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LABELING OF CHROMATOPHORE MEMBRANES AND REACTION CENTERS FROM THE PHOTOSYNTHETIC BACTERIUM *RHODOSPIRILLUM RUBRUM* WITH THE HYDROPHOBIC MARKER 5-[^{125}I]IODONAPHTHYL-1-AZIDE

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Summary

Chromatophores of the photosynthetic bacterium *Rhodospirillum rubrum* and isolated reaction centers were labeled with the lipophilic membrane marker 5-[^{125}I]iodonaphthyl-1-azide. The two smaller reaction center proteins L and M bind more label than the larger subunit H, a fact supporting the proposed localisation of the 3 subunits obtained with hydrophilic labels. Besides these integral proteins the lipids, among them mainly the pigments and the quinones, are highly labeled suggesting a hydrophobic environment around these molecules and a preferred reactivity to iodonaphthylazide. Such a hydrophobic environment may be of great importance for the function of the photosynthetic reaction centers especially for the charge separation and the primary reactions in electron transport.

Introduction

Photosynthetic reaction centers are pigment-protein complexes where light energy, absorbed by the bulk of chlorophyll, is converted into chemical energy in the form of a redox potential difference. In this process the special reaction center chlorophyll, in *Rhodospirillum rubrum P*-865, is oxidized. The electron is transferred to an electron acceptor within the reaction center complex of

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Abbreviations: *P*-865, reaction center bacteriochlorophyll (865 nm); SDS, sodium dodecyl sulphate.

a very negative redox potential. This primary reaction represents the driving part of the photosynthetic electron transport chain.

The isolation of reaction centers from chromatophores has been described for several species of photosynthetic bacteria [1—3]. The reaction center complex of *R. rubrum* contains 3 protein subunits of apparent molecular weights 21 000, 24 000 and 29 000, three pairs of pigment molecules, 2 bacteriochlorophyll 800, 2 bacteriochlorophyll 865 and 2 bacteriopheophytin and a variable amount of phospholipids [4]. The knowledge of the orientation of these proteins and the arrangement of the surrounding of the reaction center in the membrane might help to understand the basic processes of conversion of light energy into a chemical form.

Several methods have been used so far to study the localisation of the reaction centers in the membrane of photosynthetic bacteria. Since electrons from cytochromes are accepted on the inside, protons taken up on the outside of the chromatophore membrane, the reaction center complex spans through the whole membrane and has the oxidizing part on the inside, the reducing part on the outside of the membrane [5] (with *Rhodopseudomonas sphaeroides* and *Rhodopseudomonas capsulata*). Immunological reactions with antibodies against whole reaction centers as well as against the largest subunit H and chromatophore membranes give evidence that the H subunit is exposed at the cytoplasmic surface of the membrane [6—8] (all with *Rps. sphaeroides*). The treatment of membranes with proteases results in a preferential degradation of the subunit H also suggesting an architecture where subunit H is exposed on the cytoplasmic side of the membrane [9,10] (with *Rhodopseudomonas palustris*, *Rps. sphaeroides* and *Chromatium minutissimum*). Enzymatic iodination of chromatophores or spheroplasts resulted in labeling mainly subunit H, suggesting that this subunit is largely exposed to the cytoplasmic but probably also to the inner side of the membrane. On the other hand, the smaller subunits must be completely embedded in the membrane [11]. At the same time large amounts of the light harvesting pigment-protein complex were labeled, indicating that at least part of the bulk pigment protein complexes are on the cytoplasmic side accessible to iodination [12] (with *R. rubrum*).

It seemed to us of special interest to extend these localisation studies by experiments with a lipophilic marker molecule. This technique was introduced recently by Gitler and coworkers [13,14] using photoaffinity labeling with liophilic azide compounds. The marker reagent is allowed to distribute in the membrane according to its affinity to the different phases in the membrane in the absence of light. Upon ultraviolet activation covalent bonds to the components close to the label molecules were formed. The amount of label found in distinct membrane fractions gives an answer on the microenvironment in the membrane of the components in question, supposed that the reactivity of the marker molecule is the same to all components of the membrane.

Materials and Methods

In all the experiments, the carotenoidless mutant G-9$^+$ of *R. rubrum* was used. Cells were grown in light and chromatophores and reaction centers were prepared as described before [4]. The lipids were extracted in the cold with

organic solvent (acetone/methanol, 7 : 2, v/v) and analysed by thin-layer chromatography [15]. The proteins were separated on 12% SDS gels at constant voltage according to Laemmli [16]. Pigments and quinones were analysed as described earlier [4]. 5-[^{125}I]Iodonaphthyl-1-azide was prepared from 1,5-diamino-naphthaline and analysed according to Bercovici and Gitler. The specific activity of the label was in the range of 0.4 Ci/mol.

Labeling was carried out at 4°C under stirring in the dark using 10 ml of chromatophores (12—16 mg protein/ml) in phosphate buffer (10 mM, pH 7.0) or 1 ml of reaction centers (2.2 mg protein/ml) in Tris-HCl buffer (10 mM, pH 8.0). Iodonaphthylazide was added in ethanol (2 mCi for chromatophores, 1 mCi for reaction centers) in a final concentration of 1% which had no effect on the nativity of chromatophores or reaction centers as judged from their absorption spectra. The preparation was irradiated with ultraviolet light between 300 and 400 nm (Philips TW 6 W) at 4°C at a distance between sample and light source of 3 cm or with red light (above 600 nm) with Osram K 25 W lamp equipped with a filter R-62 (Balzers). After irradiation (chromatophores for $1\frac{1}{2}$ to 2 h, reaction centers for 1 h) the samples were dialyzed against bovine serum albumin buffer (5 mg protein/ml) for 48 h. A better washing procedure was achieved when the chromatophores were centrifuged (for 60 min at 305 000 $\times g$) several times in the presence of 10 mg/ml bovine serum albumin until the supernatant was essentially free of radioactivity. Gels of iodinated membranes were stained first with Coomassie Blue and then cut into slices of 1 mm. Their radioactivity was determined in a γ counter.

Results

In Table I and II, the activities found in the organic solvent fraction and in the pellet of this extraction are compared when either chromatophores or purified reaction centers were subjected to treatment with iodonaphthylazide in ultraviolet light and in red light. Furthermore, the distribution of the iodonaphthylazide label among the two fractions of reaction centers prepared from ultraviolet light treated chromatophores is given. Ultraviolet light labeling of either chromatophores or reaction centers give essentially the same distribution. The activity found in the protein containing residue after ultraviolet light labeling is around 10%. However, in reaction centers isolated from ultraviolet light-labeled chromatophores, higher amount of label was found to be bound to the proteins. When the labeling occurs in red light, conditions where the azide is not activated but the photosynthetic pigments and the quinones as components of a working electron transport are possibly in more reactive state, the activity found in the proteins is reduced to less than 1%.

As a control, ultraviolet photoaffinity labeling was also done with the extracted lipids in organic solution. Since the distribution of the label was similar as with chromatophores, very different reactivity of the compounds analysed must be assumed.

The organic fractions of the experiments in Tables I and II were further analysed (Tables III and IV). The labeling was determined in phospholipids, pigments and in quinones and the specific radioactivity of these compounds was calculated. Bacteriochlorophyll, bacteriopheophytin and quinones were

TABLE I

DISTRIBUTION OF IODONAPHTHYLAZIDE LABEL AFTER ACETONE/METHANOL TREATMENT OF CHROMATOPHORES UNDER DIFFERENT LABELING CONDITIONS

Iodonaphthylazide-labeled and washed chromatophores were extracted with 10-fold vol. ice-cold mixture of acetone/methanol (7:2, v/v), the soluble part was separated from the residue by low speed centrifugation and the radioactivity determined in both fractions.

	Labeling conditions			
	(1) Illumination with ultraviolet light for 90 min		(2) Illumination with red light for 60 min	
	cpm	%	cpm	%
Total activity in chromatophores	$8.58 \cdot 10^6$	100	$6.97 \cdot 10^6$	100
Activity in supernatant (pigments, quinones, phospholipids)	$7.72 \cdot 10^6$	90	$6.83 \cdot 10^6$	98
Activity in sediment (proteins, lipopolysaccharides *)	$8.58 \cdot 10^5$	10	$1.39 \cdot 10^5$	2

* Lipopolysaccharides may be present in *R. rubrum* chromatophores due to contamination by the outer membrane.

clearly labeled higher than the phospholipids. The bacteriopheophytin exhibited a 10-fold higher specific labeling than bacteriochlorophyll or quinones and a 100-fold higher specific labeling than the average of the phospholipids. The amount of label in the pigments and quinones compared to the one in the structural lipids was still more pronounced when red light was used to activate the system. However, labeling over a long time in the dark (14 days) by thermal inactivation of the iodonaphthylazide in chromatophores diminished these differences by a factor of 2 to 3 and strongly increased the label in the proteins (not shown).

A first unknown compound on the thin layer plates contained in all cases

TABLE II

DISTRIBUTION OF IODONAPHTHYLAZIDE LABEL AFTER ACETONE/METHANOL TREATMENT OF REACTION CENTERS UNDER DIFFERENT LABELING CONDITIONS

Labeled and dialysed reaction centers (column 1 and 2) and reaction centers isolated from labeled chromatophores (column 3) were dialysed against distilled water and then extracted following the procedure given in Table I for chromatophores.

	Labeling conditions					
	(1) Illumination of reaction centers with ultraviolet light for 60 min		(2) Illumination of reaction centers with red light for 60 min		(3) Illumination of chromatophores with ultraviolet light for 2 h and isolation and analysis of the reaction centers	
	cpm	%	cpm	%	cpm	%
Total activity in reaction centers	$5.07 \cdot 10^7$	100	$3.88 \cdot 10^7$	100	$7.94 \cdot 10^6$	100
Activity in supernatant (pigments, quinones, phospholipids)	$4.55 \cdot 10^7$	90	$3.86 \cdot 10^7$	99.5	$6.19—6.51 \cdot 10^6$	80
Activity in sediment (proteins, lipopolysaccharides)	$5.10 \cdot 10^6$	10	$1.16 \cdot 10^5$	0.5	$1.43—1.74 \cdot 10^6$	20

TABLE III

DISTRIBUTION OF IODONAPHTHYLAZIDE LABEL IN SUPERNATANT OF ACETONE METHANOL-TREATED CHROMATOPHORES AFTER SEPARATION BY THIN-LAYER CHROMATOGRAPHY

Aliquots of the acetone/methanol extract of the experiment described in Table I were separated on thin-layer plates [4]. The radioactivity of each spot was determined after extracting the silica gel removed from the plate. Specific activity was calculated with concentration values determined separately [4].

	Labeling conditions			
	Illumination with ultraviolet light for 90 min		Illumination with red light for 60 min	
	Specific activity (cpm/mmol)	% of activity of supernatant	Specific activity (cpm/mmol)	% of activity of supernatant
Phospholipids	$4.1 \cdot 10^8$	9	$8.1 \cdot 10^7$	2
Bacteriochlorophyll *a*	$9.4 \cdot 10^9$	29	$2.5 \cdot 10^9$	10
Unidentified yellow compound	—	6	—	6
Bacteriopheophytin	$7.1 \cdot 10^{10}$	13	$6.4 \cdot 10^{10}$	15
Rhodoquinone and free iodonaphthylazide label not removed by washing or dialysis	—	35	—	60
Ubiquinone	$4.9 \cdot 10^9$	6	$3.4 \cdot 10^9$	5

TABLE IV

DISTRIBUTION OF IODONAPHTHYLAZIDE LABEL IN SUPERNATANT OF ACETONE METHANOL-TREATED REACTION CENTERS AFTER SEPARATION BY THIN-LAYER CHROMATOGRAPHY

Aliquots of the acetone/methanol extract of the experiment described in Table II were separated by thin-layer chromatography and analysed following the procedure given in Table III.

	Labeling conditions					
	Illumination of reaction centers with ultraviolet light for 60 min		Illumination of reaction centers with red light for 60 min		Illumination of chromatophores with ultraviolet light for 2 h and isolation and analysis of the reaction centers	
	Specific activity (cpm/mmol)	% of activity of supernatant	Specific activity (cpm/mmol)	% of activity of supernatant	Specific activity (cpm/mmol)	% of activity of supernatant
Phospholipids	$8.2 \cdot 10^9$	6.2	$3.3 \cdot 10^8$	<1	$3.2 \cdot 10^9$	15—20 *
Bacteriochlorophyll *a*	$3.9 \cdot 10^{10}$	3.4	$3.4 \cdot 10^{10}$	4	$8.7 \cdot 10^9$	5
Unidentified yellow compound	—	n.d.	—	n.d.	—	5
Bacteriopheophytin	$3.8 \cdot 10^{11}$	19	$4.0 \cdot 10^{11}$	23	$4.3 \cdot 10^{10}$	13
Rhodoquinone and free iodonaphthylazide label not removed by washing or dialysis	—	65	—	67	—	50
Ubiquinone	—	n.d.	—	n.d.	—	3

* The phospholipid content varied from preparation to preparation due to variable amounts of bound lipids.

the highest amount of label. From its mobility on the thin layer plates, its spectrum and the fact that after reduction with borohydride similar spectral changes as in upiquinone were observed, it was identified as rhodoquinone. This compound seems to be a specific component occurring only in *R. rubrum* [18,19].

When the pool of phospholipids isolated from chromatophores or from reaction centers was separated after the labeling procedure into the main components diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine, no significant differences between the specific labeling of the three phospholipids were observed. Since their relative amounts in the membrane and in the reaction center complexes differ, the total activity found in each species reflects the abundance of each lipid in the membrane (not shown).

Besides the lipid part of the membrane, the proteins were analysed by SDS gel electrophoresis. When isolated reaction centers are treated with iodonaphthylazide, all three subunits of the reaction center as well as the remaining light harvesting protein contained the radioactive label. Strong labeling was also observed on the gels in the region of the front band due to radioactivity bound to the structural lipids surrounding the reaction center protein as well as to the highly active pigments and quinones (Fig. 1b).

When the radioactivity patterns in gels are compared with those from either intact reaction centers or delipidated reaction center proteins, the portion attributable to the lipids can be calculated (see Fig. 1). A similar labeling is

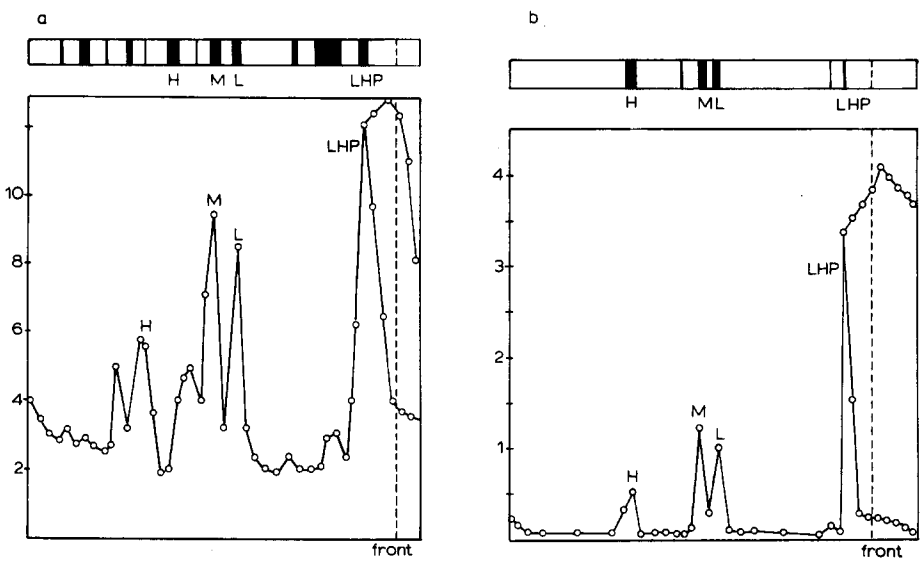

Fig. 1. Distribution of radioactivity in membrane proteins of labeled chromatophores and reaction centers after separation on SDS gels. (a) Iodonaphthylazide-labeled chromatophores. Chromatophores were solubilized with SDS and the proteins separated on SDS gels (12%) according to Laemmli [16]. The bar on top of the distribution of the radioactivity sketches the staining of the gel with Coomassie Brilliant Blue. At the front of the gel, the field between the two lines indicates the radioactivity found in the different lipids (pigments, quinones and phospholipids) obtained by comparison of gels of native (upper line) and delipidated (lower line) chromatophores (procedure as given in Table I). (b) Reaction centers were solubilized with SDS and the proteins separated on 12% SDS gels according to Laemmli [16]. Details as given in (a). LHP, light-harvesting protein; L, H, M, subunits of the reaction centre.

seen after incubation of whole chromatophores with iodonaphthylazide (Fig. 1a) and in reaction centers isolated from labeled chromatophores (not shown). Again a large part of the radioactivity is found in the light harvesting protein and the lipids and pigments at the front of the gel. Besides the three subunits of the reaction center and the light harvesting protein, several slower moving protein bands of unknown origin were labeled. Based on a 1 : 1 : 1 stoichiometry for the three subunits and a molecular weight of 21 000, 24 000 and 29 000 the specific labeling in all experiments for L and M was about equal, the one for subunit H, however, was quite lower. From these data it is concluded that all three subunits of the photosynthetic reaction center posses hydrophobic regions which are able to react with the iodonaphthylazide.

Discussion

Iodonaphthylazide, so far used as label for integral proteins in membranes, binds in our preparations preferentially to the lipids of the membrane (90—97%) although it cannot be completely ruled out that the ice-cold acetone/methanol mixture contains small amounts of proteins soluble in organic solvents. After activation with ultraviolet light, which is the usual photoaffinity labeling procedere, 10% of the radioactive iodonaphthylazide is bound to proteins. This portion is reduced to less than 1% when the activation is achieved by red light absorbed by the photosynthetic pigments. These results are obtained irrespective of whole chromatophores or isolated reaction centers used as the biological material for the labeling. In reaction center complexes isolated from ultraviolet light-labeled chromatophores, the amount of the label found in the proteins is increased to about 20%. This reflects partly the smaller lipid to protein ratio. Furthermore, the increased labeling of the reaction center proteins indicates that the membrane proteins are distributed in a nonhomogeneous manner, the reaction center proteins being clearly more labeled than the avrage of all proteins of the chromatophore membrane. Of special interest is the high labeling of the functional lipids. The pigments and the quinones show a 10 to 100 times higher specific labeling than the structural phospholipids. In spite of chemical differences among the latter, no differences in the labeling between the three main species are observed. The high labeling of the functional lipids may on one side suggest that the microenvironment of these molecules must be of a very lipophilic nature. However, these lipid molecules are labeled in a similar manner when exposed to ultraviolet light and iodonaphthylazide after extraction with methanol/chloroform. It is known [20] that nucleophile groups such as aromatic amine groups may show better reactivity to nitrenes than others. This would explain the higher specific labeling of rhodoquinone compared to ubiquinone and the high level of label in the tetrapyrrols. The differences between chlorophyll and pheophytin and the similarities among the phospholipids are not explained by this fact.

Labeling studies with red light show that a covalent binding of iodonaphthylazide is also achieved under these conditions. Similar results were reported recently by Feher et al. [21] using an azidoanthraquinone as marker. Since the label does not absorb light above 400 nm, it must be assumed that the red light is absorbed by the photosynthetic pigments and the energy then trans-

ferred to iodonaphthylazide. The higher specific labeling of the pigments in red light could then be due to this interaction between pigment and label. Such an energy transduction from pigments to iodonaphthylazide may also be possible during activation with ultraviolet light which not only activates the label used but also the absorption band of the pigment in this region. In fact, the difference between the specific labeling of the structural lipids and the pigments is highest after illumination with red light, smaller after activation with ultraviolet light and negligible after thermal degradation. However, in all cases bacteriopheophytin is labeled more strongly than bacteriochlorophyll.

It is known from spectroscopic data that bacteriopheophytin plays an important role as a transient electron acceptor in the reaction center while the quinones are components of the primary and secondary electron acceptor systems. The difference in midpotential between bacteriochlorophyll and bacteriopheophytin is in the range of 1000 mV. Since the reduction of bacteriopheophytin is 25 times faster than the transfer of the electrons from bacteriopheophytin to the iron-quinone complex and 1000 times faster than the back reaction [22,23], it must be assumed from such biophysical data that the bacteriopheophytin as well as the quinones are extremely shielded from the neighbouring components of the electron transport system, probably by an extremely lipophilic environment as suggested by the iodonaphthylazide labeling reported here. The differences in the lipophilic environment concerning the pigments are further documented by an elution diagram (Fig. 2) as first described for the lipids of *R. rubrum* by Costes et al. [24]. Bacteriopheophytin clearly eluted earlier with a more lipophilic solvent than the bacteriochlorophyll. It may be of interest that the two tetrapyrrol pigments differ in the chemistry of the side chain as well [25]. Furthermore, preliminary data suggest a different kinetic of the biosynthesis.

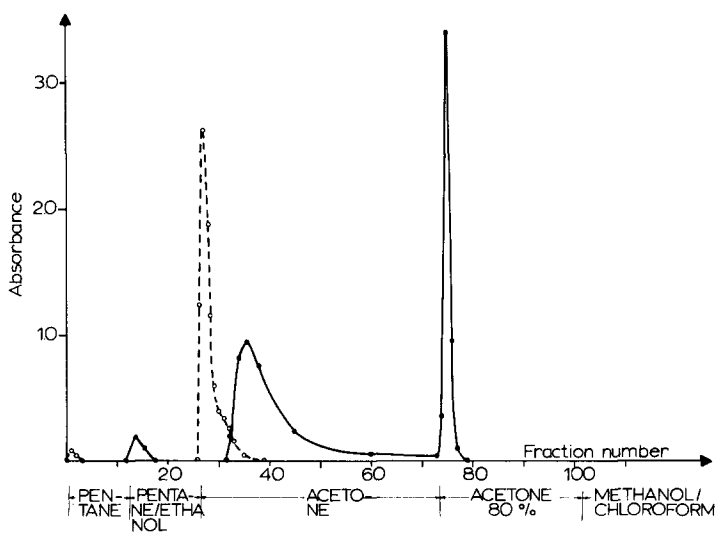

Fig. 2. Elution profile of pigments from lyophilized chromatophores with organic solvents of increasing hydrophility. 40 mg lyophylized chromatophores were packed in a chromatography column and the lipids eluted as described previously [21]. Fraction size was 15 ml. Bacteriochlorophyll was followed spectroscopically at 771 nm and bacteriopheophytin at 752 nm.

Of the three subunit proteins, the smaller L and M exhibit the higher specific labeling with iodonaphthylazide than the larger H. This agrees well with the observation that the pigments are located on the (LM)-complex [4]. The labeling pattern of the three subunits is in agreement with the present knowledge of the arrangement of the three proteins within the membrane, i.e. L and M are buried in the membrane and not exposed to a water phase while H probably spans through the membrane linking the electron donor on the inside with the proton accepting side on the outside of the chromatophore membrane.

Our results demonstrate that the method of photo-affinity labeling is not only a valuable tool for topological studies of hydrophobic membrane proteins but it can give some information about the microenvironment of functional components of lipid nature such as photosynthetic pigments and quinones. However, for such a task the shorter living and less specific acting carbenes should be preferred compared to nitrenes [26,27].

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