LABELING OF MEMBRANES AND REACTION CENTERS FROM THE PHOTOSYNTHETIC BACTERIUM *RHODOSPIRILLUM RUBRUM* WITH FLUORESCAMINE

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1. Introduction

Photosynthetic reaction centers are pigment–protein complexes in which the light energy, absorbed by the bulk of chlorophyll, is converted into chemical energy in form of a redox pair [1]. Most bacterial reaction centers described so far are composed of 3 protein subunits named L, M and H. These have molecular weights of 21, 24 and 29 × 10^3 [2]. Studies on the architecture of the components of all known energy transducing electron transport chains either in mitochondria, chloroplasts or bacteria reveal a highly ordered and asymetric structure of these membranes. To understand the function of the reaction center complex in driving the photosynthetic electron transport chain, it seems to be of great interest to obtain more information on the molecular organisation of the reaction center proteins in the membrane. Knowledge of localisation of these membrane proteins comes from different approaches: interaction with cytochromes, reaction with specific antibodies, effects of proteases, enzymatic iodination or photoaffinity labeling [3–10]. From all these experiments we can conclude that the H subunit of the reaction center complex as well as part of the light-harvesting pigment protein complexes are exposed on the surface of the cytoplasmic side of the membrane. Several results indicate further that the H subunit spans the membrane bridging the 2 hydrophilic sides through the inner lipophilic membrane part. On the other hand the subunits L and M seem to be completely embedded in the membrane. These 2 proteins are neither accessible to antibodies nor to the $^{131}I/H_2O_2$/ lactoperoxydase system. However, they are heavily labeled with the lipophilic marker [^{125}I]iodonaphthylazide. The use of fluorescamine as a marker for hydrophobic parts of the membrane is described here. This reagent forms fluorescent products upon reaction with primary amines and is well known for the determination of amino acids and of water soluble peptides [11]. Unstable in the presence of water, it has to be dissolved in organic solvents such as acetone. Therefore, when added to whole cells its affinity to the lipophilic membranes is higher than to water soluble proteins in the cytosol [12]. It reacts also with phospholipids, namely with phosphatidylethanolamine.

2. Material and methods

Chromatophores and reaction centers were prepared from cells of the carotenoidless mutant G-9 of *Rhodospirillum rubrum* as in [13]. Fluorescamine (Roche) was prepared with freshly distilled acetone and added in 20–50 μl portions under strong agitation to the membrane suspension (final conc. 0.2 mg/ml = 0.3 mg fluorescamine/mg protein, acetone conc. 2%). Lipid extraction was done with an aceton/methanol mixture (7:2) in the cold [13]. The proteins were separated on 12% SDS gels at constant voltage according to [14]. Fluorescent bands were detected by eye when irradiated with 368 nm, photographed through a 500 nm interference filter and labeled on the gel with ink. Afterwards the gels were stained with Coomassie brillant blue. Fluorescence of extracts of labeled membranes was detected in a spectrofluorimeter using 390 nm as excitation, 470 nm as emmission wavelength.

3. Results and discussion

In delipidated denatured chromatophores fluorescamine is able to react with all 3 subunits as well as with the light harvesting protein as can be seen from gels 1, 6 (fig.1). When reaction centers with their bound lipids or chromatophores are incubated with fluorescamine (gels 2–5) we find the fluorescamine label mainly in subunit M and to a lesser extent in H. The heavy fluorescence at the front represent labeled phospholipids, especially phosphatidylethanolamine. A high molecular weight band consisting of aggregates of the reaction center subunits, probably induced by the addition of the acetone is also visible.

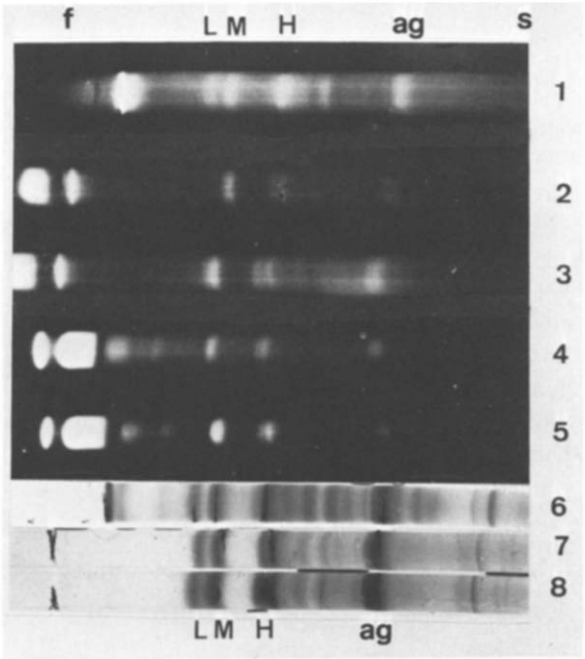

Fig.1. Fluorescence and protein pattern of SDS gels of crude reaction center preparations: (1) labeling of delipidated crude reaction centers with fluorescamine; (2) labeling native crude reaction centers with fluorescamine; (3) as (2) but labeling in the presence of 10 mM ferricyanide (*P*-865 oxidised); (4) labeling of chromatophores with fluorescamine in the dark followed by preparation of crude reaction centers; (5) as (4) but labeling of the chromatophores in the light (30 min); (6) Coomassie Blue staining of gel 1; (7) Coomassie Blue staining of gel 2; (8) Coomassie Blue staining of gel 3.
(6–7) Black ink marks indicate fluorescent bands seen before staining.
Strong fluorescence near the front of the gels is due to the fluorescence of the lipids and of the light harvesting pigment protein complexes

Abbreviations: s, start; f, front of gel; L, light subunit; M, medium subunit; H, heavy subunit; ag, aggregates of hydrophobic subunits

When gels 2,3,4 and 5 respectively, are compared, it is suggested that the labeling is stronger when the reaction center pigment *P*-870 is in its oxidized state, either induced by addition of ferricyanide (gels 2,3) or by the action of light (gels 4,5). A labeling of subunit L was never detected.

The effect of illumination on the intensity of fluorescamine labeling was examined quantitatively in reaction centers isolated from chromatophores incubated with fluorescamine in the dark and in the light. Light gave a clear stimulation of the fluorescence labeling when the incubation occurred at pH 6 (table 1). However, no differences are seen after incubation at pH 8. Labeling at more alcaline pH is always more effective due to the fact that the reagent reacts only with unprotonated amines. Furthermore, this table shows that phosphorylation conditions and the presence of an artificial electron carrier lowers the stimulation of the fluorescamine labeling induced by light. Yet different results are obtained when chromatophores are extracted with a mixture of acetone/methanol after labeling. The organic fraction contains mostly lipids and the residue mainly proteins. In both of these fractions more label is found after incubation in the light between pH 6 and 8 (fig.2).

Table 1
Effect of light incubation of chromatophores on the fluorescence labeling of extracted crude reaction centers (relative units/protein)

Conditions	pH 6	pH 8
Dark	0.35	1.12
Light (20 min preillumination, 20 min illumination during fluorescamine addition	0.63	1.10
Light as before, but in the presence of phosphorylation conditions: Mg^{2+}; ADP; P_i; PMS	0.52	0.89

Chromatophores were labeled in the dark and in the light (at 160 mW/cm^2) followed by the extraction of crude reaction centers by treatment with LDAO (0.275%). Fluorescence of the samples are corrected for variable amounts of extracted proteins

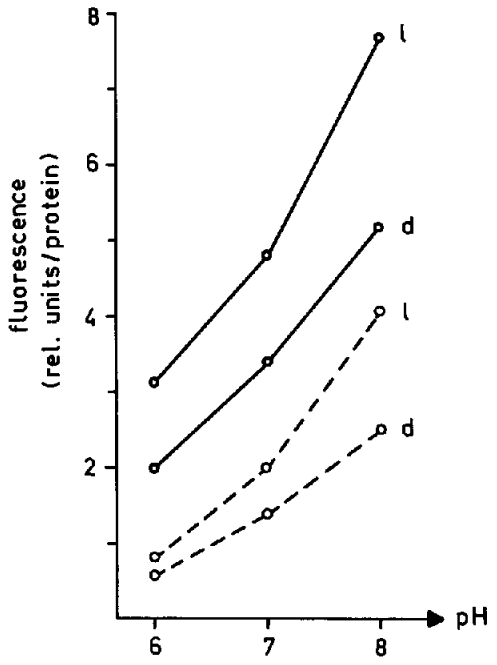

Fig.2. Dependance of fluorescamine labeling of chromatophore lipids (full line) and chromatophore proteins (broken line) on pH and illumination. Chromatophores were labeled in the light (l), (20 min preillumination, 30 min illumination during addition of fluorescamine, light intensity 160 mW/cm^2) or dark (d) and then separated into acetone/methanol-soluble and -insoluble fractions, respectively. The latter was dissolved in 2% SDS before the measurement of the fluorescence. The fluorescence of both fractions was calculated on the basis of the protein content of the chromatophores before extraction.

The observed effects of light on the labeling of the proteins and lipids of the photosynthetic membrane could be interpreted as conformational changes in the membrane including in the reaction center itself similar to the conformational changes discussed for the hydrophobic part of the coupling factor of chloroplasts CF_0 [15]. The term conformational change may also mean rearrangement of lipids in the close environment of the proteins. Such changes in lipid accessibility to fluorescamine are suggested by the differing results at pH 8 given in table 1 and fig.2. While the labeling in the total proteins and the total lipids is increased by light, no light/dark difference is obvious in a reaction center preparation isolated from similarly treated membranes. This suggests that either the fluorescence stimulation in the proteins at alkaline pH is due to other proteins than the reaction center (not substantiated in the experiments shown in fig.1) or rather a compensation of the increase of fluorescence labeling in the reaction center proteins by a decrease in labeling in the lipids in the close surrounding of the reaction center proteins. The results obtained with fluorescamine as a lipophilic membrane marker agree with the results obtained earlier with lactoperoxidase [9] and with iodonaphthylazide [10] for the position of subunits H and M in the bacterial membrane. While H is a transmembrane protein having hydrophilic as well as hydrophobic portions, M is clearly a protein buried within the membrane. The labeling pattern of subunit L obtained after treatment with lactoperoxidase or with iodonaphthylazide are characteristic for a very hydrophobic protein, yet no fluorescamine labeling is obtained. This suggests that the reactive groups of subunit L are in someway shielded from reaction with the short living fluorogenic reagent either by the surrounding lipids or by proteins such as one of the other subunits H or M or certain parts of L itself.

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