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Bacterioruberins reinforce reconstituted *Halobacterium* lipid membranes

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A model of the red membrane of *Halobacterium* was reconstituted from its total polar lipids and bacterioruberins, or diphytanylphosphatidylcholine and bacterioruberins. Bacterioruberins are much better incorporated into the lipid vesicles than zeaxanthin or even than decaprenozeaxanthin, in spite of their comparable molecular lengths. When incorporated into the vesicles, bacterioruberins decrease the water permeability and increase the rigidity of the bilayers. Thus, at least in a system related to natural archaebacterial membranes, carotenoids do play their postulated role of reinforcers.

In 1979, as part of a general theory of the molecular evolution of biomembrane constituents, we have postulated that some carotenoids might play the role of membrane reinforcer in bacteria by stabilizing both halves of the bilayer like transmembrane 'rivets' [1].

This role of carotenoids has been confirmed with model membranes [2] and in vivo [3]. We have recently shown that (i) several dipolar carotenoids such as zeaxanthin, astaxanthin and their C_{50} homologues can be incorporated, in a transmembrane manner, into dimyristoylphosphatidylcholine (DMPC) or dipalmitoylphosphatidylcholine (DPPC) vesicles [4]; (ii) the incorporation ratio is higher when the molecular length of the carotenoid corresponds to the thickness of the phospholipid bilayer [5]; and (iii) these carotenoids, when incorporated, reinforce the bilayers [5,6].

However, neither DMPC nor DPPC are major lipid constituents in bacterial or archaebacterial membranes. Kates et al. [7] have shown that *Halobacterium* species contain exclusively the phytanyl analogues of phospholipids (mainly phosphatidylglycerophosphate, with small amounts of phosphatidylglycerol and phosphatidylglycerosulfate) and glycolipids (mainly a sulfated triglycosylglycerol diether); they have also shown that these archaebacterial extreme halophiles produce largely a red membrane [8] in which the major red pigments consist of acyclic C_{50} carotenoids called 'bacterioruberins' [9–11].

To confirm the stabilizing role of bacterial carotenoids in natural membranes, we have studied *Halobacterium* lipid systems. We have first isolated bacterioruberins and total polar lipids of *Halobacterium* (Fig. 1).

We have then investigated the extent of maximal incorporation of bacterioruberins in vesicles

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composed of natural total polar lipids (mixture of different anionic headgroups) and in vesicles composed of pure 2,3-di-*O*-phytanyl-*sn*-glycero-1-phosphocholine (DPhPC) [5]. We now wish to report some biophysical properties of these systems.

Total polar lipids and bacterioruberins * (λ_{max}(tetrahydrofuran):475, 495 (ε 140000), 535 nm; Litt. $\lambda_{max}(CHCl_3)$: 475, 502, 535 nm; λ_{max}(acetone): 465, 490 (ε 140000), 525 nm [10]) were isolated from the acetone-insoluble lipid extracts of *Halobacterium cutirubrum* and the acetone-soluble lipid extracts of *Halobacterium halobium*, respectively, by procedures described earlier [8,12].

The preparation of unilamellar vesicles (sonication, ether injection or reverse phase method), their purification (elimination of external carotenoid aggregates through polycarbonate filters), and the evaluation of the yield of vesicle formation (the ratio of phospholipid concentration before and after vesicle preparation and purification) were achieved as described in Refs. 4–6. Electron microscopy was done using the negatively staining method. The dissymmetry of light scattering of the vesicles was determined in a FICA 4200 photogoniodiffusiometer equipped with a 5 mW He-Ne vertically polarized laser (SA Optilas, France) [4–6].

The concentration of the phospholipid and of the carotenoid in vesicles was measured, respectively, by phosphorus determination [13] and by ultraviolet-visible spectroscopy (Uvikon 820 Kontron spectrophotometer) [4]. Stopped-flow experiments were performed on a Durrum-Gibson stopped-flow spectrophotometer, measuring the intensity of light scattered at right angles. Rapid mixing (3 ms) of a suspension of vesicles (in 350 mM NaCl/1 mM $EDTANa_2$/5 mM NaN_3/10 mM Tris-HCl (pH 8) buffer) with the same volume of 50 mM NaCl buffer at 25 °C was followed by measuring the intensity changes of scattered light. The kinetics was characterized by the process half-time ($t_{1/2}$) [6].

We first observed a large difference in the yield

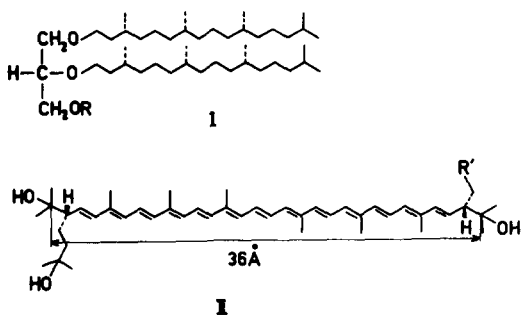

Fig. 1. Structure I represents diphytanylphosphatidylcholine (R is phosphocholine) or total polar lipids of *Halobacterium* (R is mainly 1,3-glycerol bisphosphate and 3-SO_3^--Gal-Man-Glc). Structure II represents bacterioruberin (R', CH_2-$C(CH_3)_2OH$) or monoanhydrobacterioruberin (R', $CH = C(CH_3)_2$).

of vesicle formation between DPhPC and total polar *Halobacterium* lipids with bacterioruberins added. The yield was about 10% (ether injection method at 60 °C) to 25% (sonication or reverse phase method at room temperature) for DPhPC, whereas it reached 70 to 80% with the halophilic lipids. Pure DPhPC self-organizes preferentially into a hexagonal H_{II} phase at higher temperatures (unpublished data); in contrast, the natural *Halobacterium* lipids are better adapted, presumably because of their larger headgroups, to form vesicles in an aqueous medium.

The extent of maximal incorporation of bacterioruberins was expressed as a molar percentage, i.e. by

$$s = 100 \times c_{carot}/(C_{carot} + C_{phospholipid})$$

The value of s in vesicles obtained by reverse phase evaporation is 11% in DPhPC (phosphatidylcholine headgroup) and 9% * in total polar lipids (mixture of different anionic headgroups) of *Halobacterium*. These values are much higher than the incorporation value of zeaxanthin ($s = 1.5\%$) or decaprenozeaxanthin ($s = 1.5\%$) in DPhPC vesicles [5]. These results can be explained by a better fit of the molecular dimensions of bacterioruberins and of the above mentioned lipids [5]. The length of the hydrocarbon part of decaprenozeaxanthin is similar (hydrophobic part:

* This is a mixture of bacterioruberin sensu stricto (the major constituent) and the structurally related monoanhydrobacterioruberin (11).

* To calculate this value, we used the table of lipid composition of Ref. 12.

TABLE I

WATER PERMEABILITY ($t_{1/2}$) AND ELASTICITY OF BILAYERS ($-\Delta I/I_0(Z-1)$) FOR THE VESICLES COMPOSED OF DIPHYTANYLPHOSPHATIDYLCHOLINE OR TOTAL LIPIDS OF *HALOBACTERIUM CUTIRUBRUM* WITH CHOLESTEROL OR BACTERIORUBERINS

Lipids	$t_{1/2}$ [c] (ms)	$-\Delta I/I_0(Z-1)$ (%)
DPhPC [a]	40	1.6±0.2
DPhPC + 11 mol% bacterioruberins	110	1.0±0.1
DPhPC + 5 mol% cholesterol	100	0.9±0.1
Total lipids [b] (*Halobacterium cutirubrum*)	50	2.9±0.2
Total lipids + 9 mol% bacterioruberins	110	1.8±0.2

[a] Diphytanylphosphatidylcholine.

[b] Diphytanylglyceryl ethers (mixture of different polar headgroups).

[c] $t_{1/2}$ was obtained by both osmotic shock and ^2H_2O methods. The values of both methods were the same within the experimental error of ±5 ms.

about 37 Å) to that of bacterioruberins (about 36 Å); the two large terminal cyclohexene rings of decaprenozeaxanthin probably make it too thick to be well incorporated in the diphytanyl ether lipids, whereas the acylic polar heads of bacterioruberins can be accommodated into the aqueous phase.

In a previous paper [6], we have shown that the stopped-flow light scattering method is well adapted to the evaluation of the mechanical properties of vesicles and of their permeability to water. We have confirmed a linear relationship between $\Delta I/I_0$ (relative light scattering intensity change) and $(Z-1)$ (Z: dissymmetry of light-scattering) for all vesicle samples in Table I; thus $-\Delta I/I_0(Z-1)$ is independent of vesicle size and can be used to evaluate membrane elasticity [6]. The half-time of the kinetics for water permeability, measured by the osmotic shock and the Lawaczeck ^2H_2O diffusion method [14], was the same in each sample. The water permeability and bilayer elasticity for the vesicles of different composition are summarized in Table I.

Table I shows that the incorporation of bacterioruberins, at about 10 mol%, i.e. at twice their concentration in the red membrane of *Halobacterium cutirubrum* [8], does lower the water permeability and enhance the rigidity both of DPhPC bilayers and of reconstituted total lipid membranes. Thus, at least in this system, analogous to natural archaebacterial membranes, carotenoids do act as membrane reinforcers, as predicted by the theory presented earlier [1].

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