ROLE OF LIPID IN THE FORMATION AND FUNCTION OF THE RESPIRATORY SYSTEM OF *STAPHYLOCOCCUS AUREUS*

Paul E. Goldenbaum and David C. White

Department of Biological Sciences
Florida State University
Tallahassee, Florida 32306

Anaerobically growing *Staphylococcus aureus,* when aerated vigorously, forms a membrane-bound electron transport system that consists of primary dehydrogenases, cytochromes, and cytochrome oxidases. Concomitant with this, there are changes in the composition of the membrane lipids, with increases in glucolipids, vitamin K_2 isoprenologues, and phospholipids. The inhibitors benzo(a)pyrene or piperonyl butoxide blocked both the lipid changes and the formation of the electron transport system, suggesting that the lipid changes are an obligatory part of the process. Further evidence of the involvement of lipids in the formation of the respiratory system comes from the study of mutants. A glycerol auxotroph, when deprived of glycerol, stops net phospholipid biosynthesis and forms a defective cytochrome system. Depriving a menadione mutant also forms a defective respiratory system. Present activity is directed toward isolating fractions of the membranes in which the lipid and respiratory pigment changes are occurring.

Staphylococcus aureus is an ideal organism in which to study the formation of the membrane-bound electron transport system, since it can grow glycolitically in the absence of oxygen if glucose is present, or can utilize a respiratory system if it is aerated.[1–3] If exponentially growing cells are incubated in the absence of air but the presence of glucose, no detectable respiratory system is formed.[3] If such a culture is aerated, a functional respiratory system is formed (FIGURE 1). This involves the synthesis of the primary dehydrogenases, the cytochromes, and cytochrome oxidases, which are readily detectable by difference spectroscopy.[3] Since the organism is also capable of efficient oxidative phosphorylation (P. Keyser and D. C. White; unpublished data) and active transport of amino acids,[4, 5] the necessary coupling factors are also present in the membrane. Under the conditions of growth utilized in these experiments a cytochrome system with ratios of pigments consistent with the environment of growth is fully active within about 1.5 hours after the onset of aeration.

The electron transport system consists of the pigments that form a multienzyme complex, which must have structural integrity to function. Disruption of their spatial relationships by organic solvents interrupts electron transport. The fact that absorbance changes on reduction of the pigments are a measure of function, and are readily measured in living cells, makes the function of the electron transport system a convenient multienzyme-membrane complex to study.

In addition to the respiratory system, the staphylococcal membrane also contains lipids. Methods for the quantitative assay of the phospholipids,[6, 7] fatty acids,[8] carotenoids,[9] and vitamin K_2 isoprenologues[10] of the *S. aureus* membrane were developed. With these methods in hand it was possible to

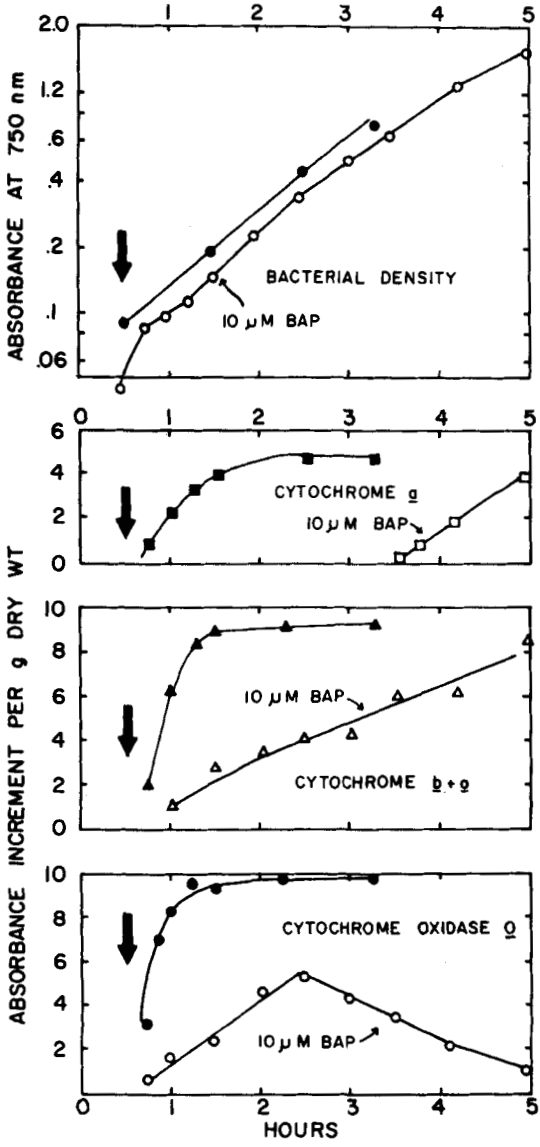

FIGURE 1. The effect of BAP on the synthesis of the electron transport system in anaerobically growing *S. aureus*. Cultures were aerated at the time indicated by the arrow in the presence (open symbols) or absence (closed symbols) of 10 μM BAP. *Cytochromes* were measured by difference spectra and the enzymatic reducibility was measured by the reduction in the presence of 20 mM L-lactate, compared to pigments reduced chemically by the addition of $Na_2S_2O_4$.

show that the formation of the respiratory pigments occurred concurrently with an increase in total phospholipids, a change in the ratio of cardiolipin (CL) to phosphatidyl glycerol (PG), and increases in vitamin K_2 isoprenologues, glucolipids, and carotenoids, especially the xanthophylls.[3, 11] These lipid changes are illustrated in FIGURES 2 and 3. Changes in both the composition and metabolism of the membrane phospholipids have also been detected when the composition of the membrane-bound electron transport system was changed in *Haemophilus parainfluenzae*.[12]

Were these changes in membrane lipid composition and metabolism an integral part of the formation of the electron transport system, or were these two processes just occurring simultaneously? In the course of other investigations, it was found that the environmental carcinogen benzo(a)pyrene (BAP) and the mixed function oxidase inhibitor piperonyl butoxide (PB), when added to *S. aureus* at the time of the switch from anaerobic to aerobic growth, showed marked effects on the synthesis of phospholipids and polar carotenoids. The effect of BAP on the lipid changes associated with the switch to aerobic growth is illustrated in FIGURES 2 and 3. The effects of PB are similar.

Since these two lipophilic inhibitors produced changes in the composition of the membrane lipids, the effects on the formation and function of the electron transport system were investigated. In the presence of 10 μM BAP, the formation of functional cytochromes *a* and *b* to steady-state levels took almost three times longer than in the control (FIGURE 4). With the inhibitor present, not only was the cytochrome oxidase formed more slowly, but only half as much was formed. Once formed, the cytochrome oxidase lost enzymatic reducibility (FIGURE 4). It remained in the membrane, as it could be detected after reduction by dithionite as the reduced carbon monoxide complex. Functional cytochrome oxidase can be reduced in the presence of L-lactate, which requires the function of the intact respiratory chain.[3] Comparison of the difference spectra of the cells grown with and without BAP shows the vast difference in the proportions of the functional respiratory pigments (FIGURE 4).

The facts that the inhibitors PB and BAP (1) produced changes in the metabolism of the membrane lipids and (2) led to defective formation of the electron transport system suggested that the lipid changes are an integral part of the formation of the respiratory system.

A second approach to the problem of the interrelationship between the function of the lipids and the electron transport system came from studies with mutants. It is possible to select mutants of *S. aureus* that are unable to form a functional electron transport system.[13] With this in mind, a mutant unable to form or utilize glycerol was isolated and generously provided by Dr. L. Mindich (Public Health Laboratories of New York City). We have been able to confirm and extend Dr. Mindich's elegant work with this mutant.[14–16]

Essentially, when the organism is deprived of glycerol, growth and DNA and protein synthesis slowly stop. RNA synthesis is slowed more rapidly. On the addition of glycerol, growth starts, after a short lag at the predeprivation rate. There is essentially no loss in viability if the deprivation is terminated within 90 min.

The lipid changes during deprivation are complex, however. There is an abrupt and immediate cessation of net phospholipid synthesis. Acetate continues to be incorporated into free fatty acids, which are longer (19–20 carbon atoms) than those formed normally (15–16 carbon atoms). These fatty acids are never esterified to the complex lipids, and a system for their catabolism is

induced. There is no turnover of fatty acids in the complex lipids or exchange with these in the free fatty acid pool. The loss of the nonacetylated glycerol of the PG continues at the predeprivation rate. During deprivation, the glycerol

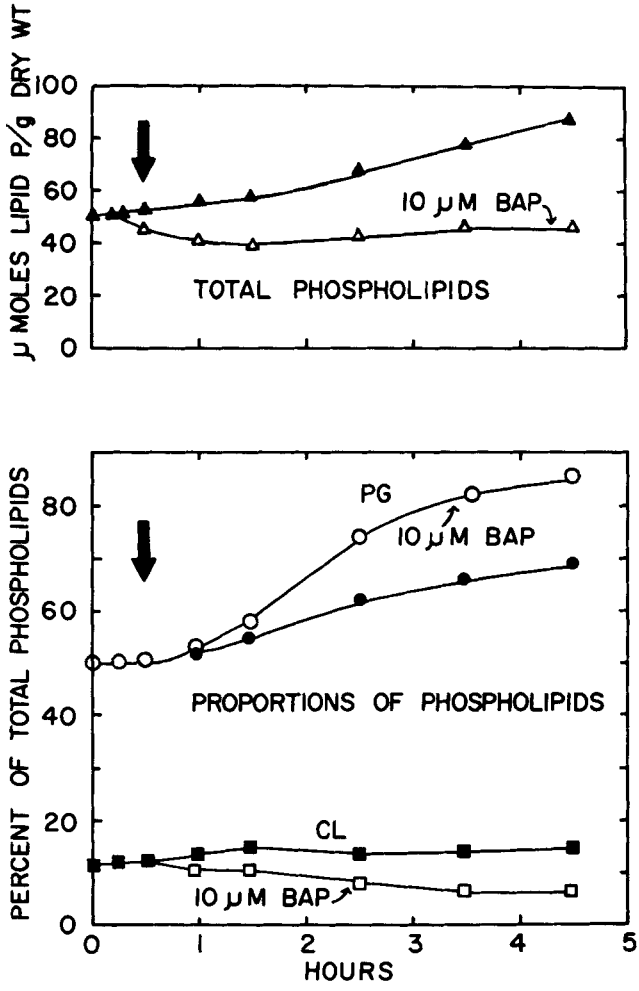

FIGURE 2. Effect of 10 μM BAP on the synthesis of phospholipids during the switch from anaerobic to aerobic growth. Aeration is indicated by the arrow. The total amount (upper graph) and proportions of phosphatidylglycerol (PG) or cardiolipin (CL, lower graph) in the control (open symbols) or with BAP (closed symobls) are indicated.

released from PG was quantitatively utilized to form the lysyl ester of phosphatidyl glycerol (LPG).[18] The total phospholipid content remained constant, but the proportions of LPG increased rapidly and the PG fell concomitantly. The CL remained constant. There was a marked slowing of the synthesis of the

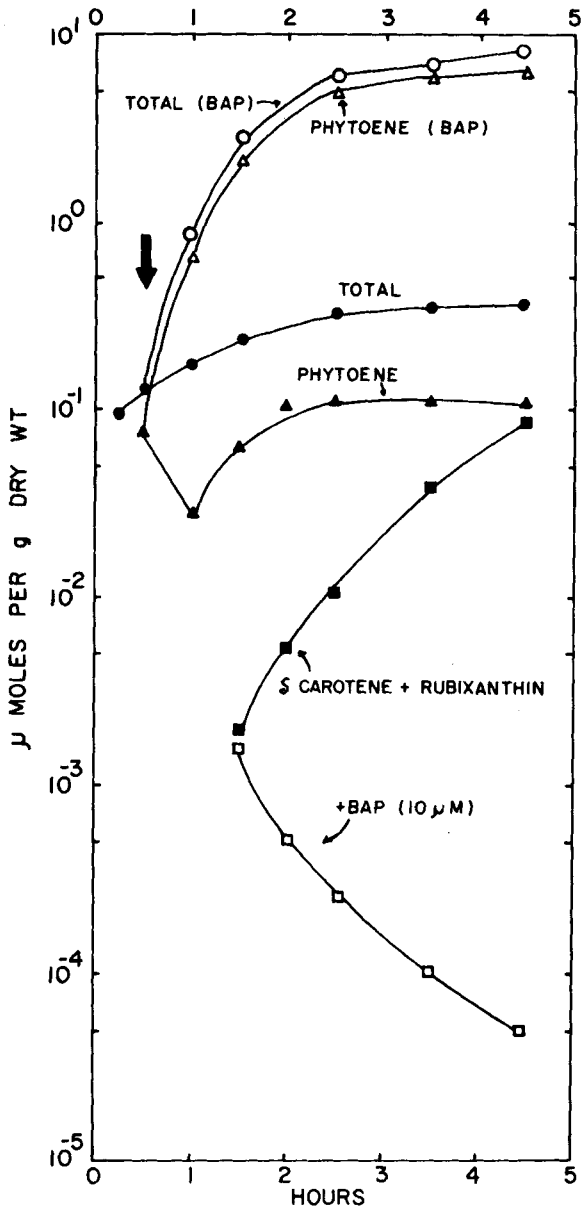

FIGURE 3. The effect of BAP on the synthesis of polar carotenoids in *S. aureus* during the switch from anaerobic to aerobic growth. Symbols are as in FIGURE 1.

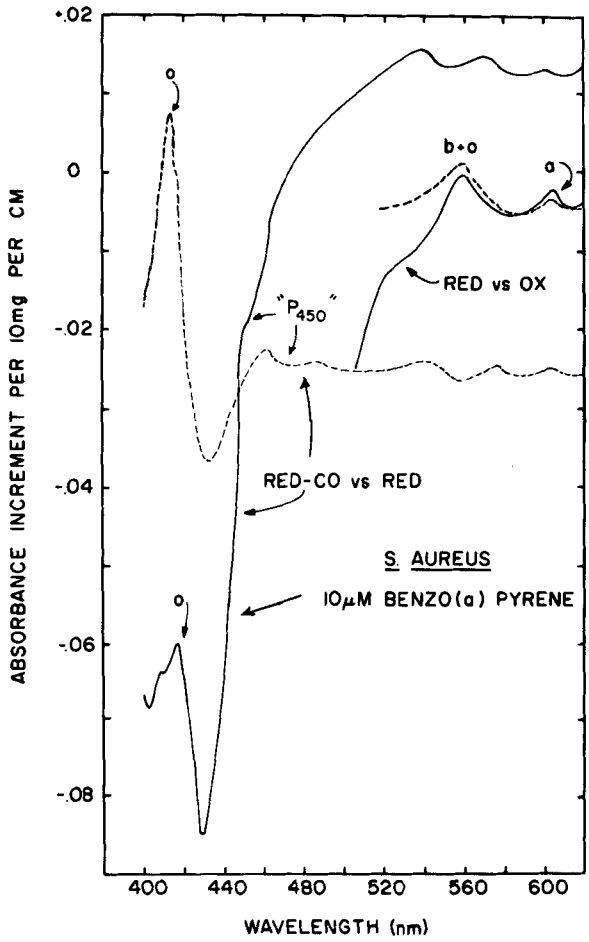

FIGURE 4. Difference spectra of *S. aureus*. Cells were harvested by centrifugation 1 hour after the initiation of aeration, washed, and suspended in 50 mM phosphate buffer (pH 7.6) at 4° C at a density of 12 mg (dry weight) per ml. L-lactate (0.1 mM, final concentration) was added to a portion of the cells to reduce the respiratory pigments. After anaerobiosis, these cells were compared to a suspension in which the pigments had been oxidized by vigorous agitation with a Vortex mixer in a Cary 14 recording spectrometer (Red vs. Ox spectrum). Another portion of the cell suspension with the pigments reduced in the presence of L-lactate was saturated with carbon monoxide, and these cells were compared to cells with the pigments reduced in the presence of L-lactate (Red-CO vs. Red spectrum). Control spectra are indicated as dotted lines. Solid lines indicate cells taken after 1 hour of growth with BAP.

glucolipids and mono- and diglycerides. There were changes in the synthesis of the neutral lipids during glycerol deprivation. The formation of the phytoene and carotene carotenoids was stopped after a lag, and the synthesis of xanthophylls (rubixanthins) was markedly slowed. The formation of the vitamin K_2 isoprenologues was stopped abruptly, immediately after glycerol deprivation. With the addition of glycerol to deprived cultures there is an immediate rectification of the altered lipid composition, which apparently can be initiated without protein synthesis.

Since the deprivation of the glycerol auxotroph results in altered lipid metabolism, the effect on the function of the electron transport system was assayed. Mindich [14] showed that the formation of an efficient active transport system for amino acids was depressed during glycerol deprivation. We established that no new functionally active amino acid transport system was formed during glycerol deprivation.[16] Active transport of amino acids in *S. aureus* requires the function of the electron transport system.[4] We have been able to show that the ability to form ATP by oxidative phosphorylation is progressively depressed as glycerol deprivation continues (P. Keyser and D. C. White; unpublished data), and preliminary evidence suggests that in the absence of glycerol, the cytochrome oxidase *o* can be added to the membrane but is not functional. These membrane function defects can apparently be corrected within 5 min, on the addition of glycerol in the absence of protein synthesis.

The fact that glycerol deprivation led to an abrupt cessation of vitamin K_2 synthesis suggested that the isolation of a vitamin K_2 auxotroph could be interesting. Such a mutant has been selected. This mutant requires manadione for growth. When deprived of menadione, there are subtle changes in the phospholipid composition. CL is apparently formed more rapidly than the bulk of the PG, but gross changes in the phospholipid concentration were not detectable. The deprived menadione auxotrophs show decreased electron transport activity, and this defect can be corrected by added menadione in the absence of protein synthesis.

If *S. aureus* is deprived of glycerol in the presence of BAP, there is a marked effect on the electron transport system. Very little functional respiratory system is formed, although some appears to have been left in the membrane in a nonfunctional state. Apparently the presence of lipid in the membrane protects somewhat against the disorganizing effects of the lipophilic carcinogen BAP.

Hopefully, these mutants and others can add further understanding to the role of lipids in the formation and function of the membrane-bound electron transport system.

If the formation of a functional electron transport membrane requires lipid metabolism, it might be possible to isolate areas of rapid lipid metabolism with their associated specific proteins from the rest of the membrane. This necessitates fractionation of the membranes into functional subunits. The membranes of *S. aureus* are readily prepared from the intact cells by lysostaphin treatment, lysis, and differential centrifugation.[4] Preliminary fractionations by the elegant Daniels Technique of chromatography on precipitated detergent crystals [17] has indicated a reproducible pattern of lipid and BAP distribution that suggests heterogeneity in membrane localization (FIGURE 5). With this and other techniques, the lipid and respiratory pigment changes can be shown not only to occur simultaneously and to be inhibited by BAP, PB, or glycerol deprivation, but also to occur in the same areas of the membrane.

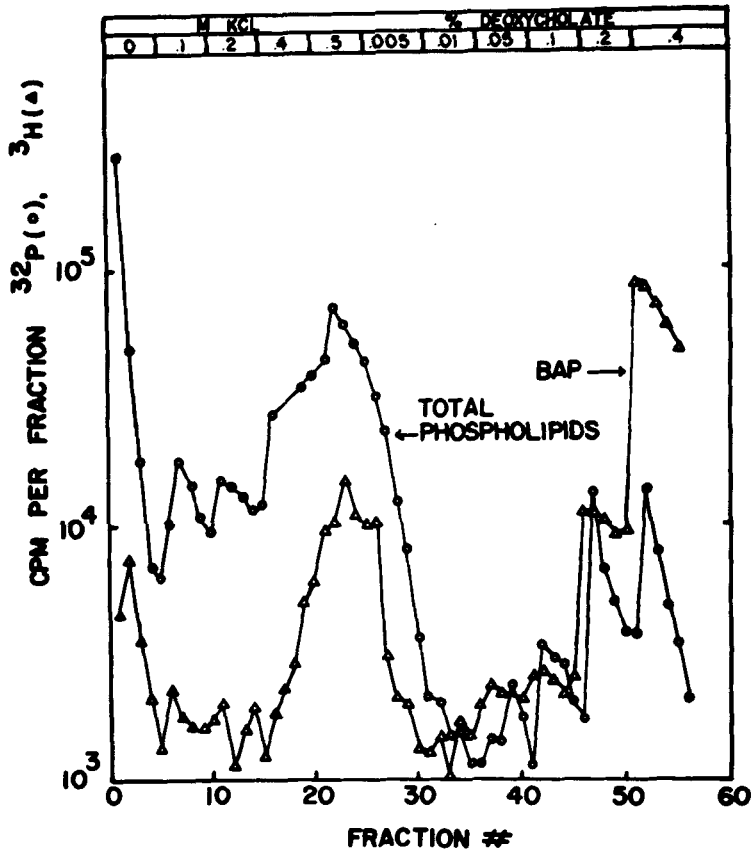

FIGURE 5. Chromatographic separation of membranes prepared from *S. aureus*. Bacteria were harvested after growth with $H_3{}^{32}PO_4$ and BAP-^{3}H and membranes were prepared after lysis by lyostaphin.[4] The membranes were added to a cadmium sarcosinate column[17] and eluted with the solutions of increasing molarity of KCl and deoxycholate, as indicated at the top of the FIGURE. The ^{3}H and ^{32}P in each fraction was then determined.

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DISCUSSION OF THE PAPER

DR. JURTSHUK (*University of Houston, Houston, Texas*): Is the cytochrome P-450 component membrane-bound in your system?

DR. WHITE: It is.

DR. JURTSHUK: Which functions did you measure that are cytochrome P-450-dependent, besides, say, the oxygenation of the carotenoids (which you indicated does involve cytochrome P-450)?

DR. WHITE: Primarily, we measured its CO spectra.