Photosynthetic Electron-Transfer Reactions in the Green Sulfur Bacterium *Chlorobium vibrioforme*: Evidence for the Functional Involvement of Iron–Sulfur Redox Centers on the Acceptor Side of the Reaction Center†

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ABSTRACT: The green sulfur bacterium *Chlorobium vibrioforme* was cultured in the presence of ethylene to selectively inhibit the synthesis of the chlorosome antenna BChl *d*. Use of these cells as starting material simplified the isolation of a photoactive antenna-depleted membrane fraction without the use of high concentrations of detergents. The preparation had a BChl a/P_{840} of 50, and the spectral properties were similar to those of preparations isolated from cells grown with a normal complement of chlorosomes. The membrane preparation was active in $NADP^+$ photoreduction. This indicated that the fraction contained reaction centers with complete electron-transfer sequences which were then characterized further by flash kinetic spectrophotometry and EPR. We confirmed that cytochrome c_{553} is the endogenous donor to P_{840}^+, and at room temperature we observed a recombination reaction between the reduced terminal acceptor and P_{840}^+ with a $t_{1/2}$ = 7 ms. Oxidative degradation of iron–sulfur centers using low concentrations of chaotropic salts introduced a faster recombination reaction of $t_{1/2}$ = 50 μs which was lost at higher concentrations of chaotrope, indicating the participation of another iron–sulfur redox center earlier than the terminal acceptor. Cluster insertion using ferric chloride and sodium sulfide in the presence of 2-mercaptoethanol restored both the 50-μs and 7-ms recombination reactions, allowing definitive assignments of these centers as iron–sulfur centers. Following the suggestion of Nitschke et al. [(1990) *Biochemistry 29*, 3834–3842], we associate these two kinetic phases to back-reactions between $P840^+$ and iron–sulfur centers F_X and F_AF_B, respectively. The iron–sulfur cluster degradation and reconstitution protocols also led to inhibition and restoration of $NADP^+$ photoreduction by the membrane preparation, providing unequivocal evidence for the function of the centers F_X and F_AF_B in the physiological electron-transfer sequence on the acceptor side of the *Chlorobium* reaction center. At 77 K we observed a recombination reaction of $t_{1/2}$ = 20 ms that we suggest occurs between F_X^- and P_{840}^+. Degradation of the iron–sulfur clusters resulted in replacement of the 20-ms phase with a faster reaction of $t_{1/2}$ = 80 μs that was most likely a recombination between the early acceptor A_1^- and P_{840}^+ or decay of $^3P_{840}$. Analysis of the iron–sulfur centers in the preparation by EPR at cryogenic temperature supports the optical measurements. EPR signals originating from the terminal acceptor(s) were not observed following treatment of the membrane preparation by chaotropes, and a modified signal was restored following cluster reinsertion. The reinsertion of iron–sulfur clusters in the *Chlorobium* preparation did not require the addition of any polypeptides, and the inclusion of *psaC* and *psaD* gene products that are required for the reconstitution of photosystem 1 did not increase the extent of the *Chlorobium* reconstitution. Kinetic interactions between the redox centers are discussed with reference to the model of the *Chlorobium* reaction center proposed by Nitschke et al. [(1990) *Biochemistry 29*, 3834–3842].

The green bacteria are a unique group of photosynthetic procaryotes characterized by the organization of their main antenna pigment BChl[1] *c*, *d*, or *e*, depending on species or isolate, into chlorosomes (Olson, 1980; Blankenship, 1984). The chlorosomes are oblate spheroidal structures arranged on the cytosolic surface of the plasma membrane in contact with the reaction centers (Staehelin et al., 1980). In addition to BChl *d* the green bacteria also contain BChl *a* which serves as an antenna pigment on the reaction center core and as a "base plate" between the chlorosome and reaction center (Feick & Fuller, 1984). Efficient transfer of electronic excitation energy occurs between the pigments in the chlorosomes and the reaction centers (Van Dorssen & Amesz, 1988; Mimuro et al., 1989).

Two main groups of green bacteria have been described: (1) the strictly anaerobic green sulfur bacteria (Chlorobiaceae) and (2) the facultative aerobic gliding bacteria (Chloroflexaceae) which are capable of photoautotrophic and chemotrophic growth. Important differences between the two groups lie in components of their reaction centers and asso-

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[1] Abbreviations: BChl, bacteriochlorophyll; DPIP, 2,6-dichlorophenolindophenol; EPR, electron paramagnetic resonance; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide electrophoresis; TMPD, *N,N,N',N'*-tetramethylphenylenediamine; FNR, ferredoxin–$NADP^+$ oxidoreductase; PMS, phenazine methosulfate; Fd, ferredoxin.

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ciated photochemistry. The reaction center of the aerobic gliding green bacteria, as typified by *Chloroflexus aurantiacus*, has been shown to be very similar to that of the photosystem 2 of cyanobacteria and green plants. For operational convenience we wish to designate this class of reaction centers as "type 2". In contrast, the reaction center of the anaerobic green sulfur bacteria has been suggested to be similar to that of photosystem 1 and the Heliobacteria. We designate these reaction centers as "type 1", and their main features include an ability to reduce $NAD(P)^+$ directly (Buchanan & Evans, 1969), possession of iron–sulfur redox centers as reaction center intermediates (Knaff & Malkin, 1976; Jennings & Evans, 1977; Swarthoff et al., 1981b; Nitschke et al., 1987, 1990), and a large (ca. 65 kDa) molecular mass reaction center core polypeptide (Hurt & Hauska, 1984). The evolutionary relationships between the various type 1 and type 2 reaction centers have been recently reviewed (Mathis, 1990).

Following an extensive EPR study Nitschke et al. (1990) recently reported on the properties of the iron–sulfur centers in two species of green sulfur bacteria *Chlorobium limicola f. thiosufatophilum* and *Chlorobium phaeobacteroides* 4903, and resolved the signals into those arising from three distinct iron–sulfur centers. The *g*-values and orientation dependencies of the EPR signals were observed to be very similar to those of the centers F_X, F_A, and F_B of photosystem 1. The findings of Nitschke et al. (1990) suggest the participation of the *Chlorobium* iron–sulfur centers F_X, F_A, and F_B in charge stabilization on the acceptor side of the reaction center, and they proposed a model for the sequence of electron transfer directly analogous to photosystem 1.

The purpose of this study was to investigate the *Chlorobium* reaction center via flash absorption spectrophotometry and EPR to identify the redox centers functionally involved in electron transfer at physiological temperature and to determine their kinetic interactions. We elected to conduct the study using membrane preparations that were active in $NAD(P)^+$ photoreduction, thereby ensuring functional integrity of the electron-transfer sequence on the acceptor side. This paper reports on the spectroscopy of the membrane preparation as isolated and following the oxidative degradation of the iron–sulfur redox centers and their reconstitution.

Experimental Procedures

Growth of Bacteria. Chlorobium vibrioforme f. thiosulfatophilum 8327 was grown in a medium described by Rieble et al. (1989) but containing 500 μL/L Ethrel, a commercial plant regulator containing 21.7% (2-chloroethyl)-phosphonic acid obtained from Union Carbide Agricultural Products, to inhibit the synthesis of antenna BChl *d* as reported by Ormerod et al. (1990). The medium was sterilized in 2.4-L bottles which were immediately filled and screw-capped after autoclaving. Sterile Na_2S, CO_2-saturated HCO_3^-, Ethrel, and 500 μL/L 5 N NaOH to adjust the pH of the medium were introduced just prior to inoculation which was conducted under a stream of nitrogen. The cells were grown under a combination of fluorescent and incandescent lamps for 4 days (incident intensity of 80 W m^{-2}) at a temperature of 28 °C. The cells in the late exponential phase were harvested by continuous flow centrifugation, washed thoroughly with 50 mM Tris-HCl, pH 8.0, and stored at −80 °C. Cultures for inoculation were stored in the same medium minus Ethrel in darkness at 4 °C and retained viability for at least 6 months.

Preparation of the Membrane Fraction. Chlorosome-depleted membranes were prepared from cells that had been stored at −80 °C by a modification of the procedure described by Schmidt (1980). In a typical preparation 25-g wet weight cells were suspended in 160 mL of 50 mM Tris-HCl, pH 8.0, containing 10 mM sodium ascorbate and broken by passage through a French pressure cell at 20000 psi three times. The resulting homogenate was centrifuged at 12000*g* for 10 min and the pellet discarded. The supernatant was layered on a sucrose step gradient and ultracentrifuged using a high-capacity fixed-angle rotor at 300000*g* for 3 h. The sucrose concentrations in the step gradient for a Beckman Ti 50.2 rotor were 5 mL of 55%, 5 mL of 45%, 4 mL of 40%, and 4 mL of 35% and 8 mL of supernatant. The sucrose solutions were w/w and contained 50 mM Tris-HCl, pH 8.0, 10 mM sodium ascorbate, and 0.05% v/v Triton X-100. The fraction recovered for routine study was obtained from the 45% layer and the lower portion of the 40% layer. The 35% layer contained residual chlorosomes, but a fraction with high reaction center photoactivity was found just beneath. The 40–45% fraction which contained 25–35% of the BChl *a* was stored at −80 °C in the sucrose–ascorbate buffer.

Photosystem 1. Photosystem 1 was prepared from commercially grown spinach according to the method of Anderson and Boardman (1966). The D_{144} fraction was used for control experiments in the iron–sulfur cluster reconstitution protocols.

Spectrophotometry. Absolute absorbance and oxidized minus reduced difference spectra were obtained using a Varian Instruments (Sunnyvale, CA) Cary 219 double-beam spectrophotometer and a Hewlett-Packard Model 8452A diode array spectrophotometer. Spectra of samples maintained close to 77 K were obtained in a single-beam instrument comprised of a tungsten–iodide source, a Spex Industries, Inc. (Metuchen, NJ), 220-mm Spectramate double monochromator, and an EMI 9558 photomultiplier. The apparatus was interfaced by On-Line Instrument Systems, Inc. (Jefferson, GA), to an IBM PS2 Model 30-286 computer which controlled the acquisition of spectra and data reduction. For observation, samples in 50% glycerol were slowly frozen in liquid nitrogen using an apparatus of the type described by Estabrook (1956).

Flash Kinetic Spectrophotometry. Flash-induced absorbance transients were detected at 833 nm using a measuring beam from a 20-mW diode laser (Spectra Diode Labs, San Jose, CA, Model SDL-1400-H1) driven by a 12-V marine battery operated at 60–70 mA. The detector, PIN 10D (UDT), was placed 1.25 m from the sample to minimize fluorescence artifacts. The measuring beam in the visible range was isolated from a tungsten iodide source using a 200-mm focal-length Jobin Yvon Model H-20 monochrometer (Instruments SA, Inc.). The signal detection, signal averaging, and laser flash excitation were as described previously (Biggins et al., 1989). The response time of the apparatus was limited by the DC to 300-kHz bandwidth of the detector amplifier. The same spectrophotometer was used for flash transient analysis at 77 K using the low-temperature apparatus described above for absolute absorbance measurements. The samples contained 50% glycerol and were cooled slowly in liquid nitrogen prior to flash activation. Data fitting in all cases was carried out using Sigmaplot, version 4.0 (Jandel Scientific).

EPR Measurements. EPR X-band measurements were performed at 13 ± 1 K using a Varian E-9 spectrometer equipped with an Air Products low-temperature accessory. The samples (A_{810} = 15–20) were suspended in 250 mM glycine, pH 10.8, containing 100 mM sodium dithionite to produce strongly reducing conditions (Nitschke et al., 1990) and were contained in sealed tubes under argon. Spectra were measured in the dark at 13 ± 1 K after dark adaptation and

following 20-min continuous illumination of the samples at 200 K (ethanol/dry ice mixture). An additional 20-min illumination did not increase the intensity of the signals. A 300-W xenon arc lamp filtered through 5 cm of water saturated with $NaNO_2$ was used for the illumination. The spectra presented (photoaccumulated minus dark adapted) were the average of eight scans (3 min/scan). The EPR instrument conditions were 20-mW power and 10-G modulation amplitude. The *g*-factors were calibrated versus Mn/SrO.

NADP⁺ Photoreduction. The photoreduction of $NADP^+$ was followed spectrophotometrically by measuring the change in absorbance at 340 nm. The measuring beam was modulated at 1 kHz and detected by a Princeton Applied Research (Princeton, NJ) Model 126 lock-in amplifier. The excitation beam, 6×10^3 W·m^{-2}, comprised wavelengths longer than 665 nm. The reaction mixture was 50 mM Tris-HCl, pH 8, containing either 0.5% 2-mercaptoethanol or 10 mM ascorbate plus 50 μM TMPD as donor, 500 μM $NADP^+$, 3 μg/mL Fd, 0.0125 IU/mL FNR, and *Chlorobium* membranes (A_{810} = 0.2–0.6). Spinach Fd and FNR were obtained from Sigma Chemical Co. (St. Louis, MO).

Oxidative Degradation of Reaction Center Iron–Sulfur Centers and Their Reconstitution. The procedure for the destruction and reinsertion of iron–sulfur clusters was a minor modification of the method developed for photosystem 1 by Golbeck and co-workers (Parrett et al., 1989, 1990; Mehari et al., 1991; Li et al., 1991) as follows. After treatment of the membrane fraction by chaotropic agents at 4 °C in air to destroy the iron–sulfur centers, the samples (A_{810} = 1–2) were rapidly desalted by passage through a Sephadex G-75 column equilibrated with 50 mM Tris-HCl, pH 8.0. The preparations were then concentrated using a Pharmacia LKB 100 K stirred cell pressurized with nitrogen. The 1- or 2-mL samples were stirred in a serum bottle fitted with a serum cap equipped for the introduction of reagents and gas lines. The reaction mixture was purged with argon for 1 h at room temperature in the dark, and 2-mercaptoethanol was then added through the septum to give a final concentration of 0.5% (v/v). After 10 min of additional purging, 30 mM $FeCl_3$ was introduced to give a final concentration of 150 μM followed by 30 mM Na_2S, 150 μM final concentration, and the mixture was slowly stirred for 30 min. For the observation of $NADP^+$ photoreduction the same cluster insertion protocol was conducted in an optical cuvette fitted with a serum cap. The supplementary Fd, FNR, and $NADP^+$ were injected immediately preceding the photoreduction assay which was conducted in the same cuvette under a positive argon pressure. For EPR experiments the cluster insertions were conducted on a larger scale, and the reconstituted membrane preparation was then ultracentrifuged to concentrate the samples and remove the excess $FeCl_3$, Na_2S, and 2-mercaptoethanol.

Extinction Coefficients. The following extinction coefficients were used: $\Delta\epsilon_{842}$ = 100 mM^{-1} cm^{-1} for P_{840}, $\Delta\epsilon_{553}$ = 20 mM^{-1} cm^{-1} for cytochrome c_{553}, and $\Delta\epsilon_{603-645}$ = 27 mM^{-1} cm^{-1} for BChl *a* (Olson, 1981).

Results

Biochemical Characterization of the Membrane Fraction. The room temperature absorption spectrum of the chlorosome-depleted membrane preparation is presented in Figure 1 and shows contributions from the antenna BChl *a* at 809 and 602 nm and residual chlorosome antennae BChl *d* at 730 nm and BPh *d* at 672 nm. The reaction center BChl *a* can be seen as a shoulder on the long-wavelength side of the BChl

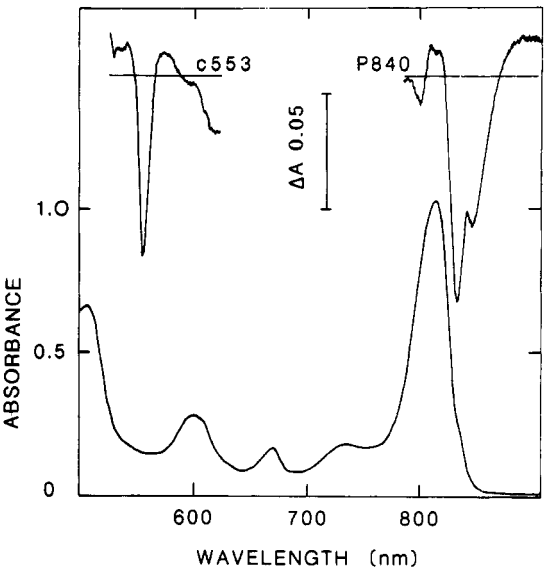

FIGURE 1: Room temperature absorption spectrum of the *Chlorobium* membrane preparation. Oxidized minus reduced difference spectra of P_{840} and cytochrome c_{553} of the same preparation are shown in the upper portion of the figure. The samples were oxidized using 5 μM potassium ferricyanide and reduced with 10 mM sodium ascorbate plus 50 μM TMPD.

a maximum and was resolved further at 77 K as will be shown below in Figure 2. We found that it was possible to deplete the concentration of antenna BChl *a* further by detergent treatment but only at the expense of the functional integrity of the acceptor side of the reaction center.

The oxidized minus reduced difference spectra in the upper portion of Figure 1 show P_{840} and cytochrome c_{553}. The P_{840} spectrum shows minima at 841, 830, and 792 nm, and a photoinduced difference spectrum was similar (not shown). The spectrum of the α-band of the endogenous donor cytochrome c_{553} for the same sample is shown on the left. We noted that although P_{840} was fully reducible by 10 mM ascorbate, the complete reduction of the cytochrome required the addition of a lipophilic mediator such as TMPD (50 μM). This may have been due to either inaccessibility of a fraction of the cytochrome c_{553} pool to ascorbate because of the vesicular structure of the preparation (Olson et al., 1976b) or simply a kinetic limitation in the rate of reduction of the cytochrome. Further reduction of samples using dithionite introduced an additional absorption band at 562 nm, most likely due to a *b*-type cytochrome in confirmation of Fowler (1974).

Figure 2 shows the absorption spectrum and its fourth derivative of the *Chlorobium* preparation in the BChl *a* region taken at 77 K. The 809-nm absorption band observed at room temperature was resolved into four components centered at 808.5, 817.5, 826, and 835 nm. We assume that these arise from pigments in the crystalline antenna BChl *a* located on the base plate linking the chlorosome to the reaction center, and the 835-nm shoulder arises from the reaction center BChl *a* (Olson & Thornber, 1978). In *Prosthecochlorus* the reaction center absorption band was shown to comprise two components at 4.2 K (Swarthoff & Amesz, 1979), but we were not able to resolve these two bands in the fourth derivative of the 77 K spectrum due to the poor signal to noise ratio and the differencing intervals. These data confirm the results of Olson et al. (1973), Swarthoff and Amesz (1979), and Vasmel et al. (1983) and show that the membrane preparation from

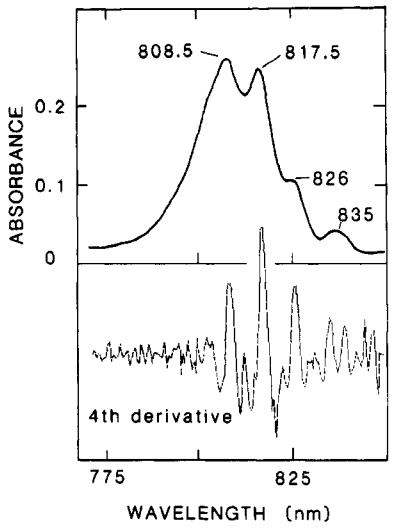

FIGURE 2: Absorption spectrum of the *Chlorobium* membrane preparation measured at 77 K. The sample was suspended in 45% sucrose and 50% glycerol. The maxima indicated were taken from the fourth derivative of the spectrum shown in the lower panel.

Table I: Photoreduction of $NADP^+$ by the *Chlorobium* Membrane Fraction

conditions	velocity [μmol of $NADP^+$ reduced·(mg of BChl *a*)$^{-1}$·h^{-1}]
complete, anaerobic	23.1 ± 0.12
complete, aerobic	0.08
minus $NADP^+$	0
minus Fd + FNR	0

Ethrel-grown cells was optically very similar to the photoactive preparations derived from cells containing their normal complement of chlorosomes.

Quantitative data from a series of such spectral measurements showed that the membrane preparation was almost devoid of BChl *d*, and from the difference spectra the reaction center P_{840} to BChl *a* ratio was 50. The content of cytochrome c_{553} was found to be between 6 and 7 per reaction center.

$NADP^+$ Photoreduction. Our method for preparation of the chlorosome-depleted membranes preserved $NADP^+$ photoreduction activity when the samples were assayed with supplementary Fd and FNR isolated from spinach. Table I shows that in a complete enzyme assay the preparation photoreduced 23 μmol of $NADP^+$·(mg of BChl *a*)$^{-1}$·h^{-1} with control experiments showing an absolute dependence on Fd, FNR, and $NADP^+$ and anaerobic conditions. Although these rates of photoreduction were an order of magnitude lower than $NADP^+$ photoreduction by photosystem 1 preparations, they were higher than those reported initially by Buchanan and Evans (1969) when recalculated and expressed on a BChl *a* basis.

Flash Kinetic Spectrophotometry at Room Temperature. The demonstration that the chlorosome-depleted membrane preparations were active in $NADP^+$ photoreduction indicated that a significant proportion of the reaction centers had complete secondary electron-transfer sequences on both the donor and acceptor sides. The preparation was then investigated by flash kinetic spectrophotometry to identify the redox centers involved in the electron-transfer reactions and to elucidate their kinetic interactions at physiological temperature. The preparation as isolated and stored in 10 mM ascorbate was active in P_{840} photobleaching and showed a very complex signal recovery after activation via a laser flash (not shown). Extensive data fitting showed the signal to be a composite of

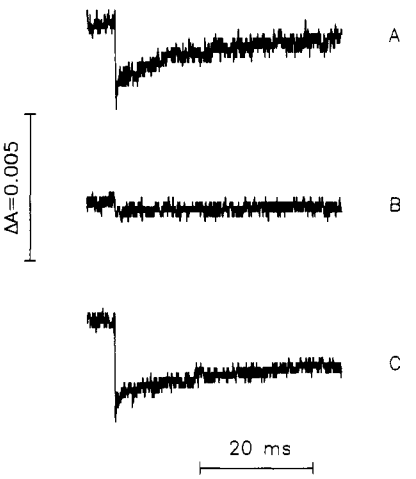

FIGURE 3: Flash kinetic spectrophotometry of the *Chlorobium* membrane preparation. The signals due to P_{840} were acquired at 833 nm at room temperature, and each transient was the average of 4–10 signals. (A) Sample immediately after reduction with 10 mM ascorbate and 50 μM TMPD followed by Sephadex G-75 gel filtration to remove the excess reducing agents. (B) Same sample with 100 μM benzyl viologen. (C) Same sample with 2 mM sodium ascorbate and viologen.

multiple microsecond and millisecond kinetic phases, presumably due to several recombination reactions between P_{840}^+ and reduced acceptors and reactions on the donor side. Accordingly, an experimental protocol was developed to examine the recombination reactions on the acceptor side in the absence of the faster competing reaction(s) on the donor side. This proved to be complicated because, in the absence of any external reducing agent such as ascorbate, P_{840} became oxidized in air-saturated buffer.

The method adopted for routine investigation was a modification of the classical procedures developed by Ke and coworkers for analysis of the acceptor side of photosystem 1 (Hiyama & Ke, 1971; Ke, 1972, 1973) as illustrated in Figure 3. First the preparation was prereduced using 20 mM ascorbate containing a lipophilic mediator such as TMPD to completely reduce P_{840} and the endogenous donors. The sample was then rapidly gel filtered through a small (2 × 15 cm) Sephadex G-75 column to removal all exogenous reducing agents and then immediately analyzed by flash kinetic spectrophotometry. Figure 3A shows a reversible absorbance transient due to P_{840} oxidation, followed by a slow recovery with a $t_{1/2}$ = 7 ms obtained as soon as possible after the column treatment. The transient represents a recombination reaction between P_{840} and an acceptor of the reaction center because no exogenous acceptors or donors were present. Because the preparation was physiologically competent in $NADP^+$ photoreduction, we suggest that the acceptor was F_AF_B, consistent with the model of Nitschke et al. (1990). The signal slowly disappeared with time as the P_{840} became progressively oxidized in the air-saturated buffer ($t_{1/2}$ = 20 min). Use of an anaerobic column and sample collection procedure did not significantly increase the amplitude of the signal (Figure 3A), indicating that the concentration of P_{840} participating in the reaction in this time range was most probably close to maximal.

Addition of an autoxidizable exogenous acceptor, such as benzyl viologen, eliminated the absorbance change (Figure 3B). No P_{840} signal was detected because the benzyl viologen interacted efficiently with the reduced terminal acceptor and suppressed the recombination reaction shown in Figure 3A. As no exogenous donor was present, P_{840}^+ accumulated during

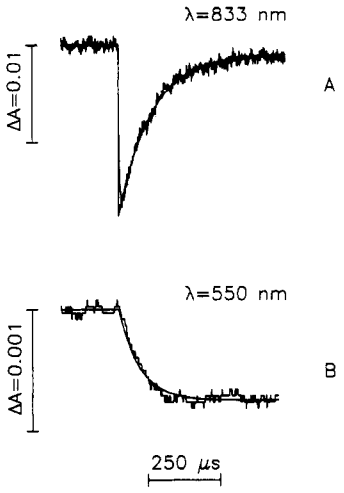

FIGURE 4: Flash kinetic spectrophotometry of the *Chlorobium* membrane preparation. The signals were acquired at room temperature for P_{840} and for cytochrome c_{553} as shown in traces A and B, respectively. The transients shown were the average of 4–10 signals. The samples contained 2 mM sodium ascorbate and 50 μM TMPD.

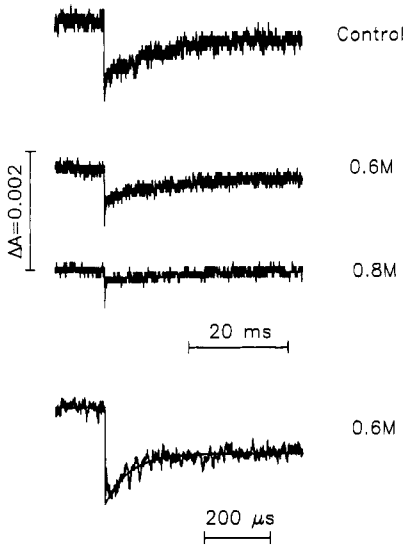

FIGURE 5: Flash kinetic spectrophotometry of the *Chlorobium* membrane preparation following oxidative degradation of the iron–sulfur centers. Analytical conditions are as described for Figure 3, trace A. Control: prereduction followed by gel filtration to show the recombination between the terminal acceptor and P_{840}. Second trace: same as control, but incubation of the preparation with 0.6 M NaI on ice for 30 min followed by gel filtration. Third trace: incubation of the preparation with 0.8 M NaI on ice for 30 min followed by gel filtration. Fourth trace: the preparation after the 0.6 M NaI treatment on a faster time scale showing the data fit for two components of $t_{1/2}$ = 50 μs and 7 ms.

the repetitive flash sequence in the experiment.

To investigate the kinetic interactions on the donor side of the reaction center in the absence of recombination reactions, the sample was poised in weakly reducing conditions by including 2 mM ascorbate in the presence of benzyl viologen as the exogenous acceptor (Ke, 1973). Figure 3C shows the flash absorbance transient obtained from such a sample. The half-time of the P_{840}^+ reduction was observed to be quite long (200 ms) compared with the recombination reaction (Figure 3A). As expected, the half-time was found to be dependent on the concentration of the exogenous donor (Hiyama & Ke, 1971). With increasing concentrations of ascorbate the P_{840}^+ reduction became much faster, and at 10 mM ascorbate the $t_{1/2}$ was 300 μs (not shown). However, as was found for the chemical reduction of cytochrome c_{553}, the addition of a lipophilic mediator was required to induce maximal signal amplitude and kinetics. The signal obtained by including ascorbate and TMPD is shown in Figure 4A, and the $t_{1/2}$ was ca. 50–80 μs. This half-time was found to be a minimal value, and the reaction could not be accelerated further using higher concentrations of TMPD or PMS. This suggested some kinetic barrier between the exogenous donors and P_{840}^+ such as the tightly bound cytochrome c_{553}. This was confirmed as illustrated in Figure 4 which compares the P_{840}^+ recovery with that of cytochrome c_{553} oxidation measured on the same time scale under similar conditions. The kinetics of P_{840}^+ reduction (Figure 4A) and those of the cytochrome c_{553} oxidation (Figure 4B) are comparable, and these data are consistent with previous reports that cytochrome c_{553} is the secondary endogenous donor in the reaction center (Fowler et al., 1971; Olson et al., 1976a,b; Prince & Olson, 1976; Swarthoff & Amesz, 1979; Swarthoff et al., 1981c).

Oxidative Degradation of Iron–Sulfur Centers and Their Reconstitution. As an experimental approach to prove that the terminal acceptor is an iron–sulfur center and to identify earlier acceptors in the *Chlorobium* reaction center, we chose to systematically degrade and then reconstitute the acceptor side using techniques than have proven incisive in the analysis of photosystem 1. Golbeck and co-workers (Parrett et al., 1989, 1990; Mehari et al., 1991; Li et al., 1991) have exploited techniques of iron–sulfur cluster destruction using chaotropic agents and detergents followed by cluster reinsertion into the residual apoproteins. Therefore, the effect of chaotropic agents on the functional integrity of the *Chlorobium* reaction center was followed by flash kinetic spectrophotometry using the protocol developed for observation of interactions on the acceptor side (Figure 3).

Figure 5 (upper trace) is the absorption transient due to P_{840} in a control sample showing the characteristic $t_{1/2}$ = 7-ms decay kinetics, which represents the recombination between the terminal acceptor $F_AF_B^-$ and P_{840}^+. Incubation of the membrane preparation with low concentrations of NaI in air at 4 °C resulted in modifications of the absorbance signal as shown in Figure 5 (second trace), which is also shown on a faster time scale with the data fit in the lower trace. It can be seen that a faster decay component of $t_{1/2}$ = 50 μs was introduced by the NaI treatment. With increasing concentrations of NaI both the 7-ms and the 50-μs decay phases were progressively lost as shown in Figure 5 (third trace). The loss of the 7-ms kinetic phase following treatment of the membranes by the chaotrope is consistent with our conclusion that the terminal acceptor is an iron–sulfur center, most likely F_AF_B. The introduction of a faster recombination reaction of 50 μs, and its subsequent loss upon treatment of the preparation with higher concentrations of chaotrope, indicates the probability of another iron–sulfur acceptor earlier than F_AF_B.

Evidence for reconstitution of the electron-transfer sequence by reinsertion of iron–sulfur clusters is presented in Figure 6 (upper trace), which shows restoration of the recombination reaction between P_{840}^+ and the terminal acceptors. The lower trace, which is of the same reconstituted sample on a faster time scale with the data fit, shows that both the 50-μs and 7-ms kinetic phases were restored. The signal amplitude was usually greater than that of the control shown above (Figure 5, upper trace), and an elaboration of this point will be made below. Omission of Fe^{3+} and S^{2-} from the cluster insertion protocol resulted in a similar signal of much lower amplitude (not shown), indicating the presence of some residual Fe^{3+} and S^{2-} in the preparation. Figure 6 (second trace) shows the effect of adding 100 μM benzyl viologen, and the 7-ms decay was

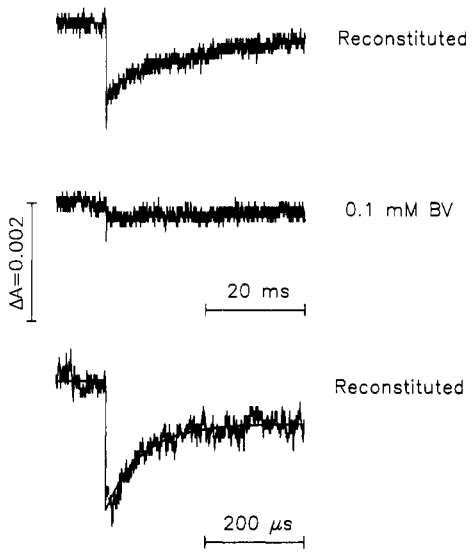

FIGURE 6: Flash kinetic spectrophotometry of the *Chlorobium* preparation after reconstitution of the iron–sulfur centers. The preparations were oxidatively degraded using 0.8 M NaI and then the iron–sulfur clusters were inserted using Fe^{3+} and S^{2-} in the presence of 2-mercaptoethanol as described under Experimental Procedures. Reconstituted: a sample after cluster insertion followed by rapid gel filtration on Sephadex G-75 to remove all reducing agents and reactants in the cluster insertion protocol. Second trace: the reconstituted sample in the presence of 0.1 mM benzyl viologen. Third trace: the reconstituted sample as shown in the upper trace but on a faster time scale showing the data fit for two components of $t_{1/2}$ = 50 μs and 7 ms.

lost, confirming that this phase corresponds to the back-reaction between the terminal acceptor and P_{840}^+. These results allow positive assignment of these components on the acceptor side in recombination with P_{840}^+ as iron–sulfur centers. As suggested previously, the $t_{1/2}$ = 7-ms phase resulted from recombination between $F_AF_B^-$ and P_{840}^+. We now suggest that the $t_{1/2}$ = 50-μs kinetic phase resulted from a recombination reaction between F_X^- and P_{840}^+.

The cluster insertion and restoration of the optical signals signifying reconstitution of F_AF_B in the membrane fraction did not require the addition of any polypeptide component(s). This indicates that if the *Chlorobium* reaction center is analogous to photosystem 1 and the terminal F_AF_B centers are located on a small subunit, the apoprotein was not removed by the 0.8 M NaI treatment of the preparation. The polypeptide composition of the preparation was analyzed by SDS–PAGE before and after the chaotrope treatment (data not shown), and we did not detect any significant changes, particularly in the low molecular mass region (<25 kDa) as would have been the case for photosystem 1 (Parrett et al., 1989). Accordingly, the mechanism of iron–sulfur center destruction was most likely via disruption of hydrogen bonding in the iron–sulfur cluster regions of the polypeptide by the NaI, leading to increases in susceptibility of the clusters to air oxidation. Presumably the resulting apoprotein(s) remained with the reaction center core in the preparation.

As will be discussed below, extensive damage to the acceptor side of the majority of the reaction centers in the membranes most likely occurred during preparation of the membrane fraction. The damage could have included both loss of the F_AF_B-containing subunit, if it is a constituent of the *Chlorobium* reaction center, and/or large-scale oxidative degradation of the iron–sulfur centers. Both possibilities were tested by additional reconstitution experiments. First, we attempted to increase the concentration of iron–sulfur centers in the control membrane preparation using the cluster insertion protocol on the *Chlorobium* preparation directly. We routinely observed an increase in the amplitude of the optical signal due to the $F_AF_B^-/P_{840}^+$ recombination reaction, indicating that additional sites in the preparation could be reconstituted. The increases were significant, and even a doubling of the control value was observed in some preparations (data not shown).

Table II: Photoreduction of $NADP^+$ by the *Chlorobium* Membrane Fraction after Degradation of Iron–Sulfur Centers and Cluster Insertion

conditions	velocity [μmol of $NADP^+$ reduced·(mg of Bchl *a*)$^{-1}$·h^{-1}]
0.8 M NaI, aerobic (cluster destruction)	0
after FeS insertion	12.05 ± 0.14
FeS insertion minus Fe^{3+} + S^{2-}	0.045
FeS insertion, aerobic	0
FeS insertion + 50% glycerol	0

Second, we attempted further reconstitution of the control membrane preparation by cluster insertion in the presence of the photosystem 1 *psaC* and *psaD* gene products that had been expressed in *Escherichia coli*. These products were generously provided by Drs. D. A. Bryant and J. H. Golbeck, who have recently demonstrated their role in the reconstitution of the F_AF_B clusters in the photosystem 1 complex of *Synechococcus* sp PCC 6301 (Li et al., 1991). In control experiments the two polypeptides were used to successfully reconstitute the centers of F_AF_B in a spinach D_{144} photosystem 1 preparation after treatment with 6 M urea. The $t_{1/2}$ = 2 ms recombination reaction between P_{700}^+ and F_X^- in the urea-treated preparation (Parrett et al., 1989) was replced by a $t_{1/2}$ = 30-ms reaction following cluster insertion (data not shown). The $t_{1/2}$ = 30-ms reaction was due to the recombination reaction between P_{700}^+ and $F_AF_B^-$ and is diagnostic of the reconstitution of the terminal centers (Li et al., 1991). However, the same reconstitution procedure using the photosystem 1 gene products did not increase the extent of reconstitution of F_AF_B centers in the *Chlorobium* membrane preparation possibly due to species incompatibility. Alternatively, the *Chlorobium* reaction center may not have a small subunit bearing the terminal iron–sulfur centers, F_AF_B, or the major damage to the acceptor side of the *Chlorobium* preparation occurred at a point prior to F_X^-.

$NADP^+$ Photoreduction Following Cluster Degradation and Reinsertion. As shown in Table II, the $NADP^+$ photoreduction activity was totally absent in samples of *Chlorobium* membranes that had been treated with NaI in air to destroy the iron–sulfur centers F_AF_B and F_X, and the activity could be restored by cluster insertion. Control experiments show conclusively that restoration of the $NADP^+$ photoreduction was dependent on rebuilding the iron–sulfur centers because omission of either Fe^{3+} or S^{2-}, or introduction of air, did not result in any activity. The magnitude of the reconstitution was significant because over 50% of the control activity was restored. The effect of glycerol was studied because it was required in the medium for flash kinetic spectrophotometry at 77 K (see below), and it can be seen that no reconstitution occurs.

EPR Measurements. Experiments were then performed to establish that the procedures used for the degradation and reconstitution of the iron–sulfur centers in the samples were accompanied by changes in the EPR signals of the clusters. Figure 7A shows an EPR spectrum of the control at 13 K following continuous illumination of the sample at 200 K in strongly reducing conditions. The conditions (100 mM dithionite, pH 10.8) promoted a reduction of $P840^+$ and allowed the accumulation of reduced terminal acceptors (Nitschke et al., 1990). Although the signals were weak due to the low concentration of reaction centers with functional terminal acceptors (see Discussion below), the control spectrum is

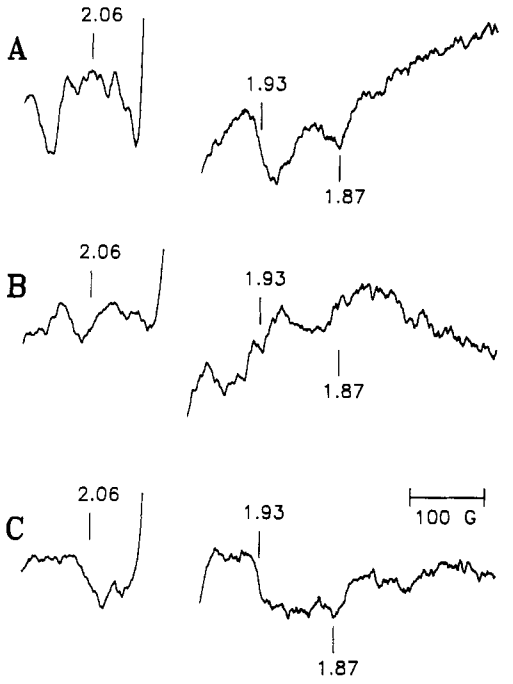

FIGURE 7: EPR spectra of the *Chlorobium* membrane preparation at 13 K. The spectra shown are difference spectra obtained at 13 K between samples that were preilluminated at 200 K for 20 min minus samples maintained in darkness. The samples were in highly reducing conditions as described under Experimental Procedures. (A) Spectrum of the control membrane preparation. (B) Spectrum of the membrane preparation after treatment with 0.8 M NaI to degrade the iron–sulfur centers. (C) Spectrum of the NaI-treated preparation followed by cluster insertion using $FeCl_3$ and Na_2S in the presence of 2-mercaptoethanol.

characteristic of an iron–sulfur center with g values at approximately $g_x = 1.87$ and $g_y = 1.93$. However, we were unable to resolve the resonance expected at $g_z = 2.06$. The spectrum was stable in the dark below 200 K, and it resembles those reported previously for samples observed under similar conditions (Swarthoff et al., 1981b; Nitschke et al., 1990). Nitschke et al. (1990) concluded that the spectrum originates from the iron–sulfur centers F_AF_B.

Treatment of the preparation with 0.8 M NaI, which inhibits $NADP^+$ photoreduction and the recombination reactions between the terminal acceptors and P_{840}^+, also results in loss of the EPR signal as expected (Figure 7A), and after iron–sulfur cluster insertion an EPR signal reappeared (Figure 7C). The EPR signal observed following illumination of the reconstituted sample at 200 K (Figure 7C) shows a pronounced broad feature at midfield centered at about $g = 1.87$. Again, the weakness of the signal and baseline irregularities in the spectrum preclude definitive assignment of the signal to that seen in the control (Figure 7A). However, taken with the other data above on the reconstitution of $NADP^+$ photoreduction and the flash kinetics, the EPR signal most likely reflects the reconstitution of functional iron–sulfur centers in preparations following the cluster insertion protocol.

Flash Kinetic Spectrophotometry Conducted at 77 K. Figure 8 shows flash absorption transients for samples that were prereduced using ascorbate/TMPD and then slowly frozen to 77 K in the presence of 50% glycerol. The upper traces (control) show the fast P_{840} photobleaching and recoveries on two time scales for the control membrane preparation. It can be seen that the major kinetic component had a $t_{1/2} = 20$ ms with a minor (<10%) component of $t_{1/2} = 80$ μs. Nitschke et al. (1990) did not observe the oxidation of $F_AF_B^-$ by P_{840}^+ in a back-reaction at cryogenic temperature.

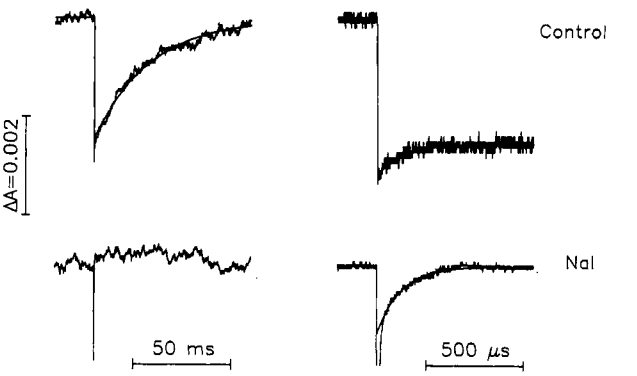

FIGURE 8: Flash kinetic spectrophotometry of the *Chlorobium* membrane preparation at 77 K. Control: the preparation was prereduced with 10 mM sodium ascorbate and 50 μM TMPD, adjusted to 50% glycerol, and cooled in liquid nitrogen. The reversible transient at 833 nm is shown on two time scales. NaI: the lower traces show the transients due to P_{840} in the preparation after 0.8 M NaI treatment and gel filtration as in Figure 5, third trace, followed by concentration of the sample using a 100 K stirred cell and addition of glycerol to 50%. The transients in the control and NaI-treated samples were the average of 50 signals.

Therefore, we suggest that the 20-ms recombination reaction at 77 K (Figure 8, upper traces) must be between F_X^- and P_{840}^+. Because of uncertainties in the optical path length of the frozen sample, we could not quantitate the concentration of P_{840} turning over in this reaction but estimate that it was between 25% and 35% of the photoreactive P_{840} observed in this time range at room temperature.

The two lower traces in Figure 8 (NaI) show the flash transient at 77 K in a sample treated with 0.8 M NaI to oxidatively degrade the iron–sulfur centers, i.e., conditions identical to those used for the generation of data at room temperature as shown previously (Figure 5, third trace). It can be seen that the 20-ms kinetic phase was totally replaced by the faster decay component of $t_{1/2} = 80$ μs. This result is consistent with assignment of the 20-ms component in the control (upper traces) to the iron–sulfur center, F_X, and the 80-μs kinetic phase to a back-reaction between a redox center prior to F_X and P_{840}^+. By analogy to photosystem 1 we suggest that the 80-μs phase represents the back-reaction between A_1^- and P_{840}^+. Alternatively, the 80-μs phase may be due to decay of triplet P_{840}, and this possibility will be discussed further below.

We unsuccessfully attempted to restore the 20-ms decay component observed in the control by reconstitution of the iron–sulfur centers using the same protocol that led to the results shown in Figure 6. However, as was indicated in Table II and the section of the restoration of $NADP^+$ photoreduction, the iron–sulfur clusters could not be inserted in samples containing 50% glycerol which was mandatory for the spectrophotometry of the samples at 77 K.

Discussion

The growth of *Chlorobium* in the presence of ethylene to inhibit the synthesis of the bulk of BCl *d* provided excellent starting material for the rapid isolation of a membrane fraction that had functional reaction centers with low antenna size without the use of high concentrations of detergents or chaotropes. Spectral analysis of the membrane preparation showed that the BChl *a* antenna and reaction centers were optically similar to cells grown with a full complement of chlorosomes. The membrane preparation was active in the photoreduction of $NADP^+$, indicating that a significant fraction of the reaction centers was physiologically intact,

thereby permitting the unambiguous identification of the recombination reaction between the terminal acceptor, $F_AF_B^-$, and P_{840}^+. Of the total amount of reaction center P_{840}, as determined from the chemically induced oxidized minus reduced difference spectra, the P_{840} turnover in the recombination reaction with F_AF_B was ca. 5%. The turnover of P_{840} as determined from reactions on the donor side was ca. 33%. Assuming that all the reaction centers in *Chlorobium* in vivo are normally functional in electron transfer to F_AF_B, this indicates that the terminal acceptors are particularly susceptible to damage during sample preparation.

A variety of photochemically active preparations isolated from green sulfur bacteria have been studied previously by flash absorption spectrophotometry. Our results confirm the reports of Prince and Olson (1976) and Swarthoff et al. (1981c) that cytochrome c_{553} is the physiological donor to the reaction center P_{840}. There is reasonable agreement between all groups that the reduction of P_{840}^+ by cytochrome c_{553} occurs with a half-time of 50–90 μs at room temperature. However, Prince and Olson (1976) reported a biphasic reduction of P_{840}^+ with a fast phase of 5 μs which would not have been detected in our experiments.

This report is the first to document flash-induced recombination reactions between P_{840}^+ and reduced terminal acceptors in the electron-transfer sequence of green sulfur bacteria at room temperature. Swarthoff and co-workers observed recombination reactions at cryogenic temperatures in a preparation that had been treated with Triton X-100 and reported half-times for P_{840}^+ reduction of 13 ms at 5 K (Swarthoff et al., 1981a), 20 ms at 120 K, and 25–30 ms at 80 K (Swarthoff et al., 1981c). It is possible that the 25–30-ms component they observed at 80 K corresponds to the 20-ms decay we detected at 77 K for the membrane preparation (Figure 8, upper traces). As mentioned above, we suggested that this reaction represents the recombination between P_{840}^+ and F_X^-, and, if correct, this would imply that F_X remained functional in the Swarthoff et al. (1981c) preparation after 1% Triton X-100 treatment.

Swarthoff and co-workers have also reported extensively on the BChl *a* triplet that forms in preparations from *Prosthecochlorus aestuarii* and concluded that it originates from P_{840} (Swarthoff et al., 1981c). The triplet lifetime was reported to be 90 μs at 295 K, 165 μs at 120 K (Swarthoff et al., 1981c), and 280 μs at 5 K (Swarthoff et al., 1981a). We observed an 80-μs decay at 77 K for NaI-treated membranes, and although we have suggested that this corresponds to the back-reaction between P_{840}^+ and the early acceptor A_1^-, further studies will be required to determine whether this kinetic phase is of the triplet.

The functional involvement of the iron–sulfur centers F_AF_B and F_X in *Chlorobium* was supported in this study by cluster degradation and reinsertion experiments. These protocols were introduced originally by Malkin and Rabinowitz (1966) for the reconstitution of apoferredoxin and successfully applied by Golbeck and co-workers for the reconstitution of reaction center iron–sulfur clusters in photosystem 1 (Parrett et al., 1989; 1990; Mehari et al., 1991; Li et al., 1991). We have now extended these observations to another group of procaryotes with type 1 reaction centers and to a photosystem 1 preparation from eucaryotes. This report also demonstrates for the first time the full restoration of electron transfer to the physiological acceptor $NADP^+$ in such experiments, confirming the validity of the optical and EPR signals used routinely as criteria for monitoring these iron–sulfur center reconstitutions.

The *Chlorobium* preparation was found to be very sensitive to oxidative degradation using chaotropic agents and detergents. In contrast to photosystem 1, we observed the complete destruction of the centers F_AF_B and F_X using a concentration of chaotrope that did not result in the removal of a detectable fraction of the low molecular mass polypeptides from the preparation. If the redox centers F_AF_B in *Chlorobium* are located on a small subunit of the reaction center as postulated by Nitschke et al. (1990), then it was not detached from the reaction center core in the protocols used in this study because cluster insertion occurred without the addition of any polypeptides. We showed that the amplitude of the optical signal diagnostic of the recombination reaction between $F_AF_B^-$ and P_{840}^+ could be increased by applying the iron–sulfur cluster insertion protocol directly to the preparation. This indicates that a significant number of acceptor sites were available for reconstitution in the preparation as isolated. However, the signal amplitude could not be increased further by including the *psaC* and *psaD* gene products required for reconstitution of photosystem 1. This implies that either the entire complement of endogenous acceptor apoproteins was present in the preparation and only required the iron–sulfur cluster or that the cyanobacterial photosystem 1 gene products were not compatible with the *Chlorobium* reaction center core. Alternatively, the *Chlorobium* F_AF_B redox centers may not be localized on a subunit that is analogous to photosystem 1.

As discussed above, the optical measurements indicate that over 90% of the terminal acceptors in the reaction centers in the preparation were inactive, making the acquisition of EPR data with high signal to noise ratios in the iron–sulfur center region extremely difficult. However, the spectra obtained were reproducible and comparable to those reported previously (Swarthoff et al., 1981b; Nitschke et al., 1987, 1990). The low-temperature EPR spectrum of the *Chlorobium* membranes after photoaccumulation at 200 K was similar to that observed by Swarthoff et al. (1981b) and Nitschke et al. (1990). The photoinduced EPR signals were not detected in preparations that had been treated with NaI and reappeared following cluster insertion. These data confirm the optical and enzymological studies and provide additional evidence that the terminal iron–sulfur centers are functional in electron transfer.

The EPR spectrum of the sample reconstituted by cluster insertion showed a broad resonance centered at around $g = 1.89$. We tentatively suggest that this represents the terminal iron–sulfur cluster(s) ineracting in an environment that has been modified by the chaotrope treatment we used to destroy the centers prior to the reconstitution experiment. For precedence we cite the report of Li et al. (1991), who observed modified EPR signals from the terminal acceptors reconstituted from the photosystem 1 F_X-core heterodimer in the absence of the *psaD* gene product. The signals they observed were also broad, and they did not resolve the high-field resonances due to F_A and F_B. However, inclusion of the *psaD* gene product in the reconstitution protocol resulted in normal EPR signals, indicating the extreme sensitivity of the magnetic interactions of the centers to the protein environment.

Our results extend the model of the *Chlorobium* reaction center as proposed by Nitschke et al. (1990) and are summarized in Figure 9, which shows a scheme of electron-transfer reactions with the kinetic parameters we have determined at room temperature and 77 K. The kinetics of the recombination reactions between the reduced acceptors and P_{840}^+ in *Chlorobium* are about 5 times faster than the analogous reactions in photosystem 1 and can only be detected if special precautions are taken to eliminate the competing reaction between

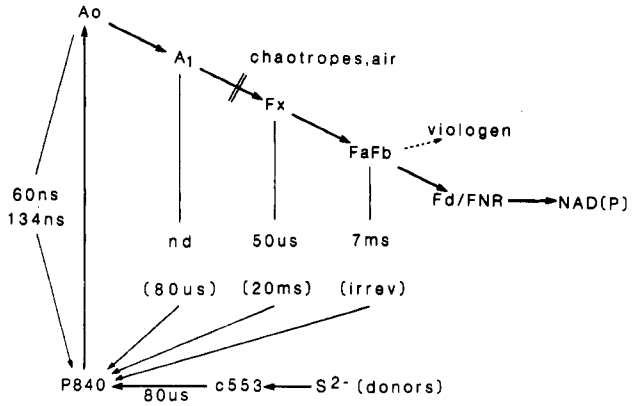

FIGURE 9: Scheme of the kinetics of electron transfer in the reaction center of *C. vibrioforme*. The half-times measured at 77 K are shown in parentheses. The assignment of the nanosecond reactions is tentative. nd = not determined.

reduced cytochrome c_{553} and P_{840}^+ on the donor side. The turnover of P_{840} was observed at 77 K, indicating back-reactions with early acceptors. The participation of F_X was inferred from both the reported irreversibility of the recombination between $F_AF_B^-$ and P_{840}^+ at low temperature (Nitschke et al., 1990) and the sensitivity of the 20-ms kinetic phase to cluster destruction (Figure 8). The 80-μs phase observed at 77 K after cluster destruction we ascribe to the reaction between A_1^- and P_{840}^+ and reiterate that it may also be due to the decay of triplet P_{840} resulting from the species $A_1^-P_{840}^+$ or from the radical pair $A_0^-P_{840}^+$.

The kinetic data in this paper were limited to the microsecond time range and, as noted above, account for about 30% of the total reaction center P_{840}. The remaining reaction enters were either photochemically inactive or turning over in the submicrosecond range. Preliminary nanosecond determinations monitoring optical signals at 920 nm, now in progress with Dr. J. H. Golbeck, University of Nebraska, have shown that two kinetic components of 60 ns and 134 ns may account for the residual photochemical turnover of P_{840}. It is likely that these components represent recombination reactions between very early acceptors and P_{840}^+ in reaction centers that were totally degraded on the acceptor side. On the other hand, the 134-ns kinetic phase in particular may result from a reaction in forward electron transfer. These points will be resolved when we complete a more extensive analysis of components undergoing absorption changes in the NIR.

Added in Proof

Feiler et al. (1992) have recently reported on the subunit composition of a highly purified reaction center complex from *Chlorobium limicola f. thiosulfatum* that contained the terminal FeS centers F_A and F_B. Using SDS–PAGE for analysis, they did not observe a polypeptide of 8.9 kDa analogous to the *psaC* gene product that binds F_A and F_B in photosystem I.

Acknowledgments

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Binding of Carbon Dioxide to Phosphoenolpyruvate Carboxykinase Deduced from Carbon Kinetic Isotope Effects†

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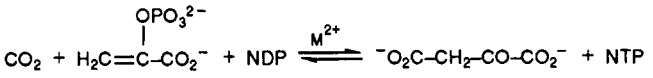
ABSTRACT: Phosphoenolpyruvate carboxykinase [ATP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.49] from *Chloris gayana* Kunth has been purified by a combination of ammonium sulfate fractionation, ion exchange, gel filtration, and affinity chromatography on agarose–hexane–ATP. In the direction of OAA formation, the specific activity of the enzyme was 33 μmol/(min·mg of protein). The carbon isotope effect on carboxylation was measured by successive analysis of remaining CO_2 over the course of the reaction. At 22 mM PEP and 1.3 mM MgADP, pH 7.5, the isotope effect is 1.024 ± 0.001. When the concentration of PEP was reduced to 1 mM, the isotope effect rose to 1.034 ± 0.004; when the concentration of MgADP was reduced to 60 μM, the value rose to 1.040 ± 0.006. The variation of the carbon isotope effect on carboxylation with both substrate concentrations indicates that the enzyme operates by a random kinetic mechanism. This in turn requires that the enzyme have a binding site for substrate CO_2; this is one of the first enzymes for which such a site has been demonstrated.

Phosphoenolpyruvate (PEP)[1] carboxykinase [ATP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.49] catalyzes the nucleoside diphosphate-dependent carboxylation of phosphoenolpyruvate to form oxalacetate and a nucleoside triphosphate (Utter & Kolenbrander, 1972):



PEP carboxykinase is ubiquitous in living things, but it serves different roles in plants and in animals. In vertebrates, the enzyme catalyzes the decarboxylation of OAA to PEP as the first committed step in gluconeogenesis. In PEP carboxykinase-dependent C_4 plants, the decarboxylation of OAA by PEP carboxykinase is coupled to photosynthetic carbon fixation by the Calvin cycle (Furbank & Hatch, 1987; Hatch, 1976).

Analogy with PEP carboxylase suggests that the mechanism of PEP carboxykinase should involve two distinct steps (Scheme I): The first step is transfer of phosphate from PEP to the nucleoside diphosphate substrate, a process which occurs with inversion of configuration at phosphorus (Konopka et al., 1986). The other product of this step is the enolate of pyruvate, which readily reacts with CO_2 to form OAA. CO_2, rather than HCO_3^-, is known to be the substrate (Utter & Kolenbrander, 1972). The stepwise mechanism is consistent with isotope exchange studies (Krebs & Bridger, 1980; Miller & Lane, 1968).[2]

From the point of view of kinetic mechanism, PEP carboxykinase is one of the best studied carboxylases. The preponderance of kinetic evidence for the enzyme from animal sources suggests that the order of substrate binding is random. Measurements of product inhibition and isotope exchange at equilibrium are consistent with random binding of substrates (Jomain-Baum & Schramm, 1978; Chang et al., 1966). Proton relaxation rate measurements on the avian enzyme reveal that all three substrates (PEP, CO_2, ITP) can bind to the E–Mn^{2+} complex (Hebda & Nowak, 1982); however, the kinetic competency of all these complexes has not been dem-

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[1] Abbreviations: NDP, nucleotide 5′-diphosphate; NTP, nucleotide 5′-triphosphate; OAA, oxalacetic acid; PEP, phosphoenolpyruvate; EPR, electron paramagnetic resonance; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HEPES, *N*-(2-hydroxyethyl)-piperazine–*N*′-2-ethanesulfonic acid; HPC, hydroxypropylcellulose; BSA, bovine serum albumin; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EPPS, *N*-(2-hydroxyethyl)-piperazine-*N*′-3′-propanesulfonic acid; DTT, dithiothreitol.

[2] A referee has pointed out that convincing mechanistic evidence for the stepwise mechanism is not available, and a concerted mechanism (involving simultaneous carboxylation and phosphate transfer) is feasible. We agree that no specific evidence to eliminate a concerted mechanism exists, but we believe that the stepwise mechanism is correct. Our conclusions would not be altered if the mechanism were concerted.

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