Biochimica et Biophysica Acta 901 (1987) 250–254
Elsevier

BBA 73604

Acyl-chain-dependent incorporation of chlorophyll and cholesterol in membranes of *Acholeplasma laidlawii*

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(Received 7 April 1987)

Key words: Membrane stability; Thylakoid membrane; Chlorophyll; Cholesterol; Acyl chain; Unsaturated acyl chain; (*A. laidlawii*)

Lipid compositions in thylakoid membranes from photosynthetic organisms and plasma membranes of the cell-wall-less bacterium *Acholeplasma laidlawii* are very similar. The ability of chlorophyll *a* to affect lipid packing was investigated by incorporation into acyl-chain modified *A. laidlawii* membranes during growth. Cholesterol was used as a reference. *A. laidlawii* grows well with mono- and diunsaturated, but not at all with triunsaturated fatty acids. However, both chlorophyll and cholesterol allowed growth with linolenic acid. Hence, in linolenoyl media, *A. laidlawii* is dependent on these membrane additives for survival. Incorporated amounts of chlorophyll, and to a lesser extent cholesterol, rose dramatically upon increased acyl-chain unsaturation. The absorbance maximum of chlorophyll in *A. laidlawii* membranes was similar to that in lipids from photosynthetic membranes. The major membrane lipids, monoglucosyldiacylglycerol and diglucosyldiacylglycerol, form reversed hexagonal and lamellar liquid-crystalline phases in water, respectively. From the regulation of these lipids as a response to the presence of chlorophyll or cholesterol, it is concluded that chlorophyll stabilizes and cholesterol destabilizes the lamellar phases in unsaturated membrane lipids.

Introduction

Compositions of polar lipids in photosynthetic membranes and membranes of the cell-wall-less fatty-acid auxotrophic bacterium (mycoplasma) *Acholeplasma laidlawii* are surprisingly similar [1,2]. This similarity extends to the physical properties and the phase equilibria formed by these lipids. The major lipids from thylakoids and *A. laidlawii*, monogalactosyldiacylglycerol and monoglucosyldiacylglycerol, respectively, both form reversed hexagonal liquid-crystalline phases (H_{II}) in excess water [3–5]. The other sugar lipids, digalactosyldiacylglycerol and diglucosyldiacylglycerol, as well as the phospholipids and sulpholipids, form lamellar liquid-crystalline phases only [1–5]. Likewise, a large ratio between the mono- and disugar lipids, a low lipid hydration and high temperatures, favour the formation of reversed cubic phases at the expense of the lamellar phases in mixtures of these lipids [3–5].

In *A. laidlawii*, the molar ratio monoglucosyldiacylglycerol/diglucosyldiacylglycerol is extensively regulated in vivo as a function of conditions or factors that govern the packing of lipids into different aggregate structures, i.e., the hydrocarbon chain volume and length and the hydrocarbon/water interfacial area [2,5–7]. This regulation aims at compensating for any packing disturbancies brought by, for example, different fatty

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acyl chains, changes in temperature, and the presence in the membrane of different foreign molecules such as hydrocarbons, alcohols, detergents and sterols [5–7]. Thereby, similar phase equilibria and an optimal lamellar stability are maintained for the lipids irrespective of the disturbant conditions [5]. For a specified condition, the addition of a foreign molecule will cause a compensating metabolic change in the monoglucosyldiacylglycerol/diglucosyldiacylglycerol ratio. The sign and magnitude of this change are related to changes in the phase equilibria, for example, a large decrease in the ratio is caused by molecules destabilizing a lamellar phase and promoting cubic or reversed hexagonal phases [6,7]. The amounts of the phospholipids are also regulated so that a constant surface charge density and potential is always maintained [8].

During greening of dark-grown plants the etioplast develops to chloroplast. The development is initiated by chlorophyll biosynthesis. Simultaneously the prolamellar body structure is transformed first into prothylakoids and later to thylakoids. The prolamellar body has an organization similar to those proposed for bicontinuous cubic lipid phases [3]. During the first hours of greening, chlorophyll molecules are less tightly bound to proteins [9]. NMR studies have also indicated that a pool of chlorophyll molecules is associated with the lipid portion of thylakoid membranes [10]. Can chlorophyll affect the packing of lipids and perhaps influence the transformation of the prolamellar body? We therefore had *A. laidlawii* to incorporate chlorophyll into its membrane, and from the metabolic compensations occurring in the monoglucosyldiacylglycerol/diglucosyldiacylglycerol ratio we could analyze the packing disturbances brought about by this foreign molecule. The importance of lipid acyl-chain composition could also be analyzed by feeding *A. laidlawii* with different fatty acids for lipid synthesis (compare Refs. 2,5–7). For comparative purposes, parallel experiments were done with cholesterol.

Materials and Methods

A. laidlawii, strain A, was grown in a lipid-depleted bovine serum albumin/tryptose growth medium supplemented with different fatty acids [11] with and without chlorophyll or cholesterol. 20 units/l of avidin were added in order to prevent endogenous saturated fatty-acid synthesis. The following fatty-acid supplements (from ethanolic stock solutions) were used. (i) 120 μM palmitic acid plus 30 μM oleic acid; (ii) 150 μM oleic acid; (iii) 150 μM linoleic acid; and (iv) 150 μM linolenic acid. 20 μM chlorophyll *a*, from *Anacystis nidulans* (Sigma Chemicals), or 20 μM cholesterol was mixed with the fatty acids, sterilized by filtration (0.2 μM) and then added to the medium in order to prevent precipitation. In the growth medium used, the lipid-depleted bovine serum albumin acts as a carrier for the hydrophobic additives. Lipids were labelled by adding 30 μCi/l of [^{3}H]palmitic acid and/or 10 μCi/l of one of ^{14}C-labelled unsaturated fatty acids in (i–iv) above. 30 μCi/l of ^{3}H-labelled cholesterol was used. The growth media were stored frozen in the dark at $-20\,^\circ$C. Cells were adopted to the different supplemented media by 5–7 consecutive inoculations.

25 ml batches (2% inoculum) were grown in screw-capped tubes 20 h at $30\,^\circ$C in darkness. Growth was analyzed by measurement of pH and the turbidity at 524 nm, and by phase-contrast light microscopy. Cells were harvested and washed, membranes prepared, and lipids extracted, purified and analyzed as described [11]. Chlorophyll was extracted from washed membranes with 80% acetone, and determined by absorbance measurements [12]. Extraction and analysis were performed in dim light.

Results and Discussion

Cell growth

In the growth media used, *A. laidlawii* is forced to use the supplemented fatty acids for lipid synthesis [11]. Cell-growth yields with different fatty acids, as analyzed by culture turbidity and membrane amounts, were in the order palmitoyl > oleoyl > linoleoyl enriched media, respectively. No growth occurred in the linolenoyl medium. Chlorophyll and cholesterol substantially increased cellular yields in the linoleoyl media. Furthermore, chlorophyll and cholesterol both allowed growth to occur in the linolenoyl media,

albeit with cellular yields lower than in the other fatty-acid media. Visual inspection of washed cells or membrane pellets from chlorophyll-supplemented media revealed a strong green colour, the intensity of which increased with increasing unsaturation of the supplemented fatty acids.

Incorporation of chlorophyll

Absorbance measurements of washed membranes verified that chlorophyll was incorporated into the *A. laidlawii* membranes and that the amounts increased with increased acyl-chain unsaturation, see Fig. 1. The difference in chlorophyll content between the cells must be even more pronounced than shown (Fig. 1), since growth yields were lowest for linolenoyl cells (see above). The absorbance maxima found, i.e., around 670 nm, are similar to the maxima observed for chlorophyll *a* in intact etiochloroplast membranes or in lipid vesicles made of native as well as synthetic lipids (compare Refs. 13–15). Note the absence of a shoulder on the curves around 700 nm, which is typical for the aggregation of chlorophyll due to a low solubility of the latter in certain lipid matrixes [13–15]. Analysis of absorbance spectra from extracted chlorophyll in aqueous acetone verified the difference in incorporation indicated in Fig. 1. These spectra were similar to that of a chlorophyll *a* reference sample with no signs of phaeophytin formation. Furthermore, the incorporated chlorophyll *a* had not lost the phytol chain, since extracted chlorophyll *a* showed a mobility in thin-layer chromatography [11] similar to a reference sample and different from chlorophyllide.

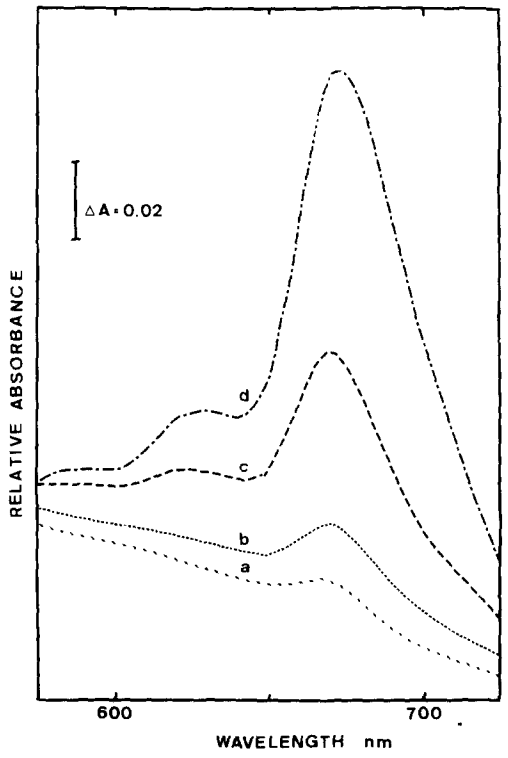

Fig. 1. Absorbance of membranes from *A. laidlawii* grown in media with 20 μM chlorophyll and (a), 120 μM palmitic acid plus 30 μM oleic acid; (b), 150 μM oleic acid; (c), 150 μM linoleic acid; (d) 150 μM linolenic acid. Similar amounts of membranes were used. The absorbance maxima (nm) were (a), 669; (b), 669; (c), 671; and (d), 674 nm, respectively.

Membrane lipid composition

Lipid analysis of the membranes revealed several interesting features, see Table I. With an increasing extent of acyl-chain unsaturation in *A. laidlawii*, an extensive increase in the molar fraction of chlorophyll in the membrane lipids occurred. Similar trends were observed for cholesterol, but with a lesser span over the series (Table I). It has been shown in model systems that the solubility of chlorophyll is much lesser in saturated lipids [16] than in unsaturated lipids [13,15], indicating that the lipid solubility is an important factor governing chlorophyll incorporation. When maximum solubility is exceeded, chlorophyll segregates into a separate phase [16], but this was obviously not the case here, as indicated from the absorbance curves, compare above. The hydrophobic volume of cholesterol is larger than that of the chlorophyll isoprenoid chain. We have previously noted that the solubility of cholesterol in *A. laidlawii* lipids in vitro is larger in dioleoyl lipids than in palmitoyl-oleoyl lipids [17]. The large amounts of cholesterol found in linolenoyl membranes (Table I) are more than the amounts soluble in *A. laidlawii* oleoyl lipids [17]. This suggests that solubility in lipids is an important factor governing incorporation of chlorophyll and cholesterol into membranes in vivo.

Lipid regulation and membrane stability

Decreased amounts of monoglucosyldiacylglycerol (vs. diglucosyldiacylglycerol) in *A. laid-*

TABLE I

LIPID AND CHLOROPHYLL COMPOSITION IN MEMBRANES OF *A. LAIDLAWII*

Mean of three or four experiments. n.g., no growth. The ratios of membrane proteins/lipids are similar in the different membranes, data not shown.

Supplement to the growth medium	Chlorophyll [a] (mol/100 mol total lipid)	Cholesterol (mol/100 mol total lipid)	Ratio monoglucosyl-diacylglycerol/diglucosyl-diacylglycerol (mol/mol)	Fraction charged lipids [b] (mol/100 mol polar lipid)
120 μM Palmitic acid				
+ 30 μM oleic aicd [c]			1.20	29.9
+ 20 μM chlorophyll	0.22		1.30	31.5
+ 20 μM cholesterol		10.5	1.25	29.2
150 μM Oleic acid [d]			0.88	37.6
+ 20 μM chlorophyll	2.4		0.75	39.3
+ 20 μM cholesterol		21.9	0.37	46.6
150 μM Linoleic acid [e]			0.54	41.1
+ 20 μM chlorophyll	15.8		0.63	39.5
+ 20 μM cholesterol		29.4	0.30	48.1
150 μM Linolenic acid [f]			n.g.	
+ 20 μM chlorophyll	65.0		0.72	43.5
+ 20 μM cholesterol		40.4	0.45	50.7

[a] From the molar absorbance of extracted chlorophyll *a* at 663 nm. Chlorophyll amounts in membranes were corroborated by the amounts left in the growth medium supernatants after centrifugation of the cells.

[b] Phosphatidylglycerol and the glycerophosphoryl derivates of monoglucosyldiacylglycerol and diglucosyldiacylglycerol.

[c] Acyl-chain composition was approx. 80% palmitoyl plus 20% oleoyl chains as determined by gas-liquid chromatography and liquid-scintillation counting

[d] 95% oleoyl chains as per footnote c.

[e] 95% linoleoyl chains as per footnote c.

[f] 90% linolenoyl chains, as per footnote c.

lawii membranes is a metabolic compensation to conditions or factors that promote the formation of nonlamellar phases of the reversed type, as previously predicted [2] and experimentally verified for a variety of membrane acyl-chain compositions and perturbant molecules [5–7]. Consequently, increased amounts of monoglucosyldiacylglycerol occur as a response to factors promoting the formation of lamellar phases [2,5–7]. We have previously noticed that perturbant molecules stabilizing a lamellar phase yield a small increase in the monoglucosyldiacylglycerol/diglucosyldiacylglycerol ratios, whereas those molecules that destabilize the lamellar phase often yield large decreases in the ratio [6,7]. This probably depends on the closeness of the entire polar lipid mixture to a lamellar–nonlamellar transition [5]. According to the monoglucosyldiacylglycerol/diglucosyldiacylglycerol ratios in Table I chlorophyll stabilized a lamellar phase for the *A. laidlawii* lipids in all instances, except with oleoyl chains. For cholesterol a destabilization was very prominent with oleoyl and linoleoyl acyl chains. Cholesterol increase the molecular order parameter in oleoyl-enriched *A. laidlawii* membranes [18], which correlates with a concomitant reduction in the passive permeability of the membranes [19]. The permeability increases dramatically with increased acyl-chain unsaturation [19]. Perhaps chlorophyll and cholesterol can increase the molecular order in linoleoyl and linolenoyl membranes by imposing a more extended conformation of the supposedly highly disordered di- and triunsaturated acyl chains, thereby reducing a too large permeability and promoting (or allowing) cellular growth, see results above. Due to their different molecular structures, chlorophyll and cholesterol affect the average hydrocarbon volume

and chain length and the hydrocarbon/water interfacial area, and thereby the phase equilibria, in different ways, as indicated from the differential responses in monoglucosyldiacylglycerol/diglucosyldiacylglycerol ratios (Table I). However, with linoleoyl and linolenoyl acyl chains, very common in thylakoid lipids, chlorophyll has a stabilizing influence on the lamellar phases of *A. laidlawii* lipids. This is corroborated by recent findings showing that large amounts of chlorophyll *a* (46%, mol/mol), compare above, in polyunsaturated monogalactosyldiacylglycerol changed the reversed hexagonal phase formed by this lipid towards a lamellar-like phase [20]. Such properties are the ones expected for the transformation of a bicontinuous cubic phase to a lamellar one and thus perhaps contribute to the transformation of the prolamellar body to thylakoids.

In *A. laidlawii*, the amounts of charged lipids are regulated so that a constant lipid surface charge density and surface potential are maintained during conditions with varying lipid lateral packing areas (i.e., acyl-chain unsaturation) and quenching of the lipid surface charge with ions [8]. Table I shows that charged lipid amounts were only marginally affected by chlorophyll, whereas cholesterol substantially increased charged lipid amounts in unsaturated membranes. Most likely, this is due to the larger and more bulky hydrophobic part of cholesterol compared to the isoprenoid chain in chlorophyll.

Conclusions

The amounts of chlorophyll and cholesterol incorporated into membranes of *A. laidlawii* are dependent upon the extent of lipid acyl-chain unsaturation. Both molecules release the growth inhibition caused by linolenoyl acyl chains, probably by increasing the molecular order in the membranes. According to the impact on the monoglucosyldiacylglycerol/diglucosyldiacylglycerol ratio, and hence, on the phase equilibria, chlorophyll promotes a lamellar phase in linoleoyl and linolenoyl membranes, whereas cholesterol promotes non-lamellar phases in unsaturated membranes, respectively.

Acknowledgements

We thank Viola Tegman for excellent technical assistance, Agneta Ehman for typing and the Swedish Natural Science Research Council for financial support.

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