous squalene becomes bound to the catalytic site of the epoxidase system. Fig. 3B shows the time course of epoxidation in the presence of SPF of the squalene that had been previously incorporated to microsomes in the presence of SPF and PI. As expected, no lag phase was observed in this case.

Previous studies (14,15) have shown that [^{14}C]squalene synthesized anaerobically from [^{14}C]mevalonic acid in the postmitochondrial fraction of rat liver is bound tightly to microsomes recovered from the reaction mixture. The labeled squalene thus synthesized *in situ* from mevalonic acid was found to be effectively converted to oxidosqualene when NADPH and SPF were added to the microsomes under aerobic conditions. PI was not effective in supporting the epoxidation occurring in these microsomes. The epoxidation in the presence of SPF did not show any lag phase (Fig. 3C). It can, therefore, be concluded

that the squalene synthesized in situ is already bound to the proper catalytic site and can undergo epoxidation if NADPH and SPF are supplied. Another conclusion that can be drawn is that acidic phospholipids are not required for squalene epoxidation in vivo.

Much is, however, left unelucidated about the roles of soluble factors in the microsomal squalene epoxidase system.

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