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REVIEW

Lipid Requirement of Membrane-Bound Enzymes

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Introduction

The work carried out in the past years on the interaction between lipids and enzymes in biological membranes has clearly shown that lipids are not only a hydrophobic matrix to which enzymes may be bound, but that they also play an active role in determining and regulating most of the membrane-bound enzymatic activities. The length of this review does not permit an examination of all these lipid-requiring enzymes, which have been extensively reported in earlier reviews [1–5]. The purpose of this review, rather, is (1) to critically evaluate the investigations which have been made on some membrane enzyme systems with regard to their lipid requirement, and (2) to report some recent advances concerning the molecular mechanisms of activation and regulation of membrane enzymes by lipids.

The effects of lipids on enzymatic activity have been observed in two ways: by disassociation and reassociation of lipids with membrane-bound enzymes; and by relating changes in the enzymatic activity with changes in the structure of the lipid phase of the membrane. One should also consider that the concept of lipid-requiring enzymes may not only be related to the direct lipid effect on enzymes, since it has been shown in some instances that lipid can be a solvent for the substrate [6], or even to activate the substrate [7].

Keeping these conditions for lipid–enzyme interactions in mind, we discuss now the main aspects of past investigations on lipid-requiring enzymes.

Lipid Removal

We examine first the procedures involved in removing lipids which are associated with a membrane enzyme. Typically, this is done either by degradation of the lipids, or by extraction of the lipids or the proteins from the membranes.

Degradation with Phospholipases

Phospholipases can effectively be used to degrade lipids and thus eliminate their influence on the membrane enzyme being studied [8, 9]. There are problems associated with phospholipase procedures, and these should be taken into account for a correct interpretation of the results. Phospholipase A generates lysocompounds and fatty acids as hydrolysis products. These can maintain the activity of some membrane-bound enzymes, such as the $(Ca^{2+} + Mg^{2+})$-ATPases of sarcoplasmic reticulum [10] and erythrocytes [11], and the microsomal UDP-glucuronyltransferase [12]; and inhibit some membrane-bound enzymatic activities, such as the mitochondrial NADH-CoQ reductase [8, 13]. True deletion effects may not be seen until these hydrolysis products are removed, and this can be effectively accomplished by washing the system with bovine serum albumin [8]. Membrane delipidation using this procedure has resulted in inactivation of several membrane-bound enzymes [for review, see ref. 2].

Phospholipase C has often been preferred to phospholipase A since it produces degradation products which do not seem to affect the activity of membrane enzymes [14]. However, it should be considered that the diglycerides left in the membrane after digestion with phospholipase C could cause a rearrangement of the remaining phospholipids or affect their charge properties [15]. This phenomenon may explain why the lipid requirements for the erythrocyte $(Ca^{2+} + Mg^{2+})$-ATPase appear to be quite different, depending on the hydrolase used [11, 16].

Additionally, in the case of the microsomal glucose-6-phosphatase, it has been shown that endogenous phospholipase A or acylhydrolases can produce fatty acids during the digestion of the lipids with phospholipase C, and in this way alter the meaning of the results [17]. Finally, it should be noted that the hydrolysis products from phospholipase A_2 treatment of biological membranes can cause solubilization and release from the membrane of bound enzymes such as the mitochondrial β-hydroxybutyrate dehydrogenase [18], NADH dehydrogenase [19, 20], and others [21, 22].

Extraction with Detergents

The interaction of detergents with biological membranes causes disruption of membrane structures and solubilization of their components [23]. Although detergents can inactivate membrane enzymes, these effects are generally not irreversible when nonionic or weakly ionic detergents are used [24]. Treatment of membrane enzymes with these milder detergents, though, usually does not result in complete delipidation of the enzyme. Dean and Tanford [25] have reported that 4 moles phospholipid/mole of sarcoplasmic reticulum ATPase is the minimum amount of lipid which they can find after polyethyleneglycol treatment. Others have found that about 4 moles phospholipid/mole cytochrome oxidase remain after solubilization and purification of the enzyme with Triton X-100 and cholate–ammonium sulfate purification [26, 27].

It is important that detergent removal be complete in subsequent lipid reactivation studies. This can now be achieved for most weakly ionic or non-ionic detergents, either by dialysis [28], or various column or batch procedures [29–31].

Extraction with Organic Solvents

More drastic treatments involving organic solvents can insure complete lipid removal. Caution must be taken when using such procedures, however, because these treatments can cause partial or total denaturation of the enzyme. This can happen even with relatively mild organic solvents, such as 10% aqueous acetone and *n*-butanol, which in some systems cause irreversible denaturation [23] and in others leave the enzyme's activating capabilities intact [32–34]. In spite of such difficulties, most of the early studies on the effect of lipids on membrane-bound enzyme activities, such as those on the mitochondrial respiratory chain [33] and the lipid-synthesizing enzymes of the endoplasmic reticulum [35, 36], have been carried out by extraction of the lipids with organic solvents.

Results from such studies, though, may be unclear, as in the instance of the cytochrome oxidase system. Acetone treatment of this enzyme complex has been used to remove matrix and boundary lipids, leaving tightly bound lipids with an enzyme which is capable of being reactivated. However, when further extraction with chloroform–methanol–ammonia mixture is used to extract the remaining lipids, irreversible denaturation of the enzyme occurs [26]. The reason for this observation may be either because those tightly bound lipids were essential for the structural integrity of the enzyme, or because the treatment of this enzyme with the strong organic solvents had irreversibly denaturated the enzyme.

Recently successful extractions with strong organic solvents under strict extraction procedures have been reported. The lipid products from phospholipase treatment of the $(Na^+ + K^+)$ATPase have been removed without irreversibly denaturating the enzyme by using a dry ether extraction procedure [37]. The ATPase can also be delipidated with chloroform–methanol, and if the extraction is carried out at $-70°C$, the enzyme can be subsequently reactivated by lipid [38]. The membrane-bound 2:3-cyclic nucleotide 3-phosphodiesterase activity can also be retained after treatment with chloroform–methanol if the extraction procedure is carried out in the complete absence of water [39].

Lipid Activation

One of the basic criteria used to define a lipid-requiring enzyme is its reactivation after addition of lipids [33]. Reactivation can be obtained with the enzyme still associated with the original membrane [8, 40] or with the enzyme extracted from the membrane in purified form [26, 41–43]. In the latter case, the molecular interaction between lipid and enzyme can generally be better studied.

A comparison of the kinetic parameters of the enzyme, such as K_m, in the original and in the reactivated system is essential to show that the active conformational state found in the native enzyme has not been altered. This is necessary because many enzymes undergo changes in the apparent K_m for the substrate during delipidation procedures. For instance, after delipidation the apparent K_m has been found to markedly decrease in the mitochondrial cytochrome oxidase [40], microsomal glucose-6-phosphatase [44], and other bound enzymes [45]; and it increases in the case of the *E. coli* pyruvate oxidase [42].

Nevertheless, in those studies where the K_m has been measured both in the native and reactivated systems, it has been found to be approximately the same [46–48]. In some cases, though, the type of phospholipid used in the reactivation can influence the K_m, as seen in the mitochondrial β-hydroxybutyrate dehydrogenase [49] and the *E. coli* pyruvate oxidase systems [42].

In terms of evaluating enzyme conformation, Warren et al. [50] have argued that similar conformations are demonstrated for the $(Ca^{2+} + Mg^{2+})$-ATPase of sarcoplasmic reticulum when a stoichiometry is achieved between Ca^{2+} transport and ATP hydrolysis in the reconstituted ATPase system that is the same as in the native system.

General Lipid Requirements for Enzyme Activity

Many systems have only the general requirement of a hydrophobic region in which the active enzyme or its substrate may thermodynamically favorably exist. This general requirement for a hydrophobic environment may be satisfied by several different types of phospholipids [50–52, for review see ref. 2], fatty acids [43], or even detergents [25, 27, 53].

The general lipid effect on membrane-bound enzyme can apparently relate to the secondary structure of membrane proteins. Circular dichroism studies of the inner mitochondrial membrane which had been depleted of more than 95% of the lipid by treatment with aqueous acetone–ammonia showed a large decrease in the alpha helical structure of the proteins. The addition of phospholipids restored the helicity to that found in the intact membrane [54]. No change in helical structure, though, was noted when about 70% of the lipids of the inner mitochondrial membrane were removed with phospholipase A_2 [55]. It is thought that the organic solvents were able to remove tightly bound lipids which are essential to the functional structure of the proteins, while the phospholipases could not [54].

Apart from their role in preserving the active conformation of the membrane enzymes, lipids can be considered hydrophobic solvents for the substrates. This seems to be the case for the membrane-bound enzymes connected with lipid metabolism, where most of the substrates, intermediates, and products are lipid soluble [14, 36]. For the bacterial C_{55}-isoprenoid alcohol phosphokinase, it has been shown that one role of lipids is to allow the diffusion of the lipid substrate rather than the maintenance of an active enzyme structure [6]. In the functioning of the mitochondrial respiratory chain, lipids provide a medium which allows for the interaction of the mobile carriers, CoQ and cytochrome c, with the other components of the respiratory chain [2, 56, 57]. This, however, is not the only effect lipids have in the activity of the respiratory chain. They can also directly control the activity of succinate dehydrogenase [58] and cytochrome oxidase [59], and influence the binding of inhibitors [60].

In the mitochondrial NADH–CoQ reductase, it has been shown that even though there is a general lipid requirement for enzyme activity, the lipids which can reactivate interact differently with the enzyme complex [61]. Using a lipid exchange procedure, it has been found that phosphatidylcholine and phosphatidylethanolamine will bind at one site on the enzyme, and that they are easily removed from the enzyme with cholate. Diphosphatidylglycerol binds to a second site and is not removed with cholate [61].

Finally, it should be mentioned that partial reactions in multistep

enzyme mechanisms can be affected by lipids [59, 62, 63]. In the $(Ca^{2+} + Mg^{2+})$-ATPase of sarcoplasmic reticulum it has been found that lipids are not required for the formation of the phosphoenzyme intermediate, but rather for the subsequent breakdown of the phosphoprotein [62, 64]. Similarly, it has been shown in the mitochondrial cytochrome oxidase complex that the electron transfer between cytochromes a and a_3 may be the lipid-requiring step in the reaction [59].

Specific Requirements for Enzyme Activity

There are many examples of membrane-bound enzymes requiring lipids with specific polar head groups in order to function. Although there has been much discussion about the lipid head group requirement of the $(Na^+ + K^+)$-ATPase [for review see 65], Roelofsen and van Deenen [37], using careful phospholipase procedures, have clearly demonstrated that this ATPase requires an acidic phospholipid for function, especially phosphatidylserine. This finding has since been confirmed by others, using detergent extraction procedures [47, 66]. Negatively charged phospholipids do not activate the $(Na^+ + K^+)$-ATPase simply by forming a stable lipid–enzyme complex; because other phospholipids, such as phosphatidylethanolamine, form the same stable complex with the enzyme but are unable to activate it [67]. In the adenyl cyclase system from cat myocardium, it has been found that phosphatidylinositol will promote catecholamine activation while phosphatidylserine will enhance glucagon and histamine activation [46, 68, 69]. The acetylcholinesterase in erythrocytes has a specificity for cardiolipin [70], while the $(Ca^{2+} + Mg^{2+})$-ATPase of erythrocytes has been found to function best in the presence of phosphatidylserine [11, 71]. Using both phospholipase and protein purification procedures, enzyme II of the PEP-phosphotransferase system in bacteria has been found to be reactivated by anionic lipids, especially phosphatidylglycerol [72, 73].

In terms of lipid-requiring enzyme systems, the mitochondrial β-hydroxybutyrate dehydrogenase system is one of the most extensively studied. This purified lipid-depleted enzyme requires the specific quaternary ammonium moiety of phosphatidylcholine for activity [74–77]. A precisely structured lipid hydrocarbon portion is not important, although unsaturated lecithins reactivate better than the saturated forms [41, 78]. The properties of the bilayer are not essential for activity, because the enzyme is active in the presence of short-chain lecithin molecules below their critical micelle concentration [41]. The role of phospholipids in the functioning of this enzyme is related to the binding of NADH. The enzyme must be combined with the lipids in an active complex for this binding

with the coenzyme to occur [79]. A role of lipids in the regulation of the binding of a cofactor has also been shown in the case of the pyruvate oxidase, where lipids seem to regulate the binding of the adenine pyrophosphate [80].

The $(Ca^{2+} + Mg^{2+})$-ATPase from sarcoplasmic reticulum has been shown to have maximal activity in the presence of phosphatidylethanolamine [50, 81]. However, it should be noted that optimal enzyme activity conditions may differ from optimal functional conditions. In reconstituted sarcoplasmic reticulum ATPase systems, the best coupling efficiency between Ca^{2+} transport and ATP hydrolysis occurs with a mixture of dioleoylphosphatidylcholine and -ethanolamine [50, 82]. There may as well be a specific fatty acyl chain requirement for the sarcoplasmic ATPase. Warren et al. [51] have found that unsaturated lecithins activate this enzyme better than saturated lecithins. When using the saturated lecithins, acyl chains must be greater than 14 carbons long to promote a stable enzyme complex. With the 12-carbon acyl chain dilauroyllecithin the ATPase–lipid complexes are rapidly and completely inactivated.

The technique of lipid replacement, which has been developed by the group of Warren et al. [83, 84], warrants comment because of its applicability to studies involving displacement of those lipids directly around the membrane enzyme. The procedure is straightforward, involving adding the exogenous lipid to a purified lipid–protein complex in the presence of detergent. The two lipid pools equilibrate so that the lipid environment around the protein reflects the composition of the entire lipid pool. A two- or three-fold cycling of this procedure will result in replacement of essentially all of the lipid which is associated with the protein. This technique has since been applied to studies on the mitochondrial β-hydroxybutyrate dehydrogenase [85] and the NADH–CoQ complex [61].

Lipids can also have a more direct interaction with the substrate than the role of substrate solvent. The galactosyl-transferase involved in the synthesis of the lipopolysaccharides of Gram-negative bacteria requires phosphatidylethanolamine. This lipid specificity seems to be related to the ability of phosphatidylethanolamine to form an active complex with the lipopolysaccharide substrate, which can then interact with the transferase [7, 86].

Membrane Fluidity and Enzyme Activity

The effect of lipids on a membrane-bound enzyme can be investigated by relating the activity of the enzyme with changes in the physical state of the surrounding lipids [2, 3, 5, 87].

Temperature-Associated Effects

Arrhenius plots of the activity of many membrane-bound enzymes show sharp changes in the energy of activation at characteristic temperatures [5, 88–90]. These discontinuities in Arrhenius plots have been related to temperature-caused changes in the fluidity of the lipid phase as detected by following the mobility of spin-labeled molecules within the bilayer [88–92]. Such measurements of changes in lipid fluidity, however, do not usually correspond to the midpoint of the broad fluid-solid phase transition of membrane lipids as detected by differential scanning calorimetry [93, 94] or x-ray diffraction [95]. Using these latter techniques lipid phase transitions in natural membranes are usually found to occur at lower temperatures than those observed with spin labels. Examples of membrane enzymes that do show changes in the energy of activation at temperatures which correspond to the temperature transition of the lipids are, the stearyl-CoA desaturase [96], the $(Na^+ + K^+)$-ATPase [91], and the NADPH oxidase [97].

Since in natural membranes the bulk lipids, as detected by differential scanning calorimetry, are often in a fluid state when discontinuities in the Arrhenius plots of enzyme activity are observed, then changes in the physical state of lipids which are restricted to a small area of the bilayer must be responsible for the function of the enzyme. These changes can be related to pretransition phenomena such as phase separations [98] or clustering of lipids [94, 99], as well as to the physical state of the lipids which are in direct contact with the enzyme [100, 101]. In fact, it is known that integral membrane proteins are surrounded by a boundary or annulus of lipids whose mobility is restricted with respect to the bulk lipids [83, 101–103], and these boundary lipids are apparently related to the functioning of the enzyme [100, 102]. For example, the association of increasing amounts of lipids with a purified mitochondrial cytochrome oxidase has been studied with spin-labeled fatty acids [102], and more recently with spin-labeled phosphatidylcholine [104]. It has been found that maximum enzyme reactivation occurs when the enzyme's lipid annulus, which contains lipid with immobilized fatty acyl chains, is filled. The addition of more lipids will produce an adjacent fluid region, whose components were found to be in equilibrium and exchangeable with the boundary lipids [104].

The involvement of boundary lipids in the functioning of the sarcoplasmic reticulum $(Ca^{2+} + Mg^{2+})$-ATPase has also been reported. It has been shown that an annulus of about 30 lipid molecules per ATPase is required to maintain maximal ATPase activity [51]. Furthermore, this enzyme interacts with the lipids in such a way so as to change their

transition temperature. Hesketh et al. [100] have studied the effects of thermal changes on the activity of the $(Ca^{2+} + Mg^{2+})$-ATPase, complexed with dipalmitoyllecithin (PC 16:0) at different ratios. They have shown that as more ATPase is incorporated into the bilayer, the sharp temperature transition at 41°C, as measured with spin-labeled fatty acids in the pure PC 16 : 0 lipid bilayer tends to disappear, and a new break at 30°C is formed. At a ratio of about 30 moles PC 16 : 0 per ATPase, that is, when all the lipid molecules in the bilayer are contained as annulus lipids only the lower transition is observed. The increase in fluidity of the annular lipids at 30°C coincides with the development of ATPase activity at this temperature, showing that annular lipids are the primary determinant of the ATPase activity. The bulk lipids do, however, exert some influence on the enzyme activity. When Arrhenius plots of enzyme activity at high ratios of PC 16 : 0 to ATPase were calculated, two discontinuities, at 30°C and 39°C, were noted. The sharp break at higher temperature was eliminated when the ratio of lipid to protein decreased, and all the lipid assumed an annulus role. It therefore seems that the presence of the ATPase in the bilayer causes perturbation of a restricted region of lipid, which will then show a different phase transition than the one of the bulk lipids. This could be considered a normalizing effect of the protein, because in the natural membranes the effect of the protein is to raise the lipid temperature transition, while in the saturated fatty acid phospholipids system is to lower it.

The discontinuities in Arrhenius plots for the activity of membrane-bound enzymes have also been interpreted as the result of lipid pre-transition phenomena associated with the liquid to solid phase transition. As the temperature of the system is lowered, before the formation of the solid phase, aggregates of homogeneous or heterogeneous clusters of lipids start forming [105]. The formation of such clusters in the membrane has been associated with discontinuity in Arrhenius plots. Lee et al. [99] have presented data that the discontinuity in the Arrhenius plot of a pure sarcoplasmic reticulum ATPase which has been complexed with dioleoyl-lecithin is observed at 29°C. At this temperature they also noted changes in the partitioning of the spin-labeled TEMPO between the water phase and the fluid lipid phase, which indicated the onset of cluster formations. These data, however, should be considered with caution since other researchers have not detected any discontinuities in the Arrhenius plot of the ATPase–dioleoyllecithin complex in a temperature range between 3°C and 40°C [64]. Changes in membrane-bound activities have also been correlated with lateral phase separation of the lipid in the membrane. Linden et al. [106] have reported that the two breaks found in the Arrhenius plot of the sugar transport activity of *E. coli* correspond to the

upper and lower temperature boundaries of the process of lateral phase separation occurring in the membrane.

Interactions more specific than general fluidity requirements can also be deduced from these types of studies. The discontinuity in Arrhenius plots for C_{55}-isoprenoid alcohol phosphokinase has been correlated with the temperature phase transition in the lipid bilayer [6]. But circular dichroism studies have shown that little change in enzyme conformation occurs during the lipid phase change. This combination of data suggests that the discontinuity in the Arrhenius plot may be due to the influence of the fluidity of the hydrocarbon region on the diffusion of a substrate.

Effects of Fatty Acid Manipulation

Another approach to study the effects of lipid fluidity on membrane-bound activities is to modify their fatty acid composition. This has been done using microorganisms such as mycoplasma [107, 108] and the unsaturated fatty acid auxotrophs of *E. coli* [109] and yeast [110], which can incorporate into their phospholipids the fatty acids that are added to the growth media. Using the fatty acid auxotroph of *E. coli* grown with elaidic, oleic, or linoleic acids, it has been shown that the Arrhenius plots of the D-glucoside transport show distinct changes in the slopes at temperatures corresponding to the thermal lipid transitions detected by spin-labeled probes [109]. Mavis and Vagelos [111] have studied the rate–temperature profile of three membrane-associated enzymes involved in lipid biosynthesis of an unsaturated fatty acid auxotroph of *E. coli*. They found that the glycerol-3-phosphate acyltransferase and 1-acyl-glycerol-3-phosphate acyl transferase were dependent on the fluidity of the membrane, whereas the glycerol-3-phosphate dehydrogenase was not.

Membrane fluidity can also be altered in eucaryotic cells by changing the dietary fatty acids (or cholesterol), which in turn alters the fatty acid composition of the cell membranes [112]. Using this approach it has been reported that changes can be caused in the activity of the $(Ca^{2+} + Mg^{2+})$-ATPase of sarcoplasmic reticulum and erythrocyte membranes [113, 114], as well as other membrane-bound enzymes [112].

Generally, this type of study which involves changes in fatty acid composition of the membrane, has shown that fatty acids which can maintain a certain level of fluid lipid phase are needed for the functioning of membrane-bound activities [3].

Conclusion

It can be seen from the body of this review that many lipid-requiring enzymes have been successfully and thoroughly studied. The criteria for

precise lipid removal, lipid reactivation of enzymes, and corresponding studies of lipid fluidity effects have been adequately examined for several enzyme systems. In conclusion, it seems reasonable to discuss those aspects of lipid-requiring enzymes that to date have not been clarified. A major limitation to any such investigation is the ultimate purification of the membrane enzymes. Only when these proteins can be removed from their associated lipids and cofactors can the impact of each of these on the enzyme activities be evaluated.

There are two general categories of effect of lipids on enzyme activity: (1) the effect of the lipid on the microenvironment of the enzyme and (2) the effect of the lipid on the enzyme in terms of a precise molecular interaction between the two. The maintenance of enzyme activity by generating the appropriate fluid hydrophobic environment is an example of the first category, and this general requirement has been examined in several instances. The contribution of the lipid to the polar microenvironment, however, has not been studied in detail. It is known that changes in the fluidity of the paraffinic chains can alter the hydration of the lipid polar head groups [115, 116]. Conversely, variations of pH [117], metal ions [117, 118], and protein concentrations [118, 119] can affect the lipid polar head groups and cause changes in the fluidity of the hydrocarbon region. Although precisely how these sorts of interactions affect the enzyme in question is unclear, the occurrence, for example, of membrane enzymes which show a requirement for acidic phospholipids indicates that such interactions do occur. Such considerations, of course, may have to be extended to other factors such as substrate lipid interactions in order to suggest answers.

Direct effects, meaning strong molecular interactions, with specific binding sites on the membrane enzyme for the lipids, are also largely unknown. There are indications that some hydrophobic effects may be the result of direct interactions. For instance, the tightly bound lipids of the cytochrome oxidase complex, which are not sufficient to keep the enzyme in active state but seem to be essential for enzyme stability, apparently have direct interactions with the enzyme molecule [26]. In terms of polar interactions, the mitochondrial β-hydroxybutyrate dehydrogenase system, which has an absolute requirement for lipids with a choline head group, may be an example of a direct interaction between lipid polar head groups and the enzyme molecules [41].

It could therefore be concluded that substantial information about lipid-requiring enzymes has been obtained, and that significant areas in the knowledge of their interactions remain to be investigated.

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