Photoaccumulation in Photosystem I Does Produce a Phylloquinone ($A_1^{\bullet-}$) Radical[†]

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ABSTRACT: Previous work has challenged the assignment of a photoaccumulated EPR signal to the phylloquinone electron acceptor in photosystem I, $A_1^{\bullet-}$. Biosynthetic deuteration of the phylloquinone in the cyanobacterium *Anabaena variabilis* has been shown to narrow this photoaccumulated signal, demonstrating that the signal arises from $A_1^{\bullet-}$. The ESP signal attributed to $P700^{\bullet+}A_1^{\bullet-}$ is also narrowed by this deuteration, showing that the photoaccumulated EPR signal and the ESP signal are monitoring the same redox component. Confirmation that the photoaccumulated EPR signal comes from deuterated phylloquinone was obtained by exchanging the deuterated for protonated phylloquinone, which broadened the photoaccumulated EPR signal.

The photosystem I reaction center contains a primary electron donor, a dimer of chlorophyll *a* molecules (P700),[1] and five electron acceptors. The primary electron acceptor A_0 is thought to be a chlorophyll *a* monomer, and to donate electrons to a phylloquinone (vitamin K_1) secondary electron acceptor A_1. Subsequently electrons are transferred through three [4Fe-4S] centers, called Fe-S_X, Fe-S_A and Fe-S_B [for reviews of photosystem I, see Evans and Nugent (1993) and Golbeck and Bryant (1991)]. Recent experiments observing the reoxidation of the phylloquinone electron acceptor optically (Luneberg et al., 1994) or by transient electron paramagnetic resonance (EPR) spectroscopy (Van der Est et al., 1994; Möenne-Loccoz et al., 1994) have demonstrated conclusively that phylloquinone transfers electrons to Fe-S_X in forward electron transfer at room temperature. The reoxidation of $A_1^{\bullet-}$ ($t_{1/2}$ ∼150−250 ns) was unaffected by the removal of Fe-S_A and Fe-S_B, but was affected by the removal of Fe-S_X from the photosystem I core.

There are essentially four assignments of the term A_1 (denoting a phylloquinone electron acceptor) to experimentally observed redox components: (a) the two chemically extractable molecules of phylloquinone (Schoeder & Lockau, 1986; Biggins & Mathis, 1988); (b) an electron spin polarized (ESP) transient EPR signal shown to arise from the radical pair $P700^{\bullet+}A_1^{\bullet-}$ (Thurnauer & Gast, 1985; Rustandi et al., 1990; Snyder et al., 1991); (c) flash-induced optical transients due to the reoxidation of $A_1^{\bullet-}$ (Brettel, 1988; Mathis & Setif, 1988; Setif & Brettel, 1993; Luneberg et al., 1994) or charge recombination between the $P700^{\bullet+}$ and $A_1^{\bullet-}$ radicals (Setif et al., 1984); (d) an asymmetric EPR signal at $g = 2.00$ photoaccumulated at low temperatures (200 K) under reducing conditions (Bonnerjea & Evans, 1982), or produced by illumination while freezing in the presence of reductants (Gast et al., 1983).

However, the assignment of the asymmetric EPR signal at $g = 2.00$ to A_1 has been challenged [see Golbeck and Bryant (1991) and Nugent and Evans (1993) for reviews]. UV destruction of phylloquinone *in situ* did not prevent the photoaccumulation of the EPR signal assigned to $A_1^{\bullet-}$ (Ziegler et al., 1987; Biggins et al., 1989). However, it is not certain that this treatment was completely destroying the phylloquinone, as there are conflicting reports as to whether UV treatment abolishes forward electron transfer (Palace et al., 1987; Biggins et al., 1989). The assignment of the EPR signal of $A_1^{\bullet-}$ to phylloquinone remains challenged, however, by experiments in which cyanobacteria were grown under conditions which resulted in biosynthetic deuteration of the phylloquinone (Barry et al., 1988). The linewidth of the EPR signal they observed was not narrowed by this treatment, as might be expected when proton hyperfine interactions are lost. This led to the suggestion (Barry et al., 1988) that the photoaccumulated EPR signal does not represent phylloquinone, but rather a reporter species that is influenced by the redox state of the true A_1.

But other experiments have suggested that this EPR signal does arise from the phylloquinone. Ether extraction of phylloquinone removes the ability to photoaccumulate this EPR signal (Itoh et al., 1987; Mansfield et al., 1987). The optical spectrum of the photoaccumulated A_1 seen in parallel optical and EPR experiments (Mansfield & Evans, 1986) is similar to the flash-induced spectrum of A_1 (Brettel et al., 1986). Under reducing conditions and prolonged illumination, it has been shown that the semiquinone $A_1^{\bullet-}$ can be reduced to the quinol (Setif & Bottin, 1989, 1991), removing the optical transients associated with charge recombination between $P700^{\bullet+}$ and $A_1^{\bullet-}$. This treatment also removes the ESP signal associated with the $P700^{\bullet+}A_1^{\bullet-}$ radical pair (Snyder et al., 1991) and the ability to photoaccumulate the EPR signal attributed to A_1 (Heathcote et al., 1993). These experiments in which A_1 is double reduced suggest that the

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[1] Abbreviations: P700, primary electron donor of photosystem I; A_0, chlorophyll primary electron acceptor in photosystem I; A_1, phylloquinone secondary electron acceptor in photosystem I; Fe-S_A, Fe-S_B, and Fe-S_X, [4Fe-4S] centers of photosystem I; EPR, electron paramagnetic resonance; ESP, electron spin polarized; ESE, electron spin echo; ESEEM, electron spin echo envelope modulation; ENDOR, electron nuclear double resonance; FAB, fast-atom bombardment.

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photoaccumulation of the EPR signal is monitoring the same component (phylloquinone) as the optical and ESP measurements.

It is important to conclusively assign this EPR signal to the phylloquinone, as other spin resonance techniques such as electron nuclear double resonance (ENDOR) and electron spin echo envelope modulation (ESEEM) could be used to determine the electronic structure of the semiquinone radical and the considerable influence the protein environment is having on the redox properties and behavior of this redox component. We have therefore repeated the experiments of Barry et al. (1988) and demonstrated that biosynthetic deuteration of the phylloquinone in the cyanobacterium *Anabaena variabilis* does lead to a narrowing of the photoaccumulated EPR signal attributed to $A_1^{\bullet-}$. We have also shown that the ESP signal attributed to $P700^{\bullet+}A_1^{\bullet-}$ is narrowed upon this deuteration of the phylloquinone, as previously reported by Rustandi et al. (1990). The extent of deuteration was greater than expected, as monitored by fast-atom bombardment (FAB) mass spectrometry of the phylloquinone. We have confirmed that the photoaccumulated EPR signal arises from the deuterated phylloquinone by exchanging the deuterated with protonated phylloquinone, which leads to a broadening of the photoaccumulated EPR signal.

MATERIALS AND METHODS

A methionine auxotroph (Met-27) of *A. variabilis* (Currier et al., 1977) was grown under sterile conditions on BG-11 medium (Rippka et al., 1979) containing 80 μM protonated methionine (Sigma Chemical Co.) or [*methyl*-d_3]methionine ($CD_3SCH_2CH_2CH(NH_2)CO_2H$, 99% atom D, Aldrich Chemical Co.). Methionine was added by sterile filtration. The cyanobacteria were grown to mid-log phase and harvested, and the cells were resuspended in 0.4 M sucrose, 20 mM Hepes, and 1 mM EDTA, pH 8.0. Membrane fragments containing photosystem I were isolated following the procedure of Barry et al. (1988), up to the point where the thylakoid membranes are solubilized with lauryl maltoside. The resulting supernatant containing the photosystem I particles was centrifuged overnight at 40000*g*, and the pellets were resuspended in 20 mM Hepes and 1 mM EDTA, pH 8.0, buffer. The chlorophyll *a* concentration of the final preparations was either 4 mg/mL (protonated methionine) or 5.4 mg/mL ([*methyl*-d_3]methionine) and the chl *a*:P700 ratio 145:1 (protonated methionine) or 150:1 (deuterated methionine).

EPR samples were prepared as described in Heathcote et al. (1993). Sodium dithionite (0.2% w/v) was added under anaerobic conditions, and then the samples were incubated in the dark for 30 min and frozen in liquid nitrogen in the dark. The EPR signal attributed to $A_1^{\bullet-}$ was photoaccumulated by illumination of these samples at 205 K for 2 min (Heathcote et al., 1993). In order to exchange protonated phylloquinone (Sigma) for the deuterated quinone in photosystem I particles from cells of *A. variabilis* Met-27 grown on [*methyl*-d_3]methionine, a modification of the procedure of Rustandi et al. (1992) was used. In an EPR tube a final concentration of 200 μM phylloquinone was added to the photosystem I particles, which were then illuminated at 4 °C for 10 min at an ambient photon flux density of 500 μmol m^{-2} s^{-1} under a stream of oxygen-free argon. Sodium dithionite (0.2% w/v) was added, and the samples were frozen in liquid nitrogen after 30 min incubation in the dark. Control samples were prepared in exactly the same way, but substituting ethanol at the same volume for the phylloquinone added in ethanol.

EPR spectra were recorded on a JEOL RE-1X spectrometer fitted with an Oxford Instruments ESR 900 helium flow cryostat. Spectra were stored on a Dell microcomputer using software written in this laboratory. All *g* values and linewidths were calculated with reference to a powdered manganese oxide standard sample.

The pulsed EPR spectra were obtained using the experimental setup described in detail by Moënne-Loccoz et al. (1994). Samples were maintained at 4 K by an Oxford Instruments CF935 flowing gas cryostat. Samples were excited by 532-nm laser pulses of 10-ns duration (doubled Nd-YAG laser, Spectra Physics DCR-11). The repetition rate was 10 Hz, and flash energy ($2-3$ mJ/cm^2) was set to prevent sample degradation or photoaccumulation of reduced electron acceptors.

Spectra were recorded on a Bruker ESP380 X-band pulsed spectrometer with a variable *Q*, dielectric resonator (Bruker Model 1052 DLQ-H 8907). For a 16-ns pulse in an $S = {}^1/_2$, $g = 2.002$ system, the maximum microwave magnetic field generated by a 1-kW traveling wave tube amplifier was approximately 6 G within the 10-mm homogeneous region of the resonator.

The following microwave pulse echo sequence was used with quadrature detection (in-and-out-of-phase channels): ($p-\tau-2p-\tau-echo$) (Norris et al., 1980; Moënne-Loccoz et al., 1994). Spectra presented were recorded in the in-phase channel; there were no signals in the out-of-phase channel. Field-swept electron spin echo (ESE) spectra of photoaccumulated radicals were accumulated with a pulse sequence of p (96 ns)$-\tau$ (224 ns)$-2p$ (192 ns). The same pulse sequence was also used in recording flash-induced ESE spectra in order to improve spectral resolution by using larger microwave pulses of lower energy, and also to avoid moving ESE signals to the out-of-phase channel (see Moënne-Loccoz et al., 1994). The first pulse in the sequence was 128 ns after the laser flash. Since the ESP signal detected in the flash-induced spectra decays on the microsecond time scale (Moënne-Loccoz et al., 1994), it did not decay significantly during the pulse sequence.

Phylloquinone was extracted from thylakoid membranes of *A. variabilis* by the methods described by Schoeder and Lockau (1986). However, a Cyclobond (polyhydroxylated) bonded HPLC column (supplied by Technicol, Stockport, U.K.) was used. Mass spectra were recorded on a Kratos MS5ORF mass spectrometer. The sample was ionized by fast-atom bombardment (FAB) using a matrix of nitrobenzyl alcohol and a 7 keV argon beam. The accelerating voltage was 8 keV, and positive ions were detected.

RESULTS

It has been demonstrated that the 5-methyl group of methionine is the C_1 donor of the 2-methyl group of phylloquinone (Jackman et al., 1967; Barry et al., 1988). Solution ENDOR data on napthoquinone anion radicals (Barry et al., 1988) suggest that the methyl coupling will dominate the proton hyperfine contribution to the EPR spectrum of the semiquinone radical of phylloquinone.

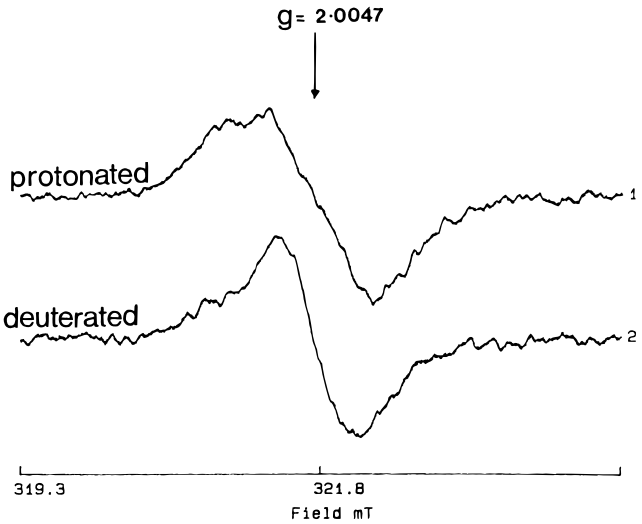

FIGURE 1: The EPR signal photoaccumulated at 205 K in photosystem I preparations from *A. variabilis* grown on protonated and [*methyl*-d_3]methionine. Samples were frozen in the dark in the presence of dithionite as described in Materials and Methods. The spectra above are light minus dark spectra following 2 min illumination at 205 K, and are the average of 2 scans. EPR conditions: microwave power, 5 μW; modulation width, 0.2 mT; recording temperature, 75 K.

Therefore, growth of *A. variabilis* (Met-27) on [*methyl*-d_3]-methionine should narrow the photoaccumulated EPR spectrum attributed to A_1—if it does arise from the phylloquinone.

Figure 1 presents the EPR spectra photoaccumulated by 2 min illumination at 205 K of photosystem I particles from *A. variabilis* Met-27 grown in media supplemented with protonated or deuterated methionine. The pH of the samples was at 8.0 not 10.0, in order to avoid double reduction of A_1 to the quinol (Heathcote et al., 1993). The EPR spectra were recorded at microwave powers (5 μW) that are nonsaturating at the recording temperature (75 K).

The photoaccumulated EPR spectrum of protonated photosystem I particles (Figure 1, top spectrum) resembles that obtained in spinach digitonin photosystem I particles under similar conditions (Heathcote et al., 1993). The signal is centered at $g = 2.0047$, a g value characteristic of semiquinone anion radicals (Hales & Case, 1981), and similar to the value of $g = 2.0048$ reported in digitonin photosystem I particles (Heathcote et al., 1993). The linewidth (ΔH_{ptp}) of 0.88 mT is similar to that reported for digitonin spinach photosystem I of 0.95 mT and close to the 0.85 mT reported for vitamin K_1 in ethanol (Hales & Case, 1981). There are, however, differences in the spectrum from the cyanobacterial photosystem I and digitonin photosystem I from spinach (Heathcote et al., 1993); most notably, the feature on the low-field side of this asymmetric signal is more pronounced in the cyanobacterial sample. It is also important to note that although the g value and linewidth of the EPR signal presented in Figure 1 (top) are close to those reported for vitamin K_1 in ethanol, the lineshape of the signal is very different in the cyanobacterial photosystem I. The lineshape of the signal in Figure 1 (top) is much more asymmetric than that of vitamin K_1 in ethanol, presumably reflecting the effect of the protein.

In contrast to the protonated sample, the photoaccumulated signal in the deuterated cyanobacterial photosystem I (Figure 1, lower spectrum) particles is very different. Although centered at the same g value, the signal has lost the feature

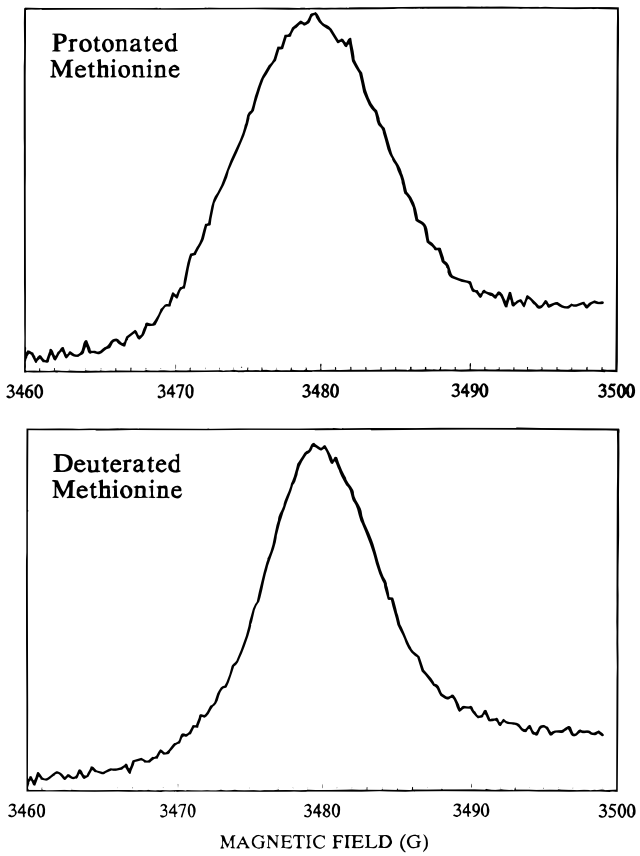

FIGURE 2: Field-swept ESE spectra of the EPR signal photoaccumulated at 205 K in photosystem I preparations from *A. variabilis* grown on protonated and [*methyl*-d_3]methionine. See Materials and Methods for the preparation of samples. These are the same samples as those presented in Figure 1, following illumination at 205 K. The signals were detected in the in-phase channel, using long microwave pulses ($\pi/2$ pulse = 96 ns) of low intensity; 0.025 mT/point, 40 added sequences/point; recording temperature, 4 K.

at low fields, which probably arises from unresolved proton hyperfine, and this has led to a spectrum that overcompensates at the low-field side. As predicted, the linewidth (ΔH_{ptp}) has narrowed from the 0.88 mT seen in protonated samples, although the linewidth has reduced to 0.63 mT, which suggests more extensive deuteration of the phylloquinone than the deuteration of the C-2 methyl group reported by Barry et al. (1988).

In order to demonstrate that the narrowing of the photoaccumulated EPR signal reflected deuteration of the A_1 redox component, we decided to monitor the ESP signal arising from the $P700^{\bullet+}A_1^{\bullet-}$ radical which had already been shown to narrow when the quinone was deuterated (Rustandi et al., 1990). We monitored the ESP signal using pulsed EPR (Moënne-Loccoz et al., 1994). Initially, we acquired field-swept electron spin echo (ESE) spectra of the photoaccumulated $A_1^{\bullet-}$ in photosystem I particles isolated from *A. variabilis* Met-27 grown on protonated or deuterated methionine (Figure 2). These spectra, which are presented as absorption spectra (rather than the first derivative EPR spectra), confirm that the biosynthetic deuteration of phylloquinone is narrowing the photoaccumulated EPR signal attributed to $A_1^{\bullet-}$. Again, the loss of unresolved proton hyperfine at low fields is noticeable. A similar narrowing of the ESP signal arising from the radical pair $P700^{\bullet+}A_1^{\bullet-}$ should be seen (Rustandi et al., 1990). ESP spectra were acquired from samples of the same preparations which had

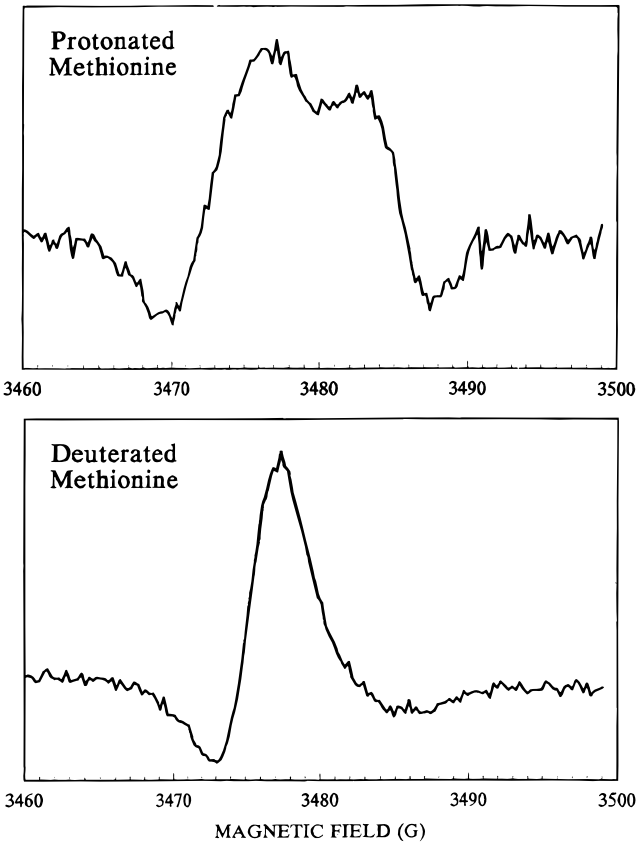

FIGURE 3: Field-swept ESE spectra of the laser-induced signal (on−off, t_0 = 128 ns) in prereduced photosystem I particles from *A. variabilis* grown on protonated and [*methyl*-d_3]methionine. Samples were frozen in the dark in the presence of dithionite as detailed in Materials and Methods. Laser repetition rate, 10 Hz; pulse energy, 2−3 mJ/flash; other conditions as in legend to Figure 2.

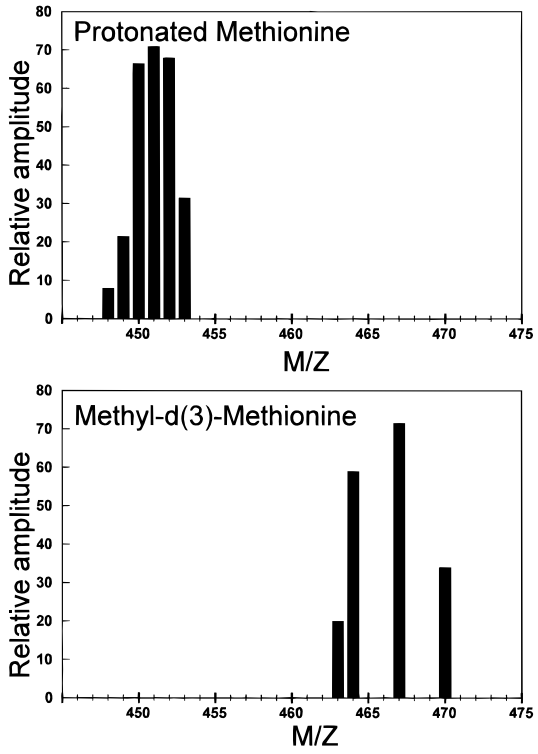

FIGURE 4: Mass spectra of phylloquinone in photosystem I particles isolated from a methionine auxotroph of *A. variabilis* grown on media supplemented with protonated or [*methyl*-d_3]methionine. See Materials and Methods for experimental details.

not been illuminated at 205 K but were otherwise prepared by the same procedure as detailed in Materials and Methods (Figure 3). The ESP and ESE spectra are being acquired with relatively long microwave pulses of low energy (see Materials and Methods) in order to improve spectral resolution and to avoid moving the ESP signal into the out-of-phase channel, a phenomenon first observed by Thurnauer and Norris (1980). It was recently studied by Moënne-Loccoz et al. (1994) and discussed by Tang et al. (1994). The ESP spectrum from *A. variabilis* grown on protonated methionine shows the characteristic emission/absorption/emission pattern, although it differs in some respects from the ESP spectra presented from light modulated transient EPR studies (Thurnauer & Gast, 1985; Rustandi et al., 1990) since they are absorption spectra. However, they are similar in some if not all respects to spectra obtained from the cyanobacterium *Synechococcus* 6301 using time-resolved transient EPR and direct detection (Van der Est et al., 1994), which like those presented in Figure 3 are not field modulated and therefore not first derivative spectra. The major difference is that a significant feature on the low-field side of the absorption signal seen by Van der Est et al. (1994), and attributed by them to unresolved hyperfine structure, is nearer the middle of the high-field side in our spectrum. The reasons for this are unknown. The ESP spectra from the deuterated methionine grown samples show a dramatic narrowing of the spectrum, showing that the ESP signal (which contains contributions from both partners in the $P700^{\bullet+}A_1^{\bullet-}$ radical pair) and the EPR signal attributed to $A_1^{\bullet-}$ are monitoring the same redox component. The out-of-phase ESP signal seen at shorter (higher power) microwave pulses (Moënne-Loccoz et al., 1994) is also narrowed by this biosynthetic deuteration (results not shown).

Since the ESP spectrum (at least at X-band) cannot be considered as simply a combination of the spectrum of $P700^{\bullet+}$ and $A_1^{\bullet-}$ [see Snyder and Thurnauer (1993) for a review of ESP] signals, a more detailed analysis of the changes in the spectra is beyond the scope of this investigation. Simulating the ESP signal from the EPR spectra and hyperfine couplings of $P700^{\bullet+}$ and $A_1^{\bullet-}$ is also not possible (see Discussion).

In Figure 4 we present FAB mass spectra of phylloquinone extracted from thylakoid membranes isolated from *A. variabilis* cells grown on protonated or deuterated methionine. The parent ion has a mass of 451 when phylloquinone is isolated from cells grown on protonated methionine, as would be predicted and has been previously reported (Barry et al., 1988). To our surprise, when phylloquinone is isolated from cells fed [*methyl*-d_3]deuterated methionine, the most intense peak is found at 467, with significant peaks at 463, 464, and 470. This suggests that considerably more deuteration has occurred than expected, with deuteration occurring in the phytyl chain as well as the 2-methyl group. This is in contrast to the results of Barry et al. (1988), who reported a 65% deuterium incorporation into the methyl group of the phylloquinone only.

This observation suggests that there is a possibility that photosystem I components other than phylloquinone could be deuterated in these preparations although there is no evidence of this (see Discussion).

To confirm that the photoaccumulated EPR signal did arise from biosynthetically deuterated phylloquinone and not from a reporter species, an experiment was carried out to exchange

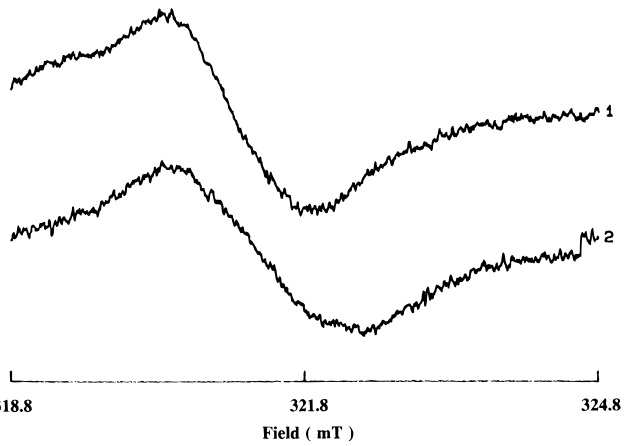

FIGURE 5: The EPR signal photoaccumulated at 205 K in photosystem I preparations from *A. variabilis* grown on [*methyl*-d_3]-methionine before (1) and after (2) incubation in the light with protonated phylloquinone. See Materials and Methods for the details of treatment of samples to exchange deuterated for protonated phylloquinone. Samples were frozen in the dark in the presence of dithionite, and subsequently illuminated at 205 K for 2 min. The spectra presented are light minus dark, and the average of 2 scans. The spectra have been shifted so the low-field peaks coincide for ease of comparison of linewidth. EPR conditions: microwave power, 5 μW; modulation amplitude, 0.2 mT; recording temperature, 75 K.

the deuterated quinone for protonated phylloquinone while monitoring the photoaccumulated signal. The exchange technique of Rustandi et al. (1992), which demonstrated exchange of quinones by monitoring the ESP signal, was used. This avoids the solvents used in extraction and reconstitution of the quinone, an experimental technique which alters the phase of the ESP signal (Rustandi et al., 1990) suggesting rebinding of the quinone into the photosystem I during reconstitution does not exactly reproduce the original situation. The results presented in Figure 5 show that, upon exchange of protonated phylloquinone with the photosystem I particles isolated from cells grown on deuterated methionine, the linewidth of the EPR signal photoaccumulated at 205 K increases from 0.63 mT in the control sample of deuterated *A. variabilis* to 0.85 mT in the sample to which exogenous protonated phylloquinone had been added. This result confirms that the photoaccumulated EPR spectrum arises from phylloquinone.

DISCUSSION

The EPR spectrum photoaccumulated in photosystem I particles isolated from *A. variabilis* grown on protonated methionine resembles that previously observed in digitonin photosystem I particles isolated from spinach (Mansfield & Evans, 1988; Heathcote et al., 1993). It has the same *g* value characteristic of a quinone, but has a slightly narrower linewidth (ΔH_{ptp} of 0.88 mT compared to 0.95 mT). The narrower linewidth and more enhanced feature at the low-field side of the spectrum (Figure 1) could reflect species differences or may indicate that the photoaccumulated $A_1^{\bullet-}$ EPR spectrum from spinach (Heathcote et al., 1993) in fact contains a slight contribution from another redox component ($A_0^{\bullet-}$?). Certainly, the linewidth seen in *A. variabilis* cells grown on protonated media is approaching that reported for phylloquinone *in vitro* (Hales & Case, 1981) although the lineshape is very different. The results presented in the previous section conclusively demonstrate that the asymmetric EPR signal centered at $g = 2.0047 \pm 0.0002$ and ΔH_{ptp} of 0.88 mT seen in *A. variabilis* arises from phylloquinone.

Biosynthetic deuteration of phylloquinone results in a narrowing of this EPR signal from 0.88 to 0.63 mT (Figure 1). At the same time, the EPR signal changes, presumably due to the loss of unresolved proton hyperfine couplings, most noticeably at the low-field side of the spectrum. The EPR spectrum of $A_1^{\bullet-}$ in the deuterated *A. variabilis* has the same linewidth and appearance as the photoaccumulated signal observed by us in photosystem I particles isolated from fully deuterated cells of *Synechococcus lividus* (the cells were kindly provided by Dr. M. Thurnauer, Argonne National Laboratory). This suggests that in fact all protons on the phylloquinone contributing to (unresolved) proton hyperfine couplings were deuterated when *A. variabilis* met27 was grown on [*methyl*-d_3]deuterated methionine. This result was subsequently supported by the FAB mass spectra of isolated phylloquinone (see below).

Since the ESP spectrum arising from the $P700^{\bullet+}A_1^{\bullet-}$ radical pair (Thurnauer & Gast, 1985; Rustandi et al., 1990; Snyder et al., 1991) contains contributions from the $P700^{\bullet+}$ and $A_1^{\bullet-}$ spectra, narrowing of the $A_1^{\bullet-}$ spectrum due to deuteration of the phylloquinone should also result in a narrowing of the ESP signal. This prediction has previously been given support by the observations of Rustandi et al. (1990, 1992) who found that replacement of the protonated phylloquinone with fully deuterated phylloquinone led to a narrowing of an ESP signal observed using light-modulated EPR. These authors did not however monitor the photoaccumulated $A_1^{\bullet-}$ EPR signal. Our results using pulsed EPR (Figures 2 and 3) show that the field-swept ESE spectra of the photoaccumulated EPR signal show narrowing upon biosynthetic deuteration of the phylloquinone and that the ESP flash-induced spectra measured with the same pulse sequence show a dramatic narrowing. Unfortunately, it is not possible to extract the $P700^{\bullet+}$ and $A_1^{\bullet-}$ spectra directly from the ESP signal at X-band [see Snyder and Thurnauer (1993) for a review]. There are currently two models for simulating ESP spectra, the correlated coupled radical pair (CCRP) model (Van den Brink et al., 1994; Van der Est et al., 1995) and the sequential electron transfer polarisation (STEP) model (Morris et al., 1995). In theory, we could use these to simulate the protonated and deuterated ESP spectra to demonstrate that the change in the photoaccumulated spectrum of $A_1^{\bullet-}$ is the cause of the corresponding change in the ESP spectra, thus equating $A_1^{\bullet-}$(photoaccumulated) = $A_1^{\bullet-}$ (ESP). However, both models employ the *g* tensors of phyllosemiquinone *in vitro*, whereas our EPR spectra of phyllosemiquinone *in vivo* show that the *g* tensors of this species/radical are different. Even if the *g* tensors were measured, neither approach at simulation actually includes hyperfine couplings in the calculations, which are only globally included as a linewidth parameter. So although we now for the first time know the proton hyperfine couplings for the phyllosemiquinone *in vivo* (Rigby et al., 1996), they cannot be used in these simulations to equate the photoaccumulated and ESP $A_1^{\bullet-}$ signals.

We expected that growth of *A. variabilis* met 27 on [*methyl*-d_3]methionine would biosynthetically label the 2-methyl group of phylloquinone with deuteration as previously reported (Jackman et al., 1967; Barry et al., 1988). The methionine would donate the deuterated methyl group to

S-adenosylmethionine, which would then act as a methyl group donor to the phylloquinone. However, the FAB mass spectra presented in Figure 4 show that considerably more deuteration of the phylloquinone is occurring. The phylloquinone extracted from *A. variabilis* growth on protonated methionine has a parent ion at a mass of 451 (Figure 4) as expected, showing that the extraction and purification have been successful. But the most intense peak in the FAB mass spectrum of phylloquinone isolated from *A. variabilis* grown on deuterated methionine is at 467 (Figure 4). There are 39 protons in the phytyl chain in addition to the three methyl group protons, and this result suggests that over a third of these are on average labeled with deuterons. In terms of the EPR spectrum of $A_1^{\bullet-}$, only the methyl protons and the methylene protons closest to the ring in the phytyl chain contribute significantly to proton hyperfine couplings and the broadening of the EPR spectrum (Rigby et al., 1996). It seems that all of these have been labeled with deuterium, as the linewidth of the deuterated spectrum is the same as the linewidth of the EPR spectrum obtained from fully deuterated *S. lividus*. This observation is supported by subsequent ENDOR studies of the protonated or deuterated $A_1^{\bullet-}$ in *A. variabilis* (Rigby et al., 1996). We believe this labeling pattern could only have happened if significant breakdown of methionine has occurred as normally the deuterated methyl group should only be transferred to *S*-adenosylmethionine because of the binding to the S atom. This raises the possibility of some nonspecific labeling of redox components other than phylloquinone. However, the spectrum of $P700^{\bullet+}$ in the deuterated samples is the same linewidth as in the protonated samples, indicating that there has been no significant deuterium labeling of chlorophyll *a* (data not shown).

To confirm that the EPR signal photoaccumulated at 205 K arises from phylloquinone and not a reporter species that has become deuterated, we carried out an experiment in which a protonated phylloquinone was exchanged for the deuterated quinone. We used a method already shown to be successful by Rustandi et al. (1992), who demonstrated quinone exchange by monitoring the ESP signal at room temperature. The photoaccumulated EPR signal in deuterated membranes broadened from ΔH_{ptp} of 0.63 to 0.85 mT (Figure 5) upon exchange, demonstrating conclusively that this EPR signal arose from the quinone. However, the photoaccumulated EPR signal after exchange was different from that seen in protonated *A. variabilis* (Figure 1) lacking the feature at low field. This might indicate that the quinone after exchange is not rebinding into the protein binding pocket in exactly the same way.

The results presented here are the opposite of those presented by Barry et al. (1988). There are however possible explanations for this difference. The EPR spectrum photoaccumulated by Barry et al. (1988) is 0.10 mT wide and centered at $g = 2.0054$, and so resembles the spectrum of $A_1^{\bullet-}$ reported in spinach photosystem I particles isolated using Triton X-100 (Mansfield & Evans, 1988; Heathcote et al., 1993). Barry et al. (1988) used Triton X-100 in the isolation of their photosystem I particles. In contrast, we used lauryl maltoside only (see Materials and Methods) and so obtained a spectrum for $A_1^{\bullet-}$ (Figure 1) that resembles that seen in spinach photosystem I isolated with digitonin rather than Triton X-100 (Heathcote et al., 1993). In Triton X-100 photosystem I particles from spinach, the photoaccumulation of $A_0^{\bullet-}$ gives an EPR spectrum 0.12 mT wide. It is possible that Barry et al. (1988) were photoaccumulating an EPR signal containing contributions from both $A_0^{\bullet-}$ and $A_1^{\bullet-}$. They used a photoaccumulation method (Ziegler et al., 1987) that involves illumination at pH 10.0 in the presence of dithionite at room temperature prior to freezing and photoaccumulation. These conditions could lead to some double reduction of the quinone (Setif & Bottin, 1991; Heathcote et al., 1993) and thus photoaccumulation of $A_0^{\bullet-}$. Of course, this fact was not appreciated at the time Barry et al. (1988) carried out their experiments, especially since the original observations of the EPR spectrum of $A_1^{\bullet-}$ (Bonnerjea et al., 1983; Gast et al., 1984) had involved room temperature illumination.

The greater extent of deuteration we report will also have made it easier to observe line narrowing of the photoaccumulated EPR spectrum of $A_1^{\bullet-}$. Obtaining the asymmetric "native" EPR signal of $A_1^{\bullet-}$ may have helped since this is more dramatically affected by deuteration in appearance than the signal seen from photosystem I prepared using Triton X-100. Of course, that was the only $A_1^{\bullet-}$ EPR signal reported in the literature at the time of the experiments of Barry et al. (1988). We have also found that prolonged exposure to the 20 mM MES, pH 6.0, buffer used during the preparation of photosystem I from *A. variabilis* (Barry et al., 1988) can give rise to spurious EPR signals at $g = 2.00$ during photoaccumulation at 205 K, particularly if samples are frozen at pH 6.0. These signals are Gaussian and about 0.10 mT linewidth but show no response to deuteration (unpublished work).

Assignment of the photoaccumulated EPR signal to $A_1^{\bullet-}$ has allowed more detailed study of the electronic structure of $A_1^{\bullet-}$ using ENDOR spectroscopy (Rigby et al., 1996).

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