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Dual Role of Phospholipid in the Reconstitution of Cytochrome P-450 LM2-Dependent Activities

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SUMMARY

The effect of dilauroylphosphatidylcholine (DLPC) concentration on cytochrome P-450 LM2 (LM2)-dependent reduction and monooxygenase activities was examined as a function of preincubation time. Purified NADPH-cytochrome P-450 reductase (reductase) and LM2 were reconstituted at different DLPC to LM2 ratios by preincubation of the proteins in the presence of DLPC for either 5 min or 2 hr at room temperature. After preincubation was complete, the samples were assayed for either monooxygenase activity or first-electron transfer activity. When preincubated for 5 min, overall monooxygenase activity was dependent on the [DLPC]:[LM2] ratio, beginning at a low level in the absence of phospholipid and increasing to a maximum at a 160:1 ratio. At [DLPC]:[LM2] ratios above 160:1, the rate was decreased to 80% of the maximum rate. When the samples were preincubated for 2 hr, again low monooxygenase activities were obtained in the absence of DLPC, which increased to a maximum at 160:1 [DLPC]:[LM2] ratio. Above this [DLPC]:[LM2] ratio, the rate was decreased to less than 50% of the maximum value. These changes in overall activities appear to be related to changes in the amount of functional reductase-LM2 complex formed. Similar results were found when LM2 reduction was examined. When preincubated for 5 min, LM2 reduction was shown to be diminished as the DLPC to LM2 ratio decreased below 160:1. The DLPC-dependent effect on reduction was primarily characterized by alterations in the fraction of LM2 reduced in the first phase, with the first-phase rate constant and the slow phase parameters being largely unaffected. Below a 16:1 ratio ([DLPC]:[LM2]), no phospholipid stimulation of LM2 reduction was observed. When the [DLPC]:[LM2] ratio was increased above a 160:1 ratio, only a small effect on the kinetic constants was observed, which was characterized by a 20% decrease in the fraction of LM2 reduced in the first phase. LM2 reduction was more sensitive to DLPC concentration after longer preincubations (2 hr), with a 50% decrease in the fraction of reduction in the first phase being observed at [DLPC]:[LM2] ratios above 160:1. The results are consistent with a dual role for phospholipid in the stimulation of LM2-dependent activities. First, DLPC facilitates the association of reductase and LM2 and, second, DLPC provides a matrix for the incorporation of LM2 and reductase. Facilitation of the protein association appears to be a relatively rapid process, occurring after a 5-min preincubation, whereas a 2-hr preincubation altered the protein interactions in a manner consistent with incorporation of the LM2 and reductase into the phospholipid.

The cytochrome P-450 system found in liver microsomes has been shown to require at least three endogenous components, cytochrome P-450, reductase, and a heat-stable factor (1, 2). The heat-stable factor, later identified as phosphatidylcholine, was shown to affect the activity of cytochrome P-450-dependent reactions (3), with the reaction rate being dependent not only on the phospholipid concentration but also on the fatty acid composition of the phosphatidylcholine molecule. Of the phospholipid molecules with saturated fatty acid side chains, those containing lauric acid were found to be particularly effective in stimulating cytochrome P-450-dependent activities (4).

Some cytochrome P-450-dependent activities were shown to be dependent on membrane composition. Differences were observed in several monooxygenase activities using LM2 in a reconstituted system with phospholipid vesicles of various compositions (5). In their study, a relationship was observed between the degree of negative charge on the membrane and LM2-dependent monoxygenase activities. Phospholipid was shown to affect not only enzyme activity but also the spin state of cytochrome P-450. Addition of dilauroyl-GPC to purified LM2 produced a type I difference spectrum with a peak at 387 nm and a trough at 418 nm. The magnitude of this low to high spin shift appeared to be affected by the presence of either substrate or reductase (6). More recently, the effect of phospholipid on cytochrome P-450-dependent activities was examined (7). Unlike the previous studies, which simply compared enzyme activity in the absence and presence of phospholipid,

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ABBREVIATIONS: reductase, NADPH-cytochrome P-450 reductase; LM2, cytochrome P-450 LM2 isolated from phenobarbitol pretreated rabbit liver; PBRLM5, cytochrome P-450 isoform 5 isolated from phenobarbital-pretreated rat liver (equivalent to cytochrome $P\text{-}450_{PB\text{-}B}$); DLPC, dilauroylphosphatidylcholine.

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these investigators examined the effect of changes in the phospholipid concentration, focusing on high phospholipid to protein ratios. Cytochrome P-450 and reductase purified from phenobarbital-pretreated rabbits were reconstituted with phosphatidylcholine at phospholipid to protein ratios ranging between 200:1 and 2000:1 (mol:mol). As the phospholipid to protein ratio was increased, the rate of benzphetamine demethylation was decreased, suggesting that the monooxygenase reaction was a diffusion-limited reaction at the concentrations examined. Temperature dependence studies of cytochrome P-450-dependent metabolism of benzphetamine showed a break in the Arrhenius plot at about the same temperature as the gel-liquid crystal phase transition for dimyristoylphosphatidylcholine. When a preparation of phosphatidylcholine, which did not undergo a phase transition over the temperature range tested, was used in similar studies, no discontinuity was observed. These results point to the importance of phospholipid in altering the interaction between cytochrome P-450 and reductase.

Transfer of the first electron from NADPH to cytochrome P-450 has been described as a biphasic process. The overall rate of reduction has been shown to be affected by the presence of substrate (8–13), the reductase to P-450 ratio (14, 15), temperature (16–19), the concentration of NADPH (20), and the particular isoform examined (10, 11, 20–22).

The kinetics of first-electron transfer to cytochrome P-450 were also shown to be influenced by the presence of phospholipid. DLPC was shown to enhance the rate of NADPH-supported first-electron transfer to cytochrome P-450 LM4 (21), apparently by increasing the amount of cytochrome P-450 LM4 reducible in the early phases without affecting the rate constants for reduction. Phospholipid was also shown to affect the rate of cytochrome P-450 reduction at higher phospholipid to protein ratios. As the amount of phospholipid exceeded the 200:1 phosphatidylcholine to P-450 ratio, the rate of electron transfer was decreased in a manner similar to that seen for the monooxygenation reaction (7, 14, 19). Arrhenius plots of the reduction of cytochrome P-450 showed a discontinuity very close to the temperature of the dimyristoylphosphatidylcholine phase transition. LM2 reduction has also been shown to be affected by differences in phospholipid composition. As with monooxygenase activity (23), LM2 reduction was shown to be dependent on membrane charge. Addition of phospholipids with a negative charge enhanced the rate of LM2 reduction in a reconstituted system (15).

A number of investigators have proposed that the phospholipid acts simply as a detergent, which permits complex formation between the reductase and cytochrome P-450. Low concentrations of detergent have been shown to be able to substitute for phosphatidylcholine both for reconstitution of monooxygenase activity (24) and for first-electron transfer (25). Müller-Enoch *et al.* (26) reported that phospholipid was not even required as long as cytochrome $P\text{-}450_{PB\text{-}B}$ and reductase were permitted to associate by preincubation at room temperature for 2 hr at high concentrations.

The purpose of this study was to examine the role of phospholipid in the reduction of LM2, as well as in cytochrome P-450-dependent monooxygenase activities, over a wide range of DLPC concentrations. The results show a similar dependence of both monooxygenase activity and first-electron transfer on DLPC concentration, but overall results were shown to be greatly influenced by changes in the preincubation time. The results suggest a dual role for DLPC in the reconstitution of cytochrome P-450-dependent activities.

Materials and Methods

LM2 was purified according to the method of Coon *et al.* (27), with the modifications described previously (20). Reductase was purified according to the method of Yasukochi and Masters (28). Both proteins were isolated from rabbits pretreated for 5 days with 0.1% phenobarbital in the drinking water. PBRLM5 and reductase were purified from phenobarbital-pretreated rats as previously described (29). Cytochrome P-450 concentrations were measured according to the method of Omura and Sato (30) at 450 minus 490 nm, using a difference extinction coefficient of 91 mM^{-1} cm^{-1}. The concentraiton of the reductase was measured from the absolute spectrum at 456 nm, using an extinction coefficient of 21.4 mM^{-1} cm^{-1} (31). The specific contents for both LM2 and PBRLM5 were 10 nmol/mg.

Preparation of reconstituted system. DLPC was suspended to a concentration of 8 mM in 50 mM potassium phosphate buffer, pH 7.25, containing 20% glycerol, 0.1 M sodium chloride, and 5 mM EDTA (buffer A) by sonication of the preparation until clarification was observed. Various amounts of phospholipid and reductase were mixed and incubated at room temperature for 5 min, followed by addition of LM2 and incubation for an additional 5 min. In these reconstituted systems, the reductase to LM2 ratio was maintained at 1.5:1, whereas the DLPC to LM2 ratio was varied from 1.6:1 to 1600:1. During the preliminary incubation, the component concentrations were 17.6 and 11.8 μM for reductase and LM2, respectively. When LM2 and reductase were combined in the absence of phospholipid, an appropriate amount of buffer A was added in order to maintain the protein concentrations used in the presence of phospholipid. In certain cases, the reconstituted systems were incubated for 2 hr following the addition of LM2. After the incubations were completed, the samples were diluted with buffer and the other assay components.

For some of the experiments described below, reductase and LM2 were mixed, in the absence of DLPC, at varying concentrations of the detergent Tergitol NP-10. In these studies, LM2 and reductase were preincubated for 2 hr in the presence of the indicated concentration of Tergitol (ranging from 0.005 to 0.1%). After preincubation was complete, the proteins were mixed with 100 mM potassium phosphate buffer, pH 7.25, and an NADPH-regenerating system for the examination of LM2-dependent monooxygenase activities.

Measurement of cytochrome P-450 reduction. LM2 reduction was measured by rapidly mixing the reconstituted system with NADPH under anaerobic conditions (13, 20). The progress of the reaction was followed by monitoring the formation of the carbon monoxide-ferrous complex of LM2 at 450 nm. Most of the reactions were measured using a stopped flow spectrophotometer, whereas the slower reactions were measured by mixing NADPH in the side arm of a Thunberg cuvette with the reconstituted system and monitoring the change in absorbance at 450 nm minus 490 nm.

For the rapid reactions, the reaction was initiated by rapidly mixing the contents of each of the syringes in the stopped flow spectrophotometer. The reaction temperature was 25° and the path length of the stopped flow spectrophotometer was 2 cm. The first syringe contained the reconstituted system (1.5 μM reductase and 1.0 μM LM2 in DLPC), 7 mM glucose, 90 units/ml glucose oxidase, and 2500 units/ml catalase in 100 mM potassium phosphate buffer, pH 7.25. The second syringe contained 500 μM NADPH, 7 mM glucose, 90 units/ml glucose oxidase, and 2500 units/ml catalase in 100 mM phosphate buffer. Glucose, glucose oxidase, and catalase act as a deoxygenating system that serves to ensure anaerobiosis during the reaction. For each syringe, all of the components were added with the exception of glucose oxidase and catalase, and the samples were bubbled with carbon monoxide for 1 min. Glucose oxidase and catalase were then added to the samples and bubbling with carbon monoxide continued for an additional 30 sec. The samples were then loaded into the stopped flow spectrophotometer.

A Thunberg cuvette was used for the slower reactions. The cuvette

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contained the reconstituted system, glucose, glucose oxidase, and catalase in 100 mM phosphate buffer (1.48 ml). The side arm contained NADPH and the deoxygenating system (20 μl). Samples were bubbled with carbon monoxide in a manner similar to the stopped flow studies described above. When appropriate, 1 mM benzphetamine was present in all reactant solutions before mixing. After mixing, the final component concentrations were 0.75 μM reductase, 0.5 μM LM2, 7 mM glucose, 90 units/ml glucose oxidase, 2500 units/ml catalase, and 250 μM NADPH, in 100 mM potassium phosphate buffer, pH 7.25.

Benzphetamine and *p*-nitroanisole metabolism. Benzphetamine demethylation was determined by measurement of formaldehyde formation, as previously described (29, 32). Metabolism of *p*-nitroanisole was determined by monitoring the formation of *p*-nitrophenol at 405 nm minus 490 nm, using an Aminco DW2C spectrophotometer in the dual-wavelength mode (33). The final concentrations of components were 0.375 μM reductase and 0.25 μM LM2 (in various concentrations of DLPC), 5 mM glucose-6-phosphate, 2 units/ml glucose-6-phosphate dehydrogenase, 10 mM magnesium chloride, and either 400 μM *p*-nitroanisole or 1 mM benzphetamine, in 100 mM potassium phosphate buffer, pH 7.25. The reaction was initiated by the addition of NADPH to a final concentration of 500 μM. The values for *p*-nitroanisole demethylation for a given preparation have a variation from the mean of less than ±2%.

Results

Effect of DLPC concentration on LM2 reduction. In order to quantify the effect of DLPC on LM2 reduction, reconstituted systems containing different concentrations of DLPC were prepared, and the rate of electron transfer to LM2 was examined in the absence of substrate (Fig. 1). At high DLPC to LM2 ratios, reduction was a rapid process, with the rate being minimally affected by changes in the phospholipid concentration. As the DLPC to LM2 ratio was decreased below 160:1, the overall rate of reduction was diminished. This effect was more pronounced as the DLPC to LM2 ratio was decreased below 16:1. Similar effects were observed in the presence of substrate (data not shown), except that the reaction was 90% complete after 1 sec (at optimal DLPC levels), as compared with the slower reduction observed in the absence of substrate (20).

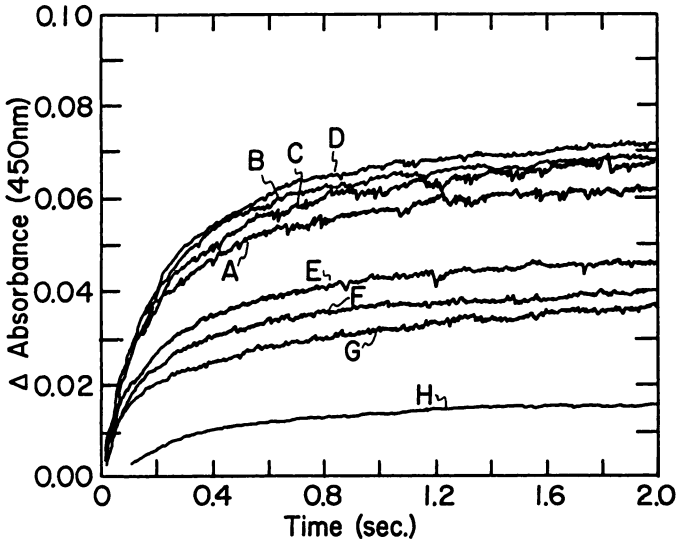

Fig. 1. Effect of [DLPC]:[LM2] on first-electron transfer to LM2. LM2 and reductase were preincubated for 5 min in the presence of DLPC at the indicated [DLPC]:[LM2] ratios. The proteins were preincubated at a high concentration (>10 μM) and at a reductase to LM2 ratio of 1.5:1. After preincubation, the samples were diluted to an LM2 concentration of 1 μM, loaded into the stopped flow spectrophotometer (2-cm path length), and rapidly mixed with an equal volume of NADPH, as described in Material and Methods. The [DLPC]:[LM2] ratios illustrated are 1600:1 (*A*), 800:1 (*B*), 320:1 (*C*), 160:1 (*D*), 80:1 (*E*), 48:1 (*F*), 16:1 (*G*), 1.6:1 (*H*).

The data in Fig. 1 were treated as two exponential processes and the kinetic constants were calculated (Table 1). Variation of the DLPC concentration did not appear to affect the rate constant for the first phase (k_{first}). However, as the DLPC to LM2 ratio was decreased below 160:1, a decline in the fraction of LM2 reduced in the first phase was observed. The fraction of LM2 reduced in the first phase was also decreased as the DLPC to LM2 ratio was increased to 1600:1, although the decrease represented only a 10% change. The rate constants and fraction of LM2 reduced in the second phase were largely unaffected by changes in the DLPC to LM2 ratio. Similar results were obtained in the presence of 1 mM benzphetamine, with the DLPC concentration affecting the fraction of LM2 reducible in the first phase. Although the first-phase rate constant appeared to be affected at a number of DLPC concentrations, the effect was small. The rate constants and fraction of reduction in the second phase were largely unaffected by changes in the DLPC concentration, with the exception of the fraction reduced in the second phase at the highest DLPC concentration. It was not possible to accurately determine the rate constants for reduction at DLPC to LM2 ratios lower than 16:1, due to the small amount of LM2 reducible in the first phase under these conditions. Because DLPC appeared to affect only the fraction of reducible cytochrome and not the rate constants for reduction, these results suggest that the amount of complex between LM2 and reductase was altered as the DLPC concentration was decreased.

Due to the extremely slow reactions observed at the lower DLPC to LM2 ratios, LM2 reduction was measured using a Thunberg cuvette, which ensured anaerobiosis over a longer time period. The results in Fig. 2 illustrate that significant reduction of LM2 did occur at low DLPC concentrations; however, the reactions were very slow. At a 16:1 DLPC to LM2 ratio, the reaction only attained about 70% completion after 1 hr. Although the majority of the reaction was extremely slow, about 35% of the cytochrome was reduced in the first minute at a 16:1 DLPC to LM2 ratio (Fig. 2A). The rate of reduction was even slower when measured in the absence of DLPC. Under these conditions, less than 10% of the LM2 was reduced in the first minute, with less than 50% reduction after 2 hr. The presence of DLPC at a 1.6:1 phospholipid to LM2 ratio did not significantly enhance the rate over that observed in the absence of phospholipid. When these experiments were repeated in the presence of 1 mM benzphetamine, certain similarities were observed (Fig. 2B). (a) In general, the rate in the absence of DLPC was substantially slower than the rates observed in the presence of the phospholipid. (b) Stimulation of LM2 reduction was not observed until the DLPC to LM2 ratio was increased to 16:1. Despite these similarities, however, there were some important differences. (a) The reactions were faster in the presence of substrate, with greater than 95% reduction occurring after 2 hr. (b) In each case, the amount of LM2 reducible in the first minute was about twice that obtained in the absence of substrate. Assuming the rapidly reducible portion of the reaction is due to reduction of preformed reductase LM2 com-

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TABLE 1

Kinetic parameters for LM2 reduction at different DLPC concentrations

The kinetic data from Fig. 1 were analyzed using an iterative fitting procedure to two exponentials. The fraction of LM2 reducible in each phase was taken as a fraction of the dithionite-reducible absorbance. Each value represents the mean ± standard error. The numbers in parentheses represent the number of determinations.

[DLPC]:[LM2]	k_{first}	Fraction in first phase	k_{second}	Fraction in second phase
	sec^{-1}		sec^{-1}	
Without benzphetamine				
1600:1 (4)	9.9 ± 1.0	0.33 ± 0.01[a]	1.7 ± 0.1	0.25 ± 0.02
800:1 (4)	8.4 ± 0.4	0.39 ± 0.02	1.6 ± 0.1	0.22 ± 0.01
160:1 (7)	8.0 ± 0.5	0.40 ± 0.02	1.4 ± 0.1	0.23 ± 0.03
80:1 (2)	7.7 ± 1.5	0.21 ± 0.02[a]	1.2 ± 0.6	0.18 ± 0.01
48:1 (5)	7.4 ± 0.7	0.25 ± 0.02[a]	1.3 ± 0.2	0.17 ± 0.02
32:1 (4)	8.7 ± 1.1	0.19 ± 0.01[a]	1.5 ± 0.2	0.16 ± 0.01
16:1 (4)	11.4 ± 2.4	0.11 ± 0.02[a]	1.1 ± 0.2	0.18 ± 0.01
+1 mM benzphetamine				
1600:1 (5)	18.2 ± 0.4[a]	0.78 ± 0.01[a]	3.0 ± 0.5	0.22 ± 0.01[a]
800:1 (5)	17.3 ± 0.8[a]	0.80 ± 0.02[a]	3.9 ± 0.8	0.16 ± 0.02
320:1 (4)	16.5 ± 0.3[a]	0.82 ± 0.03	2.6 ± 0.5	0.10 ± 0.01
160:1 (5)	20.2 ± 0.7	0.88 ± 0.03	2.8 ± 1.0	0.11 ± 0.03
80:1 (5)	17.9 ± 1.0	0.53 ± 0.02[a]	2.3 ± 0.7	0.08 ± 0.01
48:1 (4)	16.9 ± 0.2[a]	0.53 ± 0.01[a]	2.2 ± 0.2	0.11 ± 0.01
32:1 (5)	16.8 ± 0.6[a]	0.47 ± 0.01[a]	2.3 ± 0.3	0.11 ± 0.01
16:1 (7)	19.8 ± 1.4	0.27 ± 0.01[a]	2.3 ± 0.3	0.14 ± 0.01

[a] Values significantly different ($p < 0.05$) from the 160:1 [DLPC]:[LM2] value.

plex (13), then both DLPC and substrate appear to enhance the total amount of functional complex formed.

Effect of preincubation time on LM2 reduction. In the above studies, the reconstituted systems were prepared by mixing the proteins together and waiting for 5 min before dilution with the other assay components (see Materials and Methods). These studies demonstrated that the rate of LM2 reduction in the absence of phospholipid and at very low DLPC concentrations appeared to be smaller than that found in the presence of DLPC. Therefore, the effect of preincubation time on LM2 reduction was examined, to determine whether prolonged preincubation could substitute for the presence of phospholipid. As illustrated in Fig. 3, increasing the duration of preincubation does increase the initial rate of LM2 reduction, but the increase was observed at all DLPC concentrations examined. As shown in Fig. 3A, after a 5-min preincubation about 45% of the LM2 was reducible in the first second of the reaction. When the proteins were preincubated for 2 hr, the amount of LM2 reducible in the initial second was increased to about 70%. Incubation beyond 2 hr did not appear to further enhance the amount of reducible LM2 (Fig. 3A). Similar results were obtained in the presence of 1 mM benzphetamine (data not shown). Increases in the duration of preincubation also enhanced the rate of LM2 reduction at lower DLPC levels (Fig. 3B), as well as in the absence of the phospholipid (Fig. 3C). Comparison of each of the panels in Fig. 3 illustrates that both the rate of reduction and amount of LM2 reduced were increased as the phospholipid concentration was increased. Assuming that the initial rate of reduction is proportional to the amount of reductase LM2 complex formed, phospholipid appears to facilitate the formation of functional complex, in agreement with results previously reported (26). However, in contrast to their findings, incubation time was not the sole determinant of reaction rate. The rate of reduction was slower with suboptimal DLPC concentrations, even with preincubation times well in excess of 2 hr.

Effect of preincubation time on LM2-dependent monooxygenase activities. It was important to determine whether the requirement for DLPC is a characteristic of cytochrome P-450 reduction or whether this effect can also be observed for monooxygenase activities. Therefore, the effect of preincubation time on benzphetamine demethylation and *p*-nitroanisole demethylation was examined in the absence and presence of differing amounts of DLPC. As shown in Table 2, longer preincubation of the proteins did increase monooxygenase activities at each DLPC concentration, with preincubations extending beyond 2 hr having no substantial effect on the overall activity. Table 2 also demonstrates that the reaction rates obtained at optimal phospholipid concentrations could not be obtained as the DLPC concentration was decreased. These results are in contrast to those presented by Müller-Enoch *et al.* (26). It is interesting to note that the different monooxygenase reactions were stimulated to different degrees by phospholipid. Whereas the rate of *p*-nitroanisole demethylation was stimulated about 3-fold by DLPC (at 2 hr), the rate of benzphetamine demethylation stimulation was greater than 10-fold. If phospholipid produced its effects simply by facilitating formation of functional reductase P-450 complex, similar degrees of stimulation might be expected. The observed differences in the degree of stimulation for the different monooxygenase reactions indicate that phospholipid does more than simply promote the association of the microsomal proteins.

Effect of preincubation time on the dependence of P-450-dependent activities on DLPC concentration. The results obtained on the effect of DLPC concentration on LM2 reduction appear to differ from those expected based on literature reports. In those studies, increases in the phospholipid to P-450 ratio from 200:1 to 2000:1 produced decreases in monooxygenase activity ranging from 50% (23) to 85% (7, 14), depending on the phosphatidylcholine preparation used and the monooxygenase activity examined. Similar results were obtained with first-electron transfer, where a 90% decrease in the fraction of reduction in the first phase was observed (23). Because changes in both first-electron transfer and monooxygenase activities have been shown to result from longer preincubation of LM2 with the reductase (Fig. 3, Table 2), the possibility of changes in preincubation time producing different phospholipid dependencies was considered. Therefore, the ef-

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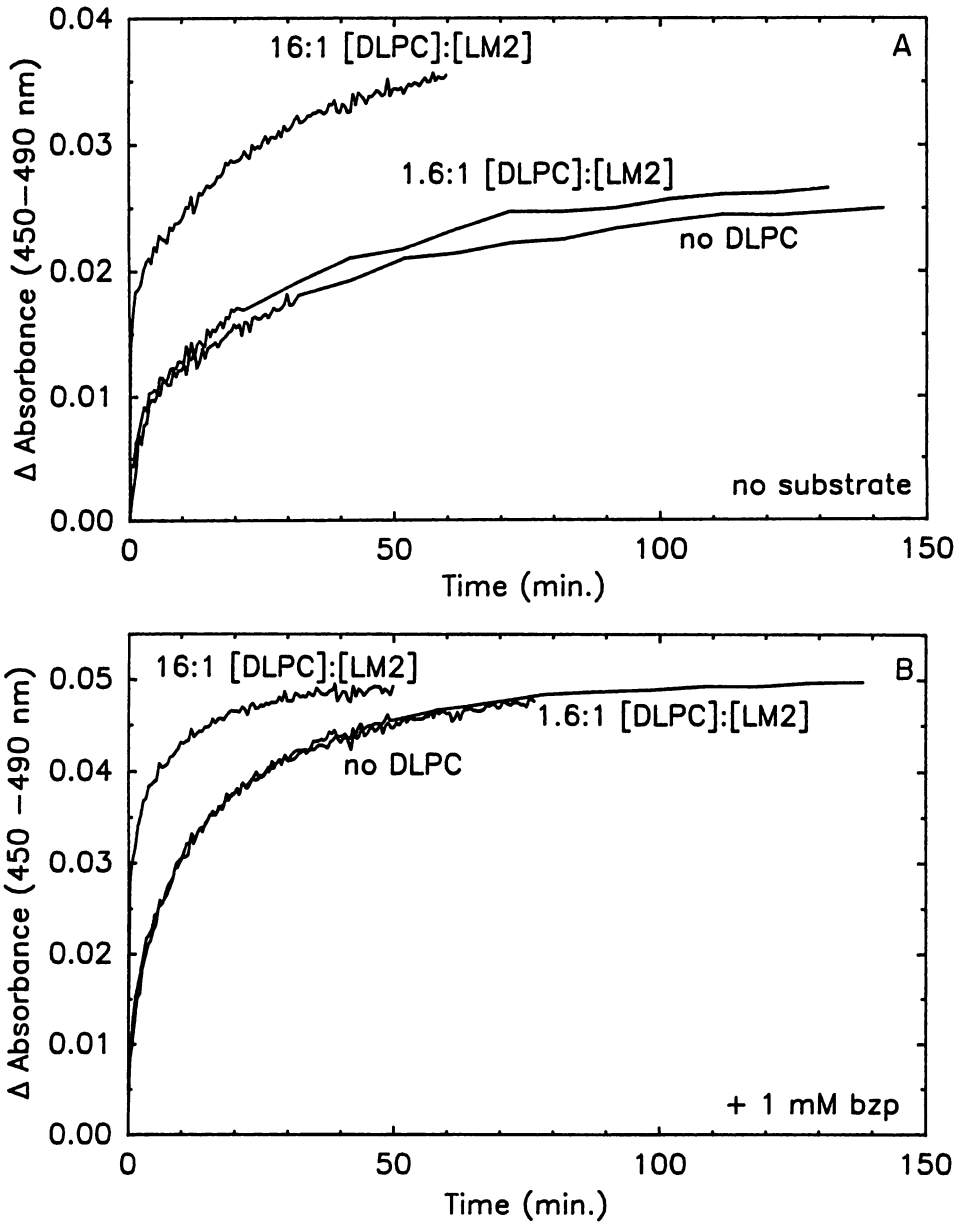

Fig. 2. Effect of [DLPC]:[LM2] on first-electron transfer at low DLPC concentrations. Reductase and LM2 were preincubated for 5 min in the presence of the indicated amounts of DLPC, as described in the legend to Fig. 1. After preincubation, the samples were diluted with phosphate buffer in a Thunberg cuvette, bubbled with carbon monoxide, and deoxygenated, as described in Materials and Methods. NADPH, present in the side arm, was mixed with the proteins and the progress of the reaction was monitored at 450 and 490 nm using a Hitachi U-3210 spectrophotometer (1-cm path length). A, Reduction in the absence of exogenous substrate; B, reduction in the presence of 1 mM benzphetamine.

fect of DLPC concentration on *p*-nitroanisole demethylation was examined after a 5-min as well as a 2-hr preincubation. The results shown in Fig. 4A after the 5-min preincubation are consistent with those obtained for LM2 reduction (Table 1) and in contrast to previously reported results (7, 14, 23). The rate in the absence of DLPC starts at a low level and increases as the DLPC concentration is increased to a 160:1 DLPC to LM2 ratio. As the DLPC concentration was further increased, there was a relatively small (20%) decrease in the reaction rate. After a 2-hr preincubation, two major differences were noted. First, the preparation was more active at the optimal DLPC to LM2 ratio and, second, the reaction rate was more sensitive to increases in the DLPC to LM2 ratio, where a 50% decrease in activity was obtained at a DLPC to LM2 ratio of 1600:1. The results after preincubation for 2 hr are consistent with other reports in the literature (7, 14, 23). Similar results were obtained when benzphetamine metabolism was examined after a 2-hr preincubation (data not shown). Although the overall rates of *p*-nitroanisole metabolism were dependent on the degree of saturation of LM2 with reductase, the overall pattern found in Fig. 4A when comparing the 5-min and 2-hr preincubations was unaffected by changes in the reductase to LM2 ratio (data not shown). A control experiment was performed to determine whether LM2 and reductase must be preincubated in the presence of phospholipid for 2 hr to mediate the slower phospholipid effect or whether a 2-hr preincubation of the proteins followed by the addition of phospholipid for 5 min would mimic the 2-hr data. When DLPC was added for 5 min following the 2-hr reductase-LM2 preincubation, the results were identical to those obtained from the simple 5-min preincubation (data not shown). These results indicate that no substantial reorganization of reductase and LM2 occurs during the simple combination of these proteins in the absence of phospholipid.

The effect of [DLPC]:[LM2] on the fraction of LM2 reduced in the first phase is shown in Fig. 4B. The results are similar to those observed for LM2-dependent monooxygenase activities

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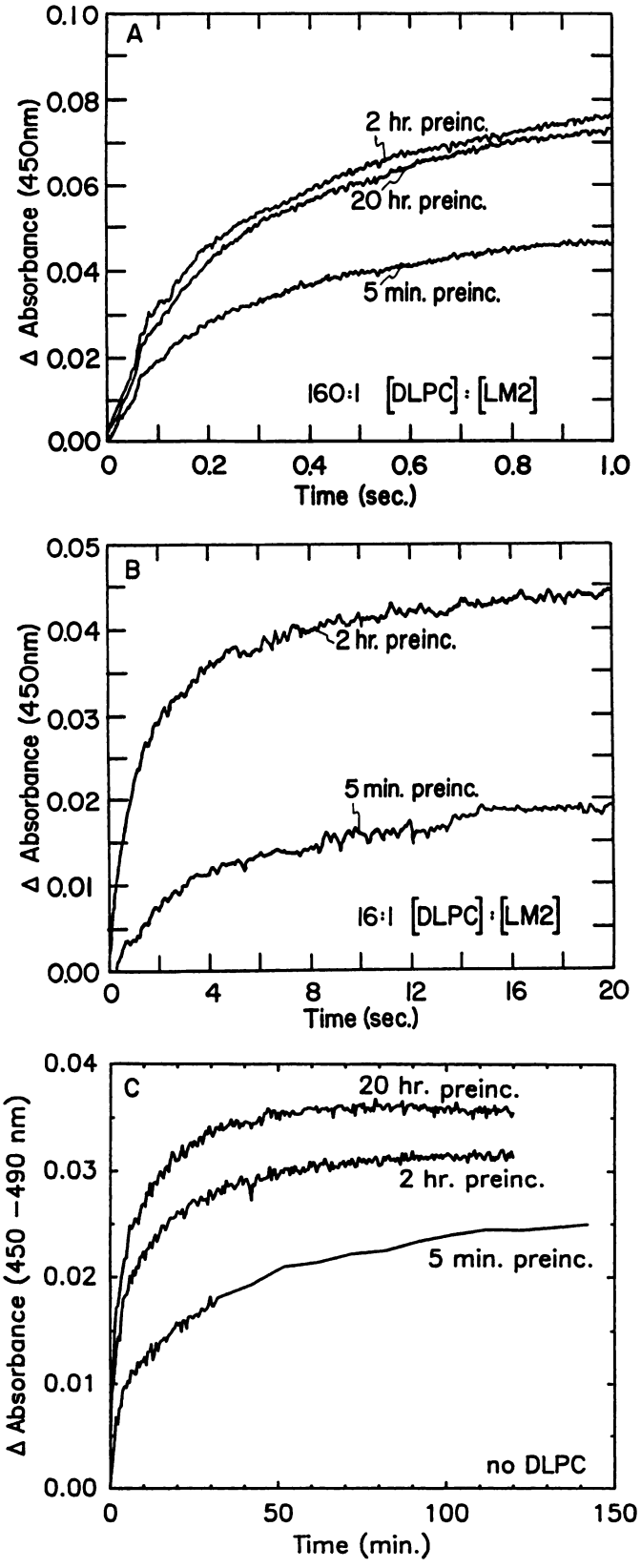

Fig. 3. Effect of preincubation time on first-electron transfer to LM2. LM2 and reductase were preincubated in the presence of the indicated [DLPC]:[LM2] for 5 min, 2 hr, and 20 hr, as described in Materials and Methods and the legend to Fig. 1. A, 160:1 [DLPC]:[LM2]; B, 16:1 [DLPC]:[LM2]; C, no DLPC. These experiments were run in the absence of exogenous substrate. The data illustrated in A and B were collected using the stopped flow spectrophotometer (2-cm path length), whereas the experiment in C was collected using the Thunberg cuvette (1-cm path length).

TABLE 2

Effect of [DLPC] on LM2-catalyzed monooxygenase activities

Reductase and LM2 were preincubated at a 1.5:1 ratio in the presence of DLPC at the concentrations indicated for 5 min, 2 hr, or 18 hr. After preincubation, the samples were diluted with other assay components, as described in Materials and Methods, and the metabolism of *p*-nitroanisole and benzphetamine was measured.

[DLPC]:[LM2]	*p*-Nitroanisole demethylation		
	5 min[a]	2 hr[a]	18 hr[a]
	nmol of p-nitrophenol formed (min)$^{-1}$ (nmol of LM2)$^{-1}$		
0	0.61	0.93	0.87
16:1	0.78	1.73	1.80
160:1	2.21	3.02	3.11
[DLPC]:[LM2]	**Benzphetamine demethylation**		
	5 min[a]	2 hr[a]	18 hr[a]
	nmol of formaldehyde formed (min)$^{-1}$ (nmol of LM2)$^{-1}$		
0	4.7	7.0	ND[b]
16:1	37.8	39.2	ND
160:1	55.7	73.8	ND

[a] Preincubation time.
[b] ND, not done.

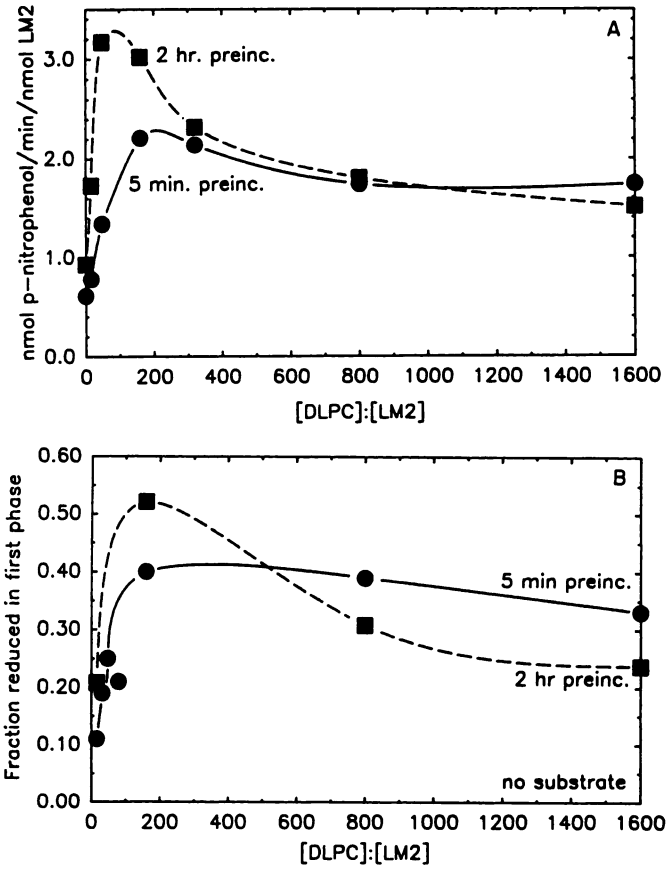

Fig. 4. Effect of preincubation time on the DLPC concentration dependence for *p*-nitroanisole demethylation and fraction of first-phase reduction. A, Effect of preincubation time on *p*-nitroanisole demethylation. LM2 and reductase were preincubated, as described in Materials and Methods, at various [DLPC]:[LM2] for the indicated times before dilution and measurement of *p*-nitroanisole demethylation. ●, 5-min preincubations; ■, 2-hr, preincubations. B, Effect of preincubation time on the fraction of LM2 reduced in the first phase was plotted as an estimate of the amount of functional reductase-LM2 complex present. ●, 5-min preincubations; ■, 2-hr preincubations.

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(Fig. 4A), in that reductase-LM2 complex formation can be "diluted" by large amounts of lipid only after prolonged preincubation times. After a 5-min preincubation in the presence of high DLPC concentrations, the fraction of reduction in the first phase was decreased less than 20% from the optimal value, whereas after a 2-hr preincubation the fraction of reduction in the first phase was decreased more than 50% (Fig. 4b), in keeping with literature reports (23). Similar results were obtained after the effect of DLPC concentration on the fraction of reduction in the first phase was measured in the presence of 1 mM benzphetamine (data not shown).

Effect of Tergitol NP-10 on cytochrome P-450-dependent monooxygenase activities. Earlier reports on the role of phospholipid in cytochrome P-450-dependent activities have suggested that phospholipid can simply act as a detergent that can be replaced by detergent addition to the reductase and cytochrome P-450 mixture (4, 25). More recent reports have suggested that monooxygenase activities comparable to those obtained in the presence of phospholipid can also be obtained in the absence of phospholipid. This was accomplished by the mixing of the microsomal proteins in concentrations of about 10 μM, with preincubation at room temperature for 2 hr before dilution with other assay components (26). As reported throughout this communication, our laboratory was unable to obtain reaction rates in the absence of phospholipid that were comparable to those obtained in its presence. In order to clarify this discrepancy, the effect of the detergent Tergitol NP-10 on *p*-nitroanisole demethylation was examined. We wanted to test whether the previously reported results (26) could be due to contamination with small quantities of detergent. Therefore, if small amounts of detergent were added to the preparation, rates comparable to those found in the presence of DLPC might be obtained. As the detergent concentration was increased, an increase was observed in the rate of *p*-nitroanisole demethylation, reaching a maximum at 0.02% Tergitol (Table 3). At that detergent concentration, the reaction rate was only about 43% that obtained in the presence of DLPC. The rate of the reaction declined as the Tergitol NP-10 concentration was increased above 0.02%.

The effect of Tergitol NP-10 concentration on the rate of *p*-nitroanisole demethylation was also examined using another cytochrome P-450 isoform, PBRLM5, which was isolated from phenobarbitol-pretreated rat liver. This isoform is equivalent to cytochrome $P\text{-}450_{PB\text{-}B}$ (29, 34) and was selected to determine whether the effect described in Table 3 is more pronounced with other cytochrome P-450 isoforms. Cytochrome $P\text{-}450_{PB\text{-}B}$ was also the isoform used by Müller-Enoch *et al.* (26). In our hands the reaction rate in the absence of DLPC was about 60% of the value obtained in the presence of the phospholipid (Table 3). Stimulation of the rate of *p*-nitroanisole demethylation was observed when low levels of Tergitol were present during preincubation, achieving rates similar to those obtained in the presence of DLPC. As the Tergitol concentration was increased further, the reaction rate decreased below the levels observed in the absence of Tergitol.

TABLE 3

Effect of Tergitol NP-10 on *p*-nitroanisole demethylation

Reductase and LM2 were preincubated for 2 hr in the presence of the indicated concentrations of Tergitol NP-10. After preincubation, the samples were diluted with the other assay components and *p*-nitroanisole demethylation activity was examined.

[Tergitol] during preincubation (%)	*p*-Nitroanisole demethylation	
	nmol of p-nitrophenol $(min)^{-1}$ $(nmol\ P\text{-}450)^{-1}$	*% of control*
LM2		
0	0.66	22
0.005	0.85	28
0.01	1.30	43
0.02	1.32	43
0.10	0.87	28
[DLPC]:[LM2] (160:1)	3.06	100.0
PBRLM5		
0	1.96	63
0.005	3.23	104
0.01	3.23	104
0.02	2.50	80
0.10	1.62	52
[DLPC]:[PBRLM5] (160:1)	3.11	100.0

Discussion

Cytochrome P-450-dependent reactions require complex formation between P-450 and its reductase. Although hydrodynamic studies have shown that both LM2 and reductase aggregate in solution (35, 36), a 1:1 functional complex appears to form between these components, which controls the rate of metabolism (37). Phospholipid constitutes a major component of the microsomal membrane, which provides the matrix for interaction of these proteins. When cytochrome P-450-dependent reactions are measured using purified proteins, they are commonly reconstituted into phospholipid. Although a requirement for phospholipid has been recognized for a number of years (2, 3), recent reports have called this requirement into question (26). Therefore, we examined both LM2 reduction and LM2-dependent monooxygenase activities over a wide range of DLPC concentrations. Both electron transfer to LM2 and LM2 monooxygenase activities were shown to be clearly dependent on the presence of DLPC. The rate of different monooxygenase activities appeared to be differentially affected by the absence of phospholipid, a result consistent with earlier reports (4). In our studies, LM2-dependent demethylation of *p*-nitroanisole in the absence of DLPC was 30% of the rate found in the presence of an optimal DLPC to LM2 ratio (160:1), whereas the rate of benzphetamine demethylation in the absence of DLPC was only 10% of the optimal value.

Monooxygenase activity was affected not only by the phospholipid concentration but also by changes in preincubation time. Although the results in Table 2 illustrate an increase in reaction rate as the preincubation was increased from 5 min to 2 hr, similar reaction rates were obtained after 2 and 18 hr. This effect was observed in both the absence and presence of DLPC, suggesting that phospholipid not only increases the rate of association between LM2 and its reductase but also increases the total amount of LM2 capable of forming a functional complex. The results further illustrate that increases in preincubation time do not always produce increases in the rate of the reaction but that the direction and degree of the response are a function of the DLPC concentration. For example, at a 1600:1 DLPC to LM2 ratio, increasing the preincubation time actually causes a decrease in the rate of the reaction. As shown in Fig. 4A, the effect of DLPC concentration on *p*-nitroanisole demethylation is dependent on the preincubation time. After a relatively long preincubation, increases in the DLPC to LM2 ratio cause a substantial inhibition of the monooxygenase activity, an effect not observed after the 5-min preincubation.

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These results suggest that extended preincubation produces a reorganization of the reductase and LM2 with respect to phospholipid, which does not occur after the short preincubation. The results shown after a 2-hr preincubation are consistent with other reports in the literature (7, 23), even though in those studies the proteins were incorporated using the cholate gel filtration (38) and cholate dialysis (14) methods, respectively.

Effects similar to those found with monooxygenase activities were found when LM2 reduction was examined. After a 5-min preincubation, the fraction of LM2 reduced in the first phase was the major parameter affected by variations in DLPC concentration. At DLPC to LM2 ratios ranging from 0 to 160:1, the fraction of LM2 reduced in the first phase was increased. Further increases in the [DLPC]:[LM2] ratio produced a relatively small decrease (less than 20%) in the fraction reduced in the first phase. After a 2-hr preincubation, a very different dependence on DLPC concentration was observed (Fig. 4B), where the fraction reduced in the first phase was decreased almost 60% as the DLPC concentration was increased to 1600:1. These results agree with those presented for *p*-nitroanisole demethylation (Fig. 4A), suggesting that similar processes are affecting both monooxygenase activity and first-electron transfer.

As stated previously, at low DLPC levels the rate of electron transfer was strongly dependent on DLPC concentration, with extremely slow rates of reduction being observed in the absence of phospholipid. Increases in the rate of reduction were not observed until the DLPC concentration exceeded 0.8 μM. The critical micellar concentration for DLPC was recently determined to be approximately 0.6 μM (39), which is close to the minimum value for phospholipid stimulation of LM2 reduction. These results suggest that micellar phospholipid may be necessary for enhancement of the interaction between reductase and LM2.

The results in Table 3 show that detergent can stimulate cytochrome P-450-dependent monooxygenase activities and that the effect is dependent on the isoform used. When reductase and cytochrome P-450 were mixed at high concentrations in the absence of either phospholipid or detergent, we were unable to obtain rates similar to those found in the reconstituted system with either isoform, in contrast to previous reports (26). These previously reported results suggesting that phospholipid was not required for maximal association of reductase and cytochrome P-450 may have been the result of contamination of the purified proteins with low levels of nonionic detergent. Tergitol NP-10 could only partially substitute for phospholipid using LM2, consistent with previous reports with 1-*O*-*n*-octyl-β-D-glucopyranoside (36). However, Tergitol produced rates similar to those obtained in the reconstituted system when PBRLM5 was used. These results point to a differential importance of phospholipid for different isoforms of cytochrome P-450. These results also demonstrate that differences in the characteristics of various preparations of a particular cytochrome P-450 isoform could be the result of differences in the amount of contaminating nonionic detergent present in the protein preparations.

In this manuscript we characterized some of the aspects involved in the incorporation of these purified proteins into DLPC membranes and have shown not only that DLPC can stimulate LM2-dependent activities in the short term but also that stimulation can be modified by its continued presence in the phospholipid. In the absence of phospholipid, both reductase and cytochrome P-450 exist as multimeric aggregates (35, 36). Mixing of these proteins in the absence of phospholipid will produce only limited numbers of mixed complexes, due at least in part to limitations in the accessibility to the protein-protein binding sites. Addition of phospholipid for 5 min appears to facilitate the association of reductase with LM2, increasing the amount of functional complex formed. This may be due to either an effect on the aggregation state of either of the proteins or an interaction of the phospholipid with these proteins, which may relax the tight multimeric complex and facilitate functional interactions between these proteins. Either phospholipid or detergent appears to be required for this process to occur, because prolonged preincubation in the absence of these compounds does not appear to facilitate formation of functional reductase-LM2 complex. The complex formed after 5 min in the presence of phospholipid appears to be tight and not readily dissociable at high DLPC concentrations (Fig. 4). Upon preincubation for longer time periods, there is a change in the interaction between the phospholipid and protein components. Preincubation for 2 hr in the presence of high concentrations of phospholipid causes a 50% decrease in reaction rates (Fig. 4). Based on studies showing that the fraction of reduction in the first phase represents the fraction of functional reductase-LM2 complex (13), prolonged preincubation in the presence of phospholipid produces an increase in the amount of complex formed under optimal conditions (Fig. 4B). These changes may be the result of increased accessibility of the reductase to LM2, due to changes in the aggregation state of the proteins as they incorporate into the phospholipid matrix. The results are consistent with the time-dependent incorporation of these proteins into the phospholipid membranes. Incorporation of these proteins into DLPC vesicles after prolonged preincubation appears to affect the affinity of the reductase-LM2 complexes such that a smaller fraction of LM2 is associated with reductase at high DLPC concentrations. These results are consistent with the lateral diffusion of LM2 and reductase in the phospholipid matrix.

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