Activation of \( C_{55} \)-Isoprenoid Alcohol Phosphokinase from Staphylococcus aureus

I. ACTIVATION BY PHOSPHOLIPIDS AND FATTY ACIDS

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The activation of \( C_{55} \)-isoprenoid alcohol phosphokinase by a variety of lipids has been investigated. A number of amphipathic lipids can serve as effective kinase activators. Both the nature of the polar and nonpolar groups are important, but kinase activation does not depend on any particular chemical structure or charge on the lipid.

The structure of those lipids which are most effective, as well as an analysis of their temperature profiles, suggests that bulk physical properties are significant. Lipids which provide a hydrated, loosely packed, highly fluid environment are often effective activators.

\( C_{55} \)-Isoprenoid alcohol phosphokinase is one of a few intrinsic membrane proteins which have been purified to homogeneity (1, 2). This enzyme, isolated for Staphylococcus aureus H membranes, catalyzes the ATP-dependent phosphorylation of \( C_{55} \)-isoprenoid alcohols. The properties of the enzyme are those one might expect of an intrinsic membrane protein. It has an unusually high content of nonpolar amino acids (58%): it is insoluble in water, but soluble in organic solvents such as butanol-1. One of its substrates is highly lipophilic, and the enzyme activity is absolutely dependent on the presence of a lipid activator. The exceptional stability of this enzyme both in the presence and absence of lipids makes this system attractive for studying lipid-protein interactions.

Earlier work from this laboratory (3) at first indicated that phosphatidylglycerol and cardiolipin were unique in their ability to stimulate \( C_{55} \)-isoprenoid alcohol phosphokinase (which will be referred to hereafter as kinase). Later it was shown that under the appropriate conditions, egg lecithin and the neutral detergent, Span 20, were also efficient activators (1). These studies are difficult to interpret since detergents such as Triton X-100 and sodium deoxycholate were included in the assay mixtures. In the present work the activation of the kinase by a variety of amphipathic lipids in the absence of detergents has been studied with a view to defining those chemical or physical properties responsible for successful kinase activation.

MATERIALS AND METHODS

\( \gamma \)-Labeled \( \text{[^{32}P]ATP and [^{14}C]ADP were purchased from New England Nuclear. Analytical thin layer chromatography was performed on precoated Silica Gel G plates (E. Merck), preparative thin layer chromatography was used in the preparation of the ficaprenol and bactoprenol substrate was performed on plates prepared with Silica Gel H (E. Merck). All solvents were Fisher ACS grade and were used without further purification with the exception of tetrahydrofuran (used in the enzyme preparation) which was redistilled prior to use. ANS was purchased from Eastman.

Both ficaprenol (from Ficus elastica) and bactoprenol (from Staphylococcus aureus H) were isolated and used as substrate (4, 5). The concentration of \( C_{55} \)-isoprenoid alcohol was determined by quantitative conversion to the acetyl ester using \( [^{14}C] \)-labeled acetate (New England Nuclear). Synthetic lecithins, saturated fatty acids, and mono-, di-, and triglycerides were purchased from Supelco; fatty alcohols, methyl esters of fatty acids, and Triton X-100 were purchased from Sigma. Unsaturated fatty acids were purchased from Nu-Chek Prep, and 12-methyltetradecanoic acid (anteisopentadecylic) was from Analabs. The endogenous \( S. aureus \) lipids as well as their fatty acid analysis were kindly provided by Dr. J. A. F. Op den Kamp, Utrecht, Holland. Other polar lipids were purchased from the sources listed in Table I. Stock solutions of the lipids were usually prepared in methanol or chloroform/methanol 1:1 and stored at -15° in the dark.

The concentrations of the phospholipids were determined by total phosphate analysis (6). The purity of all the phospholipids and glycolipids used was checked by thin layer chromatography, iodination (I\(_2\) vapor), ninhydrin, and Phosphor (Supelco) were used to visualize spots. Fatty acid analysis of the commercial lipids was performed on the methyl ester derivatives using a Perkin-Elmer model 9900GC.

Enzyme Preparation—\( S. aureus \) cells were grown and harvested at late log phase and the enzyme (Step 5) was prepared essentially as described previously (2). This material was free of lipid and was estimated to be approximately 75% kinase by sodium dodecyl sulfate gel electrophoresis. A homogeneous kinase preparation can be ob-

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Phospholipid Activation of Purified Membrane Enzyme

### Table I

<table>
<thead>
<tr>
<th>Lipid*</th>
<th>Source</th>
<th>Major fatty acid components (per cent of total)</th>
<th>Relative kinase activity b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyso PE</td>
<td>Analabs</td>
<td>14:0 16:0 16:1 18:0 18:1 18:2 18:3</td>
<td></td>
</tr>
<tr>
<td>Bovine lyso PS</td>
<td>Applied Science</td>
<td>22.4 26.6 41.6 9.3</td>
<td>3.19</td>
</tr>
<tr>
<td>Bovine PC</td>
<td>Analabs</td>
<td>17.9 11.5 26.1 5.8</td>
<td>2.71</td>
</tr>
<tr>
<td>Egg PC</td>
<td>General Biochemicals</td>
<td>9.3 14.8 67.3 14.8</td>
<td>1.15</td>
</tr>
<tr>
<td>Plant PC</td>
<td>Applied Science</td>
<td>4.0 14.8 33.8 7.4</td>
<td>0.75</td>
</tr>
<tr>
<td>Plant PI</td>
<td>Applied Science</td>
<td>2.9 33.7 14.8 33.8</td>
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<tr>
<td>Bovine CLc</td>
<td>Nutritional Biochemicals</td>
<td>8.8 3.9 15.5 71.5</td>
<td>0.23</td>
</tr>
<tr>
<td>Distearoyl-L-α-PE</td>
<td>Supelco</td>
<td>8.8 100</td>
<td>0.21</td>
</tr>
<tr>
<td>Dipalmityl-L-α-PE</td>
<td>Supelco</td>
<td>8.8 100</td>
<td>0.11</td>
</tr>
<tr>
<td>Dodecyl-L-α-PE</td>
<td>Supelco</td>
<td>8.8 100</td>
<td>0.05</td>
</tr>
<tr>
<td>Bacterial PE</td>
<td>Applied Science</td>
<td>15.7 15.7</td>
<td>0.05</td>
</tr>
<tr>
<td>Bovine PS</td>
<td>Supelco</td>
<td>15.7 15.7</td>
<td>0.03</td>
</tr>
<tr>
<td>Digalactosyl diglyceride</td>
<td>Applied Science</td>
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<td>0.10</td>
</tr>
<tr>
<td>Monogalactosyl diglyceride</td>
<td>Applied Science</td>
<td>15.7 15.7</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*The abbreviations used are: PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; CL, cardiolipin; PS, phosphatidylserine; PG, phosphatidylglycerol.

**Standard assay procedure as described in the text. Lipid concentration in all cases was 1 mM. Results presented relative to egg lecithin.

* Fatty acid composition does not include arachidonic acid, 20:4.

* Contaminated with 30% digalactosyl diglyceride.

**Kinase Assay**—The following components were added together in a test tube (3 x 50 mm) and dried in vacuo at room temperature: enzyme (in butanol/methanol 1:1, containing 1 M ammonium acetate), C17-isoprenoid alcohol (in cyclohexane), lipid activator (usually in methanol or chloroform/methanol 1:1). After the solvent was thoroughly removed, 30 μl of a buffer containing 50 mM Tris-HCl and 10 mM MgCl₂ at pH 8.5 were added. This will be referred to as the assay buffer. In some cases 4.7 mM Triton X-100 (0.3%) was also included in this buffer. After dispersing the lipid using a Vortex mixer, the reaction was initiated by adding 1 μl of a 15 mM solution of [γ-³²P]ATP. The entire mixture was then spotted on Whatman No. 3MM paper along with a single washing with chloroform/methanol 2:1. After ascending chromatography in isobutyric acid/1 M ammonium 5/3, the product, C₁₇-isoprenyl phosphate, which runs at the solvent front, was cut out and counted in a toluene-based scintillation fluid. The amount of protein present in the assay was always less than 1 μg. Enzymatic activity under these conditions was linear with time and with enzyme concentration. The assay was generally reproducible to within 15%.

**RESULTS**

**Effect of Concentration of Phospholipids on Kinase Activation**—C₁₇-isoprenoid alcohol phosphokinase can be activated by a number of phospholipids in the absence of detergents (Table I). The concentration dependence of kinase activity with all these phospholipids was very similar. The activation curves for a series of synthetic lecithins is shown in Fig. 1. Similar patterns were obtained with other phospholipids; the presence of 0.3% Triton X-100 in the assay buffer did not greatly alter the concentration dependence.

**Effects of Varying Polar Head Group in Phospholipid**—A series of commercially available phospholipids and glycolipids and the polar lipids isolated from Staphylococcus aureus H were compared in their ability to activate the kinase, all tested at 1 mM concentration. These compounds differed radically from each other in fatty acid content as well as in the identity of the polar group (Table I). Qualitatively, the order of activation by phospholipids at 25°C was as follows: lyso-

![Fig. 1. Kinase activity at 25°C measured as a function of the concentration of diacyl synthetic lecithins. The assay was performed as described in the text.](image-url)
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The addition of 0.3% Triton X-100 or 0.05% sodium deoxycholate (data not shown) resulted in large changes in the ability of many phospholipids to stimulate kinase activity. Most of the phospholipids which were inactive alone showed considerable activity in the presence of the detergents. Neither of these detergents alone can serve as a kinase activator.

Effect of Chain Length of Fatty Acid in Phospholipid—A series of synthetic lecithins was studied to explore the effect of changing the fatty acid moiety. All the lecithins studied contained 2 identical fatty acid residues. The lecithins which showed maximum activation were dicaproyl-L-α-lecithin ($C_{16}$) and dicaproyl-L-α-lecithin ($C_{18}$) (Fig. 2). Fig. 2 also shows that whereas the fully saturated lipid, distearoyl-L-α-lecithin ($C_{18}$), was not an activator at 25° the cis-monounsaturated analog, dioleoyl-L-α-lecithin was a moderately good activator.

The relative ability of the synthetic lecithins to activate the kinase was not changed by either preincubation at higher temperatures or by increasing the assay temperature. Subjecting the assay tubes to a 1-min submersion in an ultrasonic bath similarly caused no changes in this pattern. However, at higher temperatures the long chain length lecithins became kinase activators (Fig. 3). The longer the fatty acid chain length, the lower the minimum temperature required for kinase activation. At temperatures above the minimum for activation, the slopes of the activity curves as a function of temperature were similar.

This type of behavior suggested that a phospholipid mesomorphic transition (8) might be responsible for activation at higher temperatures. However, the minimum temperatures for activation did not correspond to the known transition temperatures for the various phospholipids. Similarly, in a previous study, no distinct inflections corresponding to the expected lipid transitions were seen in Arrhenius plots for the activation of kinase by several synthetic phospholipids ($C_{16}$ and $C_{18}$) (9). However, the lipid phase present in the assay tube included about 20% C$_{55}$-isoprenoid alcohol in order to obtain substrate saturation. This long chain alcohol had a considerable effect on the phase behavior of synthetic lecithins (Fig. 4). The fluorescent probe, ANS, was used to follow changes in the lipid phase as a function of temperature (10), and on addition of C$_{55}$-isoprenoid alcohol a broadening and diminution of the magnitude of the lipid transition, and a lowering of the transition temperature of dipalmitoyl-L-α-lecithin were observed. Cholesterol, which also perturbs the phospholipid transition (11), in general inhibited the kinase in the presence of good phospholipid activators and by itself, cholesterol did not activate. However, in the presence of dipalmitoyl-L-α-lecithin (a poor activator) there was a slight stimulation of kinase activity at low cholesterol concentrations (data not shown).

Activation by Fatty Acids—Certain fatty acids have been found to be effective kinase activators. The optimum concentrations for activation (5 to 10 mM) were greater than those found for phospholipids. These amounts of fatty acids were well above their monomer solubilities. These solutions were heterogeneous with a separate lipid phase partially dispersed and partially coating the glass walls of the assay tube. The maximum activity obtained usually was of the same magnitude as for the egg lecithin-activated enzyme.

Much of the data comparing the relative abilities of different fatty acids to serve as kinase activators is summarized in Table II. None of the straight chain saturated fatty acids which were examined (chain lengths 8 to 18) were capable of kinase activation. The order of activation of dicaproyl-L-α-lecithin ($C_{16}$) and dicaproyl-L-α-lecithin ($C_{18}$) was subject to variation. The arrows indicate conditions where the kinase activities are the same. This will be referred to in a subsequent paper (7).
Fig. 4. Lipid mesomorphic transition of dipalmitoyl-\(\alpha\)-lecithin in the presence and absence of C\(_{15}\) isoprenoid alcohol followed by ANS fluorescence. The ANS concentration was 2 \times 10^{-4} \text{M}. The excitation wavelength was 380 nm and the emission was recorded at 480 nm using a Hitachi Perkin-Elmer model MPP-3 Fluorimeter. The lipids were mixed together in organic solvents, dried in \textit{vacuo}, and dispersed at 55\(^\circ\)C in the kinase assay buffer. The samples were cooled to room temperature prior to the addition of ANS. The data shown were collected as the samples were being continuously heated at about 1 deg/min. After standing for 15 min at 45\(^\circ\)C the sample containing the C\(_{15}\)-isoprenoid alcohol showed a further increase in ANS fluorescence to about 80 units. Upon recouling the sample to 25\(^\circ\)C, the fluorescence remained at this high level. In the absence of the long chain alcohol the curve obtained above was reversible and showed little hysteresis. PC, phosphatidylycholine.

activation. The branched chain fatty acid, 12-methyltetradecanoic acid, however, was a relatively good activator. Table II also compares a series of C\(_{14}\) fatty acids. Whereas the saturated compound (stearic acid) and the \textit{trans}-monounsaturated fatty acids were completely incapable of kinase activation, the \textit{cis}-monounsaturated fatty acids were good activators. The position of the \textit{cis} double bond along the chain is apparently of little importance as all three positional isomers which were tested were equally good activators. Preincubation at 37\(^\circ\)C or 55\(^\circ\)C prior to assaying at 25\(^\circ\)C had no effect on the kinase activity. A brief sonication of the reaction mixture prior to assay also had no effect. Many fatty acids which were poor activators by themselves became good activators in the presence of Triton X-100.

Numerous other lipids were ineffective as kinase activators under a variety of conditions, including fatty alcohols, methyl esters of fatty acids, and mono-, di-, and triglycerides (Table II).

\textbf{Solubilization of Kinase in Assay}—One possible explanation for the ability of Triton X-100 to stimulate kinase activity when used in conjunction with other lipids is that the detergent may be aiding in dispersing the kinase, or the C\(_{15}\)-isoprenoid alcohol, or both, into solution and off the walls of the assay tube. This phenomenon will be referred to as solubilization. However, solubilization did not correlate at all with enzyme activity. Triton X-100 together with ultrasonic treatment solubilized the kinase along with the lipid substrate without any resultant activity. However, enzyme activity was fully restored when this solution was swirled over dried egg lecithin. It is demonstrated in an accompanying paper (7) that the enzyme which has been "solubilized" in this manner is in a highly aggregated form.

In the absence of Triton X-100, as much as two-thirds of the lecithin-activated kinase activity was shown to be associated with the glass wall of the assay tube. Preincubation at high temperature increased the fraction of activity dispersed in solution and also frequently increased the total activity. The use of silanized glass tubes, glass tubes presoaked in aqueous sodium hydroxide, or several varieties of plastic tubes did not reduce the amount of surface-associated kinase activity.

\textbf{Failure to Find Evidence for Phosphoenzyme Intermediate}—No evidence for an ATP-ADP exchange reaction using a variety of lipid activators either in the presence or absence of substrate could be found. Only after the product, C\(_{15}\)-isoprenyl phosphate, had been produced was there any noticeable conversion of \textit{\textsuperscript{14}}C-ADP to \textit{\textsuperscript{14}}C-ATP, probably due to a back reaction. As expected, there was also no P\(_{i}\)-ATP exchange.

A direct attempt to trap a possible covalent phosphoprotein intermediate was made. Enzyme (200 \mu\text{g}) and 15 nmol of [\gamma-\textit{\textsuperscript{32}}P]ATP (about 10\(^7\) cpm) were incubated under assay conditions in the presence of egg lecithin for 15 min at 37\(^\circ\)C. An excess of sodium dodecyl sulfate was added, and the enzyme extracted into butanol 1. After washing out excess ATP with water, the butanol-1 was removed and the protein redissolved in sodium dodecyl sulfate. Sodium dodecyl sulfate gels were run, sliced, and counted. No \textit{\textsuperscript{32}}P was present in the kinase.

\textbf{Discussion}—By characterizing the assay system and examining those lipids which are effective \textit{in vitro} one may arrive at some understanding of how the kinase is functioning and hopefully of the physical and chemical requirements of the bacterial membrane for a properly functioning kinase \textit{in vivo}. Because of the nature of the protein and its substrates, the assay system is necessarily complex. There are at least two phases present, and the enzyme may operate at the water-lipid interface. One

\begin{table}[h]
\centering
\begin{tabular}{|c|c|}
\hline
\textbf{Fatty acid} & \textbf{Kinase activity} \\
\hline
\textit{Saturated} & \\
Stearic acid (18:0) & 300 \\
Pentadecanoic acid (15:0) & 400 \\
\hline
\textit{Branched} & \\
12-Methyltetradecanoic acid (15:br) & 4600 \\
\hline
\textit{Monounsaturated} (all 18:1) & \\
Palmitoleic (cis 6) & 9000 \\
Petroselaidic (trans 6) & 300 \\
Oleic (cis 9) & 6400 \\
Elaidic (trans 9) & 400 \\
cis-vaccenic (cis 11) & 6000 \\
trans-vaccenic (trans 11) & 300 \\
\hline
\end{tabular}
\caption{Kinase activity at 25\(^\circ\)C in presence of fatty acids}
\end{table}
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The lecithins containing short, saturated fatty acids or an unsaturated fatty acid were preferred for kinase activation. Those lecithins containing these groups would be expected to have the most fluid hydrocarbon regions and be the most loosely packed. They have the lowest \( \beta \rightarrow \alpha \) liquid crystal transition temperatures (16). The temperature dependence of kinase activation by synthetic lecithins (Fig. 3) is also consistent with lipid fluidity being a critical parameter. The onset of kinase activation at successively higher temperatures by lecithins containing longer fatty acid moieties is very suggestive of a chain-melting phenomenon. The minimum temperature for activation does not correspond to the well known transition temperatures for the various synthetic lecithins, probably due to the perturbing effect of the \( \text{C}_{33}\)-isoprenoid alcohol. Large amounts of this lipid substrate were used to assure saturation. Fatty alcohols, esters of fatty acids, glycerides, and phospholipids in the crystalline phase were all poor activators and do not interact well with water (14).

The cis-monounsaturated \( \text{C}_{18} \) fatty acids were all good kinase activators in contrast to the fully saturated and the trans-monounsaturated \( \text{C}_{18} \) fatty acids, all of which were poor activators. This again follows the order expected if loose lipid packing is related to activation. It is much more difficult to pack cis-unsaturated fatty acids than trans-unsaturated fatty acids. This packing phenomenon has dramatic results in the bulk physical properties of many lipids. It will affect the liquid crystal transition temperatures of phospholipids containing these fatty acids as well as the bulk melting points of the fatty acids themselves. The presence of two or three double bonds, either cis or trans, or a branched hydrocarbon chain also opens up the structure of the lipid aggregate and destabilizes the tightly packed, highly ordered form.

The effect of Triton X-100 and sodium deoxycholate on phospholipid-activated kinase can be understood partially in the context of the previous discussion. Triton X-100 intercalates in the phospholipid bilayer and can be expected to loosen the structure of a tightly packed phospholipid (17). At high ratios of detergent to phospholipid, the Triton may remove both the phospholipid and alcohol substrate from the enzyme, resulting in lower activity.

How do the physical properties required for successful lipid activators specifically relate to the functioning kinase? The two major alternatives are that the physical properties affect (a) the kinetics for formation of an active protein lipid complex, or (b) the properties of the enzyme and substrate in this complex. However, the ability of a lipid to solubilize simultaneously both the substrate and enzyme is neither a necessary nor sufficient criterion for activation. Triton X-100 will form such a complex and yet is not a kinase activator. An understanding of the lipid activation of the kinase is best approached by considering the behavior of the enzyme and substrate within the matrix provided by the activator. This will be explored more fully in an accompanying paper (7).

The concept that lipid physical properties are critical to the functioning of intrinsic membrane enzymes is not new. Several membrane functions have been studied in membrane or lipoprotein preparations in which the lipids exhibit a thermotropic phase transition, and the temperature profiles of these enzyme activities (typically exhibited as an Arrhenius plot) frequently reflect the differences in the lipid physical properties above and below the transition temperature. Typical examples are the sodium and potassium ion-activated ATPase (18), succinate dehydrogenase (19), proline transport (19), \( \beta \)-galactoside and \( \beta \)-glucoside transport (20, 21), and some enzymes related to phospholipid synthesis (22). The addition of phospholipids to delipidated membrane enzyme preparations (23, 24), and phospholipid titrations with membrane enzyme preparations (25, 26) have also indicated in some instances a dependence of enzymatic activity on the physical state of the lipid activator. In addition, some microorganisms have altered
composition of membrane lipids at different growth temperatures with the effect of maintaining a fluid membrane state (27). Obviously, one explanation for this is the necessity of a fluid membrane for many of the membrane-associated enzyme activities. Perhaps membrane lipids are segregated into areas of differing viscosity (lateral phase separation) (28). If the S. aureus membrane is segregated in this manner then clearly the kinase must be located in a region of appropriate fluidity. The manner in which the lipid physical properties may affect the kinase activity will be explored in an accompanying paper (7).

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