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IDENTIFICATION OF THE CAROTENOID PRESENT IN THE *B*-800—850 ANTENNA COMPLEX FROM *RHODOPSEUDOMONAS CAPSULATA* AS THAT WHICH RESPONDS ELECTROCHROMICALLY TO TRANSMEMBRANE ELECTRIC FIELDS

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Summary

Mild proteolysis of *Rhodopseudomonas capsulata* chromatophores results in a parallel loss of the 800 nm bacteriochlorophyll absorption band and a blue shift in the carotenoid absorption bands associated with the *B*-800—850 light-harvesting complex. Both the light-induced and the salt-induced electrochromic carotenoid band shift disappear in parallel to the loss of the 800 nm bacteriochlorophyll absorption upon pronase treatment of chromatophores. During the time required for the loss of the 800 nm bacteriochlorophyll absorption and the loss of the electrochromic carotenoid band shift photochemistry is not inhibited and the ionic conductance of the membrane remains very low. We conclude that the carotenoid associated with the *B*-800—850 light-harvesting complex is the one that responds electrochromically to the transmembrane electric field. Analysis of the pigment content of *Rps. capsulata* chromatophores indicates that all of the carotenoid may be accounted for in the well defined pigment-protein complexes.

Introduction

It is now generally believed that the light-induced carotenoid band shift, seen in several species of photosynthetic bacteria, is an electrochromic response of the carotenoids to a transmembrane electric field (for reviews see Refs. 1 and

Abbreviation: FCCP, carbonylcyanide *p*-trifluoromethoxy-phenylhydrazone.

2). The major impetus for this view came from the experiments of Jackson and Crofts [3,4]. These workers showed that, in chromatophores from *Rhodopseudomonas sphaeroides*, carotenoid band shifts could be induced in the dark by the creation of potassium ion diffusion potentials.

However, attempts to provide a coherent physical explanation of the detailed properties of the carotenoid band shift in terms of a simple electrochromic theory have not been completely successful [5—8]. In particular the linear dependence of the amplitude of these absorbance changes upon field strength and the apparently large wavelength shift of the absorption bands have been difficult to account for.

Schmidt et al. [9] have suggested that a large permanent field, an order of magnitude greater than the light-generated field, exists in the membrane. This would then result in an apparently linear dependence of the absorption changes on the magnitude of the light induced electric field.

Holmes and Crofts [8] analysed the spectrum of the light-induced carotenoid band shift in chromatophores from *Rps. sphaeroides* GIC. They concluded that the results could be best explained by 7—11% of the total carotenoids shifting to the red by up to 7 nm.

Most recently Symons et al. [10] and De Grooth and Amesz [11] have suggested that the pool of carotenoids responsible for the band shift absorb to the red of the bulk of the carotenoid pigments. Moreover, this pool probably represents 20—35% of the total carotenoids and undergoes a progressive red shift in response to an increasing transmembrane electric field.

Carotenoids, within the photosynthetic membrane, are bound together with the bacteriochlorophylls into well defined pigment-protein complexes. It is important, therefore, to determine whether the carotenoid band shift is associated with a specific pigment-protein complex, and so give a firm basis for proposing a special responsive pool of carotenoids.

Sherman and Clayton [12] could not detect any salt-induced carotenoid band shift in the reaction centre less mutant of *Rps. sphaeroides*, PM8. They suggested that the presence of the reaction centre was somehow essential for the band shifts.

In contrast recent experiments with chromatophores of *Rhodopseudomonas capsulata* have demonstrated that the presence of a carotenoid shift is correlated with the presence of the *B*-800—850 light-harvesting pigment-protein complex [13]. A mutant of *Rps. capsulata*, strain Y142, lacking the reaction centre and the *B*-870 antenna complex still shows salt-induced carotenoid band shifts. Another strain of *Rps. capsulata*, MW 442, was constructed from a blue green mutant (which lacks *B*-800—850 and carotenoids) by the readdition of the genes responsible for the synthesis of coloured carotenoids. This mutant showed neither light-induced nor salt-induced carotenoid band shifts.

The *B*-800—850 complex from *Rps. capsulata* has a minimal functional unit of three molecules of bacteriochlorophyll and one molecule of carotenoid bound to two low molecular weight polypeptides [14]. In vivo it probably exists in an aggregated form. We have recently been able to show that mild pronase digestion of chromatophores from *Rps. capsulata* results in the selective, simultaneous loss of the 800 nm bacteriochlorophyll band and a blue shift of the carotenoid absorption bands [24]. These changes are due to the selective

degradation of part of the *B*-800—850 antenna complex prior to the digestion of the other pigment-protein complexes. It was concluded from these experiments and from those of Feick and Drews [15] that the 800 nm bacteriochlorophyll and the carotenoid are bound to the smaller of the two polypeptides (the 8000 molecular weight polypeptide) present in the *B*-800—850 complex.

In view of the experiments of Zannoni and Marrs [13] we have used mild pronase digestion of chromatophores from *Rps. capsulata* to further investigate the relationship between the *B*-800—850 carotenoid and the carotenoid band shift. The results presented below support the conclusions of Zannoni and Marrs [13] that it is indeed the carotenoid from the *B*-800—850 antenna complex which is responsible for the carotenoid band shift.

Materials and Methods

Cells of *Rps. capsulata* strain Z1 were grown photosynthetically with succinate as the sole carbon source. Harvested cells were disrupted by passage through a French pressure cell at 10 tons per square inch. Chromatophores were then isolated from broken cells by differential centrifugation [16] and resuspended in 20 mM Tris-HCl, pH 8.0.

The concentration of protein was determined by a turbidometric assay [17] and the concentration of bacteriochlorophyll by extraction into acetone/methanol (7 : 2, v/v), using the millimolar extinction coefficient at 772 nm of 75 cm^{-1} [18].

The proteolysis was carried out in the dark at 37°C, in a shaking water bath. Freshly prepared chromatophores were diluted with 20 mM Tris-HCl (pH 8.0) to give a protein concentration of 2.5 mg/ml. Then after a 15-min preincubation at 37°C the reaction was initiated by the addition of 40 μg/ml of pronase (Sigma, type VI).

At various times during the digestion aliquots of the chromatophores were removed for analysis. Their absorption spectra were recorded on a Pye-Unicam SP8000 spectrophotometer. The salt-induced (valinomycin and potassium chloride pulses) carotenoid band shift was measured using the same spectrophotometer differentially. The flash-induced carotenoid band shift was measured in a home-made single beam spectrophotometer at 496.5 nm. Actinic illumination was provided by a saturating xenon flash (45 μs width at half-peak height) filtered through two layers of Wratten 88A gelatin filters and 2 mm of a RG 695 cut off filter. The photomultiplier (EMI 9659QB) was screened with 4 mm of Corning blue glass 4—96. The signal from the photomultiplier was amplified and then displayed on an oscilloscope. The continuous light-induced carotenoid shift was measured in a similar single beam spectrophotometer at 496.5 nm where the actinic illumination was provided by light from a 55 watt quartz iodine bulb filtered as described for xenon flash. In this case the signal was displayed on a pen recorder.

The concentration of reaction centres in the chromatophores was assayed using the same in vivo differential extinction coefficient at 605 nm (19.5 $mM^{-1} \cdot cm^{-1}$) as determined in *Rps. sphaeroides* [19]. The extent of bleaching at 605 nm (which reflects the oxidation of the reaction centre bacteriochloro-

phyll) was measured following a train of saturating xenon flashes (usually at least eight flashes were required for the maximum absorption change). Light saturation was checked by varying the concentration of the chromatophores in the cuvette.

The major types and the amount of the carotenoids present in the chromatophores were determined as described by Cogdell et al. [20]. The carotenoids were identified by comparison of the R_F values on silica gel thin-layer plates and of their absorption spectra with previously published data [21—23].

Results

The effect of pronase digestion upon the chromatophores flash-induced carotenoid band shift is shown in Fig. 1. After preincubation of the chromatophores at 37°C, prior to the addition of pronase, the antimycin sensitive slow phase of the carotenoid shift is abolished (this is probably due to a rise in the ambient redox potential). Pronase digestion causes a progressive loss in the extent of the carotenoid band shift, which is complete after 80—90 min incubation. A small residual absorption change remains which will be described below.

It was shown in a previous study [24], that mild pronase digestion of chromatophores from *Rps. capsulata* initially results in the selective loss of the 800 nm bacteriochlorophyll absorption band and the removal of the carotenoids (seen as a blue shift of the carotenoid absorption bands) from the *B*-800—850 antenna complex. The 850 nm absorption band, the *B*-870 antenna complex and the reaction centre remain intact during the initial stages of digestion. We have compared the time course of the loss of the flash-induced carotenoid band

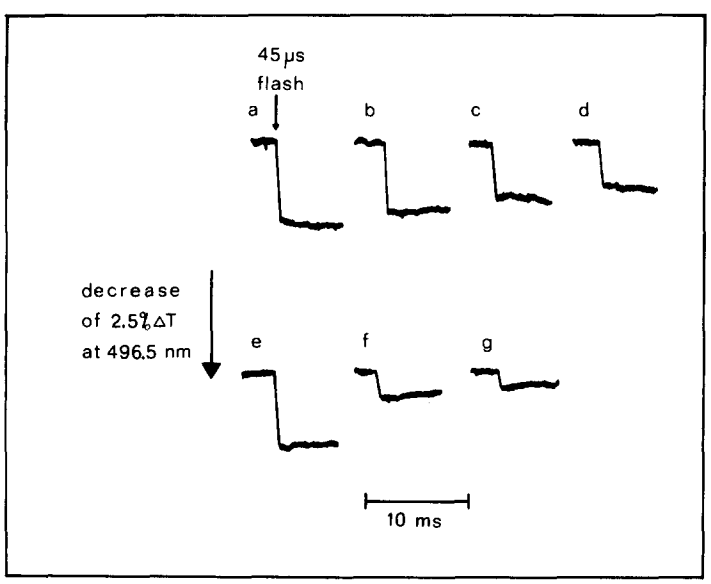

Fig. 1. The kinetics of the flash-induced carotenoid band shift at various times during the incubation of *Rps. capsulata* chromatophores with pronase. (a) 0 min, (b) 15 min, (c) 25 min, (d) 35 min, (f) 50 min, (g) 80 min incubation with pronase; (e) untreated control sample incubated at 37°C for 40 min. Each sample contained 40 µg of bacteriochlorophyll in 3.3 ml of 20 mM Tris-HCl, pH 8.0.

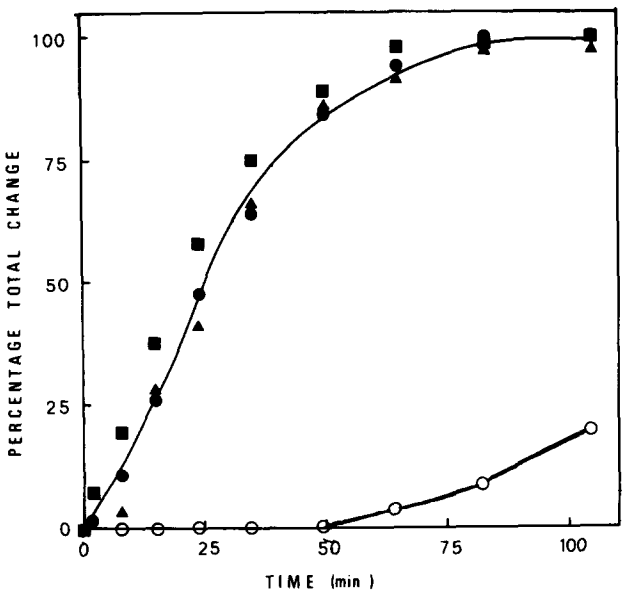

Fig. 2. Time course of the disappearance of the 800 nm bacteriochlorophyll, the 850 nm bacteriochlorophyll, the blue shift of the carotenoid absorption bands and the loss of the light-induced carotenoid band shift during pronase treatment of *Rps. capsulata* chromatophores. The total absorption changes were the maximum observed during a 6-h incubation period. The disappearance of the 800 nm bacteriochlorophyll was measured by the absorption difference 800 nm—830 nm (■) and the disappearance of the 850 nm bacteriochlorophyll by the absorption change at this wavelength (○). The blue shift of the carotenoid bands was measured by the peak to trough absorption difference 505—522 nm (●). The electrochromic band shift was measured as the amplitude of the flash-induced absorption increase at 496.5 nm (▲).

shift upon pronase treatment with the loss of the 800 nm and 850 nm absorption bands and the removal of the carotenoid from the antenna complex (Fig. 2). The decrease in the amplitude of the flash-induced carotenoid band shift, the degradation of the 800 nm absorption band and the blue shift in the carotenoid bands all show similar kinetics. During these initial stages of digestion the 850 nm absorption band is essentially unaffected (see also Ref. 24). The continuous light-induced carotenoid band shift is also sensitive to pronase digestion and its inhibition follows the same time course as the flash-induced carotenoid band shift (data not shown).

These results suggest that the carotenoid which is responsible for the electrochromic band shift is the carotenoid bound to the *B*-800—850 antenna complex. There are, however, two other possible explanations for the pronase-induced loss of the electrochromic carotenoid band shift. The pronase digestion might inhibit photochemistry and so result in the loss of the light-induced carotenoid band shift. Alternatively, the pronase treatment might cause a large increase in the ionic permeability of the chromatophore membrane. This would accelerate the decay of the carotenoid band shift and so cause an apparent inhibition of the extent of the absorption change.

Electrochromic carotenoid band shifts may also be induced in the dark by creating potassium diffusion potentials with valinomycin and potassium chloride pulses [3]. In this case the generation of the membrane potential and

the consequent carotenoid band shift are independent of light-driven electron transfer reactions. The pronase digestion of chromatophores from *Rps. capsulata* also results in inhibition of the salt-induced carotenoid band shift. The time course of this inhibition again parallels the loss of the absorption at 800 nm.

The effect of pronase digestion upon the difference spectrum of the flash-induced absorption changes from 410 nm to 500 nm is shown in Fig. 3. In the absence of pronase the absorption changes are due mainly to the light-induced oxidation of the reaction centre bacteriochlorophyll and the electrochromic carotenoid bandshift. The changes in the 410—440 nm region are primarily in response to the formation of oxidised reaction centre [25] and so give a measure of the photochemical integrity of the chromatophores. After 80 min incubation of chromatophores with pronase the electrochromic bandshift is inhibited whereas absorption changes due to the oxidation of reaction centre bacteriochlorophyll and the reaction centre carotenoid absorption changes are unaffected. As described previously [26], the reaction centre carotenoid absorbs to the blue of the major carotenoid absorption peaks. Consequently the wavelengths of the maxima and minima of the reaction centre carotenoid band shift are shifted several nanometres to the blue of the peaks and troughs of the bulk electrochromic band shift.

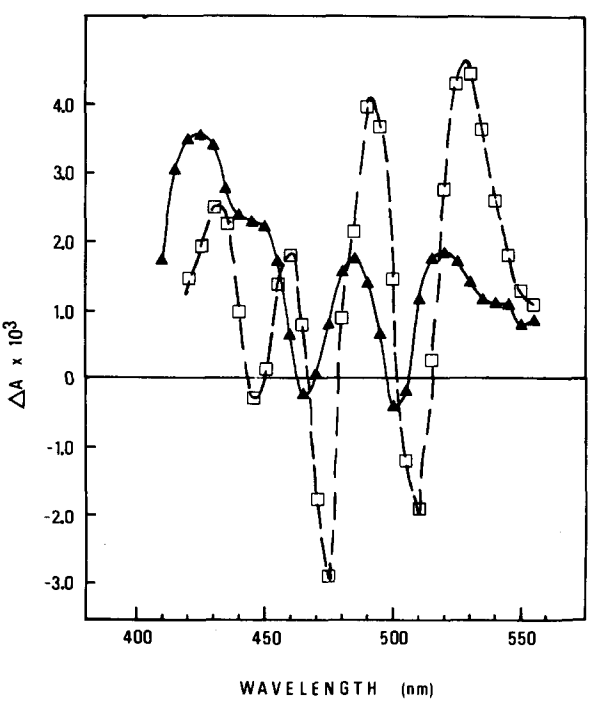

Fig. 3. Light minus dark difference spectra of pronase treated and untreated *Rps. capsulata* chromatophores. Chromatophores were incubated at 37°C in the dark for 80 min in the presence (▲———▲) and absence of pronase (□- - - - -□). The difference spectrum of the untreated control sample was identical to that for chromatophores prior to the incubation. The spectra show samples taken from the experiment depicted in Fig. 1.

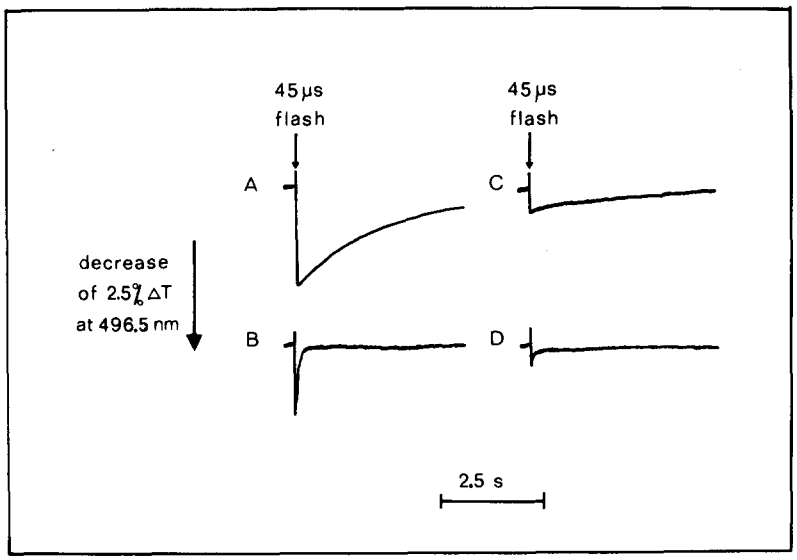

Fig. 4. The effect of pronase treatment of *Rps. capsulata* chromatophores upon the decay of the carotenoid band shift. Untreated chromatophores (A and B), chromatophores incubated with pronase for 50 min (C and D). Each sample contained 40 μg of bacteriochlorophyll in 3.3 ml of 20 mM Tris-HCl, pH 8.0. B and D contained in addition 12 μM FCCP.

The kinetics of the decay of the flash-induced carotenoid band shift provide a good indication of the ionic conductivity of the chromatophore membrane [4]. In Fig. 4 the effect of pronase digestion on the decay of the flash-induced carotenoid band shift is shown. Initially, prior to the addition of pronase, the rate of decay of the band shift is slow ($t_{1/2} \sim 1.5$—2 s). After 40 min incubation with pronase the extent of the flash-induced carotenoid band shift is only 42% of that seen in a control sample of chromatophores. However, the decay of this residual change is still slow ($t_{1/2} \sim 1.5$—2 s). Both before and after the pronase digestion, the rate of the decay of the carotenoid band shift is greatly accelerated by the addition of the uncoupler FCCP. The small fraction of the absorption changes at 496.5 nm remaining even after the addition of FCCP represents the reaction centre carotenoid band shift, which has been shown to be uncoupler insensitive [27].

The photosynthetic unit of *Rps. capsulata* consists of reaction centres and the two types of antenna complex (*B*-870 and *B*-800—850) [28]. Each reaction centre contains a single carotenoids [20] and the *B*-800—850 antenna complex contains one carotenoid per three bacteriochlorophyll molecules [14,29]. It is probable that the *B*-870 complex, in view of its similarity to the *B*-890 complexes from both *Rhodospirillum rubrum* and *Chromatium vinosum*, contains one carotenoid per two bacteriochlorophylls [30]. Schumacher and Drews [31] have demonstrated that the amount of *B*-870 per reaction centre remains relatively constant at 30 bacteriochlorophylls per reaction centre complex. Larger photosynthetic units are constructed by the addition of the *B*-800—850 light-harvesting complex. It is therefore possible, knowing the size of the photosynthetic unit, to calculate the carotenoid to bacteriochlorophyll ratio

TABLE I

THE PIGMENT COMPOSITION OF *RPS. CAPSULATA* CHROMATOPHORES

Protein, bacteriochlorophyll and carotenoids were estimated on a 4-ml sample of chromatophores. The reaction centre concentration was estimated using the in vivo differential extinction coefficient at 605 nm of 19.5 $mM^{-1} \cdot cm^{-1}$ [19]. The theoretical value of the bacteriochlorophyll:carotenoid ratio and the % total carotenoid contributed by the *B*-800—850 complex were calculated using the published values of the *B*-870:reaction centre ratio [31] and of the pigment composition of the reaction centre [20], *B*-870 [30] and *B*-800—850 antenna complexes [14,29].

Total protein (mg)	Total bacterio-chlorophyll (μmol)	Total carotenoid (μmol)	Ratio of bacterio-chlorophyll-to reaction centre	Theoretical ratio of chlorophyll to carotenoid	Experimental ratio of bacterio-chlorophyll to carotenoid	% total carotenoid contributed by the *B*-800—850
71.4	3.96	1.46	186:1	2.8:1	2.7:1	76%

% composition of carotenoid types

Spheroidene and derivatives	Spheroidenone and derivatives	Neurosporene	Others
79	16	4	1

and the percentage of the total carotenoid contributed by the *B*-800—850 antenna complex for a given sample of chromatophores.

The results of such a theoretical calculation can then be checked by extracting the bacteriochlorophyll and carotenoid pigments from the chromatophores and determining the carotenoid : bacteriochlorophyll ratio directly. Table I presents the results of such an analysis of *Rps. capsulata* chromatophores. The composition of the membrane carotenoids is similar to that previously reported in the literature [21,23]. The size of the photosynthetic unit was found to be 186 bacteriochlorophylls per reaction centre complex. On the basis of this value the expected carotenoids : bacteriochlorophyll ratio is 1 : 2.8. The experimentally determined ratio was found to be 1 : 2.7, which is in excellent agreement with the theoretical value. The percentage of the total chromatophore carotenoid contributed by the *B*-800—850 complex was calculated to be 76%.

Discussion

Mild pronase digestion of chromatophores from *Rps. capsulata* leads to the parallel loss of the electrochromic carotenoid band shift (induced by either light or diffusion potentials in the dark) and the removal of the carotenoid from the *B*-800—850 light-harvesting pigment-protein complex. During the loss of the carotenoid band shift the ionic conductance of the membrane remains at a low level and photochemistry is unaffected.

From these experiments we conclude, in agreement with the genetic studies of Zannoni and Marrs [13], that the *B*-800—850 carotenoid is the one which responds electrochromically to transmembrane electric fields.

In a similar study on the effects of pronase and trypsin digestion upon chromatophores from *Rps. sphaeroides* Hall et al. [32] found no effect of the digestion upon the electrochromic carotenoid band shift. They also showed that these mild proteolytic treatments digested the 'H' subunit of the reaction centre. In chromatophores from *Rps. capsulata* we find that over the period of time (the initial 90—100 min) when the pronase treatment removes the electrochromic carotenoid band shift none of the reaction centre subunits are affected (Webster, G.D. and Cogdell, R.J., unpublished observations). It seems probable that these species differences reflect subtle differences in the surface of these two types of chromatophores.

In the chromatophores used in the present study about 76% of the total carotenoid content is present in the *B*-800—850 antenna complex. However, several groups [8,10,11] have suggested that only about 10—40% of the total carotenoids respond electrochromically to the transmembrane electric field. It is obviously difficult to reconcile these two observations and it will be interesting to see how the computer predicted size of the responsive carotenoid pool varies with the amount of the *B*-800—850 complex in the chromatophores. However, the results presented here suggest, in view of the large discrepancy between the size of the carotenoid pool responding electrochromically to transmembrane electric fields and the *B*-800—850 carotenoid complement that only part of the carotenoid bound to the *B*-800—850 complex shows electrochromism.

The close agreement between the calculated carotenoid : bacteriochlorophyll ratio and that determined experimentally by extraction strongly suggests that all the carotenoid within the chromatophores can be accounted for in terms of the well defined pigment-protein complexes.

Acknowledgements

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