Primary photochemistry in the facultatively aerobic green photosynthetic bacterium *Chloroflexus aurantiacus*

(photosynthesis/bacteriochlorophyll/reaction center/redox potentiometry/evolution)

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ABSTRACT Photochemical activity was examined in membrane fragments and a purified membrane preparation from *Chloroflexus*. Flash-induced absorption difference spectroscopy strongly suggests a primary donor (P_{865}) that is more similar to the P_{870} bacteriochlorophyll *a* dimer found in the purple photosynthetic bacteria than it is to P_{840} found in the anaerobic green bacteria. Redox measurements on P_{865} and an early acceptor also indicate a photochemical system characteristic of the purple bacteria. The membrane preparation contains a tightly bound type *c* cytochrome, c_{554} , that is closely coupled to the reaction center as indicated by its ability to rereduce photooxidized P_{865} . *Chloroflexus* thus appears to be distinct photochemically from other families of photosynthetic bacteria and may occupy an important role in photosynthetic evolution.

Many of the electron transfer components found in the four families of anoxygenic photosynthetic bacteria are also characteristic of oxygenic photosynthetic organisms. The two families of purple photosynthetic bacteria, Rhodospirillaceae and Chromatiaceae, have an electron acceptor system strikingly reminiscent of the acceptor found in photosystem II of oxygen-evolving organisms (1). In contrast, the electron acceptor system of the anaerobic green bacteria, the Chlorobiaceae, is similar to the acceptors found in photosystem I (2-6).

Little has been reported on the primary processes of photosynthesis in the recently discovered family of the facultatively aerobic green bacteria, Chloroflexaceae. This family is unique in that it shares many features of both the green and purple bacteria. It resembles the Chlorobiaceae in that it contains two types of bacteriochlorophyll (Bchl), Bchl *c* and Bchl *a*. The Bchl *c* in *Chloroflexus* and all other green bacteria is contained in antenna-like structures called chlorosomes. The reaction center Bchl *a* in all green bacteria including *Chloroflexus* is located in an undifferentiated cytoplasmic membrane rather than in the highly differentiated intracytoplasmic membrane characteristic of all purple bacteria (7-9). Metabolically, *Chloroflexus* resembles the Rhodospirillaceae in having the capability to exist as a light-independent aerobic heterotroph (10).

Pierson and Castenholz (11) and Schmidt (12) have shown that most of the Bchl *a* in *Chloroflexus aurantiacus* has an *in vivo* absorption spectrum similar to that found in purple bacteria and unlike the Bchl *a* absorption spectrum of the green anaerobic bacteria. A continuous light-induced difference spectrum suggested the presence of a Bchl *a*-type reaction center pigment analogous to that occurring in the purple bacteria (11).

Feick *et al.* (13) recently developed a procedure for isolating from *Chloroflexus* cytoplasmic membranes that are completely devoid of Bchl *c* yet still contain photochemical activity. Using these preparative techniques, we have examined the photo-

chemistry of *Chloroflexus*, and in this paper we present evidence that the primary photoact is nearly identical to that found in the purple bacteria and unlike that found in other green bacteria.

MATERIALS AND METHODS

Preparative Methods. Organism and growth conditions were the same as described by Sprague *et al.* (14). The isolation of whole membranes and purified cytoplasmic membranes used the procedures described by Feick *et al.* (13).

Single-Flash-Induced Absorption Change Measurements. Measurements were made on a homemade single-beam laser spectrophotometer. Actinic illumination (583 nm; 1- μ sec flashes) was provided by a Candela SLL-625 flashlamp-pumped dye laser with rhodamine 590 as the dye.

Absorption and Difference Spectra. Absorption spectra were recorded on Cary 14R or Cary 219 spectrophotometers. Absolute cytochrome difference spectra were recorded with the Cary 219 using dithionite and ferricyanide as reductant and oxidant, respectively. Light-induced absorption changes in the near infrared (>900 nm) were measured on a Cary 14R spectrophotometer in the IR-2 mode.

Redox Potentiometry. Redox potentiometry was performed in a system similar to that described by Dutton (15). A double-bridged Ag/AgCl combination redox electrode (no. 7025-02-90, Ingold, Andover, MA) was used; it was calibrated before each use by measurement of a standard redox buffer solution (Ingold). Dithionite and ferricyanide were used as titrants. Mediators and titrants were prepared freshly in anaerobic solutions of either double-distilled H₂O or absolute ethanol. They were kept on ice and in the dark until used. Mediators used were phenazine ethosulfate (PES), phenazine methosulfate (PMS), *N,N,N',N'*-tetramethylphenylenediamine (TMPD), 2,3,5,6-tetramethyl-*p*-phenylenediamine (DAD), duroquinone, and vitamin K. All potentials are relative to the normal hydrogen electrode.

EPR Spectroscopy. EPR spectra were recorded at room temperature on a Varian E-9 spectrometer. Reversible light-minus-dark spectra were produced by subtraction of dark-after-light spectra from the light spectra.

RESULTS

The whole-membrane fraction (Fig. 1A) was derived from cells grown under low light conditions ($A_{740}/A_{866} = 20$) as defined by Feick *et al.* (13). Fig. 1B is an absorption spectrum of cytoplasmic membranes (CM). The low absorbance at 740 nm in-

Abbreviations: Bchl, bacteriochlorophyll; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; PES, phenazine ethosulfate; PMS, phenazine methosulfate; TMPD, *N,N,N',N'*-tetramethylphenylenediamine; CM, cytoplasmic membranes.

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FIG. 1. Absorption spectra. (A) "Whole membrane fraction" from cells grown under low light conditions to be used for cytoplasmic membrane isolation. (B) Isolated cytoplasmic membranes.

indicates that this fraction is largely free of Bchl *c*. CM preparations with $A_{866}/A_{740} \geq 5$ were used for this work.

Fig. 2 shows the flash-induced difference spectrum of purified CM at ambient redox potential. We performed a similar experiment with whole membranes between 750 and 1,000 nm (data not shown). Both whole membranes and purified CM showed bleaching at 865 and 823 nm and an absorbance increase at 805 nm. In purified CM, a smaller photobleaching at 600 nm and absorbance increases at 430 and 760 nm were apparent. These absorbance changes were not detectable in this region in whole membrane preparations because of the large absorbance of the antenna system. At this redox potential there were no absorbance changes which could be attributed to a cytochrome. The time course of flash-induced absorbance changes at 865 and 805 nm are shown in Fig. 2 *Inset*. Both absorbance changes had similar biphasic recovery kinetics. These changes were almost certainly due to the photooxidation of the reaction center Bchl and its subsequent rereduction (16). Continuous light-induced changes in the infrared region showed two peaks, at 1,160 and 1,250 nm. Both peaks were completely reversible (data not shown).

Light-induced EPR spectra in the $g = 2.0$ region are shown in Fig. 3. Both the whole membrane fraction and purified CM showed a symmetric, reversible light-minus-dark signal centered at $g = 2.003 \pm 0.001$. Both had a peak-to-peak linewidth,

FIG. 2. Laser flash-induced difference spectrum of purified CM at ambient redox potential. Each point represents the mean of 6–10 traces. The calculation was made by using data from 10 msec after the flash. (*Inset*) Representative absorbance changes at 865 and 805 nm. Each trace represents the mean of six flashes.

FIG. 3. Reversible light-induced EPR spectra of both whole membranes (200 KP) and isolated CM. Both traces are light-minus-dark after light. Modulation amplitude at 100 kHz, 4 G; receiver gain, 3.2×10^4 .

ΔH_{pp} , of 9.8 ± 0.2 G. This g factor is consistent with a Bchl *a* cation radical, and the narrow linewidth suggests that this radical probably consists of a Bchl *a* dimer (17). This EPR signal almost certainly arises from the photooxidized primary donor pigment, analogous to that found in all other photosynthetic organisms. At room temperature this photooxidation was almost completely reversible.

The results of a redox titration of P_{865} are shown in Fig. 4. This titration indicates a one-electron reaction with a midpoint potential at pH 8.1 of +360 mV. As the potential was decreased below +300 mV, the amount of P_{865} photobleaching began to decrease. This decrease in ΔA_{865} at lower potentials did not involve any interactions with the mediators as shown by its independence of mediator concentration (data not shown). The low potential decrease in ΔA_{865} fits well to a one-electron reaction with a midpoint potential at pH 8.1 of +255 mV. Both titration curves were reversible and showed little hysteresis.

Chemically induced reduced-minus-oxidized cytochrome difference spectra are shown in Fig. 5. Both whole membranes and isolated CM from anaerobic light-grown cells showed the characteristic shape of type *c* cytochrome (c_{554}) with α , β , and Soret peaks at 554, 525, and 422 nm, respectively. The difference spectrum from whole membranes of aerobic dark-grown

FIG. 4. Redox titration of P_{865} in isolated CM. The mediators were PES, PMS, DAD, and TMPD, each at 20 μ M. $\bullet$, Oxidative titration; $\blacktriangle$, reductive titration (both were done on the same sample). Midpoint potential at pH 8.1 ($E_{m,8.1}$) values are shown at each curve.

FIG. 5. Chemically induced reduced-minus-oxidized cytochrome difference spectra of whole membranes from aerobic dark-grown cells (D), anaerobic light-grown cells (L), and isolated CM from anaerobic light-grown cells. Difference spectra were taken on a Cary 219 spectrophotometer with ferricyanide and dithionite as oxidant and reductant. (Inset) Enlargement of the α region of these spectra.

cells is also shown in Fig. 5. This spectrum shows the presence of an additional cytochrome with an α peak at 562 nm, suggestive of a type *b* cytochrome. Fig. 5 Inset shows an enlargement of the α region of these spectra. We could not detect the presence of a type *b* cytochrome in photosynthetically grown cells. However, cells grown aerobically in the dark apparently contained both a type *c* cytochrome and a type *b* cytochrome. Neither cell type showed the presence of a type *a* cytochrome. The c_{554} found in the light grown cells was bound tightly to the CM because it remained in that fraction throughout the isolation.

To determine if c_{554} found in the light-grown cells is photooxidizable, a CM fraction was poised at moderately low redox potential ($+130 \pm 20$ mV), and flash-induced absorbance changes in the 550-nm region were measured. A spectrum of the change observed 10 μ sec after the flash is shown in Fig. 6. These absorbance changes had a rise time of <10 μ sec. The bleaching at 554 nm corresponds to the photooxidation of the α peak of cytochrome c_{554} . A redox titration of flash-induced cytochrome c_{554} oxidation is shown in Fig. 7. This titration indicated a reversible one-electron reaction with a midpoint potential of $+260$ mV at pH 8.1. The width of the titration region suggests only a single component.

Although we have not directly measured the rate of cytochrome photooxidation we assume that its oxidation is due to rapid electron transfer to P_{865}^+ . This is supported by the fact that absorption changes attributed to P_{865} oxidation on the millisecond time scale decrease at lower redox potentials where cyto-

FIG. 6. Flash-induced difference spectrum of cytochrome c_{554} α peak in isolated CM. The mediators in this sample were PES, PMS, DAD, and TMPD, each at 20 μ M. The potential was poised at $+130 \pm 20$ mV by addition of dithionite.

chrome c_{554} becomes reduced (Fig. 4). The redox behavior of P_{865} at lower potentials is essentially parallel to the redox behavior of cytochrome c_{554} (compare Figs. 4 and 7). Because of this close coupling between cytochrome c_{554} and P_{865} it is possible to titrate the primary acceptor indirectly by monitoring the amount of cytochrome photooxidation as a function of redox potential. The results of the acceptor titration via cytochrome c_{554} are shown in Fig. 8. The midpoint of this titration was about -50 mV at pH 8.1 for a one-electron reaction. At low redox potentials, ΔA_{554} was very small, indicating that the acceptor was completely reduced and no stable charge separation could take place.

When *o*-phenanthroline was added to whole membranes, the rate of recovery of P_{865} photobleaching was greatly increased (Fig. 9). Although both the rate of recovery and the amount of photobleaching were affected, their concentration dependences were different. The rate effect became saturated at about 100 μ M whereas the ΔA_{865} dependence still was present at 340 μ M. However, when the same experiment was performed on isolated CM, the effect of *o*-phenanthroline was greatly decreased (data not shown). The rate constant for recovery of P_{865} photobleaching was 30 sec^{-1} and was largely independent of *o*-phenanthroline concentration. The amount of P_{865} photobleaching still was somewhat dependent on *o*-phenanthroline concentration.

FIG. 7. Redox titration of cytochrome c_{554} in isolated CM. The mediators in this sample were PES, PMS, DAD, and TMPD, each at 20 μ M. $\bullet$, Oxidative titration; $\blacktriangle$, reductive titration; both were done on the same sample. $E_{m,8.1} = +260$ mV, $n = 1$.

FIG. 8. Redox titration of acceptor via cytochrome c_{554} in isolated CM. The mediators used were TMPD, PES, PMS, duroquinone, and vitamin K, each at 20 μ M. ■, Oxidative titration; ×, reductive titration; both directions were done on the same sample. $E_{m,8.1} = -50$ mV, $n = 1$.

DISCUSSION

The results presented in this paper verify the unique intermediate nature of the photosynthetic apparatus of *Chloroflexus aurantiacus*. Previous work has shown the similarity of the Chlorobiaceae and the Chloroflexaceae (9, 12–14). However, most of that work was focused on the chemical and structural composition of the antenna systems and the morphological characteristics of the photosynthetic membranes. Our results clearly distinguish *Chloroflexus* from the Chlorobiaceae on the basis of its primary photochemical processes and its subsequent electron transport. In these aspects *Chloroflexus* bears more resemblance to the purple bacteria than to the other green anaerobic bacteria.

The flash-induced absorption difference spectrum for *Chloroflexus* (Fig. 2) shows virtually the same absorbance changes that have been attributed to the oxidation of P_{870}^+ in many purple bacteria (16). The absence of the two negative peaks at 830 and 842 nm, typical of the photooxidized primary donor in the green anaerobic bacteria (9), indicates a different environment of the reaction center Bchl in *Chloroflexus* compared to the Chlorobiaceae. The fact that both whole membranes and purified CM show the same near-infrared absorption changes suggests that the reaction center complex is relatively undisturbed by the isolation procedures used in this work. It is interesting that the continuous light-induced changes in the near-infrared region

FIG. 9. The effect of *o*-phenanthroline on whole membranes (200 KP). ●, Rate constant for the recovery of P_{865} photobleaching; ■, ΔA_{865} . The *o*-phenanthroline was in absolute ethanol and was allowed to equilibrate for 10 min before measurements were made.

have peaks at the same wavelengths found previously in both the green anaerobic bacteria, 1,157 nm (2), and the purple bacteria, 1,245 nm (18, 19). In both organisms the peaks have been attributed to the oxidized form of the reaction center Bchl *a* dimer. It is possible that one of those peaks corresponds to the oxidation of antenna Bchl in an oligomeric form as suggested by Gomez *et al.* (20). The light-induced EPR spectra in the $g = 2.0$ region also indicates a primary electron donor similar to the donor found in most of the purple bacteria as well as in the green anaerobic bacteria (2, 17).

Although the primary donor, P_{865} , is spectrally similar to the primary donor in the purple bacteria, its redox behavior is somewhat different. The midpoint potential of P_{865} is 100 mV less than that found in all other purple bacteria and 100 mV higher than that found in the Chlorobiaceae. Thermodynamically, the primary donor in *Chloroflexus* thus is quite different from the donor found in either the purple or green bacteria, with a midpoint potential nearly halfway between the two.

The chemically induced reduced-minus-oxidized difference spectrum of light-grown cells of *Chloroflexus* reveals only a single type *c* cytochrome. Bartsch (21) was able to detect only a single type *c* cytochrome from photosynthetically grown cells. However, Pierson (22) recently reported evidence for the presence of both *b* and *c* cytochromes in cell-free preparations from light grown cells. We see no evidence for the presence of a type *b* cytochrome in the membranes of these light-grown cells.

The type *c* cytochrome we find in *Chloroflexus* is analogous to the high-potential c_{555} found in *Chromatium* (21). In both

FIG. 10. Rudimentary scheme of cyclic electron transport in *Chloroflexus aurantiacus* and the currently accepted schemes of cyclic electron transport in *Chromatium* and *Chlorobium* (1). The vertical axis represents redox potential in volts.

Chloroflexus and *Chromatium* these cytochromes are strongly associated with the cytoplasmic membrane and remain firmly attached throughout the isolation of fractions enriched in reaction centers. In both organisms these cytochromes donate electrons to P_{865}^+ in less than 10 μ sec at room temperature. Our preparation shows no flash-induced oxidation of a low-potential type *c* cytochrome. However, we cannot rule out the possibility of a low-potential type *c* cytochrome involved in some noncyclic electron-transfer system as is the case in *Chromatium* (21).

The acceptor system in *Chloroflexus* appears to be different from that found in the other green anaerobic bacteria. Redox titrations indicate a primary acceptor with midpoint potential of -50 mV at pH 8.1, in contrast to the -550 mV found in *Chlorobium* (2). The effect of *o*-phenanthroline on the rate of P_{865}^+ recovery also suggests a two-quinone acceptor system analogous to that found in all other purple bacteria studied to date (23). Chemical analysis of the quinones in *Chloroflexus* has shown that it contains only menaquinone and lacks ubiquinone (unpublished data). All purple bacteria contain ubiquinone, and *Chromatium* contains, in addition, menaquinone which functions as the primary acceptor. *Chlorobium* lacks ubiquinone but contains menaquinone of unknown function (23).

Fig. 10 shows a rudimentary scheme of cyclic electron transport in *Chloroflexus* developed from results presented in this paper. It is shown in comparison with the currently accepted schemes of cyclic electron transport in *Chromatium* and *Chlorobium*. The tightly bound type *c* cytochrome found in *Chloroflexus* is reminiscent of the tightly bound high-potential type *c* cytochrome in *Chromatium*. However, the CM absorption spectrum (Fig. 1B) and light-induced difference spectrum (Fig. 2) suggest a close similarity with *Rhodospseudomonas sphaeroides*.

Our results indicate that, in its primary photochemistry and electron transport, *Chloroflexus* has many characteristics of the purple bacteria whereas, in the chemical and structural composition of its antenna system and the morphological characteristics of its photosynthetic membrane, it is a classic example of a green bacterium.

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