The Photosynthetic Apparatus of *Ectothiorhodospira halochloris* 3. Effect of Proteolytic Digestion on the Photoactivity

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Photosynthetic membranes of *Rhodopseudomonas viridis* and *Ectothiorhodospira halochloris* were treated with proteinase K. The photochemical activity (light minus dark difference spectra) were compared to the polypeptide composition (SDS-polyacrylamide gel analysis). In *E. halochloris,* difference bands appear at 806 (+), 838 (+) and 854 nm (−). All three decrease in intensity upon incubation with proteinase K., but this decrease is much slower than the proteolysis of both the reaction center and antenna related polypeptides. Photochemical activity remains high as long as a small part of the RC and two lower molecular weight polypeptides M* (22.0 kDa) and B* (15.3 kDa) are present. The M subunit is the most stable polypeptide in the RC of *Rp. viridis* too, and the photochemical activity is related to the remainder of this and to the one newly formed polypeptide (15.3 kDa), but doesn't show the typical absorption shift of the antenna (B 800/1020 → B 800/960). The results are discussed quantitatively and compared to those obtained from Bchl *a* containing organisms.

Introduction

Much interest has been focused recently on the photosynthetic apparatus of bacteriochlorophyll *b* (bchl *b*) containing bacteria [1–3]. Morphologically, they are distinct by a 2-dimensional crystallin membrane system, which has been investigated by high-resolution electron microscopy [4–7]. A detailed 3-dimensional structure on the molecular level has been obtained by x-ray analysis for the reaction center of *Rhodopseudomonas (Rp.) viridis,* which was the first membrane protein to be cristallized [8, 9]. Reaction centers of bchl *b*-containing bacteria are biochemically distinct from (the much more abundant) bchl *a*-containing species, by the presence of a fourth (cytochrome *c*) subunit [8–10] in addition to the three polypeptides (H, M and L) found in the reaction center of bchl *a* containing bacteria [11].

In view of this higher complexity, the question arose, what is the minimal size of a bchl *b* containing RC? This is of particular interest, since there are several reports, that bchl *a* containing RCs retain their photochemical activity after partial proteolytic degradation [12–13], removal of the H-subunit, or by reductive elimination of one of the four RC bacteriochlorophylls [14].

Here, we wish to report proteolytic studies on the photosynthetic membranes from *Ectothiorhodospira (E.) halochloris* and *Rp. viridis.* The degradation has been followed by SDS-PAGE, and the functioning of electron transfer at the same time by light-minus dark difference spectroscopy. Electron transport is retained if the largest RC-related polypeptide is 17 kDa, with most of the subunits degraded even further. At the same time, this is the first partial characterization of RCs from *E. halochloris.*

Material and Methods

E. halochloris was grown anaerobically in the light as described earlier [15]. *Rp. viridis* was grown in Pfennigs medium (DMS medium 27, see [16]), in 151 flaskes. Cells were harvested by centrifugation at $10{,}000 \times g$ and washed twice with tris buffer (10 mM, pH 7.5). Chromatophores or rather thylakoids were prepared according to the method of Feher and Okamura [11] and adjusted to an absorbance of 50 cm^{-1} at 1020 nm. They were stored at ≤ −18 °C until use.

For the proteolytic experiments 500 µl of the chromatophores were incubated either with different concentrations of proteinase K for a defined time, or

Abbreviations: Rp., Rhodopseudomonas; E., Ectothiorhodospira; Rs., Rhodospirillum; bchl, bacteriochlorophyll; MW, molecular weight; RC, reaction center; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonylfluoride.

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with a constant concentration for different times. At defined times, one aliquot of the incubation mixtures was diluted and then used immediately for absorption and light-minus-dark difference spectroscopy. In the remainder, the digestion was stopped as in earlier investigations [17] by adding PMSF (saturated solution in acetone). It was then analyzed by SDS-PAGE (modified after Laemmli [18]) using a linear, 11.5–16.5 acrylamide gradient. For calibration in the molecular range >19 kDa, a standard set of hydrophyllic proteins was used (bovin serum albumine, hen egg albumine, lactoglobulin, pepsin, trypsinogen and lysozyme). In the low molecular weight range, calibration was done with hydrophobic polypeptides from photosynthetic antenna systems of known sequences (*Rp. spheroides* B 800/850 antenna complex; [19]). The gels were scanned after staining with Coomassie brilliant blue G on a TCD scanner (Vitratron). Silver staining was performed by the method of Dion and Pomenti [20].

The photochemical activity of reaction centers within the membrane was determined by light-minus-dark difference spectroscopy on a DMR 22 (Zeiss Oberkochen) or a ZWS II spectrophotometer (Sigma, Berlin) connected with a BS 8000 intelligent recorder (Bryans, Mitcham). Both instruments were equipped for cross illumination in order to allow measuring and illuminating at the same time (excitation with 606 nm interference filter, sampling >700 nm by use of a 695 nm low-pass filter; both Schott, Mainz). Light induced difference spectra in the latter instrument were limited to <950 nm due to the photomultiplier response.

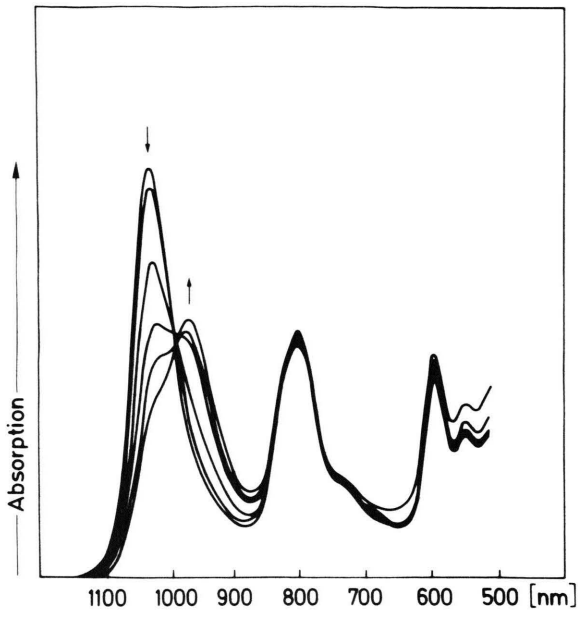

Fig. 1. Kinetics of the digestion of *E. halochloris* chromatophores with proteinase K. Absorption spectra recorded at 5 min intervals.

Results

Absorption spectroscopy

Thylakoids of *E. halochloris* have two absorption bands in the long wavelength region. The one around 1020 nm is typical for bchl *b* containing bacteria, the other one is only found in the closely related species, *E. abdelmalekii* [21]. When chromatophores of *E. halochloris* are treated with proteinase K in buffered solution in the dark, the 1020 nm absorption band is shifted to 960 nm [15] and its intensity decreased by appx. 40%. The transformation gives an isosbestic point at about 1000 nm (Fig. 1).

The second near infrared band at 800/830 nm and other bands are not significantly affected by this treatment. At a proteinase K concentration of 0.4 µg/ml and pH = 8.0, the half-life is about 70 minutes (equal absorptions of the native 1020 nm and of the 960 nm form).

The same spectral shift was obtained upon titrating chromatophores with proteinase K in the light. However, the 960 nm form of bchl *b* is much more photolabile and reacts further to chl *a* related oxidation products of bchl *b* ($A_{max} \approx 680$ nm) [22]. During this last conversion, an intermediate shift from 960 to 940 nm was observed. It should be noted, that a similar shift is sometimes found in aged RCs of *Rp. viridis* (Hajek [23]) or during cristallization of the latter (H. Michel, pers. com.) and may be related to lipid interactions [24].

When chromatophores of *Rp. viridis* are treated with proteinase K the 1020 nm absorption form is transformed directly into the absorbing pigments at 680 nm (Fig. 3). No intermediates like in *E. halochloris* could be identified during this process except for a slight absorbance increase between 900 and 950 nm. The reaction of *Rp. viridis* is much more sluggish and requires appx. twofold amounts of proteinase K for a similar decrease around 1020 nm.

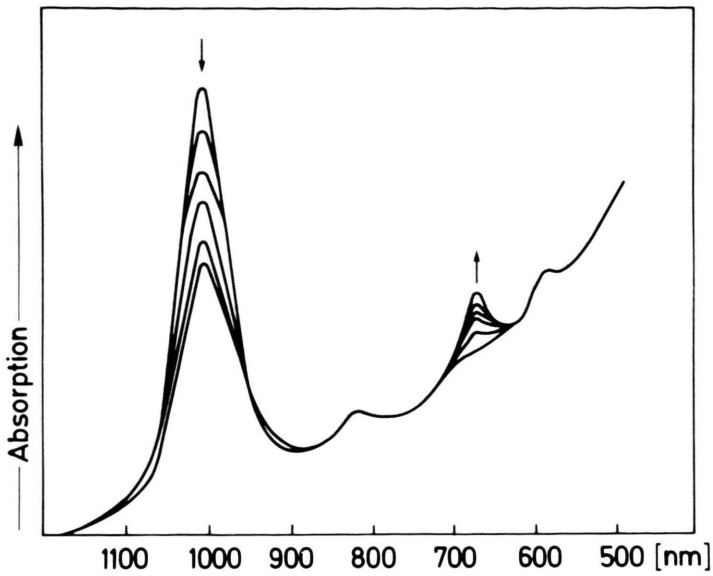

Fig. 2. Kinetics of the digestion of *Rp. viridis* chromatophores with proteinase K. Absorption spectra recorded at the same intervals as in Fig. 1.

Light-minus-dark difference spectroscopy of thylakoids

Intact thylakoids

The light-minus-dark difference spectrum of *E. halochloris* shows in the wavelength range from 700 to 900 nm three bands, two positive extrema at 806 and 838, and a negative one at 854 nm (Fig. 3A). The two extrema at longer wavelengths form an "S"-shaped feature. This difference spectrum is somewhat similar to that of *Rp. viridis* (Fig. 3B, extrema at 812 and 850 nm) but with more fine-structure in the 800 nm region. This spectrum of *Rp. viridis* is in agreement with earlier reported data [25]. It should be noted, that the absorption difference in *E. halochloris* are smaller by factor of 4–5 than in *Rp. viridis* if normalized to the 1020 nm absorbance. The difference spectra in the longer wavelength region are qualitatively similar ($A_{max} \approx 1000$ nm), but could not be evaluated quantitatively in the dual wavelength mode with the photomultiplier detection system used. In *Rp. viridis* the same difference spectra were obtained as in the light upon chemical oxidation of *Rp. viridis* chromatophores with potassium ferricyanide.

E. halochloris, however, showed some spectral shifts (data not shown): The 806 nm peak shifts to 795, the 838 band to 858 nm. This may at least in part be related to the interference of an intense negative band ($A_{max} \approx 960$ nm) arising from the recently mentioned reactions of the antenna [15].

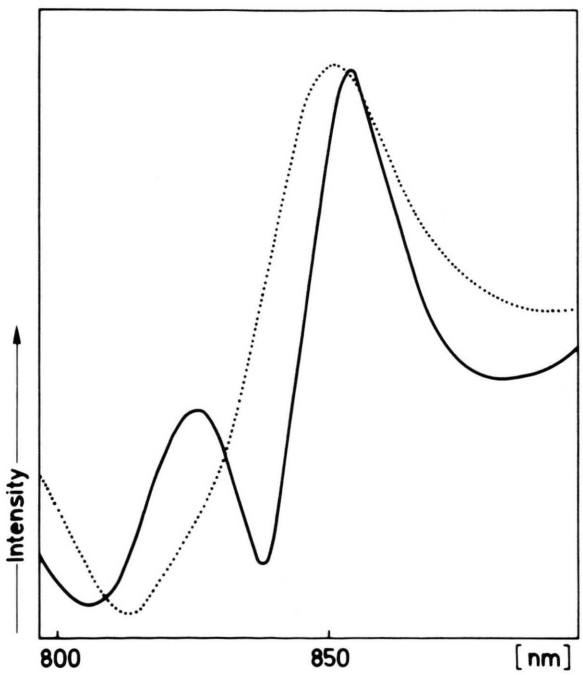

Fig. 3. Light-minus-dark differences spectra of chromatophores ($A_{1020} = 1.0$) from *E. halochloris* (−) and *Rp. viridis* (ooo). The scale of (−) → (ooo) increases fourfold as compared to (ooo).

Proteolytically digested thylakoids

All three absorption difference peaks of *E. halochloris* thylakoids decrease in intensity upon incubation with proteinase K. Indentical final absorption changes were obtained irrespective of whether the incubation was carried out with a high concentration of the enzyme (approx. 0.02 mg/ml, $A_{1020}=50$), or whether the thylakoids were titrated with proteinase K (0–0.02 mg/ml, Fig. 4) about one hour. The "S"-shaped signal (838 and 854 nm band) decreases to a limiting value of its starting amplitude in both cases – constant time (33.6%) and constant protease concentration (35.5%). The kinetics are linear to about 105 minutes (=38% decrease) and levels off, while the samples incubated with different proteinase K concentrations show a logarithmic dependence. It should be noted, that the proteolytic treatment causes a small, but distinct long wavelength shift of the difference extrema: 806 to 809 nm, 838 to 840 nm and 854 to 859 nm. The same results were obtained irrespective of an incubation of thylakoids in the light or in the dark.

For comparison the same experiments were done with chromatophores of *Rp. viridis* (Fig. 5). Here the "S"-shaped signal decreases linearly to proteinase K concentrations <0.012 mg/ml with $A_{1020}=50$. These samples show a saturation at higher concentrations. There was no shift in the maxima with increasing proteinase K concentrations, as in *E. halochloris*.

Polyacrylamid gel electrophoresis

In parallel to the absorption difference spectra described above, the changes in the polypeptide composition of the thylakoids were monitored by SDS-PAGE. Particular care was taken to ensure, that the polypeptide composition of the samples was not changed during the different procedures to be carried out with them prior to spectroscopy and electrophoresis, respectively. After several tests using different cell pathlengths and/or concentrations, the method described in the experimental part was developed. For absorption and difference spectroscopy, it involes a 50fold dilution of the incubation mixture, after which the spectra are recorded immediately. For SDS-PAGE, which requires a more lengthy sample preparation, the proteolysis was stopped by a large excess of PMSF, and the proteins were precipitated at the same time with acetone used as solvent for the reagent.

Chromatophores ob bchl *b* containing organisms show only a rather small number of protein bands in the SDS gels (<10). It is therefore possible, to distinguish well between the original bands and new ones arising from proteolysis. A comparison of the apparent molecular weights of both *Rp. viridis* and *E. halochloris* chromatophores has been described earlier [17]. The changes in the polypeptide pattern during a digestion of *E. halochloris* thylakoids are shown in Fig. 6. The most labile band of the *E.*

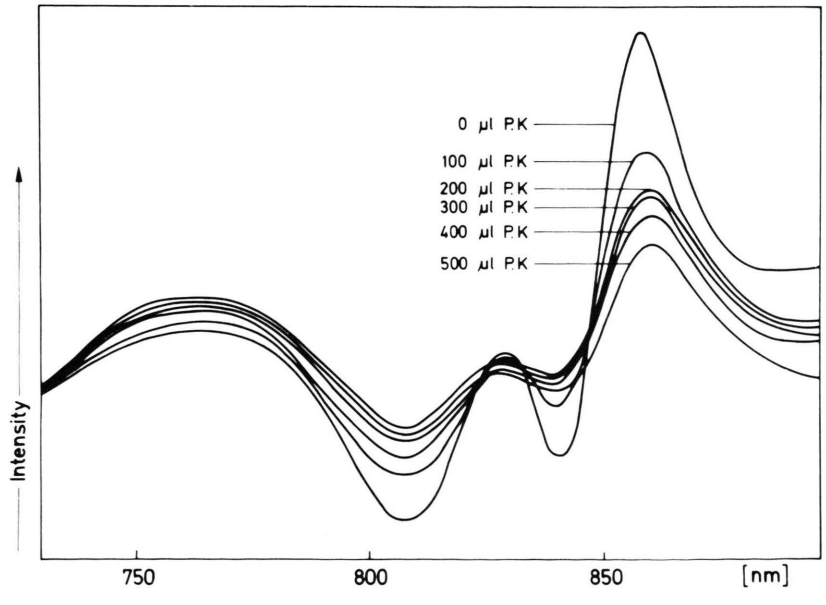

Fig. 4. Titration of the chromatophores from *E. halochloris*. (A_{1020} = 1.0) Light-minus-dark difference spectra of samples incubated for 60 minutes with increasing amounts of proteinase K (2 mg/ml) in the light.

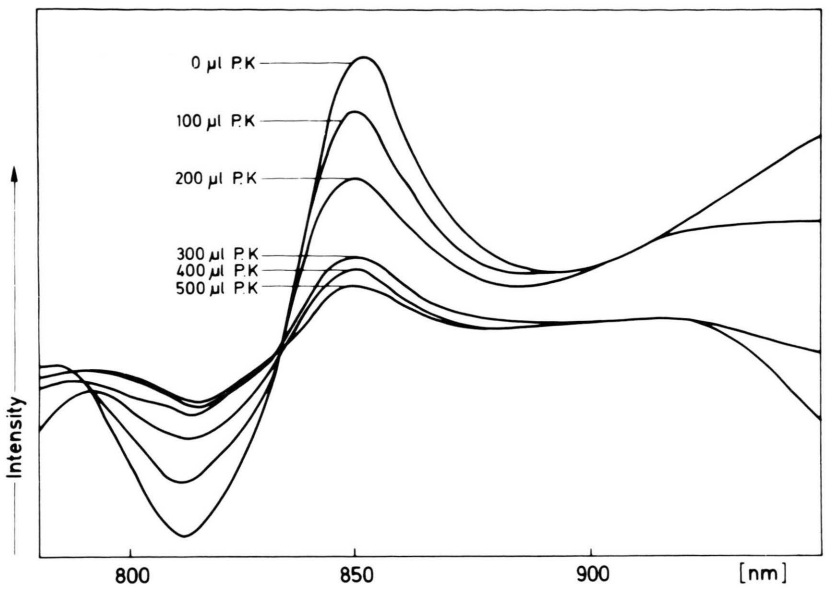

Fig. 5. Titration of chromatophores of *Rp. viridis* with proteinase K. Conditions as in Fig. 4.

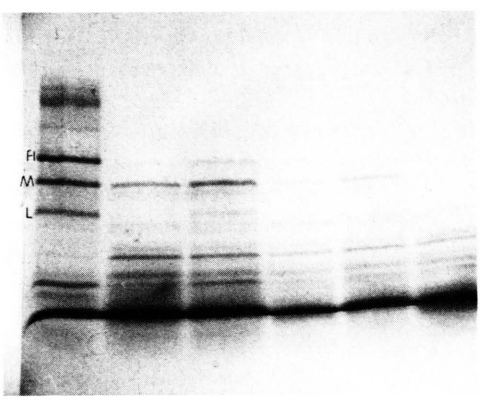

Fig. 6. SDS-PAGE of digested *E. halochloris* thylakoids.

halochloris is the one assigned to the cytochrome band (34.0 kDa). This band is present in varying amounts in thylakoids of *E. halochloris* due to losses during their preparation. The band assigned to the H subunit (28.0 kDa) of the RC disappears next. It is no longer detectable after incubation with 0.004 mg proteinase K/ml for 90 minutes, or with 0.02 mg/ml for 5 minutes. The L subunit of the RC disappears also very quickly. A quantitation is difficult, for it is weakly stainable already in the original chromatophores. A kinetic evaluation was therefore not possible. The M subunit (24.0 kDa) seems to be the most stable one of the RC (Fig. 7). There is a rapid decrease in the first 15 minutes of the incubation (71.4%) to a nearly constant level at 120 minutes amounting to 21.5% of the original intensity.

Three new bands with sizes >15 kDa arose during the incubation. The highest MW band (approx. 60 kDa) visible in some gels is an aggregate of proteinase K (MW of the monomer ≈ 19.0 kDa), as shown by blanks. The other two newly formed bands arise from fragments of the RC polypeptides. The first one moves between the M and the L subunit.

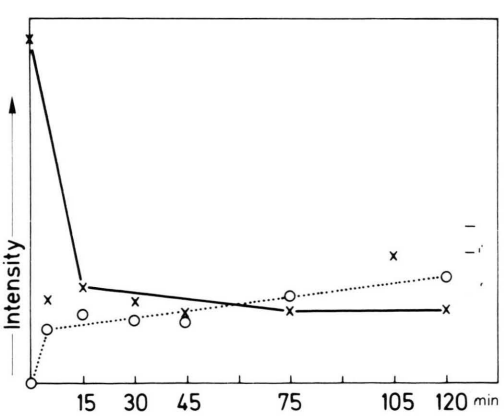

Fig. 7. Decrease of the M subunit (−) and the increase of M* (− − −) from the RC of *E. halochloris* during the digestion with proteinase K. The ordinate gives the integrated intensity of the respective band after staining with Comassie Brillant, normalized to the intensity of M at time zero.

The apparent molecular weight of this band designated "M*" is 22.0 kDa. Just like M, M* is rather stable and can be found until the end of the incubation (Fig. 8). Theoretically, M* can originate from M or H but we tend more to the former for the kinetic of its appearence shows a relative sharp increase during the first minutes, which is similar to the decrease of the M-subunit, described above. The second new band arises in the region between the RC and antenna bands. It has been termed "B*", and has an apparent molecular weight of 15.3 kDa. It appears just after beginning the incubation (like M*) and reaches a maximum intensity of 15%, relative to that of M before the experiment.

The low molecular weight polypeptides related to the antenna complexes, are labile to proteolysis, too. The first change is a slightly increased mobility of the 6.0 kDa-peptide (β-subunit) corresponding to a decreased size (approx. 5.3 kDa). At higher proteinase concentrations an additional more mobile band appears below the former. These three bands remain visible throughout the incubation, and are only slowly degraded further.

The whole membrane of *Rp. viridis* shows similar polypeptide bands as *E. halochloris*, but all apparent MWs are somewhat larger (see above). The H subunit and the cytochrome band of the RC are digested within less than five minutes even at the smallest proteinase K concentration used. The M subunit is detectable up to 0.06 mg proteinase K/ml with $A_{1020} = 50\ cm^{-1}$. There was no new band in the RC region, but between RC and antenna region a new band appears (MW ≈ 19.0 kDa) which then was digested upon addition of more proteinase (Fig. 9). Compared to the results obtained with *E. halochloris*, there is a similar order of stability among the RC polypeptides. In both organisms M seems to be the most stable RC subunit. In the region between the RC and antenna related polypeptides both bacteria show a new band (15.3 and 19.0 kDa respectively), which remained detectable during the entire incubation period. The main difference is the new M* band in *E. halochloris* and the changes in the antenna region, which were not observed in *Rp. viridis*.

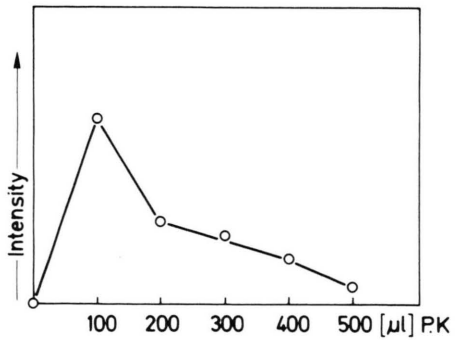

Fig. 8. Increase of newly formed polypeptide (19.0 kDa) from *Rp. viridis*, during a titration with proteinase K. Details as in Fig. 7.

Correlation SDS-PAGE and absorption

In all experiments, the light-minus-dark difference spectra of *E. halochloris* showed a decrease of the photoactivity of both components, the 806 nm band and the "S"-shaped signal. The loss of photochemical activity is, however, much slower than the digestion of even the most stable RC polypeptide. The photoactivity is *e.g.* still as high as 90% when the cytochrome, H and L subunits are no longer detectable and the most stable M-band is digested to an amount of 25% of its original value. It is noteworthy, however, that the combined intensities of the new bands (22.0 and 15.3 kDa) were relatively constant during the incubation.

This data show, that not only whole RC polypeptides, but also fragments thereof are capable of typical photoreaction. H does not seem to be necessary for the photochemistry, if we assume, that both the M* and B* peptide did not originate from it.

The changes in the antenna region can be discussed in relation to the energy-transfer, too. Although they are not as dramatically as in the RC region, the digestion of the antenna peptides can principally also induce a decrease of the photoactivity, due to uncoupling from the RCs. *Rp. viridis* showed the same tendency as *E. halochloris* (data not shown); photoactivity here is combined with a part of the M subunit and a characteristic new polypeptide (19.0 kDa).

Discussion

Albeit we were not (yet) able to isolate the RC of *E. halochloris*, the light-induced difference spectra and SDS-PAGE data indicate that they are similar to the ones from *Rp. viridis*. The light-minus-dark difference spectrum of *E. halochloris* shows maxima at 806 (+) and 838 (+) and 854 (−) nm; the latter two form an "S"-shaped signal. This indicates, that the intact RCs of *E. halochloris* have an absorption in

this region. Whereas a similar "S"-shaped signal is present in *Rp. viridis*, an analogon to the positive 806 nm peak could not be found. This band seems to be characteristic for the *E. halochloris* RC, or it is hidden under the other bands of *Rp. viridis*. In addition to the difference bands <900 nm, both species show a large absorption decrease at 1000 nm related to the bleaching of the primary donor P980 [25] or P990 [26].

The exact position of this band is difficult to determine, and seems to be rather sensitive to the environment [25, 26, 11, 24 and refs. cited there in]. In view of the low photomultiplier response of our apparatus, this band was not investigated quantitatively.

Partial controlled proteolytic digestion caused changes in the polypeptide pattern and also the absorption spectra. There are in principal two possibilities for a decreased photochemistry in thylakoids upon proteolysis:

1. the RC itself is inactivated, *e.g.* functionally important parts of the RC are digested and

2. the energy transfer from the antenna to the RC is blocked, viz. functionally important parts of the antenna are digested (too). Both *E. halochloris* and *Rp. viridis* show a continous decrease of the photoactivity. It remains high, however, even after extensive proteolysis of *both* the antenna and the RC. This means, that both the energy transfer from the antenna *and* the electron transfer remain efficient under these conditions. It should be noted, that electron microscopy also shows only a small disordering of the originally α-dimensionally crystallin membrane system (R. Steiner *et al.* in prep.). In the context of this work, only the changes related to the RC polypeptides shall be discussed in more detail.

The cytochrome and the H-subunit are not necessary for photochemistry in both bchl *b* containing species, *E. halochloris* and *Rp. viridis*. This is in line with bchl *a*-containing species, where the H subunit also does not seem to play an essential role for the photoactivity [27]. The H subunit has been shown to be located close to the antenna and the L and M-subunits of the RC: crosslinking experiments [28] show a linkage of H with the antenna and the M and L subunit of the RC [29]; no chromophore is found in H of *Rp. spheroides* [11] or *Rp. viridis* [9], in RCs of *Rs. rubrum* H is partly digestable while the photochemical activity remains high [30], in *Rp. viridis* the H subunit is peripher [9]; in *E. halochloris* an antenna complex has been isolated, that contains the H subunit [15].

From these results it seems a general feature, that H does not play a crucial role in the overall photoactivity (but see Feher for kinetics of secondary electron transport).

In view of the intact energy transfer, H seems also disposable for this function, and its role remains to be defined. Of the two remaining subunits (L, M), the former one is also lost very rapidly, and even the latter (M) is not required intact for the primary charge separation to take place. The necessary polypeptides appear to be two lower MW ones designated M* and B*, which both are most likely to arise from M. The mobilities are somewhat different in *E. halochloris* and *Rp. viridis*, but otherwise they show very similar responses.

This behavior is principally again similar to bchl *a*-containing species. However, in the latter fragments of the L-rather than of the M-subunit are required for photochemistry.

The necessary polypeptides are a small part of M and two lower molecular weight polypeptides M* and B*. *Rs. rubrum* showed its whole photoactivity as long as the L subunit and a characteristic 18.0 kDa peptide is detectable [30]. In *Rp. capsulata*, L is the most stable subunit, too, with respect to proteolysis [12].

From these data is seems to be clear, that only a small part of the whole RC is necessary for the charge transfer and that in both tested bchl *b* containing organisms the M subunit, together with two smaller peptides, are necessary for the photochemistry.

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