AN OPTICAL AND ESR INVESTIGATION ON THE ACCEPTOR SIDE OF THE REACTION CENTER OF THE GREEN PHOTOSYNTHETIC BACTERIUM *PROSTHECOCHLORIS AESTUARII*

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1. Introduction

There are several indications that the acceptor chain of green photosynthetic bacteria is basically different from that of purple bacteria. Like photosystem I, green bacteria are capable of direct photoreduction of pyridine nucleotides [1,2], a reaction that is dependent on a ferredoxin. Titrations of the ability to photooxidize cytochrome c_{553} indicate that the oxidation–reduction midpoint potential (E_m) of the first stable acceptor is about −540 mV [3]. A membrane-bound iron–sulfur protein was reported in *Chlorobium* [4], with an E_m of about −550 mV, low enough to be a component in the electron-transport chain on the reducing side of the light reaction. ESR measurements in [5] at low temperature suggested the photoreduction of an iron–sulfur protein in 'chromatophores' of *Chlorobium*, but the experiments in [6] were at variance with this conclusion. These ESR experiments, however, were seriously hampered by the extremely high absorbance of the preparations, which have a high antenna BChl to reaction center ratio. We have isolated the photochemically active photosystem pigment (PP) and reaction center pigment–protein (RCPP) complexes from *Prosthecochloris aestuarii*, with antenna BChl *a* to reaction center ratios of ~75 and 35, respectively [7]. In contrast to the membrane vesicle complex I [8,9], the PP complex could be concentrated without aggregation of the sample. Thus, we were able to obtain concentrated samples of the PP complex with good optical properties, that were suitable for ESR work.

This paper presents a study on the acceptor side of the reaction center of *P. aestuarii*, by means of ESR and optical methods. Two distinct iron–sulfur centers (X_2 and X_3) are identified, which appear to function serially as secondary acceptors in the electron-transport chain. X_2 receives the electrons from an earlier reduced acceptor, which acceptor (X_1) might be a porphyrin. If this acceptor is reduced, illumination at 5 K produces the triplet state of P840 in all reaction centers. Evidence reported in [10] indicates that the triplet is formed by the radical pair mechanism [11, 12]. This suggests the existence of an electron acceptor I, which acts before X_1 in the reaction center.

2. Materials and methods

Prosthecochloris aestuarii, strain 2K, was grown anaerobically in a mixed culture originally known as '*Chloropseudomonas ethylica*' [13] as in [14]. The membrane preparation complex I and the reaction center preparations PP and RCPP complex were prepared as in [7]. Light-induced absorbance changes were measured as in [15]. ESR experiments were performed with a Varian E-9 spectrometer, having a response time of 20 μs, as in [16]. Continuous illumination with white light provided by a 1000 W projection lamp, filtered by 5 cm water and a Balzers Calflex C filter or Xenon flashes (duration at half-maximum intensity 5 μs) were used as actinic illumination. The samples were contained in quartz tubes of 3 mm internal diameter. Samples were frozen to 80 K in a nitrogen flow cryostat, before freezing to 5 K. Preillumination with white light of the samples was carried out in the nitrogen gas flow cryostat ('high light' conditions, ~350 mW/cm^2). In some cases illumination was provided through a NG 9, 2 mm neutral density filter ('low light' conditions, 2 mW/cm^2). For

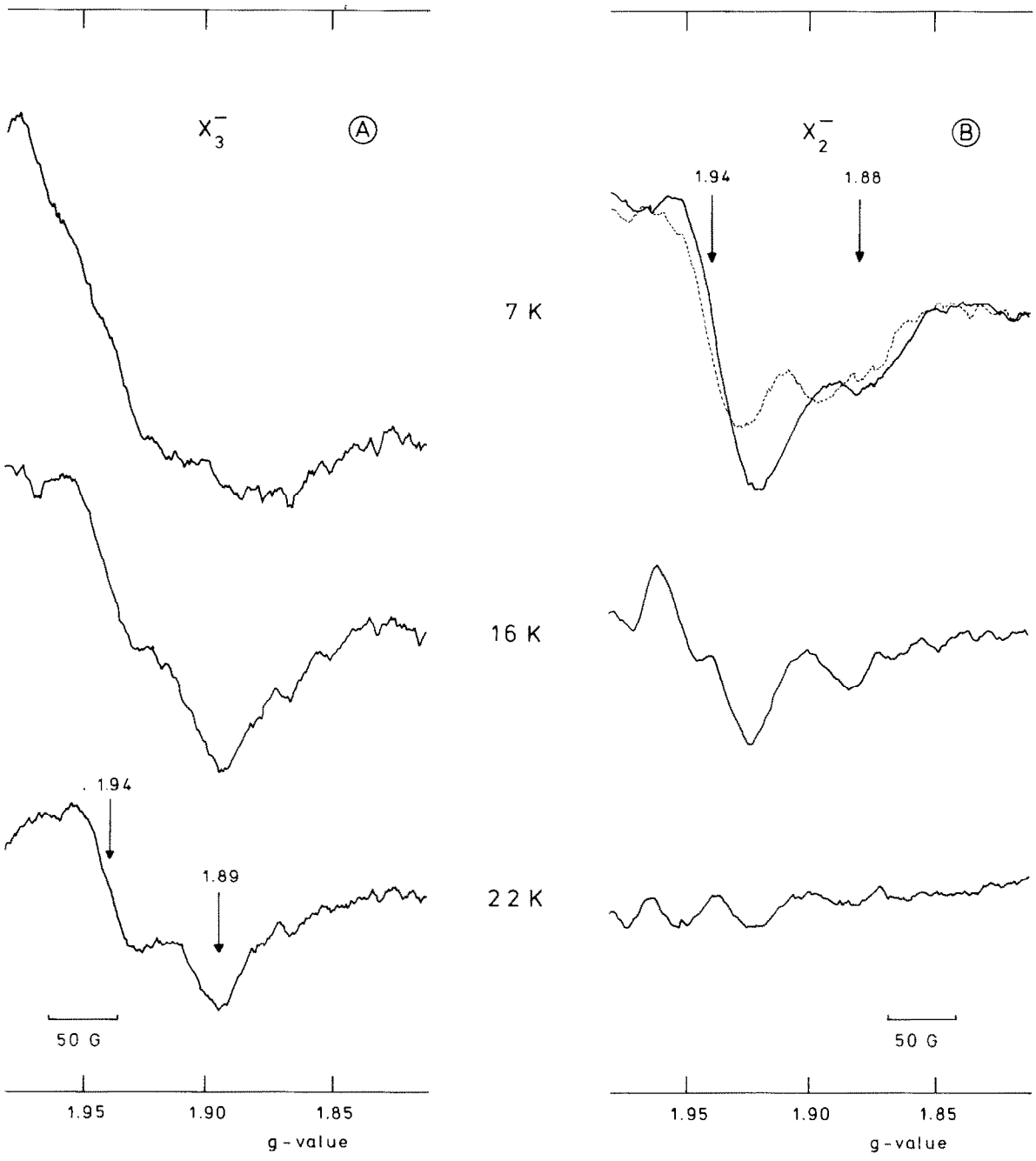

Fig.1. (A) ESR spectrum of the reduced electron acceptor X_3, measured at 7,16 and 22 K. The spectra are the difference between the spectra of the PP complex at E_h = +70 mV and at E_h = −420 mV. (B) ESR spectra of the reduced electron acceptor X_2, measured at 7,16 and 22 K in the PP complex at E_h = −420 mV. The spectra are the difference between the spectra obtained when the sample was frozen in the dark and when frozen under 'low light' conditions (solid curves) or under 'high light' conditions (broken curve). The PP complex (A_{810} = 2.1 mm^{-1}) was suspended in 20% sucrose (w/w), 0.025% Triton X-100, 5 mM ascorbate, 5 mM phosphate, 50 mM glycine (pH 7.7). The experiment at E_h = −420 mV was done in the presence of 10 mM dithionite and 100 μM PMS, flushing the sample with nitrogen gas before cooling. All samples contained 50% glycerol (v/v). The spectra are the average of 4–16 scans: modulation amplitude, 20 G; microwave power, 10 mW.

measurements at low temperatures the samples contained glycerol (50% v/v) to prevent crystallization upon cooling.

Low redox potentials in the samples were obtained by addition of dithionite, which was added from a freshly prepared 0.5 M stock solution in 0.5 M glycine (pH 9.5). The redox potential (E_h) was measured under anaerobic conditions with a Pt-electrode in a homemade cuvette, connected by a salt bridge to a Ag/AgCl reference electrode. The samples which were used to determine E_h were identical to those used for the optical and ESR measurements, but contained in addition 50 μM of the mediators methylviologen, 1,1′-trimethylene-2,2′-dipyridyl dibromide and 1,1′-trimethylene-4,4′-dimethyl-2,2′-dipyridil dibromide. The latter two mediators were a kind gift of Dr B. Ke, Yellow Springs (OH). At $E_h > -480$ mV methylviologen was used; at lower E_h the sample contained all 3 mediators.

3. Results and discussion

In [17] illumination of the PP complex at <120 K resulted in the formation of a charge separation between $P840^+$, the photo-oxidized primary electron donor and X^-, an unknown electron acceptor, in 40–50% of the reaction centers. In the other half the triplet state of P840 was formed. ESR measurements at 5 K [10] showed that P840 is a dimer of BChl *a* and that the BChl *a* triplet was formed via the radical pair mechanism [11,12], yielding a spin-polarized ESR spectrum.

If the PP complex was frozen in the dark at a redox potential (E_h) of +70 mV, ~20% of $P840^+$ appeared to be irreversibly photo-oxidized at 5 K. The remaining part of $P840^+$ had a decay time $t_{1/2}$ = 13 ms, presumably due to a charge recombination between $P840^+$ and X^-. After lowering the E_h with dithionite to about –420 mV, P840 oxidation was completely reversible, probably due to reduction of a secondary electron acceptor by dithionite [10]. Fig.1A shows the differences between the ESR spectra of samples that were frozen in the dark at E_h = –420 and +70 mV, respectively. The spectra obtained indicate that the component reduced by dithionite is an Fe–S center with anisotropic *g*-values at 1.94 and 1.89, as can be most clearly seen in the spectrum obtained at 22 K. Lowering the temperature to 7 K leads to broadening of the 1.89 peak, resulting in a less well-resolved spectrum. A weak component near $g = 2.05$ in the spectrum of X_3^- was largely obscured by a strong background signal from the quartz dewar observed at high microwave power, which produced subtraction artefacts around $g = 2.05$. Taking into account that in ~50% of the reaction centers the triplet is formed upon illumination, we conclude that in the PP complex at E_h = +70 mV, electron transfer to this Fe–S center, which we call electron acceptor X_3, occurs at 5 K only in ~10% of the reaction centers. For complex I the irreversible component in the photo-oxidation of P840 at 5 K was ~50% at E_h = +70 mV. Again, the photo-oxidation was completely reversible at E_h = –420 mV. We assume that a Fe–S center similar to X_3 is responsible for the complete irreversibility of $P840^+$ observed in 'chromatophores' from *Chlorobium* [5,6]. Apparently, reduction of X_3 is progressively lost upon the preparation of complex I and the PP complex. However, the light-induced ESR signal observed in 'chromatophores' of *Chlorobium* [5] does not appear to result from X_3^-; it may be due to a reduced Rieske Fe–S protein as suggested [6].

Under conditions that X_3 was reduced by dithionite (E_h = –450 mV at pH 7.4), we were able to accumulate another reduced acceptor in the light, when *N*-methyl-phenazonium methosulfate (PMS) was added, a fast electron donor to keep P840 reduced. Fig.2 shows the light-induced difference spectrum at room temperature of the PP complex, obtained in this way. The spectrum of this component, which we call electron acceptor X_2, showed a broad minimum at 445 nm with shoulders at 400 and 475 nm, which suggests that it is an Fe–S center, with a spectrum resembling that of the Fe–S center measured in [18], using photosystem I particles. Methylviologen (10 μM) completely abolished the absorbance changes, probably by rapid reoxidation of X_2^-. From the amplitude of the absorbance changes at 445 nm in saturating light a differential extinction coefficient of about 5 $mM^{-1} \cdot cm^{-1}$ is calculated, if the reduction of 1 Fe–S center X_2/reaction center is assumed. The absorbance changes near 670 and 800–850 nm might be ascribed to electrochromic absorbance changes of BPh *c* and BChl *a*, accompanying the reduction of X_2.

At room temperature the photoreduction of X_2 was completely reversible in the presence of dithionite; below –30°C, however, the absorbance changes were irreversible. This offered the possibility to measure the ESR spectrum of X_2^-. In order to accumulate X_2^-, the sample was illuminated with 'low light' intensity at –5°C for 1 min, followed by rapid cooling to 80 K

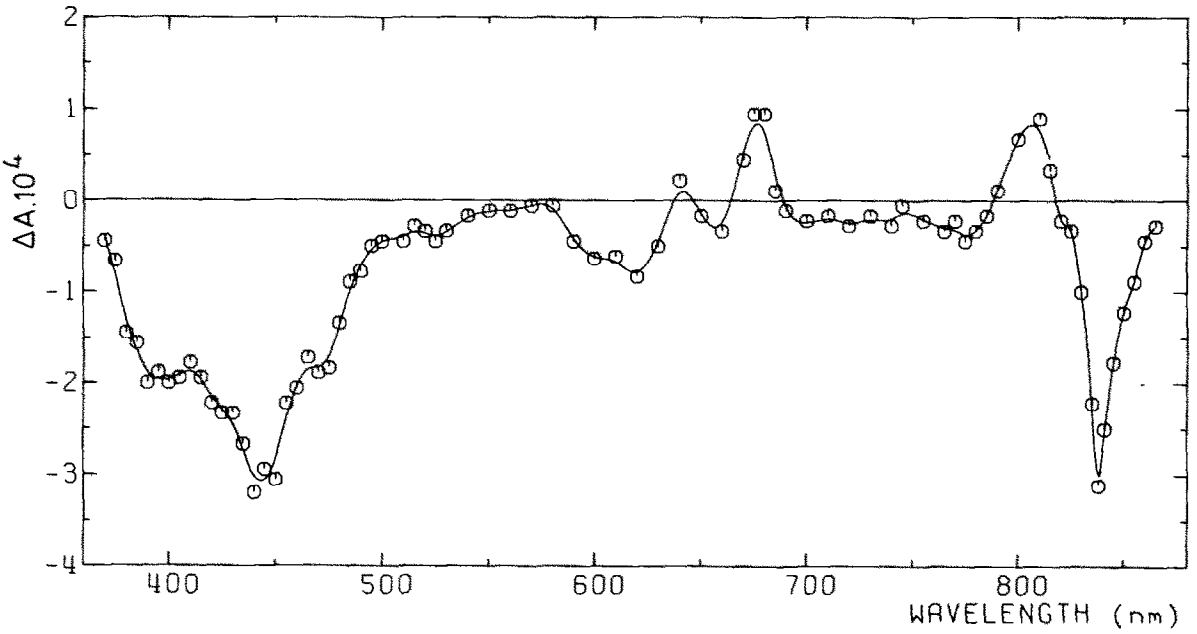

Fig.2. Absorbance difference spectrum of the PP complex (A_{810} = 0.80) due to reduction of the electron acceptor X_2. Illumination for 3 s with orange light provided by a Schott AL 606 interference filter (0.9 mW/cm²) for the spectral region 670–870 nm or with near-infrared provided by a combination of Schott RG 715 and RG 780 filters (0.9 mW/cm²) for the region 360–690 nm. The sample contained 5 mM ascorbate, 0.05% Triton X-100, 10 mM dithionite, 10 μM PMS, 35 mM phosphate (pH 7.4) and catalase, glucose and glucose oxidase to maintain anaerobis. The E_h was about −450 mV. Each point is the average of 4 measurements.

in the light. Fig.1B (——) shows the difference between the ESR spectra of the PP complex, obtained in this way at 7,16 and 22 K and of the same sample frozen in the dark. The spectra confirm our conclusion that X_2 is an Fe–S center. It shows anisotropic g-values at 1.94 and 1.88, as can be most clearly seen in the spectrum at 7 K. Like in the case of the spectrum of X_3^-, a weak signal near g = 2.05 was largely obscured by subtraction artefacts. The same result was obtained when X_2 was photoreduced in the same way as an E_h value of about −520 mV at pH 9.5 X_2^- was reoxidized upon warming to room temperature in the dark. Lowering of the E_h to about −620 mV at pH 10.0 resulted in chemical reduction of X_2, as judged from the absence of light-induced absorbance changes of this acceptor. We therefore assume that the E_m of X_2 is close to −560 mV. An ESR spectrum similar to that of X_2^- was obtained [4] in 'chromatophores' of *Chlorobium* by chemical reduction at E_h = −590 mV. The spectrum of this component, like that of X_2^-, could only be observed below 20 K, in good agreement with our results.

Upon continuous illumination or flash excitation at 5 K of a sample frozen at E_h = −420 mV and at 'low light' intensity, and which according to our interpretation should be in the state P840 $X_2^-X_3^-$, no signal due to $P840^+$ could be detected. The formation of a spin-polarized triplet was, however, still observed, which indicated the existence of a more primary acceptor. This acceptor could be accumulated together with X_2^-, if the PP complex was illuminated at −5°C and during cooling to 80 K with 'high' instead of 'low' light intensity. Fig.3 shows the difference between the ESR spectra obtained by freezing under 'high' light conditions and freezing in the dark, measured at 10 K. The spectrum shows a free radical signal, symmetrically centered at g = 2.0040 ± 0.0005 with a peak-to-peak linewidth ΔH_{pp} = 14–15 G. We assume that this spectrum is due to reduction of an electron acceptor X_1. Both the g-value and the linewidth suggest that the spectrum of X_1^- is due to a monomeric porphyrin anion [19]. The spectrum is somewhat broader than that of a free porphyrin anion [19,20]. This broadening might, however, be caused by mag-

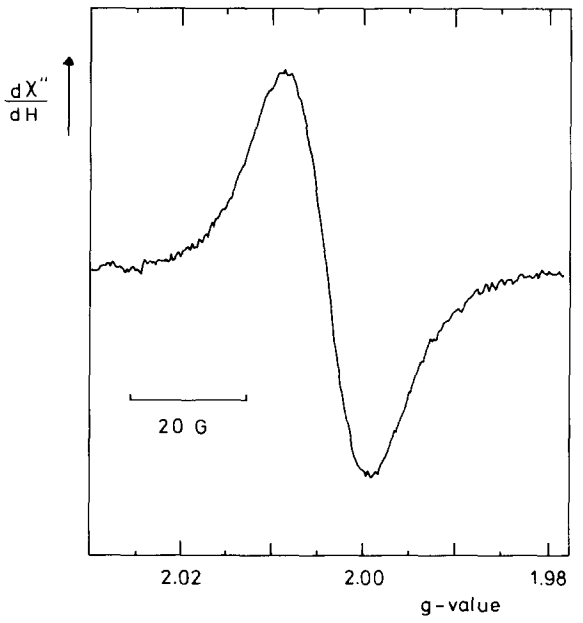

Fig.3. ESR difference spectrum at 10 K of the PP complex (A_{810} = 2.1 mm^{-1}) at E_h = −420 mV (conditions as for fig.1), due to the reduced electron acceptor X_1. The spectrum is the difference between the spectra obtained when the sample was frozen in the dark and when frozen under 'high light' conditions: modulation amplitude, 5 G; microwave power, 0.2 mW; av. 4 scans.

netic interactions with the reduced Fe–S center X_2. The spectrum of the Fe–S center X_2^- obtained under 'high' light conditions (fig.1B, . . .) is somewhat different from that obtained under 'low' light conditions, which might be due to interactions with X_1^-. Besides, the spectra of fig.1B suggest some heterogeneity in X_2^-. Similar observations have been made with photosystem I, where the ESR spectrum of the reduced Fe–S center A is modified upon reduction of center B [21]. Like X_2^-, X_1^- was reoxidized upon warming up in the dark.

Upon illumination at 5 K of the sample frozen under 'high light' conditions, i.e., in the state P840 $X_1^-X_2^-X_3^-$, no light-induced signals could be detected in the g = 2 region. Surprisingly, formation of the spin-polarized triplet was still observed, but in an amount that was 2–2.5-times the amount formed upon illumination of the sample frozen in the dark. This suggests that an even earlier acceptor than X_1, which we call I, is present in the reaction center. Recombination of $P840^+$ and I^- then would generate the triplet state of P840 by the radical pair mechanism [11,12]. When the forward electron transport to X_1 is blocked, the triplet state of P840 would be formed in all reaction centers instead of in 40–50%, in agreement with the observed increase of the triplet signal. Optical experiments are in progress to obtain more information about the identity of both X_1 and I.

4. Conclusions

A scheme representing the electron transport chain at 5 K in the reaction center of the green bacterium *P. aestuarii* is shown in fig.4. At low temperature, electrons are irreversibly transferred to X_3, an Fe–S center with principal g-values at 1.94 and 1.89, with an E_m value >−420 mV. In the PP complex this electron transport occurs only in about 10% of the reaction centers. In about 50% of the reaction centers the electrons do not go further than X_2, while in the remaining part the triplet state of P840 is formed. The E_m of

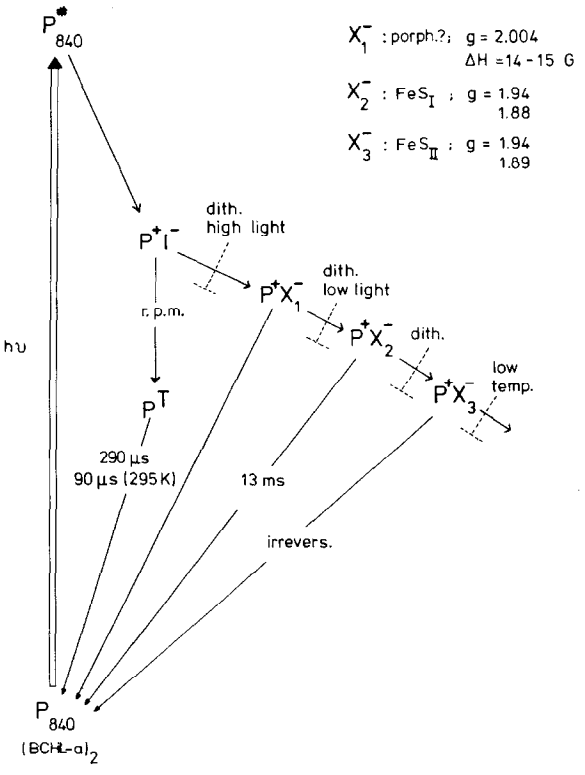

Fig.4. Schematic representation of the electron transport in the reaction center of *P. aestuarii* at 5 K; r.p.m., radical pair mechanism.

X_2 is lower than that of X_3, probably about −560 mV; it is an Fe–S center with principal g-values at 1.94 and 1.88. X_2^- and $P840^+$ recombine with a halftime of 13 ms at 5 K. X_2 can be accumulated in the reduced form at 'low' light intensity in the presence of PMS and dithionite. Accumulation of X_1^- together with X_2^- is achieved upon illumination at −5°C with 'high light' in the presence of PMS and dithionite. The spectrum of X_1^-, with $g = 2.0040 \pm 0.0005$ and $\Delta H_{pp} = 14–15$ G suggests the formation of a monomeric porphyrin anion radical; optical measurements will have to confirm this hypothesis. If X_1 is reduced, only the triplet state of P840 is observed upon illumination, formed by recombination between $P840^+$ and I^-, the primary electron acceptor of as yet unknown identity.

Our results demonstrate quite clearly that the electron-transport chain in green bacteria is basically different from that of purple bacteria, especially at the reducing side of the reaction center. This is reflected in the capability of direct NAD^+ reduction by green bacteria [1,2], whereas reduction of NAD^+ by purple bacteria is ATP-dependent [3,22]. Similarities with photosystem I of higher plants and algae, which is capable of direct reduction of $NADP^+$, are obvious. Like the charge separation between P700 and center A or B [21], formation of $P840^+X_3^-$ is irreversible at 5 K. If centers A and B are reduced, formation of $P700^+$ is reversible at low temperatures [21], similar to the formation of $P840^+X_2^-$ when X_3 is reduced. It is possible that X_1, which might be a porphyrin, corresponds to A_1 in photosystem I [23]. This then would suggest that the oxidation–reduction midpoint potentials of the acceptors in the reaction center of green bacteria are substantially higher (100–150 mV) than those in photosystem I. However, an acceptor more primary than A_1 has not yet been found in photosystem I.

From our previous paper [10] on the oxidizing side and the present one on the acceptor side of the reaction center of *P. aestuarii*, it appears that the primary photoprocesses of green bacteria resemble those of purple bacteria, whereas the remaining part of the acceptor chain resembles that of photosystem I, a situation that would be quite interesting from an evolutionary point of view.

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