Communication

Supernatant Protein Factor Facilitates Intermembrane Transfer of Squalene*

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Squalene epoxidation of microsome-associated squalene is stimulated by a soluble protein termed "supernatant protein factor" (SPF) (Saat, Y. A., and Bloch, K. E. (1976) J. Biol. Chem. 251, 5153—5160). In the absence of SPF, the initial rate for microsome-bound squalene epoxidation is rapid for 5 to 10 min but falls off sharply thereafter. SPF does not affect the rapid initial epoxidation rate of reaction but maintains it for longer periods. This SPF effect on enzyme kinetics indicates that SPF facilitates the otherwise rate-limiting access of squalene to the epoxidase site.

Trypsin treatment of microsomes totally inactivates squalene epoxidase. When such trypsin-treated squalene-containing microsomes are incubated with normal, squalene-free, enzymatically active microsomes, formation of squalene epoxide occurs rapidly. Lowering the temperature from 37°C to 22°C abolishes the SPF effect in assay systems containing either normal or trypsin-treated plus normal microsomes. These findings show that SPF promotes the transfer of squalene from one microsome population to another, i.e. intermembrane transfer of substrate.

The late stages of hepatic cholesterol biosynthesis beginning with the formation and conversion of squalene are catalyzed by membrane-associated enzymes. Several of these microsomal transformations are stimulated by soluble, presumably cytoplasmic, liver proteins (1—6). For the mechanism of action of two of these proteins, supernatant protein factor (SPF) and sterol carrier protein (SCP), promoters of both squalene epoxidase and squalene-2,3-oxidase-lanosterol cyclase (3, 3, 6), a substrate carrier role was originally proposed (4). According to this earlier hypothesis, the soluble protein activators aid the entry of the isobutyl substrate into the microsomes (3, 4). However, the physiological relevance of this carrier mechanism has been questioned since both the lipophilic intermediates and the enzymes involved in their transformations are associated with microsomal membranes. Currently more favored is the hypothesis that SPF and SCP act within the microsomal membrane, facilitating the access of substrate to specific enzyme sites (7—9).

In support of the latter hypothesis, SPF has been shown to promote the enzymatic conversion not only of exogenous squalene or squalene epoxide, but also of substrate previously incorporated or previously generated in the microsomal membrane (7—9). We now show directly that SPF mediates squalene transfer within the microsomal membrane space or between discrete membrane particles or regions.

EXPERIMENTAL PROCEDURES

Materials—Female rats (strain CD) were obtained from Charles River Breeding Laboratories. Phosphatidylglycerol was purchased from Avanti Biochemicals. Sigma Chemical Co. supplied Tris, NADPH, FAD, and soybean trypsin inhibitor. Trypsin was purchased from Boehringer Mannheim. AMO-1618 was supplied by Calbiochem.

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\[\text{[H]Squalene was purchased from New England Nuclear.} \]

\[\text{tris-2,3-Oxidosqualene was synthesized according to the method of Nadeau and Hanzlik (10) by Dr. A. K. Lala in this laboratory. Rat liver microsomes were prepared as previously described (11).} \]

\[\text{The abbreviation used is: SPF, supernatant protein factor; SCP, sterol carrier protein, designated by Srikantaiah et al. (3) for stimulatory activity of the conversion of squalene to sterols; AMO-1618, (5-hydroxycarvacryl) trimethylammonium chloride 1-piperidine carboxylate.} \]

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buffer and centrifuged at 10,000 × g for 10 min. Following resuspension of the sediment particles (5 ml of buffer), the microsomes were again sedimented by centrifugation and finally resuspended in the original volume of 0.1 M Tris-HCl, pH 7.5.

**Epoxidase Activity in Mixtures of Trypsin-treated and Normal Microsomes—** Assay mixtures contained 0.05 ml of trypsin-treated, squalene-loaded microsomes and 0.05 ml of normal, squalene-free microsomes (1 mg of protein). The total volume was 0.5 ml. Other ingredients were as described. Reactions were started by addition of trypsin-treated, squalene-containing microsomes.

**RESULTS AND DISCUSSION**

The squalene epoxidase assay using microsome-associated substrate throughout eliminates the possibility that squalene uptake from the aqueous medium is rate-limiting. Shown in Fig. 1 is the time course of conversion of microsome-associated squalene in the presence or absence of SPF. In the absence of SPF, the production of 2,3-oxidosqualene is linear for little more than 4 min, decreasing rapidly thereafter and leveling off at approximately 0.02 of the initial rapid rate. SPF did not affect the initial rate but maintained it for longer periods and prevented it from falling off as sharply. The observation that SPF has no effect on the initial conversion rate of membrane-bound squalene has two implications. First, modulation of squalene epoxide activity by SPF is ruled out. Secondly, a fraction of squalene must initially be present at or near the epoxidase site, whether SPF is present or not. The data points for the conversion of microsome-associated squalene, reported previously (8, 9), did not allow any conclusion as to SPF effects on initial rate.

To test whether the slow phase of the biphasic time course (Fig. 1) is due to an increasingly limited availability of squalene at the epoxidase site, we investigated conditions for prolonging the initial rapid rate of squalene conversion. Microsomes previously loaded with squalene were incubated for 10 min in the presence of NADPH and other cofactors in order to generate 2,3-oxidosqualene. These microsomes, containing both substrate and product, were then reisolated and washed free of cofactors. The time course of conversion of the remaining squalene was then followed and compared with the conversion rate in control microsomes which initially contained only squalene but no product (Fig. 2). In the absence of SPF, microsomes containing endogenously generated 2,3-oxidosqualene as well as squalene did not catalyze the initial rapid squalene epoxidation seen with control microsomes.

Additional product was formed at a barely measurable rate. Pure SPF, however, sharply increased the initial rate of squalene conversion in this instance. With the control microsomes which contained no 2,3-oxidosqualene at b0, the initial rate was rapid and was not altered significantly by SPF as already shown in Fig. 1. The data shown in Fig. 2 are best explained by assuming that only a small fraction of microsome-associated squalene is initially available at the enzyme site and that the slow translocation of substrate from a randomly distributed membrane pool limits the rate of epoxidation after an initial 5- to 10-min period.

Addition of high concentrations of 2,3-oxidosqualene to the assay mixture did not impair the conversion of microsome-associated squalene, eliminating the possibility that accumulating product inhibits squalene epoxidase and that SPF functions by relieving this inhibition (data not shown).

In further pursuit of the hypothesis that SPF facilitates squalene translocation between membrane regions, we have taken advantage of the trypsin sensitivity of squalene epoxidase. Microsomes preloaded with squalene and then treated with trypsin are totally devoid of epoxidase activity either in the absence or presence of SPF. Such microsomes fully retain previously bound squalene (Table I). When such trypsin-treated, squalene-containing microsomes are incubated with untreated and squalene-free microsomes, formation of squalene epoxide is minimal. However, if pure SPF is included in this assay, the amount of squalene converted to 2,3-oxidosqualene increases strikingly. Phosphatidylglycerol, which greatly facilitates the conversion of exogenous, detergent-dispersed squalene (2), does not aid in the utilization and conversion of squalene sequestered initially in trypsin-treated microsomes. This observation is consistent with the report (8) that anionic phospholipids do not influence the SPF effect on microsomes that already contain squalene.

The time course for squalene conversion by the mixed population of normal and squalene-containing, trypsin-treated

**FIG. 1 (left). Time course of conversion of microsome-associated squalene in the presence and absence of SPF.** Microsomes containing 0.74 nmol of bound [14C]squalene/mg of microsomal protein were assayed as described under “Experimental Procedures.” Reactions were initiated at 37°C by the addition of NADPH. ●-●-●, squalene epoxide activity in the absence of SPF; ○-○-○, squalene epoxide activity with 2.5 μg of pure SPF.

**FIG. 2 (right). Effect of prior squalene conversion on the ability of microsomes to convert the remainder of bound squalene.** Microsomes containing 0.54 nmol of bound squalene/mg of microsomal protein were separated into two batches. One batch (8 mg of microsomal protein) was incubated for 10 min at 37°C with 0.01 mM FAD, 1 mM NADPH, 0.5 mM AMO-1618, and 0.1 mM Tris buffer, pH 7.5, in a total volume of 4.0 ml. The control half of the microsomal batch containing bound squalene was resuspended with 0.1 mM Tris buffer, pH 7.5, in a total volume of 4.0 ml and incubated for 10 min at 37°C in an atmosphere of argon. The microsomes were then isolated by centrifugation and washed with 6 ml of 0.1 mM Tris buffer, pH 7.5, and aliquots were used for the various experiments. 2,3-Oxidosqualene formation was assayed as described under “Experimental Procedures.” Reactions were initiated by the addition of NADPH at 37°C. 2,3-Oxidosqualene was produced in the absence (●-●-●) and presence (△-△-△) of 2.5 μg of SPF in microsomes containing pregenerated 2,3-oxidosqualene. 2,3-Oxidosqualene was produced in the absence (●-●-●) and presence (○-○-○) of 2.5 μg of SPF in control microsomes.
Conversion of squalene epoxidation by normal microsomes alone (Fig. 3B) and by the mixed microsomal populations consisting of normal and trypsin-treated particles (Fig. 3A). In Fig. 3A, conversion is rapid initially but remains linear up to 8 min and continues at a rapid rate for at least 30 min. The production of 2,3-oxidosqualene by an aliquot of the same batch of microsomes preloaded with squalene, but not trypsin-treated, served as a control (Fig. 3B). Again, a distinct biphasic time course in the absence of SPF and the typical SPF effect were observed.

There are several significant differences between the kinetics of squalene epoxidation by normal microsomes alone (Fig. 3B) and by the mixed microsomal populations consisting of both normal and trypsin-treated particles (Fig. 3A). In Fig. 3B, conversion is rapid initially (4 min) both with and without SPF. Thereafter, the rate falls off sharply but continues at a measurable rate at the earliest time point except that reactions were initiated by the addition of squalene-bound normal microsomes before and after trypsin treatment and in combination with microsomes that were not trypsin-treated were assayed as a control. The amount of pure SPF used was 5 μg. Aliquots of washed microsomes before and after trypsin treatment and in combination with normal microsomes were washed and extracted with CHCl₃/CH₂OH (2:1), and the radioactivity of the extract was determined (microsomes-associated squalene).

**TABLE 1**

**Conversion of microsome-associated squalene after trypsin treatment**

Microsomes were preloaded with squalene in the absence of phosphatidylglycerol. A portion of these squalene-containing microsomes was treated with trypsin and assayed alone or in combination with normal microsomes not containing squalene, as described under “Experimental Procedures.” The remainder of the squalene-containing microsomes that were not trypsin-treated were assayed as a control. The amount of pure SPF used was 5 μg. Aliquots of washed microsomes before and after trypsin treatment and in combination with normal microsomes were washed and extracted with CHCl₃/CH₂OH (2:1), and the radioactivity of the extract was determined (microsomes-associated squalene).

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<th>Buffer</th>
<th>Buffer + SPF</th>
<th>nmo l</th>
<th>counts per min</th>
<th>Buffer</th>
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**Fig. 3.** Conversion by normal microsomes of squalene associated with trypsin-treated microsomes. Microsomes containing 1.3 nmol of bound squalene/mg of microsomal protein were trypsin-treated as described under “Experimental Procedures.” Reactions were initiated by adding trypsin-treated, squalene-containing microsomes to assay mixtures at 37°C containing normal microsomes and the assay ingredients described under “Experimental Procedures.” B, an aliquot of squalene-containing microsomes that were not trypsin-treated was assayed as described in the legend to Fig. 1 except that reactions were initiated by the addition of squalene-containing microsomes. □ — — — , absence of SPF; ○ — — ○, 8 μg of pure SPF.

**Fig. 4.** Temperature-dependent SPF stimulation of microsome-bound squalene conversion (A) and conversion of squalene bound to trypsin-treated microsomes by added normal microsomes (B). A, microsomes containing 0.61 nmol of bound squalene/mg of protein were assayed at either 22°C (- - -) or 37°C (——) in the absence (A, □) or presence (A, ○) of 2.5 μg of pure SPF. Reactions were initiated by the addition of NADPH. B, microsomes containing 1.6 nmol of bound squalene/mg of protein were trypsin-treated as described under “Experimental Procedures.” Reactions were initiated at either 20°C (- - -) or 37°C (——) by the addition of squalene-containing trypsin-treated microsomes to assay mixtures containing normal microsomes and the assay ingredients described under “Experimental Procedures.” ▲ and □, no SPF; ▲ and ○, 5 μg of pure SPF.
at 37°C. It remains to be investigated whether the lack of SPF activity at the lower temperature is due to changes in membrane fluidity or to a more direct effect on SPF structure or conformation.

The system consisting of a mixed population of microsomes used here, while artificial, has allowed the direct demonstration that SPF promotes the translocation of squalene from one membrane site to another.² The question remains by what mechanism a cytoplasmic protein that binds only weakly to microsomes (13), at least in vitro, can direct the movement of lipophilic substrate either across or between membrane bilayers. Since the endoplasmic reticulum represents a vast convoluted network within the cell, translocation of substrate across rather than within the bilayer may represent a more effective means of ensuring substrate availability. As for the mechanism of this transfer, it should be noted that squalene, once incorporated into microsomes, cannot be extracted from the particles by buffer, even in the presence of SPF.³ SPF-mediated transfer of squalene must therefore involve direct interaction with another membrane or particle surface. Whether this process occurs via an aqueous phase, by a collision process between two membranes, or possibly by membrane fusion remains to be investigated.

We are unaware of reports dealing with the modulation of membrane-associated events by soluble proteins similar to those described here.

REFERENCES

³ Analogous experiments have recently been carried out with the squalene epoxide-lanosterol cyclase system. Heat treatment of microsomes containing in situ generated 2,3-oxidosqualene abolishes the formation of lanosterol (1). 2,3-Oxidosqualene trapped within such heat-inactivated microsomes is not converted to lanosterol when combined with normal microsomes, unless SPF is present. The results indicate that SPF is also facilitating the intermembrane transfer of 2,3-oxidosqualene (E. J. Friedlander, unpublished results).