PHOTOREDUCTION OF THE LONG WAVELENGTH BACTERIOPHEOPHYTIN IN REACTION CENTERS AND CHROMATOPHORES OF THE PHOTOSYNTHETIC BACTERIUM *CHROMATIUM VINOSUM*

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1. Introduction

Recent results obtained by picosecond spectrophotometry have indicated that the primary charge separation occurs within 10 ps after a flash [1]. This evidence suggests that the bacteriochlorophyll dimer *P*-870 [2] becomes oxidized and an unidentified intermediate electron carrier, I, becomes reduced. Spectral evidence has given some indication that I^- may be the so-called long wavelength bacteriopheophytin (Bpheo) [3–5]. Under normal conditions I^- reduces the primary electron acceptor X, probably a quinone iron–protein complex, with a half-time of approximately 200 ps [6,7]. If X is in the reduced state before the flash, P^+-870 and I^- recombine at room temperature within several nanoseconds, forming the carotenoid triplet or, in a carotenoid-less mutant, the excited singlet state P^*-870 I [8,9]. At 77°K the back reaction between P^+-870 and I^- seems to be slightly slower, and in the case of carotenoid-less mutants, the bacteriochlorophyll triplet is then formed with a high quantum efficiency [8,10].

Recent ESR work on reaction center preparations of *Chromatium vinosum* has indicated that the reaction centers can be trapped in the state C^+-552 [*P*-870 I^-] [11] by illumination with strong continuous light under conditions in which the 'primary' acceptor is reduced. Together with the photo-oxidation of one molecule *C*-552 per reaction center two distinct ESR signals, which were ascribed to the formation of I^-, were observed. However, the chemical identity of I remains unconfirmed and therefore we have tried to obtain evidence from the measurement of optical absorbance changes reflecting the reduction of I. The results indicate that the reduction of the long wavelength bacteriopheophytin and a band shift near 800 nm, probably due to a bacteriochlorophyll, occur upon the formation of I^-. The experimental rationale (fig.1) is similar to that given by Tiede et al. [11], except that we did most of our

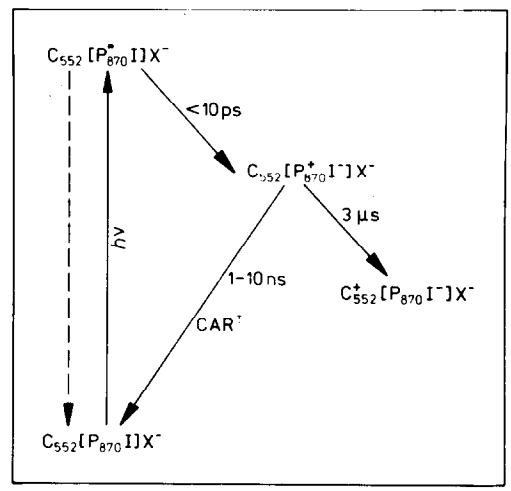

Fig.1. Scheme to explain the trapping of the state C^+-552 [*P*-870 I^-] X^- in reduced reaction centers and chromatophores of *Chromatium vinosum*.

Abbreviations: *P*-870, *P*-800, reaction center bacteriochlorophylls absorbing near 880 nm and 800 nm, respectively; Bpheo, bacteriopheophytin; *C*-552, low potential cytochrome *c* with an α-band at 552 nm; TMPD, *N,N,N',N'*-tetramethyl-1,4-phenylene diamine.

experiments at a higher temperature, approximately −15°C, where *C*-552 oxidation has a half-time of about 3 μs [12].

2. Materials and methods

Chromatium vinosum was cultured anaerobically at 30°C in a medium after Hendley [13]. After five days of growth the cells were harvested by centrifugation at 5000 × *g*. Chromatophores were prepared by sonication and differential centrifugation. Reaction centers were obtained by detergent treatment and gradient centrifugation, followed by acetone extraction to remove the remaining light harvesting pigments [14]. In these preparations at least one molecule *C*-552 per reaction center was still present.

Reaction centers or chromatophores were mixed with ethylene glycol (final concentration 55%, v/v) to keep the sample clear upon cooling. Dithionite was added shortly before freezing to a concentration of 10 mM. During some experiments a low concentration of TMPD (5 μM) was present to provide an efficient electron donor for oxidized *C*-552.

Absorbance difference spectra were recorded on a chopped double beam spectrophotometer described earlier [15] or on a split beam spectrophotometer [16]. Actinic light was provided by a 250 W quartz–iodine lamp, through an infrared transmitting filter (Kodak RG 780/3, intensity = 25 mW/cm^2) for measuring wavelengths in the region 360–680 nm, or through a combination of broad band filters (Corning CS 4-96, CS 4-97, Kodak BG 38/4 and a Calflex heat filter, intensity 42 mW/cm^2) for measuring wavelengths above 630 nm. The photomultiplier was protected from stray actinic light by a combination of infrared transmitting filters with appropriate interference filters (630–1000 nm region) or suitable band filters (360–630 nm region).

3. Results

Figure 2 shows the difference spectrum induced by 5 s of high intensity illumination in reduced reaction centers of *C. vinosum*. The difference spectrum is characterized by absorbance increases at 364 nm, 542 nm, 592 nm, 758 nm and 802 nm and absorbance increases at 423 nm, 665 nm and 785 nm. The bands at 542 nm and 758 nm in the difference spectrum

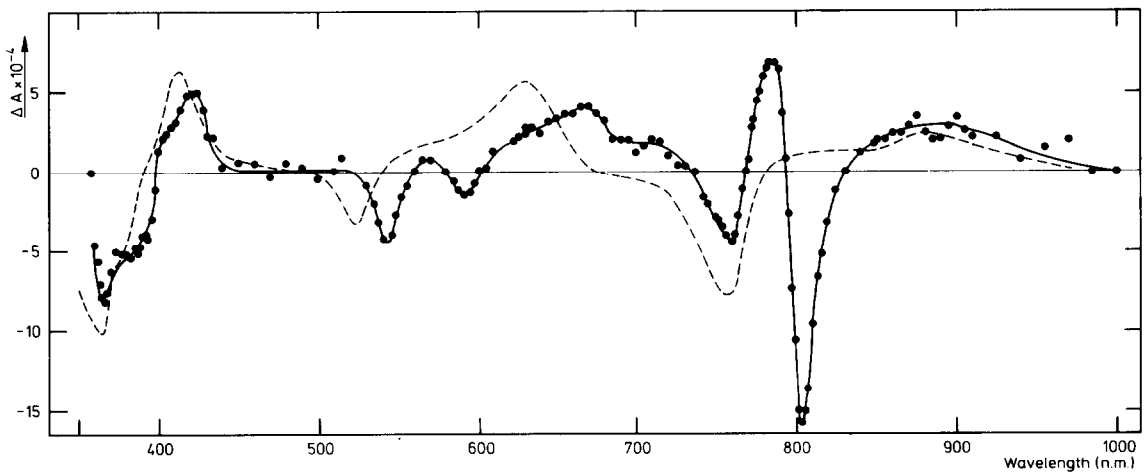

Fig.2. (•——•) Absorbance difference spectrum obtained with reaction centers of *C. vinosum* after 5 s of illumination with high intensity actinic light. The blue and the infrared parts of the spectrum were fitted by measuring the 630–680 nm region with both types of exciting light (see Materials and methods). Dark time between the illuminations, 5 min. *P*-870-concentration, 0.16 μM. Optical pathlength, 1 mm. Temperature, −15°C. Additions: sodium dithionite 10 mM; TMPD, 5 μM. Dashed line: difference spectrum for the reduction of bacteriopheophytin in vitro, calculated for the reduction of one molecule Bpheo per reaction center (data taken from ref. [3]).

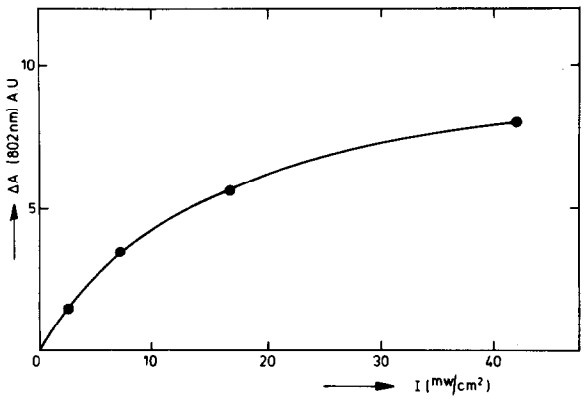

Fig.3. Amplitude of the absorbance change at 802 nm as a function of the intensity of the actinic light. Further conditions as for fig.2.

strongly suggest the reduction of the long wavelength bacteriopheophytin (Bpheo). The kinetics of the change were the same at all points of the spectrum. The half-time was approximately 2–3 s for the light-on absorbance change and several minutes for the light-off change. The signal was reproducible using a dark time of 5 min between the illuminations.

The light used was not completely saturating (fig.3) but we estimate that about 80–90% of the total amount of the Bpheo was reduced under these conditions. Assuming that one Bpheo molecule is reduced per reaction center and that no other absorbance changes occur at 542 nm, we calculated an extinction coefficient for Bpheo reduction ϵ = 25 mM^{-1} cm^{-1} at 542 nm, close to the extinction coefficient obtained for the reduction of Bpheo in vitro (ϵ = 21 mM^{-1} cm^{-1} at 530 nm [3]). The large negative peak at 802 nm can be explained by a shift of a bacteriochlorophyll absorption band at 800 nm, to a somewhat broader band at 785 nm (see Discussion). The small peak at 592 nm is probably caused by a similar shift. It should be noted that cytochrome oxidation is absent in this spectrum; apparently the TMPD is able to reduce the cytochrome efficiently, whereas the oxidized TMPD is reduced by the high concentration of dithionite which substance shows only absorption changes in the ultraviolet part of the spectrum. When the TMPD was left out or if the sample was cooled to –45°C, net cytochrome oxidation became also apparent.

Figure 4 shows parts of the visible and infrared difference spectrum obtained by 5 s of strong continuous illumination of reduced chromatophores of *C. vinosum* at –35°C. The main difference between this spectrum and that obtained with reaction center preparations in the presence of TMPD (fig.2) is that it shows the presence of absorbance changes due to the oxidation of a cytochrome, presumably *C*-552. The amount of *C*-552 photo-oxidized could be calculated using an extinction coefficient of 89 mM^{-1} cm^{-1} at 423 nm [17] and was about equal to the amount of Bpheo reduced, as calculated from the absorbance

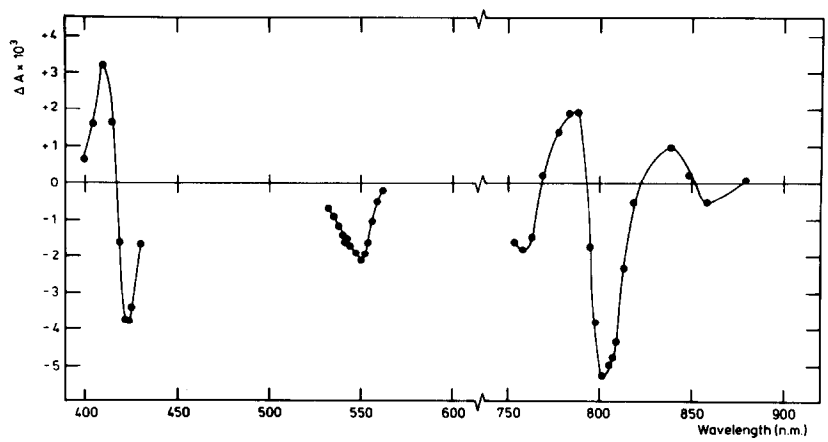

Fig.4. Absorbance difference spectrum obtained with chromatophores of *C. vinosum* after 5 s of illumination in the presence of 10 mM sodium dithionite. Bacteriochlorophyll concentration, 0.10 mM. Optical pathlength, 1 mm. Temperature, –35°C.

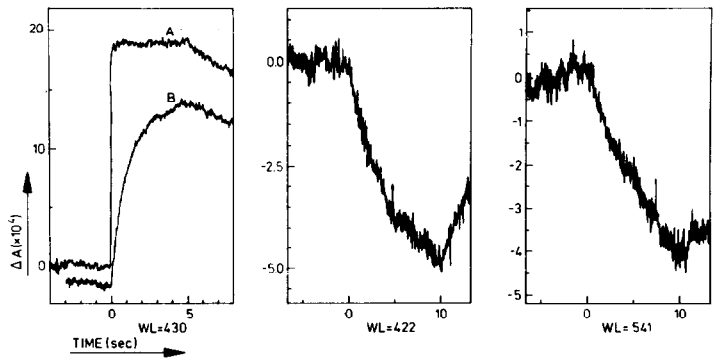

Fig.5. Kinetics of absorbance changes in chromatophores of *C. vinosum* at −15°C upon illumination with actinic light selected with a narrow band filter (maximal transmission at 875 nm, band half-width 20 nm). Left: *P*-870 oxidation at different light intensities in the presence of 0.2 mM potassium ferricyanide. Measuring wavelength, 430 nm. Trace A, 100% intensity of actinic light. Trace B, 2% intensity. Other conditions as for fig.4. Middle: *C*-552-oxidation in the presence of 10 mM dithionite. Measuring wavelength, 442 nm. Intensity of actinic light, 100%. Right: Bpheo-reduction, measured at 542 nm, under the same conditions as for the middle figure.

decrease at 542 nm, using the extinction coefficient of 25 mM^{-1} cm^{-1} obtained in the reaction center preparations. Again the amount of Bpheo reduced was approximately equal to one molecule per reaction center, indicating that the intensity of the actinic light was almost saturating.

Figure 5 shows the kinetics of the absorbance changes at 422 nm and 541 nm occurring upon illumination at −15°C of chromatophores of *C. vinosum* under reducing conditions; the actinic light was selected with a narrow band filter (maximal transmission at 785 nm, band half-width 20 nm). In the same figure can be seen the kinetics of P^+-870 formation (measured at 430 nm) with the same actinic light and also with a 50 times lower intensity. From these data, and the extinction coefficients given above, the efficiences of *C*-552 photo-oxidation and Bpheo reduction can be estimated. When this is done it is found that in reduced samples *C*-552 oxidation is 600 times and Bpheo reduction is 500 times less efficient than P^+ formation under oxidizing conditions, provided that allowance is made for the positive absorbance change of bacteriopheophytin at 422 nm (see fig.2). If it is assumed that the quantum efficiency of the primary photochemical act does not change on reduction of X [8], a calculation, based on the scheme of fig.1, results in a half-time of 6 ns for the back reaction between P^+-870 and $Bpheo^-$, which is of the same order as that found in isolated reaction centers prepared from *Rhodopseudomonas sphaeroides* R26 after a short laser flash [8,9]. A calculation based on the amount of absorbed light gave approximately the same value for this back reaction half-time.

The recovery of the state C^+-552 *P*-870 $Bpheo^-$ probably does not proceed via a direct back reaction. This is indicated by the different decay rates observed for C^+-552 and $Bpheo^-$ (fig.5). The decay rate of $Bpheo^-$ was extremely sensitive to temperature; at −35°C it seemed to be slower than 30 min (half-time), whereas at room temperature we were unable to accumulate the centers in the state C^+-552 *P*-870 $Bpheo^-$.

4. Discussion

The work described above provides support for the idea [1–8] that on illumination of reaction centers of photosynthetic bacteria an electron carrier, I, preceding the 'primary' acceptor X, becomes reduced and characterizes the spectral changes remaining after the transfer of an electron from P to I and the concomitant reduction of P^+-870 (i.e. state *P*-870 I^-). The suggestion by Faljer et al. [3] that I is identical to bacteriopheophytin, is supported by the difference spectrum obtained by us for the reduction of I. It follows from our experiments that

about one bacteriopheophytin per reaction center is reduced upon illumination. In addition the shift of a band at 800 nm to a broader band at 785 nm is observed. The shape of the difference spectrum in the infrared can be explained by making the following assumptions:

(1) There is a bleaching of a band centered at 758 nm, corresponding to the bacteriopheophytin band visible in the absolute absorbance spectrum of the reaction center [14], and

(2) A peak at 800 nm, probably a bacteriochlorophyll absorption band, is replaced by a broader band centered at 786 nm. As can be seen in fig.6 the shape of the difference spectrum synthesized using these assumptions fits well with the measured spectrum.

This difference spectrum does not support the hypothesis that the electron of I^- is shared by a bacteriopheophytin and a bacteriochlorophyll.

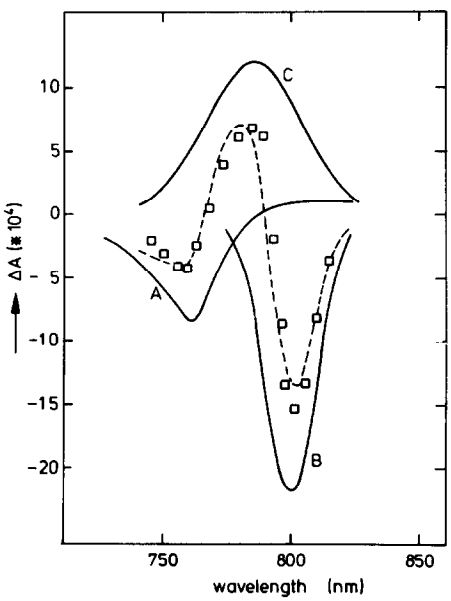

Fig.6. Analysis of the infrared region of the difference spectrum shown in fig.2. Dashed line: spectrum obtained by summation of the difference spectrum caused by the in vitro reduction of bacteriopheophytin (A, see also fig.2) and the shift of an absorption band with a maximum at 800 nm (B), to a somewhat broader band with a maximum at 785 nm (C). The halfwidth and amplitude of curve C was chosen so that the area under this curve is identical to that under curve B. Squares: Points from measured difference spectrum (shown in fig.2). Further explanation in the text.

However, since the components of the reaction center complex are tightly linked it may be expected that changes in the oxidation–reduction state of one of the constituents affect the other components by electrostatic, magnetic or conformational interactions. This might explain the two ESR signals obtained in the same species under similar conditions [11], one reflecting the reduction of the bacteriopheophytin, the other demonstrating a change of magnetic interaction between two or more of the reaction center components induced by the reduced bacteriopheophytin.

The reason for the blue shift of the 800 nm band may possibly be found in a change of the electronic configuration of the *P*-800 bacteriochlorophyll after the bacteriopheophytin has been reduced; the mechanism may be similar to that underlying the normal *P*-800 shift which is observed when *P*-870 is photo-oxidised. However the *P*-800 shift induced by the reduction of bacteriopheophytin only is not necessarily the same as that observed under conditions such that also *P*-870 is oxidised (for example shortly after a picosecond flash) because then the charge distribution on the reaction center complex may be appreciably different. Moreover, the difference spectra that have been reported from measurements on a picosecond time scale [6,8] do not include results obtained in *C. vinosum*, which makes a comparison of those measurements with the difference spectra reported here somewhat ambiguous. We are therefore trying at the moment to measure absorbance changes reflecting the bacteriopheophytin reduction in reaction centers and chromatophores of other species of photosynthetic bacteria.

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