Probing the smallest functional unit of the reaction center of *Rhodospirillum rubrum* G-9 with proteinases

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Reaction centers composed of subunits H, M, L (28 kDa, 24 kDa, 21 kDa) were isolated from *Rhodospirillum rubrum* G-9 and subjected to progressive digestion with proteinase K. Photoactivity and spectral characteristics were retained as long as subunit L and a characteristic fragment (about 18 kDa) of another subunit, most probably of subunit M, remained intact. This was shown by sodium dodecyl sulfate polyacrylamide gel electrophoresis and by the finding of only two amino-terminal sequences one of which was identical with that of subunit L. The rate of reduction of photooxidized P-870 was decreased in the digested preparation of the reaction center as compared with the control but the kinetics of the reduction of photooxidized cytochrome c_2 was unchanged. It is inferred that subunit L and a part of subunit M form the smallest functional unit of the reaction center.

Reaction center subunit *Digestion* *Proteinase K* Rhodospirillum rubrum *G-9*

1. INTRODUCTION

The reaction center (RC) of phototrophic bacteria is responsible for primary photochemistry. It was originally described as a complex composed of three polypeptides (H, M, L) with the following cofactors: 4 bacteriochlorophylls, 2 bacteriopheophytins, 1 carotenoid, 1 or 2 quinones, and a non-heme iron [1]. Subunit H can be removed from the RC without loss of photochemical activity [1,2]. Recently, the characteristic absorption spectrum and its changes upon photooxidation reflecting the primary charge separation were found also in a preparation showing one single band on SDS–PAGE at the position of subunit L [3]. However, this is not sufficient to prove that only one polypeptide is present in the preparation and that the band in the gel really contains subunit L. The preparation was obtained by ultrafiltration in the presence of LDAO and deoxycholic acid at room temperature in the absence of protease inhibitors. Hence it may well be that breakdown products of the larger subunits comigrated in the gel with subunit L (see section 3).

To get more insight what the smallest functional unit of the RC may be we digested isolated RC (H, M, L) progressively with proteinase K. A still functional preparation of the RC was obtained containing two polypeptides. One of them migrating in PAGE with an apparent molecular mass of 21 kDa was identified by amino terminal sequencing as subunit L. The other is smaller, about 18 kDa, and most probably is a fragment of subunit M. The two polypeptides were associated so intimately that they could not be separated without inducing a concomitant loss of function.

Abbreviations: RC, reaction center; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; LDAO, lauryldimethylamine oxide

2. MATERIALS AND METHODS

Chromatophores and RC of *Rhodospirillum rubrum* G-9 were prepared as in [4] except that Tris–HCl buffer was replaced by 25 mM triethanolamine buffer (pH 8), 0.025% LDAO

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(TEA-buffer). RC (802 nm, 1 cm = 2; 280/802 nm = 1.4) were digested with proteinase K (0.8 mg/10 ml) (Boehringer, Mannheim) under the conditions given in the legends to the figures. The activity of proteinase K was stopped by the addition of 4 mM phenylmethanesulfonyl fluoride from a 200-mM stock solution in ethanol. Proteinase K was separated from the digested RC by gel filtration on Sepharose 6B (column 35 × 3.2 cm) in TEA-buffer. The grey-blue fractions were pooled and further purified by adsorption on DEAE-cellulose (column 20 × 1.5 cm) followed by washing with 200 ml of the TEA-buffer, 200 ml of the buffer containing 60 mM NaCl, and 50 ml of the buffer with 100 mM NaCl. The pigmented fraction still adsorbed on the top of the column was eluted with 300 mM NaCl in the buffer. It was pooled, dialyzed against the buffer, and then used for PAGE and activity tests. Part of the same sample was dialyzed extensively against distilled water for Edman degradation. SDS–PAGE and sample preparation were performed as in [5]. Absorption spectra were measured on an Uvicon 810 spectrophotometer. Photochemical activity of the RC was measured as photooxidation of P-870 and of cytochrome c_2. Measurements were performed on an Aminco DW-2 spectrophotometer using the dual wavelength mode 600 nm–650 nm for photooxidation of P-870 and 540 nm–550 nm for photooxidation of cytochrome c_2. Samples were illuminated with light of 700 nm–900 nm (IR-filter, Balzers, Lichtenstein) at an intensity 1.5 × 10^4 erg/cm^2/s. Amino-terminal sequences were determined by Edman degradation [6].

3. RESULTS AND DISCUSSION

The near IR spectrum of the RC remained virtually unchanged when the purified RC (subunit H, M and L) were treated with the unspecific proteinase K for up to 50 min (fig.1). Only after prolonged incubation the absorption at 870 nm decreased while the absorption at 770 nm increased indicating a change of the pigment–protein interactions which parallels loss of photochemical activity.

Interestingly, the proteinase K-treated preparations, still having the characteristic absorption spectrum of native RC, had a drastically changed polypeptide pattern as shown by PAGE (fig.2).

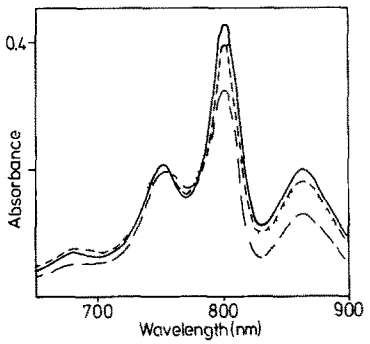

Fig.1. Near IR absorption spectra of RC incubated with proteinase K for 0 (——), 30 (···), 50 (---) and 90 (– – –) min at 25°C.

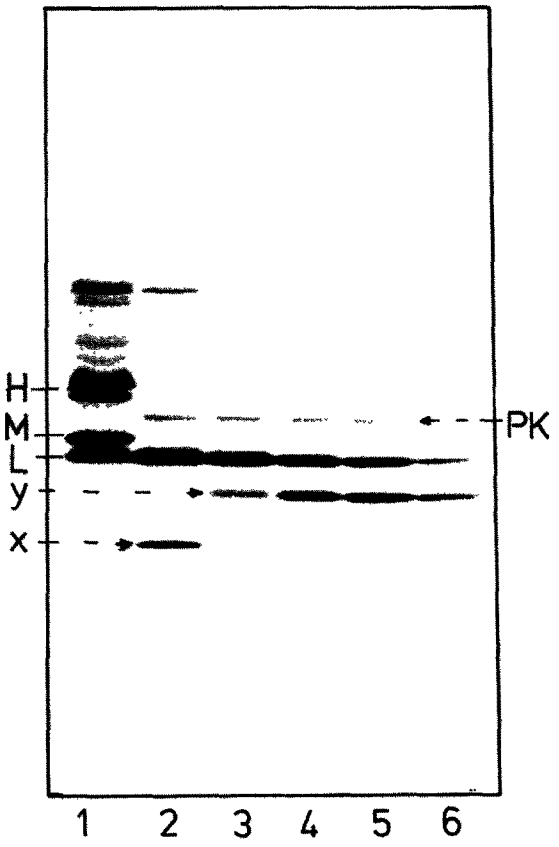

Fig.2. SDS–PAGE of RC (subunit H, M, L) incubated with proteinase K (PK). Incubation for (1) 0, (2) 5, (3) 10, (4) 20, (5) 30 and (6) 60 min at 25°C. x, unknown digestion fragment; y, supposed digestion fragment of subunit M. The formation of two bands of subunit H is due to lipopolysaccharide inference in this region of the gel [7].

After only a short incubation time subunit H and M disappeared from their original position while the staining intensity was increased in bands of polypeptides of lower molecular mass in the region of subunit L. A small polypeptide (x) appeared (fig.2, slot 2) and afterwards disappeared again. It may well be that subunit L and the digestion product increasing the band at the position of subunit L (fig.2, slot 2/3) represent the 'one-polypeptide reaction center' proposed in [3]. These authors presumed that a protease could contaminate their preparation of RC. As we could not reproduce their results the contaminating protease was probably absent in our preparation. The band at the position of subunit L regained later during digestion the original staining intensity and a new band of a smaller polypeptide (y) appeared (fig.2, slot 4). Both these bands showed a similar intensity of Coomassie blue staining. The same banding pattern as obtained with a low activity of the unspecific proteinase K is also obtained with high activities of the specific proteinases trypsin and α-chymotrypsin. This indicates that the RC digestion is not limited because of the specificity of the proteases but because of structural features of the complex.

A large preparation of a two-polypeptide RC (fig.2, slot 4) was prepared under the conditions given in the legend to fig.3. The preparation was repurified by gel filtration and ion exchange chromatography as described to remove the protease and the digestion products of the RC (fig.3, slot 2). The photochemical activity of the 3-subunit RC (control) was compared with the activity of the digested two-polypeptide RC. The reduction of oxidized P-870 in the dark is slower in the digested preparation than in the control (fig.4). In the presence of ascorbic acid the reduction rate is faster in both preparations. The kinetics of the reversible photooxidation of cytochrome c_2 are closely similar for the 2-polypeptide RC and the control (fig.5). This suggests that subunit L, the only subunit intact upon digestion, carries the binding site for cytochrome c_2. This is in agreement with the result that cytochrome c_2 was specifically cross-linked to subunit L [8]. Subunit M was suggested to carry the binding sites for quinones [9]. The change of the kinetics for reversible photooxidation by protease treatment might be due to the partial digestion of subunit M.

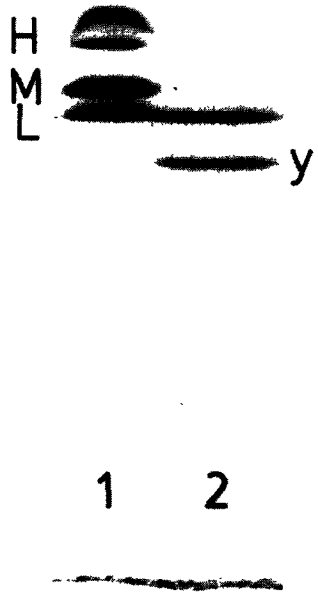

Fig.3. SDS–PAGE of the control (1) and of repurified digested RC (2) incubated with proteinase K for 30 min at 25°C; y, supposed fragment of subunit M.

For an unequivocal identification of the two polypeptides obtained after digestion and purification the amino-terminal sequences in the preparation were determined by Edman degradation. Only two main amino acid sequences were found, namely Ala, Leu, Leu, Ser, Phe, Glu, –, Lys and Gly, Val, Gly, Asp, –, Ala. The former is identical with the amino-terminal sequence of subunit L as found in *Rhodopseudomonas sphaeroides* [10] and in *Rhodospirillum rubrum* (G-9) [11]. One of the polypeptides in the digested preparation can therefore clearly be attributed to subunit L. The

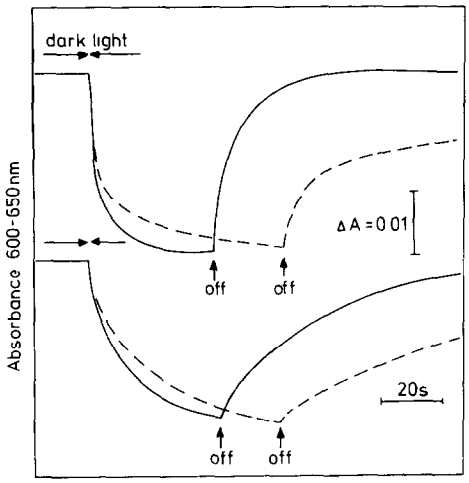

Fig.4. Reversible photooxidation of RC (802 nm, 1 cm = 0.75) in TEA-buffer. (a, top) Control without (– – –) and with (——) ascorbic acid (0.5 mM); (b, bottom) two-polypeptide RC without (– – –) and with (——) ascorbic acid (0.5 mM).

latter sequence can not yet be identified as part of another subunit as it does not correspond with any of the known amino-terminal sequences of the subunits H, M or L. It is not likely to be a fragment of subunit H since this subunit is rapidly digested, even in intact chromatophores, without producing fragments recognizable on the gel as new bands in the region of the RC bands or as intensity changes of the M or L bands [12].

Rather unlikely is also the possibility that subunit L is digested from its C-terminal end, producing in the gel the band of the smaller polypeptide (y) whilst the remainder of subunit M migrates exactly to the position of subunit L. The treatment

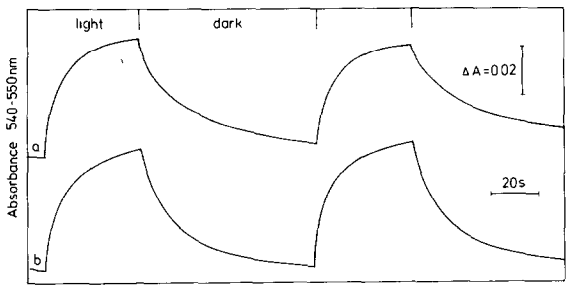

Fig.5. Reversible photooxidation of cytochrome c_2 (5 μM) with RC (1.5 μM) and ascorbic acid (5 μM) in TEA-buffer; (a) control; (b) two-polypeptide RC.

of RC with carboxypeptidase Y had no effect on any of the subunits as judged from the banding pattern on SDS–PAGE indicating that the C-terminal ends are well protected from protease. Thus the functional 2-polypeptide RC seems to be composed of the intact subunit L and subunit M shortened by around 60 amino acids of a total of about 230.

Attempts to digest selectively and without loss of activity one of the two polypeptides by other specific proteases (trypsin, α-chymotrypsin) failed. Also after gel filtration in the presence of 0.025% LDAO and 40 mM deoxycholic acid on Sephacryl S-200 (column 125 × 2.5 cm) the two polypeptides remained together. Interestingly, when chromatophore membranes are treated with proteinase K a cytosolically exposed piece of 16 amino acids is cleaved from subunit L [13] whereas this does not occur when isolated RC are treated. Possibly, in the isolated RC this piece is protected from a protease attack by detergent molecules.

From these results we propose that subunit L as well as part of subunit M form the smallest functional unit of the RC.

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