EVIDENCE FOR PHOTOREDUCTION OF MONOMERIC BACTERIOCHLOROPHYLL *a* AS AN ELECTRON ACCEPTOR IN THE REACTION CENTER OF THE GREEN PHOTOSYNTHETIC BACTERIUM *PROSTHECOCHLORIS AESTUARII*

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1. Introduction

In [1] we reported a study of the acceptor side of the reaction center of *Prosthecochloris aestuarii*. In a reaction center containing pigment–protein (PP) complex from this bacterium, the so-called PP complex [2], two distinct iron–sulfur centers (X_2, X_3) were identified, which appeared to function serially as secondary electron acceptors. In addition an electron acceptor X_1 could be accumulated in the reduced form upon illumination, yielding an ESR spectrum, measured at 10 K, which suggested that X_1 might be a porphyrin [1].

The *g*-value and linewidth of the ESR spectrum alone did not allow a determination of the identity of this component. Therefore, we measured the absorbance difference spectrum of X_1^- in the photosystem protein (PP) complex poised at a redox potential (E_h) of −620 mV, by accumulation at 0°C in the light in the presence of a fast electron donor. ESR measurements confirmed that the reduced acceptor accumulated in this way is indeed X_1^-. These experiments strongly suggest that X_1 is a monomeric bacteriochlorophyll *a* molecule, functioning as an electron acceptor preceding the iron–sulfur centers X_2 and X_3 in the reaction center of *P. aestuarri*.

2. Materials and methods

Prosthecochloris aestuarii, strain 2 K, was grown anaerobically in a mixed culture originally known as *'Chloropseudomonas ethylica'* [3] as described [4]. The reaction center preparation PP complex was prepared as in [2]. Light-induced absorbance changes were measured as in [5]. Actinic illumination was provided through a combination of Schott RG 715 and RG 780 filters (intensity ~0.1 W/cm² between 780–850 nm) for measurements in the region 380–720 nm, or through a combination of a Corning CS 4-96, a Schott BG 38/4 and a Balzers Calflex C filter (intensity 46 mW/cm²) for the region 660–850 nm. Suitable interference and absorbance filters were used to protect the photomultiplier from actinic stray light. ESR experiments were performed with a Varian E-9 spectrometer as in [6]. Continuous illumination with white light provided by a 1000 W projection lamp, filtered by 5 cm water and a Balzers Calflex C filter was used as actinic illumination. Measurements between room temperature and 80 K were performed in a nitrogen gas-flow cryostat. The samples were contained in quartz tubes of 3 mm internal diameter, or in a quartz 'flat cell' cuvette with an optical pathlength of 0.5 mm when using non-frozen samples.

Unless otherwise stated, the samples were poised at a redox potential (E_h) of ~−620 mV by dithionite at pH 10.0, to keep the iron–sulfur centers X_2 and X_3 in the reduced form [1]. Dithionite was added from a freshly prepared stock solution of 0.5 M dithionite in 0.5 M glycine (pH 9.5). The redox potential E_h was measured as in [1].

3. Results and discussion

We have reported [1] the light-induced ESR and optical spectra obtained upon reduction of X_2, an iron–sulfur center which acts as a secondary electron acceptor in the reaction center of the green bacterium *P. aestuarii*. Accumulation of X_2 in the reduced form

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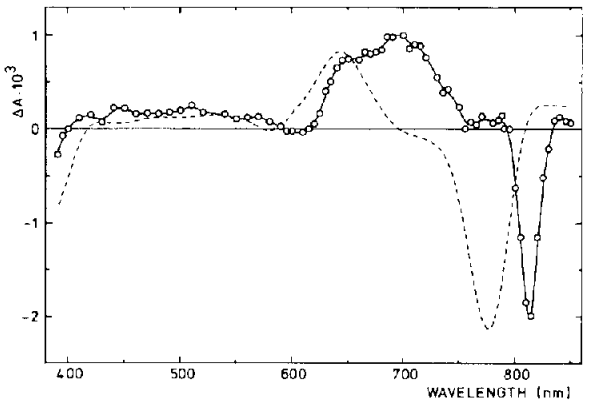

Fig.1. (——) Absorbance difference spectrum obtained upon illumination at 0°C of the PP complex (A_{810} = 0.48) due to the reduction of the electron acceptor X_1. The sample contained 5 mM ascorbate, 10 mM dithionite, 100 μM PMS, 20% sucrose (w/w), 0.025% Triton X-100 (w/w), 5 mM phosphate, 60 mM glycine (pH 10.0) and catalase, glucose and glucose oxidase to maintain anaerobiosis. The E_h was ~−620 mV. The spectra obtained with blue illumination (see section 2) were normalized to those obtained with near-infrared in the region 660–720 nm. (– – –) Difference spectrum of the reduction of BChl *a* in vitro (taken from [8]).

was achieved by illumination of the PP complex with white light of low light intensity (~2 mW/cm²) [1] at an E_h of ~−450 mV; *N*-methyl-phenazonium methosulfate (PMS), a fast electron donor, was present to keep the primary electron donor P840 reduced. Upon illumination of the PP complex with high light intensity (~350 mW/cm²) [1] at −5°C followed by cooling in the light, an ESR spectrum was obtained at 10 K, which showed a free radical signal centered at g = 2.0040 ± 0.0005 with a peak-to-peak width ΔH_{pp} = 14–15 G. The signal was attributed to the reduction of an electron acceptor X_1; its characteristics suggested that X_1^- might be a monomeric porphyrin anion. X_1 is probably not the primary acceptor, since formation of a spin-polarized triplet [7] was still observed upon illumination under conditions that X_1 was reduced, indicating the existence of still an earlier acceptor I [1].

Accumulation of X_1^- could be observed optically and with ESR at 0°C in the PP complex under conditions where X_2 was reduced by dithionite. Fig.1 (— —) shows the absorbance difference spectrum obtained upon high intensity illumination of the PP complex at 0°C and E_h = −620 mV in the presence of PMS. The spectrum shows a bleaching at 814 nm, 610 nm and 390 nm and a broad absorbance increase in the region 630–730 nm. The shape of the spectrum, when compared to the reduced minus oxidized difference spectrum of bacteriochlorophyll *a* (BChl *a*), in vitro (——), as measured [8], strongly suggests that X_1 is a BChl *a* molecule with Q_y band at 814 nm, which is converted to the BChl *a* anion upon illumination. The positions of the different bands have shifted to the red, as compared with those of BChl *a* in vitro, probably due to different surroundings. Similar observations have been reported for the anion of bacteriopheophytin *a* in purple bacteria [9–11] and for the anion of Chl *a* in photosystem I of higher plants [12]. The shape of the broad positive band between 630 and 730 nm may be affected by electrochromism of the pigment(s) absorbing at ~670 nm [2,13]. Below 400 nm the spectrum may be disturbed by absorbance changes due to the oxidation of PMS, which causes an absorbance increase at ~390 nm. As expected under the low potential condition in the presence of PMS $P840^+$ is not observed in the difference spectrum; its presence would have been clearly indicated by an absorbance decrease at 830–840 nm [2,13].

Fig.2 shows the kinetics of the absorbance changes at 815 nm and 680 nm. The kinetics of the changes were the same at all wavelengths, with halftimes of ~1 s for the light-on signal at the maximum light intensity available, and 2–5 s for the dark decay, depending on the sample. At some wavelengths irreversible absorbance changes occurred, probably due to damage of the PP complex by the intense illumina-

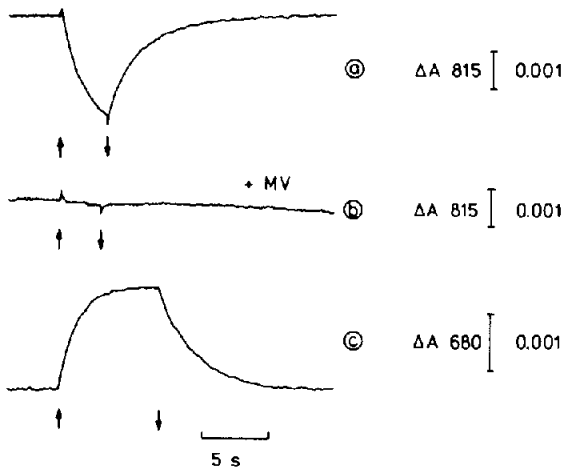

Fig.2. Kinetics of the light-induced absorbance changes at 0°C in the PP complex (A_{810} = 0.48) at 815 nm (a), at 680 nm (c) and at 815 nm in the presence of 100 μM methylviologen (b). Conditions as for fig.1.

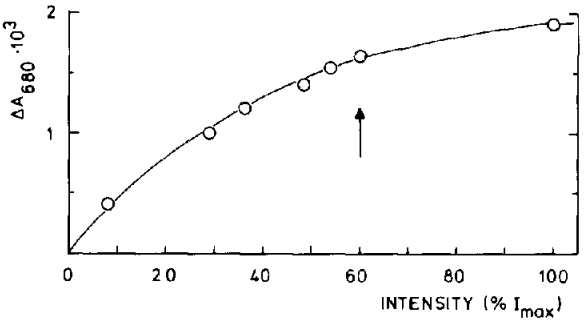

Fig.3. Intensity dependence of the light-induced absorbance changes at 680 nm at 0°C in the PP complex ($A_{810} = 0.48$). Illumination with near-infrared light for 7 s in order to reach maximum amplitude. The arrow indicates the light intensity used to measure the spectrum of fig.1.

tion. When measuring the spectrum, this effect could be minimized by illumination for a short period (1 s) followed by a dark period of ~30 s.

In the presence of 100 μM methylviologen accumulation of X_1^- was not observed, as shown by the absence of the absorbance decrease at 815 nm (fig.2(b)). Instead, an absorbance increase was observed at 600 nm, presumably due to reduction of methylviologen by the reduced acceptor X_1.

Fig.3 shows the light intensity dependence of the amplitude of the absorbance change at 680 nm upon illumination with near-infrared light. The light was not completely saturating at the highest intensity available. Extrapolation to saturating intensities, however, indicated the reduction of ~0.9 molecules X_1/reaction center, if it was assumed that the differential extinction coefficient of X_1 at 814 nm and that of P840 at 842 nm are the same.

Fig.4 (inset) shows the kinetics at 0°C of the light-induced ESR signal change in the PP complex at $E_h = -620$ mV, due to reduction of X_1. It shows half-times of ~3 s for the light-on signal and ~5 s for the dark decay. The ESR spectrum of X_1^- was difficult to obtain at 0°C, due to the low cavity filling factor when using the flat cell and the irreversible damage of the sample occurring during the long illumination times (8 min) needed to record the spectrum. However, X_1^- could be irreversibly accumulated upon illumination at 0°C for 30 s followed by rapid cooling in the light to ~−15°C; further cooling could be performed in the dark. Fig.4 shows the light-induced ESR spectrum of X_1^- at 120 K obtained in this way in a 3 mm quartz tube. Except for amplitude, the spectra were similar when measured at 255, 230, 180 and 120 K, at a microwave power of 20 μW, and showed a Gaussian free radical signal at $g = 2.0038 \pm 0.0003$ with a peak-to-peak linewidth $\Delta H_{pp} = 12.9 \pm 0.3$ G. The linewidth is in good agreement with the linewidth found for the anion of BChl *a* in vitro [14,15] and strongly suggests that X_1 is a monomeric BChl *a* molecule. The linewidth of X_1^- reported earlier at 10 K [1] was somewhat larger (14–15 G). Line broadening occurred gradually from 12.9–~15 G when cooling the sample from ~100–5 K; it was reversible upon warming. In conjunction with the broadening of ΔH_{pp}, the signal became non-Gaussian at low temperature, with excess intensity in the wings. This non-Gaussian character was more pronounced at higher microwave power (up to 2 mW) and was accompanied by a flattening of the peaks, although the value of ΔH_{pp} did not change noticeably. These observations suggest that the broadening with temperature might be caused by admixture of a doublet arising from exchange coupling of X_1^- with X_2^-, analogous to the

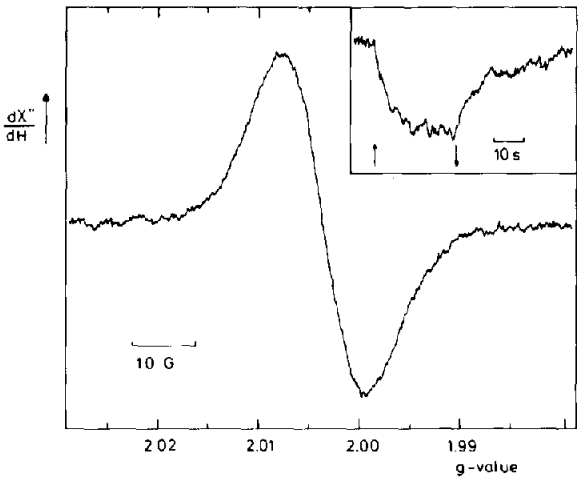

Fig.4. ESR spectrum of the reduced electron acceptor X_1, measured at 120 K in a 3 mm quartz tube. X_1^- was photoaccumulated by illumination of the sample at 273 K, followed by rapid cooling in the light to 250 K: modulation amplitude, 4 G; microwave power, 20 μW; scan time, 4 min; time constant, 1 s. The PP complex ($A_{810} = 4.7$ mm^{-1}) was suspended in a solution containing 30% sucrose (w/w), 0.05% Triton X-100, 8 mM ascorbate, 10 mM dithionite, 10 μM PMS, 8 mM phosphate, 50 mM glycine (pH 10.0) and catalase, glucose and glucose oxidase to maintain anaerobiosis. The E_h was ~−620 mV.

Inset: Kinetics of the light-induced ESR signal change at 0°C in the PP complex ($A_{810} = 4.7$ mm^{-1}; pathlength = 0.5 mm), measured at the high field peak of X_1^-: modulation amplitude, 4 G; microwave power, 20 mW; time constant, 0.3 s.

split signal observed in purple bacteria [10,16]. In our case, however, the exchange interaction would be much less, ~15 G, compared to 60–100 G in some purple bacteria [10,16]. The broadening is too large to be explained by hindered rotation of the methyl groups of the BChl *a* molecule [14].

The *g*-value found for X_1^- differs somewhat from that of the BChl *a* anion in vitro (2.0028–2.0032) as measured in [15,17]. However, values up to 2.0035 have been reported for the anion in vitro [18]. The relatively high *g*-value found for X_1^- might be due to interaction of X_1^- with the reduced iron–sulfur center X_2^-. This suggestion is supported by observations reported on the electron acceptor A_1 in photosystem I of higher plants, which is thought to be a monomeric or dimeric Chl *a*, and which might correspond to X_1 in *P. aestuarii*. In photosystem I particles in which the iron–sulfur center A_2 was reduced, *g*-values of 2.0033 [19] and 2.004 [12] have been reported for A_1^-. However, in photosystem I preparations which did not contain A_2, the *g*-value of A_1^- [20] resembled that of Chl a^- in vitro [21].

We conclude from the optical and ESR experiments that X_1 is a monomeric BChl *a*, which acts as a secondary electron acceptor in the reaction center of *P. aestuarii*. One may suppose that X_1^- corresponds to the monomeric or dimeric Chl *a* anion A_1^-, which is thought to be the primary electron acceptor in photosystem I of higher plants. However, X_1 is most probably not the 'first' electron acceptor in the reaction center of green bacteria, because upon illumination at 5 K under conditions when X_1 is reduced [1,7] it is still possible to generate the spin-polarized triplet state of P840. This is in contrast to the situation in purple bacteria [23] and in photosystem I of green plants [24], where the light-induced formation of the spin-polarized reaction center triplet is lost when the 'first' acceptor is in the reduced form before illumination.

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