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PROBING THE TOPOLOGY OF PROTEINS IN THE CHROMATOPHORE MEMBRANE OF *RHODOSPIRILLUM RUBRUM* G-9 WITH PROTEINASE K

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All the major membrane proteins of isolated chromatophore vesicles are eventually degraded upon incubation with the unspecific proteinase K. These proteins must therefore be exposed at least partially or temporarily on the cytosolic surface of the membrane which is exclusively accessible to the proteinase in intact chromatophore vesicles. That the vesicles are intact during the incubation with proteinase is demonstrated by the finding that cytochrome c_2, which is located in the interior of the vesicles, is protected from proteolytic attack. The degree of degradation of the various chromatophore proteins and the time taken for degradation differ characteristically. From the changes in intensity of the gel bands during the course of digestion it appears that reaction center subunit H is digested first, much faster than are subunits M and L. The near-infrared absorption spectrum of the chromatophores changes only after proteolytic degradation of these two pigment-carrying subunits. Fading of the band of the light-harvesting polypeptide is evident only after prolonged incubation. It seems that this is the most stable component of the chromatophore membrane. The light-harvesting polypeptide appears to be somewhat shortened eventually, leaving the protein conformation necessary for holding the pigments unchanged, as shown by the absorption spectrum. The possible topology of these major membrane components is discussed in the light of these findings.

Introduction

The topology of proteins in the chromatophore membranes of *Rhodospirillum rubrum* and *Rhodopseudomonas sphaeroides* has been studied by various techniques, such as labelling with antibodies [1], radioiodination [2–4], chemical markers [5–7] and cross-linkers [8], and by proteolytic digestion [3,4,9,10]. Intact isolated chromatophore vesicles, of which it is well known that only the cytosolic surface of the membrane is exposed [3,11,12], were labelled or incubated with proteinases and the changes in different proteins were analyzed. From the results of these studies, it is generally accepted that subunit H of the reaction center is largely exposed on the cytosolic surface. Concerning the topology of subunits M and L and the light-harvesting polypeptide, however, opinions differ as the results obtained from different labelling techniques and from digestion are inconsistent. This may be due to the specificity of all these techniques for certain properties of the membrane proteins. Radioiodination is specific for tyrosine, and the chemical markers and cross-linkers used react preferentially with the ϵ-amino group of lysine. The proteases mostly used for digestion studies are trypsin and α-chymotrypsin, which are both specific for certain amino acid linkages and scarcely active on native proteins. From this it is obvious that proteins that protrude partially from the membrane surface are not necessarily recognized by these methods.

In the case of the proteases, the difficulties encountered can be overcome by using the unspecific proteinase K which is also highly active on native proteins [13]. Hence, proteins or portions of proteins accessible to this protease on the membrane surface are degraded irrespective of their composition and conformation. This study shows that all the major proteins are partially degraded by this proteinase when it attacks the cytosolic surface of the chromatophore membrane. The time taken for degradation differs widely between the different proteins. Changes in pigment-pigment and pigment-protein interactions during digestion were followed by measuring the absorption spectra and the activity of the reaction center by photooxidation and reduction of P-870.

Materials and Methods

R. rubrum G-9 was grown phototrophically on the medium described by Ormerod et al. [14]. For isolation of chromatophore membranes, the cells (0.5 g wet wt./ml) were suspended in 10 mM phosphate buffer (pH 7) and disrupted by being passed twice through a French pressure cell at 100 bar. The homogenate was centrifuged at $18000 \times g$ for 15 min. The crude chromatophores in the supernatant were then purified by several successive centrifugations and treatment with EDTA as described previously [8] but omitting the treatment by osmotic shock. Aliquots of a freshly prepared stock solution of proteinase K (Boehringer, Mannheim, F.R.G.), 2 mg/ml in 10 mM triethanolamine buffer (pH 8), were added to a sample of equal volume of chromatophore membranes in the same buffer ($A^{1cm}_{280nm} = 36$). Incubation was carried out at 30°C. Further details are given in the legends to the figures. The activity of proteinase K was stopped by the addition of phenylmethanesulfonyl fluoride (2.5 mM) of a 20-fold concentrated stock solution in ethanol. The suspension of chromatophores was then diluted 15 times with buffer and the membranes were sedimented by centrifugation (Ti 60 rotor, $230000 \times g$, 60 min, 4°C, Kontron TGA 65 centrifuge).

Near-infrared absorption spectra of the resuspended sediments were measured with a Uvicon 810 spectrophotometer. The amount of functionally intact reaction centers in the chromatophore membranes was determined by measuring the photobleaching of P-870. Measurements were made with an Aminco DW-2 spectrophotometer, using the dual-wavelength mode (600–650 nm), by illumination with light of 700–900 nm (IR filter, Balzers, Lichtenstein) and at an intensity of $1.5 \cdot 10^4$ erg/cm^2 per s (Yellow Springs Instruments 65A). Light periods of 40 s were followed by dark periods of 100 s.

SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli [15] on slab gels with a gradient of acrylamide (8–16%). The gels were stained for *c*-type cytochromes according to the method of Thomas et al. [16] and for protein with Coomassie brilliant blue.

Results

The position of reaction center subunits H, M and L on gels after electrophoresis is already well established (Fig. 1, slot 1). The light-harvesting polypeptide – only one has been described thus far [17] but the possibility of another existing has been mentioned [18] – migrates at the front not very well separated from unknown polypeptides of apparent molecular masses around 12.5 kDa [8]. It is evident that the reaction center and the light-harvesting polypeptides constitute the bulk of the chromatophore proteins (Fig. 1, slot 1). Proteinase K attacks all the proteins of the chromatophore membranes visible on SDS-polyacrylamide gel electrophoresis (Fig. 1). High molecular mass proteins including subunit H of the reaction center and a 17 kDa protein are already cleaved after a short incubation (Fig. 1, slots 2 and 3) while subunits M and L and polypeptides in the region of 12.5 kDa are digested only after a longer incubation time (Fig. 1, slots 5 and 6). The band of the light-harvesting polypeptide is the last to disappear from its original position on the gel. A new band appears in front of the light-harvesting polypeptide which increases steadily in intensity from slot 3 to 7 and then sharply from slot 7 to 8. This band most probably contains portions of several proteins inaccessible to the protease, including the light-harvesting polypeptide, from which apparently only a short part of the sequence is cleaved. Recently, this was also shown by sequence analysis of the isolated light-harvesting poly-

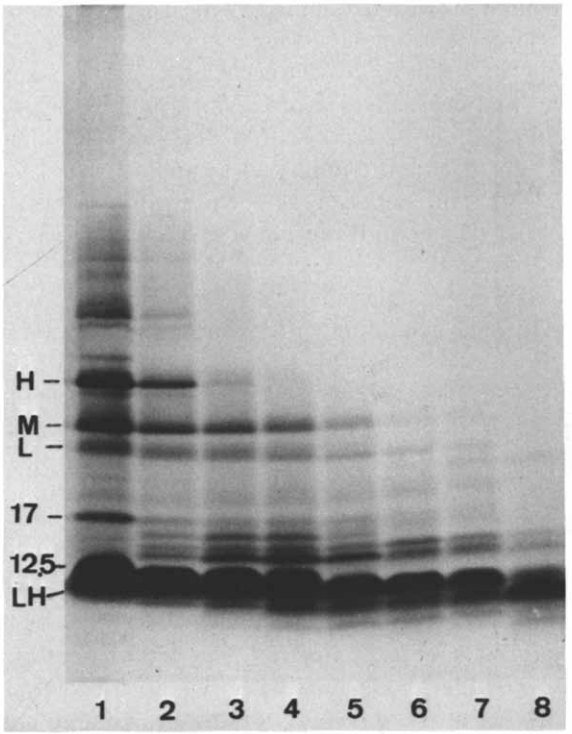

Fig. 1. Chromatophore membranes treated with proteinase K and analyzed by SDS-polyacrylamide gel electrophoresis. H, heavy; M, medium; L, light subunits of the reaction center; LH, light-harvesting polypeptide; 17 and 12.5, unknown proteins with approximate molecular masses of 17 and 12.5 kDa, respectively. (1) Untreated control, (2) 5 min, (3) 10 min, (4) 20 min, (5) 30 min, (6) 40 min, (7) 50 min, (8) 60 min incubation with proteinase.

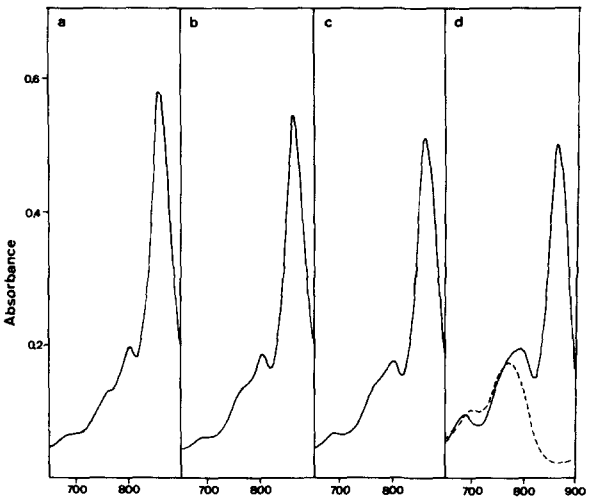

Fig. 2. Infrared absorption spectra of chromatophore membranes treated with proteinase K. (a) Untreated control identical with those incubated for 20 min (b), 40 min, (c) 60 min, (d) 135 min (———) and 15 min in the presence of 1% SDS (- - - - -) with the proteinase.

peptide after digestion of the chromatophores (unpublished results).

The near-infrared absorption spectrum of the chromatophores is a touchstone for their integrity, since pigment-protein interactions are easily disturbed, resulting in a blue shift of the absorption peaks. The absorption spectrum of the preparation in which the band of subunit H on the gel has almost disappeared (Fig. 1, slot 4) is typical for freshly prepared chromatophores (Fig. 2a). With longer digestion, the peak at 870 nm decreases steadily and the peaks at 800 and 757 nm begin to fuse, due to an increase in an absorption band between the two peaks (Fig. 2b and c). Moreover, a new peak appears at 680 nm. Finally, a reduced peak at 870 nm, a broad peak around 800 nm, and one at 680 nm are found (Fig. 2d). The peak at 870 nm is known to be due to the absorption of both the reaction center and the light-harvesting complex whereby the latter contributes most. The change of this peak from the state described in Fig. 2a to that in Fig. 2d is of the order of magnitude of the area contributed by the reaction centers. This suggests that the protease alters the light-harvesting polypeptide only in those parts protruding from the membrane on the cytosolic side of the vesicle, and not those responsible for holding the pigments. The peak at 870 nm disappears completely when SDS is added during the incubation with proteinase K, leaving a broad peak below 800 nm characteristic of the destroyed pigment-protein interaction (Fig. 2d). The change in the absorption spectrum (Fig. 2a–c) is interpreted, therefore, to be due to changes in the pigment-protein interactions of the two pigment-carrying subunits M and L of the reaction center. The proteolytic attack appears to induce structural changes in the interior of the membrane although the proteins are altered only in their exposed part. This finding holds good for the reaction center and is in contrast to that observed with the light-harvesting complex described above.

The changes in the spectra are related to altera-

tions in the activity of the reaction center. 30% of the photooxidizable P-870 is already lost after incubation for 15 min with proteinase K (Fig. 3). Addition of SDS during the incubation with proteinase K results in a complete loss of the reaction center activity (Fig. 3f).

The integrity of the chromatophore vesicles during incubation with protease was tested by measuring their content of cytochrome c_2. In intact chromatophores, all the cytochrome c_2 is contained in the interior of the vesicles where it is protected from proteolytic attack as long as the vesicles remain intact. Upon incubation with proteinase K, the cytochrome c_2 content remains constant for 1 h of incubation (Fig. 4). After 2 h of incubation, there is a small decrease in the amount of cytochrome c_2, and after $3\frac{1}{2}$ h a drastic reduction is

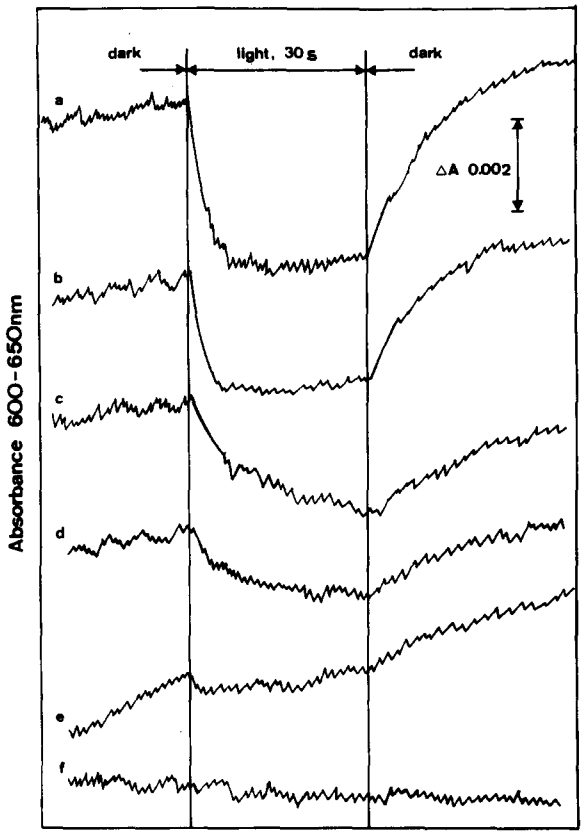

Fig. 3. Photooxidation of P-870 in chromatophore membranes treated with proteinase K. (a) Untreated control, (b) 15 min, (c) 60 min, (d) 120 min, (e) 240 min, (f) 15 min incubation in the presence of 1% SDS with proteinase.

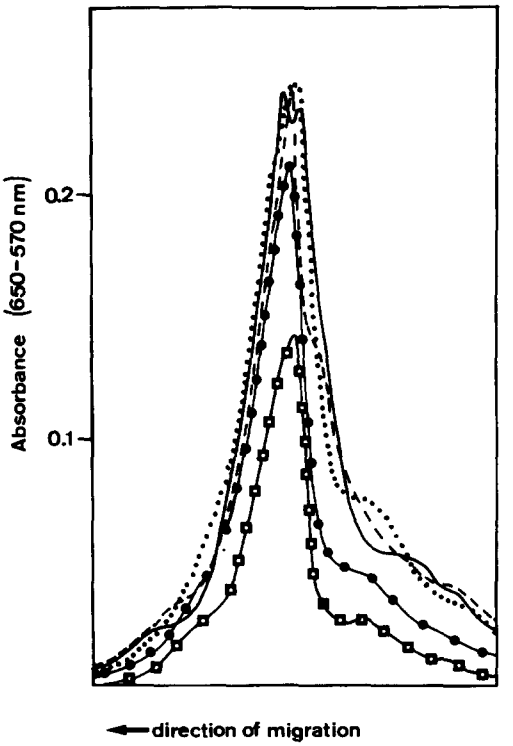

Fig. 4. Cytochrome c_2 content of chromatophores treated with proteinase K. (———) Untreated control, (— — —) 15 min, (· · · · · ·) 1 h, (●———●) 2 h, (□———□) $3\frac{1}{2}$ h incubation with proteinase. Separation and specific staining on SDS-polyacrylamide gel electrophoresis.

observed, indicating that the vesicles are ruptured. This shows that the protease acts exclusively on the cytosolic surface of the membranes for about 2 h of incubation and thereafter can also reach the opposite membrane surface.

Discussion

Treatment of chromatophores with the unspecific proteinase K reveals the accessibility of the membrane proteins independent of the composition of amino acids in the exposed part of the polypeptide chain. Very limited digestion of the chromatophore proteins was obtained only with much higher activities of the specific proteinases trypsin (165 units) and α-chymotrypsin (375 units) [3] than with proteinase K (6 units) and at a lower incubation temperature.

From our results we conclude that all of the chromatophore membrane proteins have accessible

portions on the cytosolic side of the membrane. The evidence, deriving from marker studies and from proteolysis, that only subunit H of the reaction center is exposed at the cytoplasmic surface of the membrane but not the two smaller subunits and the light-harvesting polypeptide, must be revised. Part of the amino acid sequences of all three reaction center subunits and, to some extent, also of the light-harvesting polypeptide must protrude from the membrane.

Although the chlorophyll-carrying complexes of the membrane, namely reaction center subunits M and L and the light-harvesting polypeptide, are eventually cleaved to some extent, the effect of cleavage on the pigment-protein complexes differs remarkably, as indicated by the spectra of the chromatophores. Whereas the absorption contributed by the light-harvesting complex remains unaffected, that contributed by the reaction center gradually changes. Thus, only in the case of the reaction center polypeptides does the portion of the sequences exposed to the protease on the membrane surface appear to influence the pigment-carrying part.

After a period of incubation with proteinase after which the bands of the reaction center subunits could no longer be recognized, a remaining photoactivity could still be measured. This could be explained by assuming that not all of the protein is necessary for preserving the activity of the pigment-protein complex, in particular not the sequence exposed on the surface of the membrane vesicles. Examples of such a situation are known: the partially digested membrane proteins bacteriorhodopsin [20] and erythrocyte band 3 [19] were found to retain their activity.

As the amino acid sequence of the light-harvesting polypeptide is known [17] it will be intriguing to learn now which part of the sequence of the polypeptide is degraded by the proteinase treatment and hence is exposed on the cytosolic membrane surface.

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