Lipid Dependence of the Membrane-bound \( \Delta \)-Lactate Dehydrogenase of *Escherichia coli* 

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The lipid dependence of the \( \Delta \)-lactate dehydrogenase of *Escherichia coli* plasma membranes was investigated. The delipidated purified enzyme was shown to possess a small activity in the absence of lipid or detergent. Activation, in some cases up to 7-fold the lipid-free activity, was achieved in the presence of added deoxylysophosphatidylcholines, phosphatidylcholines, some phosphatidylethanolamines, methylphosphatidic acid, phosphatidylglycerol, and phosphatidylserine. The enzyme activity was examined in the presence of a homologous series of deoxylysophosphatidylcholines with acyl chains from 6 to 22 carbon atoms long. Activation was only observed when the deoxylysophosphatidylcholine was in its micellar form. Myristoyl deoxylysophosphatidylcholine was observed to be the best activator in this series of micelle-forming compounds. Deoxylysophosphatidylethanolamines did not activate the enzyme, but increasing the degree of \( N \)-methylation increased the extent of activation. In the phosphatidylcholine series, dimyristoyl-, dipalmitoyl-, and distearoyl-, the distearoyl analog was observed to be the best activator of the enzyme and the dimyristoyl analog was the poorest. Conversely, in the corresponding phosphatidylethanolamine series, the dimyristoyl analog was the only one capable of weakly activating the enzyme. The unsaturated egg phosphatidylethanolamine and 1-palmitoyl-2-oleoylphosphatidylethanolamine were better activators than their corresponding saturated analogs, but they were, nevertheless, weaker activators than the saturated acyl chain phosphatidylcholines. The best activation was obtained by adding phosphatidylserine and phosphatidylglycerol. The activation in this case was not due to a nonspecific charge effect since the negatively charged methylphosphatidic acid was a relatively poor activator of the enzyme. The pH versus activity profile of the enzyme in the presence of several activating lipids showed that the profile in the presence of phosphatidylglycerol most closely mimicked the pH versus activity profile of the enzyme in the native plasma membrane. It is inferred that in its native state this enzyme is surrounded predominantly by phosphatidylglycerol or phosphatidylserine which are minor lipids in the plasma membrane of *E. coli*.  

\( \Delta \)-lactate dehydrogenase is one of the membrane-bound primary dehydrogenases of the respiratory chain of *Escherichia coli* (1, 2) and has been implicated in the energy-dependent transport of amino acids and sugars across the plasma membrane (2, 3). It is a flavoprotein having approximately 1 mol of flavin/mol of protein (4) and a single polypeptide chain with a molecular weight of about 72,000 which can be dislodged from its native state in the membrane by the action of "chaotropic" agents (5), detergents (4, 6), and sonication (7). The enzyme has been purified to homogeneity and obtained in a phospholipid-free form (4, 6, 7). Removal of detergents from the delipidated, purified enzyme results in an aggregation of the protein (8) and the delipidated, detergent-free enzyme has been shown to associate with added phospholipids, this association resulting in some cases in an increase in enzymatic activity (8, 9). All these characteristics justify the classification of this enzyme as an integral membrane protein (10). Antibodies prepared against the purified \( \Delta \)-lactate dehydrogenase are able to inhibit the enzyme only when the cytoplasmic surface of the plasma membrane is accessible to the antibodies, thus indicating that the enzyme is accessible to the aqueous phase only on the cytoplasmic side of the native membrane (11, 12).

The activities of several membrane-bound proteins have been shown to be modulated by their membranous environment (13–16) as may be expected from the "fluid mosaic" model of biological membrane structure (10). In particular, the phospholipids seem to be important in this regard. The classical approach to the study of modulation of membrane-bound enzyme activity by phospholipids has been to study the enzymatic activity of the purified, delipidated enzyme in the presence of added phospholipids, the so-called "reconstitution" studies. Most of the membrane-bound enzymes studied by this approach appear to be quite nondiscriminatory with regard to their phospholipid requirement but some do show varying degrees of specificity (15, 16). Some aspects of the lipid dependence of \( \Delta \)-lactate dehydrogenase have been reported by other workers (8, 9). In this communication, we present a more extensive study of the lipid dependence of this enzyme. Evidence is provided that an ordered lipid structure (such as micelles or bilayers) is essential for the activation of \( \Delta \)-lactate dehydrogenase and it is suggested that some degree of specificity may exist with regard to the phospholipid requirement of this enzyme.

**MATERIALS AND METHODS**

**Purification of \( \Delta \)-Lactate Dehydrogenase—*E. coli* strain K-125**  
(kindly given to us by Dr. P. Overath) was grown aerobically in a 25-liter fermentor (New Brunswick Scientific Co., New Brunswick, model MP-129S) to late-exponential phase using a synthetic medium (17). The cells were harvested using a continuous flow centrifuge and stored at ~70 °C until further use. The membrane fraction was prepared by the lysozyme-EDTA treatment and freeze-thawing as described by Weiner and Heppel (18). \( \Delta \)-lactate dehydrogenase was purified from the plasma membrane fraction as described by Futai (6). The final enzyme preparation represented a 340-fold purification.

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over the membrane fraction and was homogeneous using the criterion of polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (19).

Assay of D-Lactate Dehydrogenase—Enzyme assays were done using the phenazine methosulfate-coupled reduction of 3,4,5-di-ethyl-2,2,5-diphenyltetrazolium bromide as described by Futai (6). Enzyme units are defined in the same way as defined by Futai (6). When phospholipids or other amphiphiles were required to be present in the assay mixtures, the enzyme and lipid were incubated together for 30 min at the desired temperature before addition to the assay mixture.

Phospholipids—Deoxylyso-PC of varying acyl chain lengths (C<sub>n</sub>-deoxylyso-PC, C<sub>n</sub>) were synthesized as described by Eibl and Westphal (20).

Lipid Suspensions—Short acyl chain (up to C<sub>14</sub>) deoxylysophospholipids were suspended in buffer by shaking in a Vortex mixer. Deoxylysophospholipids with acyl chains longer than C<sub>16</sub> and diacyl phospholipids were suspended in buffer by sonication in a nitrogen atmosphere at a temperature greater than the lipid phase transition temperature (wherever applicable) using a Branson sonifier (Branson Instruments Inc., Danbury, CT) with a microtip. Sonication was continued until there was no more change in optical density of the suspensions at 600 nm.

Determination of Critical Micelle Concentrations—Determination of the critical micelle concentrations of the deoxylysophospholipids used in this work was based upon the observation that the fluorescence emission of the dye N-phenylphtalimide shows an increase in the quantum yield and a blue shift in the emission maximum in the presence and absence of 1 mM Cl<sub>-</sub>deoxylyso-PC are shown in Fig. 1. In the presence of deoxylyso-PC micelles, the fluorescence emission has a maximum at 420 nm (uncorrected for instrumental parameters) upon excitation at 350 nm. In the presence of deoxylyso-PC below the critical micelle concentration and have a steep slope above it. The intersection of the two phases of the plot is the critical micelle concentration. The validity of the method was verified by determination of the critical micelle concentrations of several detergents for which these values exist in the literature. The method described here is similar in principle to the dye-solubilization method for detergents (26).

RESULTS

Activation of D-Lactate Dehydrogenase by Micelle-forming Deoxylysophospholipids—We have examined the activation of d-lactate dehydrogenase by several micelle-forming deoxylysophospholipids. In Fig. 2a, we show the activation of the enzyme by deoxylyso-PCs of varying acyl chain lengths. The critical micelle concentrations of these compounds in buffer were also determined and is indicated by arrows in the upper margin of Fig. 2a. It is evident that the activation of d-lactate dehydrogenase is a function of micelle formation by the amphiphiles and that no activation is observable when micelles are not formed. This is particularly seen in the case of the C<sub>14</sub> analog where no micelles are formed up a concentration of 10<sup>-5</sup> M and no activation is observed up to this concentration. It may therefore be concluded that the activated conformation of the d-lactate dehydrogenase is obtained only when the protein interacts with deoxylyso-PC micelles and not with monomers. As seen in Fig. 2b, the chain length of the deoxylyso-PC analogs also plays a role in the activation process. The C<sub>14</sub> analog is apparently the most potent activator of the enzyme in this series of compounds.

In order to evaluate the role of the hydrophilic head group of the amphiphile in the activation process, we compared the activation of d-lactate dehydrogenase by deoxylyso-PE, deoxylyso-N-methyl-PE, deoxylyso-N,N-dimethyl-PE, and deoxylyso-N,N,N-trimethyl-PE (i.e., deoxylyso-PC) all of which had the same (C<sub>16</sub>) acyl chain. In Fig. 3a we show the activation of d-lactate dehydrogenase by these compounds. Interestingly, the activation of the enzyme is also dependent upon the nature of the hydrophilic head groups of these micelle-forming deoxylysophospholipids. In Fig. 3b the enzyme activities in presence of saturating concentrations (i.e. optimal enzyme activity) of these lipid analogs is compared. The ability of the amphiphiles to activate the enzyme increases in the order deoxylyso-PE < deoxylyso-N-methyl-PE < deoxylyso-N,N-dimethyl-PE < deoxylyso-PC. Increasing the number of methyl groups in the hydrophilic head groups of these amphiphiles increases the size of the head group relative to the hydrophobic portion and could conceivably influence the monomer packing within the micelle. A possible explanation for the differences seen in activating ability of these analogs may be the influence of steric considerations in the head group region of the lysophospholipid micelles.

Activation of the Plasma Membrane-bound d-Lactate Dehydrogenase of E. coli by Bilayer-forming Diacyl Phospholipids—It is of interest to know whether and to what extent the d-lactate dehydrogenase is activated by the phospholipids commonly found in biological membranes. We have examined three groups of phospholipids, namely, the phosphatidylcholine lines, the phosphatidylethanolamines, and negatively charged phospholipids (phosphatidylglycerol, phosphatidylycerine, and methylphosphatidic acid). Phosphatidylcholines are not-
Lipid Dependence of d-Lactate Dehydrogenase

Fig. 2. Activation of d-lactate dehydrogenase by deoxylyso phosphatidylcholines. a, activation of d-lactate dehydrogenase by deoxylyso-PC analogs with varying acyl groups. The number of carbon atoms in the acyl groups of the compounds is indicated by numbers in the right margin of the figure beside the respective arrows. The numbers and arrows indicate the critical micelle concentrations of the different analogs. For assay, the deoxylyso-PC at the desired concentration and the d-lactate dehydrogenase were preincubated at 25 °C for 30 min prior to assay at 25 °C as described under “Materials and Methods.” For the sake of clarity the experimental points have been omitted from the figure. b, relative maximal activation of d-lactate dehydrogenase by deoxylyso-PC analogs as a function of the number of carbon atoms, n, in the acyl group.

Fig. 3. Activation of d-lactate dehydrogenase by deoxylyso phosphatidylethanolamines. a, activation of d-lactate dehydrogenase by deoxylyso-PE derivatives having varying degree of N-methylation in the head group. The acyl group was palmitate in all cases. The numbers in the right margin indicate the number of N-methyl groups. The arrows and numbers in the upper margin show the critical micelle concentrations of the different analogs. The deoxylyso lipid at the desired concentration and the d-lactate dehydrogenase were preincubated at 25 °C for 30 min prior to assay at 25 °C as described under “Materials and Methods.” b, maximal relative activation of d-lactate dehydrogenase by deoxylyso-PE analogs as a function of degree of N-methylation.

Naturally found in the plasma membranes of E. coli whose main phospholipid components are the phosphatidylethanolamines and smaller quantities of phosphatidylglycerol and phosphatidylserine (27). However, the phosphatidylethanolamines are the best studied bilayer-forming phospholipids and their influence upon the activity of d-lactate dehydrogenase could be studied usefully in order to evaluate the effects of the lipid physical state upon enzymatic activity. In Fig. 4 we show the activity of d-lactate dehydrogenase in the presence of three synthetic phosphatidylethanolamines. In the series DMPC, DPPC, and DSPC, it is evident that DSPC is the best activator of the enzyme. DPPC is a poorer activator than DSPC but it is a better activator than DMPC. Since the order-disordered phase transition temperature of DMPC occurs at a convenient temperature (24 °C) with respect to the thermal stability of proteins, we have examined the influence of lipid “fluidity” upon the activation of d-lactate dehydrogenase by DMPC. As seen in Fig. 4, a slightly higher activation of the enzyme results when the protein and lipid are incubated together at a temperature which is higher than the lipid phase transition temperature. At least two explanations may be invoked to explain this observation. First, kinetics of incorporation of the protein into the bilayer may play a role. It has been shown (28) that amphiphilic proteins may be incorporated into lipid bilayers at a faster rate when the lipid is above its phase transition temperature. However, in the case of d-lactate dehydrogenase we were able to show that prolonged incubation of protein with lipid at a temperature below the lipid phase transition temperature gave us an activation curve which was identical with that seen in Fig. 4 (with incubation for 30 min). The kinetics of protein incorporation into the bilayer is therefore probably not responsible for the difference in activation seen upon incubation of protein with ordered and fluid lipid. The alternative explanation we offer is that while the protein associates with the lipid in both cases, incorporation of the protein into the bilayer when the lipid is in the ordered state may not result in the optimal conformation of the protein and its lipid environment that lead to an optimal activation of the enzyme.

The activation of d-lactate dehydrogenase by several phosphatidylethanolamines is shown in Fig. 5. The ethanolamines are, in general, not as good activators of this enzyme as the phosphatidylcholines. Maximal activation of d-lactate dehydrogenase by this series of phospholipids was seen to occur when the protein was incubated with egg-PE and the synthetic POPE. Here again, we examined the influence of the lipid physical state upon the activation of the enzyme. POPE has a broad phase transition occurring between 19 °C and 33 °C. Incubation of the protein and lipid at 17 °C prior to assay at 17 °C results in a considerably lower activation of the enzyme as compared with incubation of the protein and lipid at 35 °C prior to assay at 17 °C. Again, this may suggest that a “better”
protein-lipid complex is formed when the protein is incorporated into the lipid bilayer when the latter is in its fluid state. Contrary to what was seen in the case of activation by the phosphatidylcholines, among the phosphatidylethanolamines having saturated acyl chains, dimyristoylphosphatidylethanolamine was the only one capable of activating the enzyme and, even in this case, the activation was relatively weak. A phosphatidylethanolamine extracted from the plasma membrane of an *E. coli* fatty acid auxotroph, grown on a palmitic and elaidic acid supplement, and shown to be predominantly PEPE showed no ability to activate the delipidated D-lactate dehydrogenase regardless of whether the protein and lipid were incubated together below or above the phase transition temperature of this lipid. Comparison of the activation curves seen with POPE and PEPE strengthens our hypothesis that lipid packing may be an important factor in the activation of D-lactate dehydrogenase. It is to be expected that the bilayer formed by the lipid having the trans-unsaturated acyl chain (PEPE) is more tightly packed than the bilayer formed from the lipid having the cis-unsaturated acyl chain (POPE). Another possibility is that either one or both of these phosphatidylethanolamines may form hexagonal phases (29). In case different phases are formed by the two lipids, the direct comparison of D-lactate dehydrogenase may not be justified. The phase behavior of POPE and PEPE as a function of temperature merits study.

In order to further investigate the influence of the lipid polar head group upon the activation of D-lactate dehydrogenase, we examined the activation of this enzyme by some negatively charged phospholipids. In this series, we have used DMPG and DMPS as prototypes of the charged lipids compared with the neutral phospholipids. A, the presence of 10⁻⁵ M Triton X-100; B, in the presence of 10⁻⁵ M DMPC; C, in the presence of 10⁻⁵ M DMPG; and D, in the presence of 10⁻⁴ M DMPS. The buffers used were (©) sodium citrate; (©) sodium phosphate; (©) Tris-hydrochloride; (©) ammediol-hydrochloride. All buffers were at a concentration of 0.1 M. The activating amphiphiles and D-lactate dehydrogenase were preincubated at 20 °C for 30 min prior to assay at 20 °C as described under "Materials and Methods."
Phospholipids is almost twice as high as the maximal activation by a non-naturally occurring model for a negatively charged phospholipid. The results are shown in Fig. 6. DMPG and DMPS are seen to be the best activators in this group. In fact, the activation of D-lactate dehydrogenase by these two phospholipids is almost twice as high as the maximal activation by the neutral phospholipids. Varying the buffer salt concentration in the assay mixtures from 0.01 to 0.1 M had no influence upon activation. Electrostatic effects may therefore be ruled out in the activation of D-lactate dehydrogenase by DMPG and DMPS. Furthermore, it is evident that simple surface charge effects are not responsible for the activation seen here since DMMPA which has the same net charge as DMPG does not show the same activation properties. Evidently some specificity for phosphatidylglycerol and/or phosphatidylserine is embodied in the structure of this enzyme and this specificity is dependent upon the nature of the head group of these lipids.

**pH Dependence of D-Lactate Dehydrogenase in “Reconstituted” Systems**—In Fig. 7, we compare the pH dependence of D-lactate dehydrogenase activity in the presence of Triton X-100 and three “activating” phospholipids. In all cases, the detergent or lipid was at a concentration where activation had been previously shown to be maximal (10⁻⁴ M for Triton X-100 and 10⁻⁴ M for the lipids). Maximal activity was, in all cases, between pH 8.5 and 9.5. It is interesting to note that the pH of maximum activity seen with DMPC (pH 9.5) is the closest to the pH for maximum activity of D-lactate dehydrogenase in its native membrane (pH = about 10.0) reported by Futai (6). As reported by Futai (6), a buffer salt effect is also observed in these experiments but the cause for this effect remains unclear.

**DISCUSSION**

A systematic investigation of the D-lactate dehydrogenase of the *E. coli* plasma membrane, a phospholipid-requiring enzyme, was undertaken to explore the structural requisites for the reactivation process. In comparison with other systems, for example, the β-hydroxybutyrate dehydrogenase (16, 30, 31), the D-lactate dehydrogenase is not fully inactivated by complete lipid depletion. There is always some residual activity which amounts to about 10 to 20% of the maximal activity seen in the presence of certain phospholipids. As shown by the earlier work of others (8, 9), the addition of native phospholipids to the pure delipidated enzyme enhances enzyme activity. In this study we have used structurally well defined phospholipids of the micelle- and bilayer-forming types.

In the series of deoxyxys-PCs of different chain lengths we were able to compare monomerically dispersed derivatives with micelle-forming ones. There was no indication that deoxyxys-PC below their critical micelle concentrations can enhance the enzyme activity even at very high concentrations. Reactivation was found to depend strongly on the formation of micelles which may indicate the necessity for the formation of an apolar matrix for the insertion of the protein. This is one major result of this study. In this context, it has also been observed by Robinson and Tanford (32) that the binding of detergents and phospholipids to integral membrane proteins occurs in a highly co-operative manner at concentrations near the critical micelle concentration.

Above the critical micelle concentration in the series of deoxyxys-PCs, a pronounced chain length dependence with maximal activation by the myristoyl derivative was observed. One reason for this optimum may be a minimal chain length requirement of the enzyme which makes the myristoyl derivative a better activator than the shorter chain derivatives. Increasing the chain length above myristoyl lowers the activation of the D-lactate dehydrogenase by these compounds. This could be due to formation of “tighter” micelles by the longer chain analogs. This is in fact indicated by the behavior of the deoxyxys-PCs at the air-water interface (33). In such studies, the slope of the force-area curve gives information on the packing density of the acyl chains of these compounds. For 1-stearoyl-, 1-palmitoyl-, and 1-myristoyldeoxyxys-PC biolayer of 12, 0.4, and 0.2 dynes/cm²/molecule, respectively, were observed in the force-area curves. This indicates large differences in the packing densities of these compounds in monolayers and similar differences may be expected to exist in micelles as well. In addition, micellar shape and size considerations may also play a role in the activation process (34).

The reactivation by the deoxyxysolipids also depends upon the degree of N-methylation of the hydrophobic portion of these molecules. Among the derivatives with a palmitoyl chain, the phosphatidylcholine was found to be the best activator and the phosphatidylethanolamine was unable to activate the enzyme. In fact, an almost linear decrease in ability to activate the D-lactate dehydrogenase was observed with decreasing N-methylation. This effect may also be explained by a greater packing density of the acyl chains with decreasing surface requirement of the polar region in this series of compounds. The surface requirement of 1-palmitoyldeoxyxys-PC (50 Å²) is much larger than that for 1-palmitoyldeoxyxys-PE (35 Å²) in monolayer studies. The difference is clearly the result of the surface requirements of the respective polar portions since the apolar region was identical in all cases.

An extension of the discussion to the bilayer-forming phospholipids such as the phosphatidylcholines and phosphatidylethanolamines also shows differences in the reactivation properties of these two classes of phospholipids. As shown with DMPC, the phase transition temperature may play a small role but is not a critical factor in the reactivation process. The enzyme is reactivated by this lipid in the ordered as well as fluid phases of this lipid. In the case of the phosphatidylcholines the surface requirement for the head group (50 Å²/molecule) is considerably larger than that for the apolar portion (42 Å²/molecule), whereas in the case of the phosphatidylethanolamines, the surface requirement of the apolar portion is greater than that of the polar portion. It may thus be expected that the packing of the acyl chains in bilayers of phosphatidylethanolamines is tighter than the packing of acyl chains in bilayers of phosphatidylcholines. In keeping with this, the phosphatidylcholines are better activators than the phosphatidylethanolamines. The relatively weak activation seen with egg-PE and POPE may be due to a nonlamellar phase structure of these phospholipids under conditions in which the enzyme assays were done (29). It is remarkable that a phosphatidylethanolamine isolated from the membrane of an *E. coli* fatty acid auxotroph does not activate the D-lactate dehydrogenase. This may indicate that in the native state the microenvironment of this enzyme is devoid of phosphatidylethanolamines despite the fact that this lipid constitutes more than 80% of the total lipid content of the membrane. In addition to the phosphatidylethanolamines, smaller amounts of negatively charged phospholipids are also found in the native membrane. We therefore included several negatively charged phospholipids in the reactivation studies. It is clear from the results that these phospholipids are strong activators of the enzyme. Negative charge alone, however, does not explain the potency of these lipids as activators since the negatively charged DMMPA is a relatively poor activator of the enzyme in comparison with DMPG and DMPS. Some

3 A. Nickisch and H. Eibl, manuscript in preparation.
specificity for the polar region of these lipids therefore would seem to exist. In view of the fact that the phosphatidylethanolamines, which are the major lipids of the plasma membrane of E. coli, are poor activators of the d-lactate dehydrogenase, whereas phosphatidylglycerol and phosphatidylserine, which are minor constituents, are strong activators, it seems reasonable to suggest that the negatively charged lipids form the microenvironment of the enzyme in its native state. Further support for this suggestion comes from the pH versus activity profiles for this enzyme in its native state (6) and in bilayers of DMPC and DMPS (see Fig. 7). In consideration of membrane electrostatics (35), a negatively charged microenvironment may be expected to shift the apparent optimal pH to higher values. This is indeed seen to be the case in the native membrane and in bilayers of DMPC and DMPS.

In summary, the activation of d-lactate dehydrogenase is dependent upon the availability of an appropriate lipid matrix since only lipids above their critical micelle concentration are able to activate the enzyme. The best phospholipid activators were found to be phosphatidylglycerol and phosphatidylserine. Phosphatidylethanolamines, which constitute the bulk of the E. coli membrane were shown to be poor activators, if at all, for this enzyme. A comparison of the pH optimum of the enzyme in its native membrane and the pH optima in the presence of DMPC and DMPS suggests that in the native membrane the d-lactate dehydrogenase has a preference for domains which are rich in phosphatidylglycerol and/or phosphatidylserine.

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