CARDIOLIPIN METABOLISM IN GROWING AND SPORULATING *BACILLUS SUBTILIS*

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Received 2 February 1978
Revised version received 8 March 1978

1. Introduction

Phospholipids are involved in maintenance of structural integrity and in many functions related to membrane activities. In Gram-positive as well as in Gram-negative bacteria, diphosphatidylglycerol (cardiolipin), normally a minor component, is unique in that its concentration can undergo wide changes, depending on experimental conditions [1–3]. As most phospholipids are in association with membrane proteins, these changes in concentration may induce modifications in some structural proteins and lead to activation or inhibition in enzymatic activities [4].

An important membrane function is the participation in *Bacillus* sporulation. Mutations leading to an early block of sporulation of *Bacillus subtilis, spo*0 mutants [5,6] show pleiotropic effects related to an alteration of the cytoplasmic membrane. It has been observed that in these *spo*0 mutants, the relative amount of phosphatidylethanolamine is much lower than in the parent strain or in the revertants, even during exponential growth [7].

When organic solvents, such as ethanol, are added to the growth medium of *Escherichia coli* the structure and the functions of the bacterial membrane [8,9] are disturbed producing pleiotropic effects. In *Bacillus subtilis* grown with alcohol, sporulation is inhibited at an early stage as in *spo*0 mutants [10] and phospholipid metabolism and fatty acid distribution are affected (in press).

We now report that in *Bacillus subtilis,* cardiolipin metabolism is not only sensitive to physiological changes but also is closely related to growth phase and sporulation. Under conditions which normaly allow rapid cardiolipin accumulation, at the expense of phosphatidylglycerol there is no cardiolipin accumulation in cells committed to sporulation (t_1 cells).

2. Materials and methods

2.1. *Bacterial strains and media*

Bacillus subtilis SMY, the indole-requiring mutant 168 and the sporulating negative mutant, *spo*0A 5 NA strain derived from it were obtained from Dr P. Schaeffer. The cells were grown at 37°C in nutrient broth medium [11]. For reproducible results during exponential growth, the medium is exhausted of rapidly metabolized substrates by a preliminary culture, up to 0.300 A_{600} increase, followed by sterile filtration. Conditioned medium is used throughout this work [12].

2.2. *Analytical techniques*

^{32}P-Labelled phospholipids were extracted, separated and estimated as in [12].

2.3. *Chemicals*

[^{32}P]Orthophosphate carrier free was from the Department of Radioelements CEA, France, nutrient broth from Difco and lysozyme from Sigma. All other products were reagent grade.

3. Results

When lipids are extracted from growing bacteria, the amount of cardiolipin fluctuates considerably, depending on technical conditions of treatment. If cells are harvested by centrifugation washed, then extracted, as much as 10–30% phospholipid molecules are cardiolipin. If the culture is directly acidified with perchloric acid before centrifugation, cardiolipin represents only 2–3% extracted phospholipids.

When cultures of *Bacillus subtilis* are not well aerated (by vigorous shaking) cardiolipin accumulation begins according to the kinetic pattern shown (fig.1). Such an accumulation under anaerobiosis has been described, in *Bacillus stearothermophilus* [3]

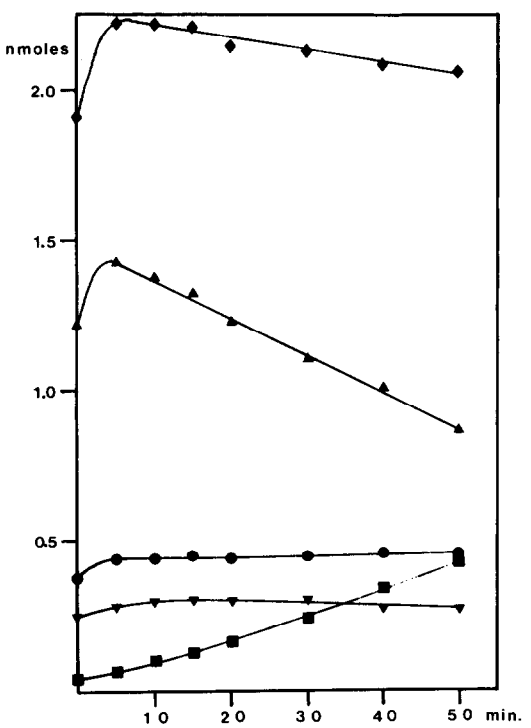

Fig.1. Cardiolipin accumulation during anaerobiosis in *B. subtilis* 168. Cells are incubated without shaking (anaerobiosis) in their growth medium, then analyzed for their phospholipid content after perchloric acid precipitation. The results are expressed in nmol phosphate/ml bacterial suspension (mid. log phase): (◆———◆) phospholipids; (▲———▲) phosphatidylglycerol; (●———●) phospatidylethanolamine; (▼———▼) lysylphosphatidylglycerol; (■———■) diphosphatidylglycerol or cardiolipin.

with concomitant quantitative conversion of phosphatidylglycerol to cardiolipin. In our experiments (fig.1) decrease in phosphatidylglycerol and lysylphosphatidylglycerol concentration correlated well with increase in cardiolipin.

In contrast to these findings, when cells of the sporulating strain are taken 1 h (t_1 cells) after cessation of exponential growth (t_0), there is no cardiolipin accumulation, even if aeration is stopped for 30 min (table 1). Since the substrate, phosphatidylglycerol, is present and the reaction does not require energy, the easiest explanation for this lack of accumulation could be inhibition of the cardiolipin synthetase or its inactivation at the end of exponential growth, as is known for other enzymes [13]. While preparing mesosomes from another spore-forming bacteria, *Bacillus licheniformis* ATCC 9945, we observed a very important cardiolipin accumulation in protoplasmic membrane as well as in mesosomes, following lysozyme treatment. Accumulation was observed in cells at any stage of the growth (B. L., unpublished results).

We applied this lysozyme treatment to *Bacillus subtilis* entering stationary phase and sporulation. As can be seen in table 1, t_1 cells incubated with lysozyme do accumulate cardiolipin, even more than exponentially-growing cells. This proves that the synthetase is present and if inhibited in non-treated cells, can be completely reactivated.

Cells from non-spore-forming mutants examined during exponential growth for cardiolipin accumulation, react to anaerobiosis like those from the sporulating strain. In contrast with sporulating strains, there is no inhibition of accumulation in mutant cells at t_1 stage (table 2).

This unexpected consequence of the mutation leading to early block in sporulation shows that in the *spo*0 mutants, cardiolipin metabolism is not under the same control as in sporulating strains.

Another hypothesis for cardiolipin accumulation could be advanced if the specific phospholipase D [14,15] in *Haemophilus parainfluenzae*, a Gram-negative bacteria, were present in spore-forming Gram-positive bacteria. Cardiolipin would accumulate because of lipase inhibition or decreased activity rather than activation of the synthetase. Since activity of the specific phospholipase D seems to depend on energy [3,16], under O_2 limitation by anaerobiosis

Table 1
Phospholipid distribution in *Bacillus subtilis* SMY during anaerobiosis in relation to the growth phase

Growth phase incubated cells	PE		lysyl PG		PG		CL	
	%	nmol	%	nmol	%	nmol	%	nmol
t_{-1}	21.3 23.8	–	10.8 11.9	+ 0.18	66.9 54.7	– 1.03	1.0 9.7	+ 0.59
t_0	16.2 18.9	–	12.4 12.9	– 0.09	70.6 62.6	– 1.04	0.8 6.8	+ 0.30
t_1	12.5 14.8	–	10.5 10.7	– 0.23	76.1 72.9	– 0.61	0.7 1.7	+ 0.07
t_1 lysozyme	12.7 10.7	+ 0.16	10.5 7.7	– 0.19	71.7 31.9	– 2.4	1.7 43.6	+ 2.4

–, unchanged

Cells are incubated without shaking in their growth medium for 30 min. Phospholipids are expressed in: %, percentage before and after anaerobiosis; nmol, changes in nmol/ml suspension 1.5 A_{600}. For incubation with lysozyme, 100 μg lysozyme is added to 1 ml bacterial suspension. t_0 is the time at which exponential growth stops; other indications (t_{-1}, t_1) express the number of hours before or after t_0.

or during the stationary phase (in non-sporulating bacteria or mutants) cardiolipin accumulates as the lipase is inactive.

We attempted to examine this possibility by addition of 0.7 M ethanol. At this concentration, no cardiolipin accumulates under anaerobiosis, neither in the normal strain (fig.2d) nor in the *spo*0 strain. Such a result can proceed from ethanol inhibition of the cardiolipin synthetase, stimulation of a cardiolipin specific phospholipase D, or inhibition of both activities.

Table 2
Cardiolipin accumulation during 30 min anaerobiosis in different *B. subtilis* strains

Strains	t_{-1}		t_0		t_1	
	PG	CL	PG	CL	PG	CL
SMY	[a] 67.1	1.3	70.6	1.2	76.1	0.7
	[b] 54.7	9.7	62.6	6.8	72.7	1.7
5 NA R	[a] 65.9	2.8	66.0	1.2	70.4	0.7
	[b] 51.6	9.2	58.2	6.9	66.7	1.0
5 NA	[a] 75.8	1.3	79.7	1.4	83.4	1.7
	[b] 66.7	18.7	63.9	16.3	72.0	13.3

[a] results; % before treatment
[b] results; % after treatment

Cells are incubated during 30 min 5 NA R is a sporulating revertant from 5 NA

A second experiment was set up: growing bacteria were put under anaerobiosis for 45 min. During that time, cardiolipin accumulated. The suspension was then divided among 4 test tubes. The first was left under anaerobiosis while the second was aerated. To the third and forth, 0.7 M ethanol was added, one tube remaining under anaerobiosis, the other being well aerated. Phospholipid analysis showed (fig.2) that decrease in cardiolipin concentration occurs only in aerated suspensions. Ethanol inhibits accumulation under anaerobiosis and stimulates cardiolipin degradation once cells are aerated.

It can therefore be concluded that cardiolipin accumulates under anaerobiosis mainly because degradation is inhibited while synthesis proceeds. Since, as we have seen, there is a cardiolipin synthe-

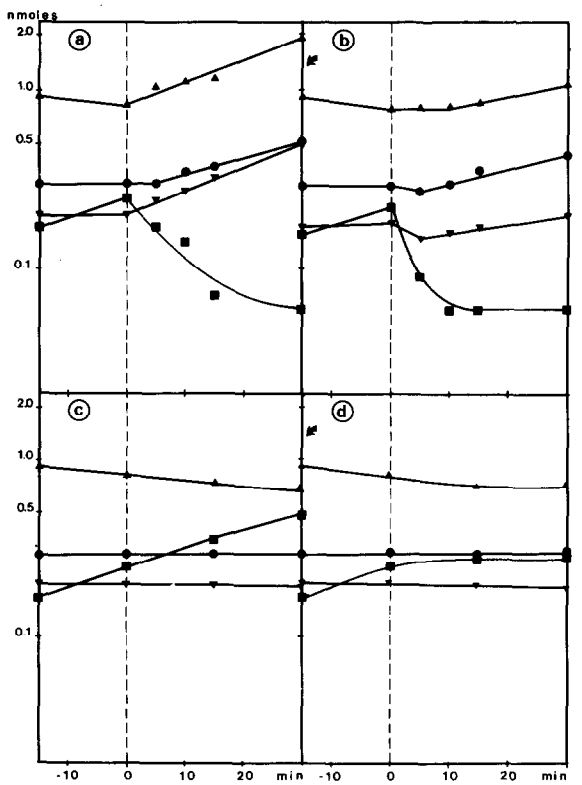

Fig.2. Effect of ethanol on cardiolipin metabolism in strain SMY. Cells are in anaerobiosis 45 min before 0 time and then aerated (ⓐ) and (ⓑ) or not (ⓒ) and (ⓓ). Ethanol 0.7 M is added 15 min before 0 time as indicated by the arrows curves (ⓑ) and (ⓓ). Phospholipids are expressed in nmole/ml bacterial suspension. (▲——▲) PG; (●——●) PE; (▼——▼) lysyl PG; (■——■) cardiolipin. A 15 min lag is observed before maximum ethanol effect on growth or cardiolipin accumulation can be measured.

tase in sporulating t_1 cells, we must assume that during sporulation, the degradative enzyme is active even under anaerobiosis while in non-sporulating cells at t_1 the degradative enzyme is not active.

4. Discussion

We have shown that in *Bacillus subtilis*, cardiolipin metabolism is very sensitive to the physiological state of the cell. Not only cardiolipin accumulates rapidly under such laboratory routine conditions as harvesting by centrifugation (results not shown) but also under anaerobic conditions or during lysis by lysozyme. This can explain why high amounts of cardiolipin were reported in most papers dealing with phospholipid distribution in Gram-positive bacteria or protoplasts.

Since the discovery of cardiolipin in *Micrococcus lysodeikticus* [17] its synthesis from phosphatidylglycerol has been well documented [1,18,19] and its degradation by a specific phospholipase D, described in *Haemophilus parainfluenzae* [15] and in *Bacillus stearothermophilus* [3].

Accumulation of cardiolipin in the stationary phase of growth seems to be universal among bacteria. The only apparent exception was described in *Bacillus licheniformis* [20] another spore-forming bacterium. In this work, cells were harvested by centrifugation and the pellet suspended in phosphate buffer before analysis. When cells were collected during exponential growth, up to 25% phospholipids were cardiolipin. When cells were collected after transition to stationary phase, cardiolipin accounted for less than 10% and at a later stage less than 5%.

During centrifugation, the cells are under anaerobiosis; we checked that cardiolipin accumulated during centrifugation of exponentially-growing *B. licheniformis*. With bacteria reaching the stationary phase as we have seen for sporulating *B. subtilis*, no cardiolipin accumulates. It would be interesting to know if in other spore-forming bacteria, cardiolipin does not accumulate under anaerobiosis after entering sporulation. This leaves open the question: why is cardiolipin degradation inhibited under anaerobiosis in growing cells but not when cells are committed to sporulation?

In *Bacillus stearothermophilus*, cessation of aeration resulted in quantitave conversion of phosphatidylglycerol to cardiolipin [3]. Cardiolipin breakdown was dependent on some metabolic reaction [3]. These observations can be extended to the sporulation of negative strains of *Bacillus subtilis* and to exponentially-growing cells of the sporulating strain.

If in *B. subtilis* there is a phosphatidylglycerol cardiolipin cycle as in *Escherichia coli* [21–23] then some unstable or transient effector required for the degradative enzyme, normally synthesized during exponential growth must have accumulated or arose from another source even under anaerobiosis in cells committed to sporulation.

In t_1 cells, it should be possible to identify the effector and see how it is related to sporulation. If such an effector is necessary for a specific cardiolipin phospholipase D activity, the negative results [24] with Gram-positive bacteria could also be explained.

Acknowledgements

This investigation was supported by grants from the CNRS and DGRST.

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