Cyanide Binding at the Non-Heme Fe^{2+} of the Iron–Quinone Complex of Photosystem II: At High Concentrations, Cyanide Converts the Fe^{2+} from High ($S = 2$) to Low ($S = 0$) Spin

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ABSTRACT: The primary electron acceptor complex of photosystem II, Q_AFe^{2+}, can bind a number of small molecules at the iron site, including cyanide [Koulougliotis, D., Kostopoulos, T., Petrouleas, V., & Diner, B. A. (1993) *Biochim. Biophys. Acta 1141*, 275–282)]. In the presence of NaCN (30–300 mM) at pH 6.5, the reduced state, $Q_A^-Fe^{2+}$, produced either by illumination at ≤200 K or by reduction in the dark with sodium dithionite, is characterized by a $g = 1.98$ EPR signal. The light- or dithionite-induced $g = 1.98$ signal decays with increasing pH above 6.5 and is almost totally absent at pH 8.1 and NaCN concentrations above 300 mM. However, at high pH (8.1), the $g = 1.98$ signal still forms transiently before it decays with a $t_{1/2}$ of approximately 30 min in spinach BBY preparations treated with 100 mM NaCN. Complementary to the disappearance of the $g = 1.98$ signal with increasing pH or incubation time, a new EPR signal develops at $g = 2.0045$. This signal has the characteristics of the semiquinone, Q_A^-, uncoupled from its magnetic interaction with the iron. Prolonged incubation of a high pH, high cyanide treated sample in a cyanide-free medium at pH 6 restores the ability of the sample to develop the cyanide-induced $g = 1.98$ signal at pH 6.5. This indicates that the iron is not physically dissociated during the high pH cyanide treatment. The high pH, high cyanide effects are accompanied by the conversion of the characteristic $Fe^{2+}(S = 2)$ Mössbauer doublet [isomer shift (Fe) = 1.19 mm/s, quadrupole splitting = 2.95 mm/s] to a new one with parameters (isomer shift = 0.26 mm/s, quadrupole splitting = 0.36 mm/s) characteristic of an $Fe^{2+}(S = 0)$ state. This explains the loss of the magnetic interaction of Q_A^- with the iron. The present results, combined with the earlier study, suggest a progressive binding of two or three cyanides at the iron site. The $g = 1.98$ to $g = 2.0045$ conversion (reflecting the high- to low-spin conversion of the iron) develops as a function of increasing CN^- concentration at high pH, with a K_d of approximately 1.2 mM. If we assume that CN^- is the active species for the earlier steps too, the respective K_d's are 0.1–0.2 mM (development of the $g = 1.98$ signal) and 10–20 μM (competition of cyanide with approximately 300 μM NO for binding to the iron).

A common property of the photosynthetic bacterial reaction center and that of photosystem II (PSII)[1] of green plants is the presence of an iron–quinone complex, which acts as the terminal electron acceptor. A number of spectroscopic similarities and sequence homologies suggest that in PSII [for a recent review, see Diner et al. (1991)], as in the bacteria (Deisenhofer & Michel, 1989; Feher, 1992), the linear quinone–non heme iron–quinone arrangement spanning 18 Å is conserved. Where the analogy deviates significantly is in the coordination and the properties of the iron. While in both systems the iron is coordinated by four histidines, two from each of two subunits, there are important differences in the 5 and 6 coordination positions. In bacteria, these are occupied by a glutamate protein residue that acts as a bidentate ligand (Deisenhoffer & Michel, 1989). In PSII, at least one of these positions is occupied by bicarbonate (Diner & Petrouleas, 1990). A number of carboxylate anions (glycolate, glyoxylate, oxalate, etc.) (Deligiannakis et al., 1994; Petrouleas et al., 1994) or NO (Petrouleas & Diner, 1990; Diner & Petrouleas, 1990) can bind to the PSII iron in competition with bicarbonate. In bacteria no ligand replacement has been observed.

We have recently shown that cyanide binds at the non-heme iron site of PSII (Koulougliotis et al., 1993). At pH 6.5, cyanide competes with NO for binding to the iron with an approximate effective K_d of 10 mM [CN^- + HCN]. At higher concentrations (approximate K_d, 50–80 mM), cyanide modifies the $g = 1.82$–1.9 $Q_A^-Fe^{2+}$ EPR signal to a new form at $g = 1.98$. This has been taken as evidence that at least two cyanides can bind at the iron site. We extend these studies to higher pH values. The results show drastic new effects of cyanide. At high pH, the $g = 1.98$ form ($Q_A^-Fe^{2+}$ state) gradually converts to a $g = 2.0045$ signal, accompanying the conversion of Fe^{2+} to a low-spin state. This indicates the binding of an additional cyanide on the iron under these conditions.

MATERIALS AND METHODS

Photosynthetic Membranes. BBY thylakoid membrane fragments (Berthold et al., 1981) were prepared from spinach with some modification of the original procedure (Ford & Evans, 1983). After the final centrifugation step, the membranes were suspended at 7–8 mg of Chl/mL in 0.4 M sucrose, 15 mM NaCl, 5 mM $MgCl_2$, and 5 mM MES–NaOH (pH 6.5) and frozen in liquid N_2 until use.

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[1] Abbreviations: PSII, photosystem II; BBY, thylakoid membrane fragments isolated by a modification of the method of Berthold et al. (1981); Q_A, the primary quinone electron acceptor of the iron–quinone complex; EPR, electron paramagnetic resonance; Tyr Y_D^+, tyrosine radical responsible for the EPR signal II_{slow}; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 4-morpholineethanesulfonic acid; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; cyt, cytochrome; Chl, chlorophyll.

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BBY membrane fragments for Mössbauer measurements were isolated from spinach grown hydroponically in a ^{57}Fe-enriched medium.

Chemical Treatments. The buffers used were MES–NaOH (pH 6.0–6.5), HEPES–NaOH (pH 7.0–7.5), and Tricine–NaOH (pH 8) at 100 mM. The pH of the cyanide solution was adjusted prior to the addition of sample from a concentrated stock suspension at the same pH. Chemical reduction was performed at the end of the incubation period with cyanide (typically 60–90 min or variable) by treatment with 50 mM sodium dithionite for 5–10 min. The ambient potential was measured with a Metrohm combined microelectrode (Ag/AgCl/KCl, 3 M) connected to a Fluke 8840A multimeter. All solutions were well buffered, and the pH of the samples was also checked after the measurements. No deviations were observed. All treatments were carried out at approximately 10 °C.

EPR Spectroscopy. EPR spectra were obtained using a Bruker ER 200D-SRC spectrometer equipped with an Oxford ESR 9 cryostat, a Bruker 035M NMR–gaussmeter, and an Anritsu MF76A microwave frequency counter. Data were collected on a PC interfaced to the spectrometer. The concentration of the samples was typically 3 mg of Chl/mL in 4 mm o.d. EPR tubes.

Mössbauer Spectroscopy. Mössbauer spectra were obtained using a constant acceleration spectrometer and a ^{57}Co(Rh) source. The sample was a dense suspension in a disk-shaped holder containing approximately 12 mg of Chl total.

RESULTS

Illumination of PSII preparations at 200 K results in single-electron transfer and formation on the electron acceptor side of the reaction center of the $Q_A^-Fe^{2+}$ state. This state is characterized by two alternative EPR signals at $g = 1.90$ (Rutherford & Zimmermann, 1984) and $g = 1.82$ (Nugent et al., 1981). The same state can also be formed in the dark by reduction with sodium dithionite. Earlier we showed that illumination at 200 K of PSII preparations treated with 30–300 mM cyanide at pH 6.5 induces a signal at $g = 1.98$ (Koulougliotis et al., 1993). This signal is presented in Figure 1A. Here we show that a full size signal can also be produced by reduction with sodium dithionite in the dark at an ambient potential, E_h, of −390 mV (Figure 1B), while the signal is vanishingly small at $E_h > 30$ mV (not shown). These observations support the previous assignment of the $g = 1.98$ signal to the $Q_A^-Fe^{2+}$ state, modified by the binding of cyanide. Dithionite eliminates the donor side contributions at $g = 2.0$ (primarily Tyr Y_D^+) due to chemical reduction of this species and therefore allows for a more accurate estimation of the $Q_A^-Fe^{2+}$ signals.

Dependence of the g = 1.98 Signal on the pH, the Cyanide Concentration, and the Time of Incubation. In a previous study, we examined the dependence of the $g = 1.98$ signal intensity on the NaCN concentration at pH 6.5. The $g = 1.98$ signal increased in the concentration range 0–360 mM NaCN, with an apparent K_d for cyanide binding in the range 50–80 mM. Here we extend these studies to higher pH values. Figure 2 examines the dependence of the $g = 1.98$ signal intensity on the NaCN concentration at three representative pH values. Pronounced changes are observed. The data at pH 6.5 show dependence similar to that observed earlier and have been included here for the sake of comparison. At higher pH, the $g = 1.98$ signal intensity drops drastically, and at pH 8.1 and 300 mM NaCN the signal is barely generated at all.

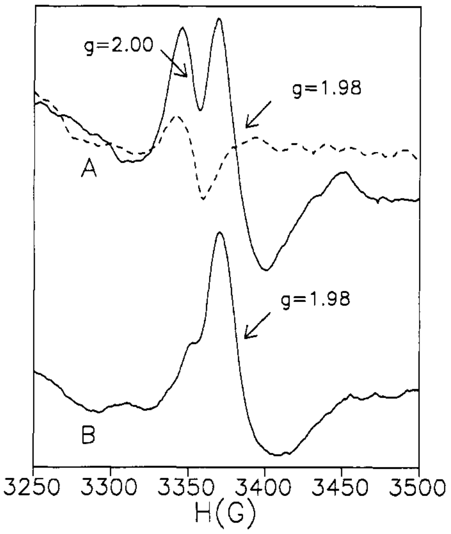

FIGURE 1: The $g = 1.98$ signal in BBY membranes treated with 340 mM NaCN, at pH 6.5, induced by (A) illumination at 200 K (dashed line, spectrum prior to illumination) and (B) reduction in the dark with sodium dithionite. The contribution from signal II at $g = 2.0$ is eliminated in the latter spectrum due to chemical reduction of this species by dithionite. EPR settings: T, 13 K; mod. amp., 16 G; microwave power, 200 mW; microwave frequency, 9.42 GHz.

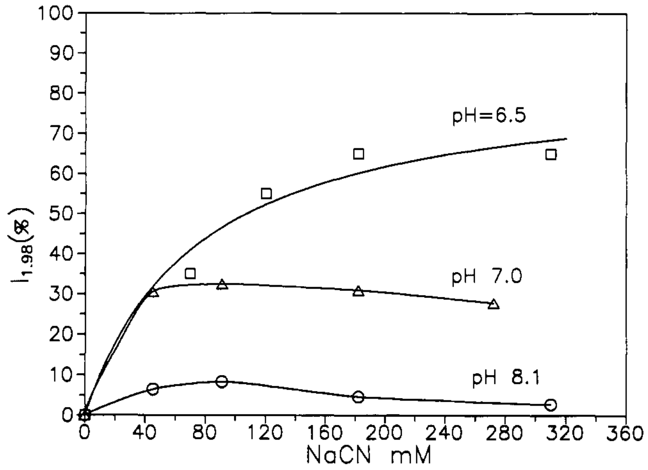

FIGURE 2: Dependence of the $g = 1.98$ signal on the NaCN concentration (90 min incubation at ~10 °C) at three pH values. The $Q_A^-Fe^{2+}$ state was produced by dithionite reduction in the dark. The signal intensity scale is normalized approximately to the fraction of centers contributing to the signal. See text for details. EPR settings as for Figure 1.

It is notable that at both pH 7 and pH 8.1 the $g = 1.98$ signal goes through a weak maximum at relatively low NaCN concentrations.

An examination of the dependence of the $g = 1.98$ signal on the time of incubation with cyanide provides insights into the suppression of the $g = 1.98$ signal at high pH. A series of samples was incubated with 100 mM NaCN at pH 8.1 and approximately 10 °C for various times and subsequently illuminated at 200 K or incubated for an additional 5 min with 50 mM sodium dithionite, in order to produce the $Q_A^-Fe^{2+}$ state. Figure 3 shows the evolution of the $g = 1.98$ signal with time and compares it with the maximum signal obtained at pH 6.5 and 340 mM NaCN. No difference in the $g = 1.98$ signal was observed between the illuminated and the dithionite-reduced samples, and therefore the two sets of data are not distinguished in the plot. Notably, the $g = 1.98$ signal is formed transiently at a significant intensity at short incubation times (<2.5 min) and then decays to the equilibrium value at

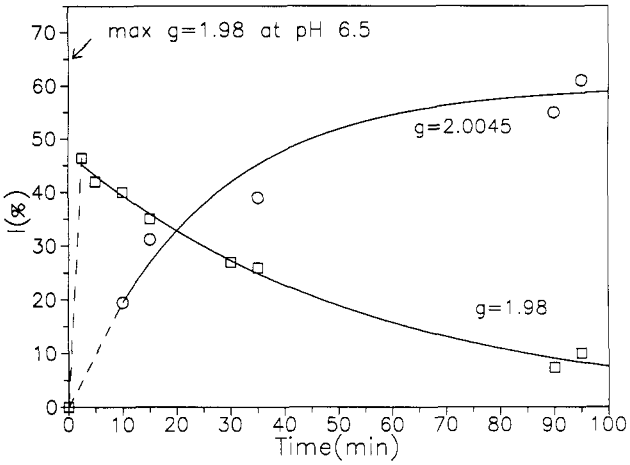

FIGURE 3: Dependence of the $g = 1.98$ and $g = 2.0045$ (see Figure 4) signals on the time of incubation of BBY samples with 100 mM NaCN, at ~10 °C, at pH 8.1. The $Q_A^-Fe^{2+}$ state is produced by dithionite reduction in the dark (points at 10, 15, 35, and 95 min) or by illumination at 200 K (see text). The signal intensity scale is normalized approximately to the fraction of centers contributing to each signal.

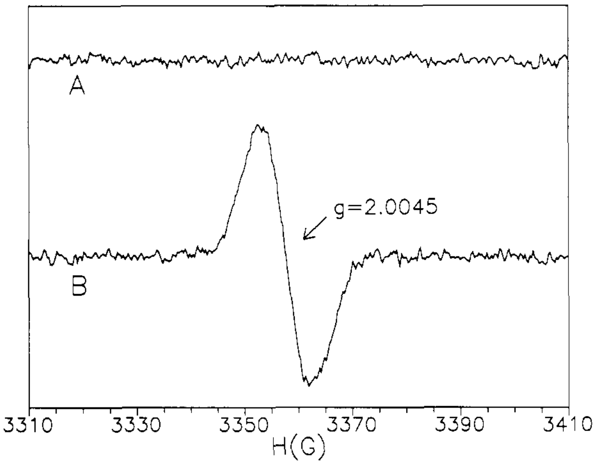

FIGURE 4: EPR spectra in the $g = 2$ region of BBY membranes, pH 8.0, reduced with dithionite in the dark after incubation for 1.5 h with (A) 340 mM NaCl and (B) 340 mM NaCN. EPR settings: *T*, 13 K; microwave power, 200 nW; mod. amp.; 4 G; microwave frequency, 9.42 GHz.

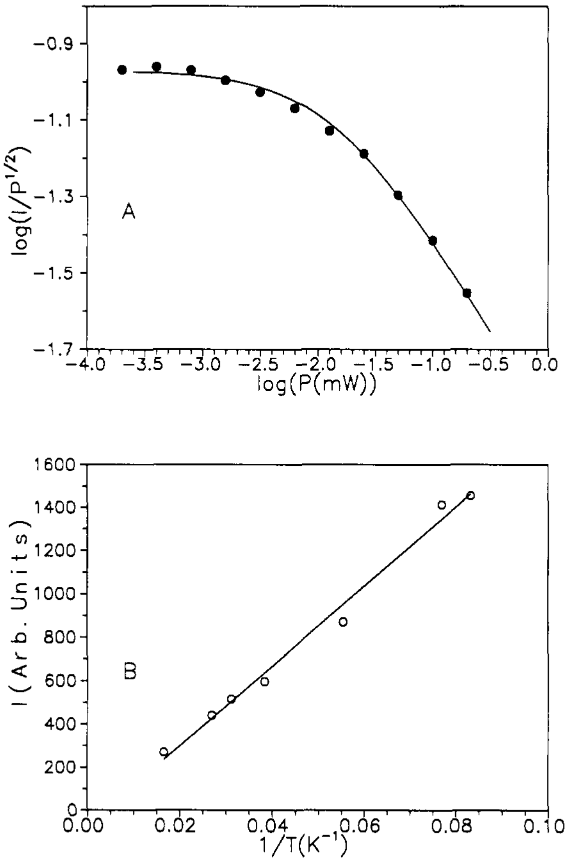

FIGURE 5: (A) Microwave power dependence of the $g = 2.0045$ signal at 13 K. The solid line is a theoretical simulation (see text). (B) Temperature dependence of the $g = 2.0045$ signal. EPR settings other than those specified in the plots were as for Figure 4.

this pH (compare Figure 2) with an approximate half-time of 45 min. Apparently, long incubation with cyanide at high pH results in a modification of the $Q_A^-Fe^{2+}$ state to one that does not produce the $g = 1.98$ EPR signal.

Cyanide-Induced EPR Signal at g = 2.0045. We have searched for alternative $Q_A^-Fe^{2+}$ EPR signals under the conditions of the disappearance of the $g = 1.98$ signal at high pH. No new signals could be detected in the $g < 2.0$ region, where all known $Q_A^-Fe^{2+}$ signals occur. Figure 4 compares the $g = 2.0$ region of a BBY sample treated with 340 mM NaCN at pH 8.0 (Figure 4B) with a control sample treated with 340 mM NaCl at the same pH for 1.5 h (Figure 4A). Both samples were subsequently treated with 50 mM sodium dithionite in darkness for 10–15 min to produce the $Q_A^-Fe^{2+}$ state. In the control sample, a $g = 1.9$ signal is induced (not shown), while the $g = 1.98$ signal vanishes at this high pH in the cyanide-treated sample. Examination of Figure 4 shows that a narrow signal with Gaussian line shape (9.50 ± 0.5 G width) is present at $g = 2.0045$ in the cyanide-treated sample, but not in the control sample. It is likely that this signal results from the $Q_A^-Fe^{2+}$ state modified by the high cyanide, high pH treatment.

We have examined the saturation properties and temperature dependence of the $g = 2.0045$ signal (Figure 5). A qualitative examination of Figure 5A shows that the signal saturates easily, unlike the signals at $g = 1.82$ or $g = 1.9$ in the untreated system or at $g = 1.98$ in the cyanide-treated system (Koulougliotis et al., 1993), which do not saturate easily due to the interaction of Q_A^- with the paramagnetic $Fe^{2+}(S = 2)$. We have fit the data with the function $\log(I/P^{1/2}) = \log(kC/(1 + P/P_{1/2})^{0.5b})$, where I is the amplitude of the EPR absorption, P is the microwave power in milliwatts, $P_{1/2}$ is the power for half-saturation in milliwatts, C is the concentration of spins, k is an apparatus-dependent constant, and b is the "inhomogeneity parameter" (Styring & Rutherford, 1988; Rupp et al., 1978). The results yielded $P_{1/2} = 15\ \mu W$ and $b = 1.0$. For comparison, the $P_{1/2}$ for the $g = 1.98$ signal at 13 K (Koulougliotis et al., 1993) is much higher than 200 mW. The temperature dependence of the signal recorded under nonsaturating conditions follows Curie behavior (Figure 5B). The g-value of 2.0045, the narrow width, the saturation behavior, and the temperature dependence are all consistent with the assignment of this signal to a free ($S = {}^1/_2$) radical. As this signal most likely results from the $Q_A^-Fe^{2+}$ state, we must assume that under the conditions of high pH, high cyanide concentration, Q_A^- behaves as a free semiquinone (see also Discussion).

Evolution of the g = 2.0045 Signal with Increasing pH, Cyanide Concentration, and Time of Incubation. If the $g = 2.0045$ signal develops at the expense of the $g = 1.98$, then it should evolve concomitantly with the decay of the $g = 1.98$

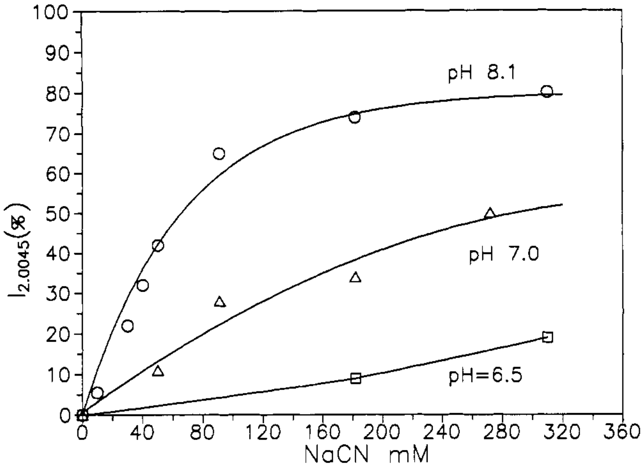

FIGURE 6: Dependence of the $g = 2.0045$ signal on the NaCN concentration (~90-min incubation at ~10 °C) at three pH values. The $Q_A^-Fe^{2+}$ state is produced by dithionite reduction in the dark. The signal intensity scale is normalized approximately to the fraction of centers contributing to the signal. EPR settings as for Figure 4.

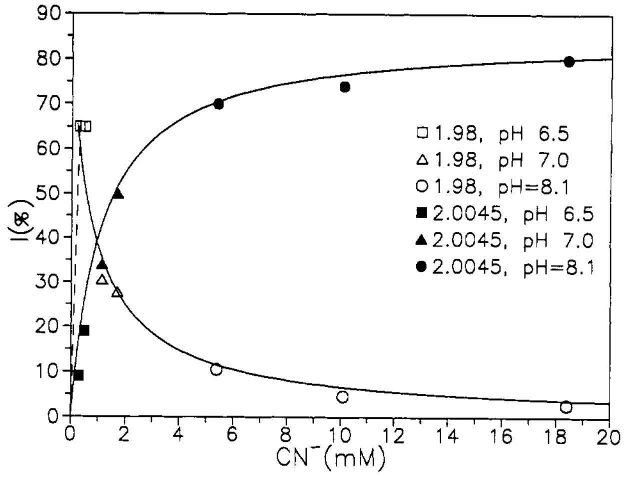

FIGURE 7: Plot of the $g = 1.98$ and $g = 2.0045$ signals as a function of the CN^- concentration. The data points are those of Figures 2 and 6 for NaCN concentrations ≥100 mM (see text). Both sets of data were fit with the same $K_d = 1.2$ mM, assuming quantitative conversion of the $g = 1.98$ to the $g = 2.0045$ form.

signal in the experiment in Figure 3. Indeed, such a trend is observed in both the illuminated and the dithionite-reduced samples. As the illuminated samples contain significant contributions from signal II, and possibly also from other free radical components (spectra not shown), quantitation of the $g = 2.0045$ signal is based mainly on the dithionite-treated spectra. The time evolution of the $g = 2.0045$ signal, also included in Figure 3, clearly shows an evolution complementary to that of the $g = 1.98$ signal.

Complementary behavior should also be exhibited by the pH dependence of the $g = 2.0045$ signal. Figure 6 shows the evolution of the $g = 2.0045$ signal as a function of the cyanide concentration at the three pH's of Figure 2. Comparison of Figures 2 and 6 shows, approximately, a complementary pH dependence for the $g = 1.98$ and 2.0045 signals. We have also checked that neither of the two signals saturates in this pH range under the microwave powers used (200 nW for $g = 2.0045$ and 200 mW for $g = 1.98$).

The above results are also qualitatively confirmed in Tris-treated BBY samples, which are devoid of the Mn complex and certain polypeptides, and in spinach chloroplast membranes. The $t_{1/2}$ for the conversion of $g = 1.98$ to $g = 2.0045$ at pH 8.0 is, however, significantly shorter in chloroplasts (approximately 5 min) while prolonged incubation with cyanide at this pH results in a decline of the $g = 2.0045$ signal. The latter effect is currently under investigation.

Quantitation of the $g = 1.98$ and $g = 2.0045$ Signals. Figures 2 and 6 show that at pH 8.1 and >300 mM NaCN concentration the $g = 1.98$ signal is quantitatively converted to the $g = 2.0045$ form. We have integrated the $g = 2.0045$ signal area and compared it with the maximum signal II_{slow}-(Y_D^+) obtained by prolonged room-temperature illumination. Assuming that under these conditions approximately all centers have Y_D oxidized, we have found that the $g = 2.0045$ signal, obtained by the incubation of BBY particles with 300 mM NaCN at pH 8.1 for 90 min, represents approximately 70–80% of the reaction centers. This estimate is in agreement with the Mössbauer results to be presented next. It is likely that the fraction of centers represented by the $g = 1.98$ signal level at pH 6.5 and 300 mM NaCN concentration is somewhat smaller, as under these conditions close to 20% of the centers already show the $g = 2.0045$ signal (Figure 6). These estimates, which are approximate (±10%), have been taken into consideration in indicating the 100% signal level in the various figures.

About the Active Species (CN^- or HCN?). The pK_a for HCN is approximately 9.3. Accordingly, a factor of ~37 increase in the CN^- concentration is expected at pH 8.1 relative to pH 6.5, while the HCN concentration remains practically constant. The drastic changes in the signals $g = 1.98$ and $g = 2.0045$ with increasing pH (Figures 2 and 6) can, to a first approximation, be attributed to the increasing CN^- concentration. A closer examination of the data in Figures 2 and 6 shows, however, that the pH dependence of the $g = 1.98$ and $g = 2.0045$ signals at relatively low NaCN concentrations is complicated. This is probably due to the known difficulty in displacing bicarbonate at high pH (Deligiannakis et al., 1994). A significant fraction of the centers is represented accordingly by the $g = 1.8$–1.9 signals (not shown), which are difficult to quantitate, particularly in the presence of the sharp 1.98 signal. At high NaCN concentrations, the sum of the $g = 1.98$ and $g = 2.0045$ signals appears to be independent of the pH, and we can assume that the $Q_A^-Fe^{2+}$ state is almost exclusively represented by these two signals. This is also supported by the experiment in Figure 3. It would be instructive to plot (Figure 7) the data at high cyanide concentrations (≥100 mM NaCN) as a function of the CN^- concentration. The $g = 1.98$ and $g = 2.0045$ signals do indeed show smooth complementary behavior, confirmed by the simulation with a single K_d. Furthermore, the data suggest that the active species for the conversion from the $g = 1.98$ to the $g = 2.0045$ form is CN^-, with an apparent K_d of approximately 1.2 mM.

It is considerably more difficult to assign the active species for the evolution of the $g = 1.98$ signal because, as was mentioned earlier, the data at the rising side of the signal (low cyanide concentrations) are perturbed by the pH dependence of the bicarbonate binding. In the previous study (Koulougliotis et al., 1993), an approximate K_d of 40–80 mM CN^- + HCN was suggested at pH 6.5. The present data show that even at pH 6.5 part of the centers show the $g = 2.0045$ signal, and this implies that the K_d may be somewhat higher. If we assume that CN^- is the active species for the development of the $g = 1.98$ signal too, then the K_d for that is approximately 0.1–0.2 mM. It is also likely that CN^- is the species competing with NO at low cyanide concentration. An approximate K_d of 10–20 μM for this effect at pH 6.5 can be estimated from

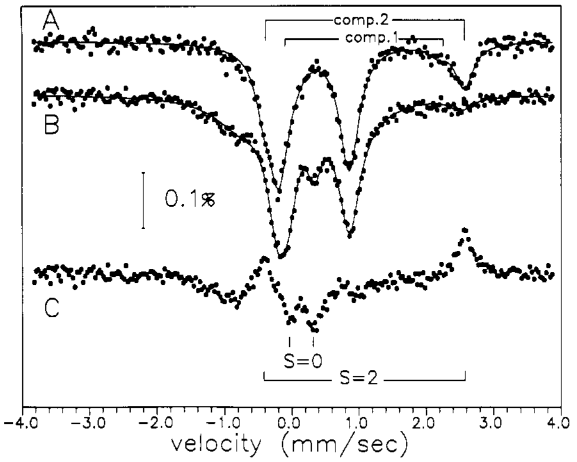

FIGURE 8: Mössbauer spectra at 80 K of BBY membranes, pH 8.1, treated with (A) 300 mM NaCl and (B) 300 mM NaCN (see text for details). Solid lines are theoretical simulations using Lorentzian line shapes. (C) Difference of B (experimental) minus A (theoretical).

the cyanide concentrations required to observe this effect (Koulougliotis et al., 1993).

Iron Is Not Dissociated by the High pH Cyanide Treatment. The conversion of the $Q_A^-Fe^{2+}$ EPR signal to that of the free semiquinone at high pH indicates the loss of the magnetic interaction between Q_A^- and the iron. Either the iron has dissociated, or its electronic configuration is severely modified by the cyanide treatment. We examine the first alternative here and the second one in the next section.

One test of the retention of the iron would be to examine whether the $g = 2.0045$ signal can be reversed to the precursor $g = 1.98$ signal. As the latter signal is indicative of a magnetic interaction of the iron with the quinone (Koulougliotis et al., 1993), it would be diagnostic of the intactness of the iron. Ideally, one could also check for the reversibility of the untreated $Q_A^-Fe^{2+}$ signals, but this might be much more difficult as cyanide may not be fully extractable. These signals are also too weak to quantitate accurately.

We have found that the $g = 2.0045$ signal, once developed by treatment with 300 mM cyanide at pH 8, cannot be easily reversed to the precursor $g = 1.98$ signal. Complete washing out of the cyanide and incubation at pH 6 and 4 °C for 48 h is required before the $g = 1.98$ signal can be observed again. Incubation of such a cyanide-depleted sample at pH 6.5 and 300 mM cyanide produces a $g = 1.98$ signal appproximately 60% of the maximum signal size at this pH. While the reversibility can probably be further improved by prolonged treatments, it is clear that the majority (most likely all) of the iron remains in place during the high pH cyanide treatment. This conclusion is further supported by the Mössbauer measurements to be presented next.

Mössbauer Spectroscopy Investigation. We have examined the high pH effects of cyanide on the iron by Mössbauer spectroscopy. The spectrum of the untreated BBY membranes at pH 8.0 (Figure 8A) shows the components that have been discussed earlier (Petrouleas et al., 1992). The non-heme iron is characterized by two components with the same isomer shift (IS), 1.19 mm/s, and quadrupole splittings (QS), 2.05 (comp 1) and 2.95 mm/s (comp 2), at 80 K. As has been noted, the relative areas of these components, which probably correspond to the $g = 1.82$ (comp 1) and $g = 1.9$ (comp 2) EPR signals for the $Q_A^-Fe^{2+}$ state, vary with the preparation and the conditions. Usually comp 1 is much weaker than comp 2.

The BBY sample above was diluted to 1 mg of Chl/mL, pH 8.0, and incubated with 300 mM NaCN for 1 h. The sample was subsequently centrifuged to form a pellet, and the Mössbauer spectrum was recorded (Figure 8B). Pronounced changes in the non-heme Fe^{2+} components are observed. The absorption intensity in the region 2–3 mm/s decreases by 80% or more, and a new peak appears at about 0.2 mm/s. Other changes become apparent by subtraction of the two spectra. In order to reduce the noise, we have subtracted the theoretical simulation of spectrum A from the experimental data of spectrum B. The resulting difference spectrum, Figure 8C, is quite revealing. The doublet of the untreated non-heme $Fe^{2+}(S = 2)$, appearing as two negative peaks (pointing upward in Figure 8C), is quantitatively converted to a new doublet with parameters (determined from a least-squares fitting) IS(Fe) = 0.26 mm/s and QS = 0.36 mm/s. These parameters are consistent with $Fe^{2+}(S = 0)$ (Greenwood & Gibb, 1971). The difference spectrum is also characterized by a shoulder at −1 mm/s and a weakly defined feature at +1 mm/s. Both of these features result from oxidized cytochrome b_{559}, which may have been converted to a low-potential form during the cyanide treatment. At 80 K the spectrum of the oxidized cyt b_{559} is severely broadened as a result of relaxation effects at this temperature (Petrouleas & Diner, 1982).

These results indicate that the conversion of the $Q_A^-Fe^{2+}$ EPR signal to that of the free semiquinone is due to the transformation of the non-heme iron at high pH and high cyanide concentration from the high-spin state ($S = 2$) to the low-spin state ($S = 0$), thereby eliminating the magnetic interaction with the spin of the semiquinone.

DISCUSSION

We present here a number of important new observations that extend an earlier study on the effects of cyanide on the iron–quinone complex (Koulougliotis et al., 1993).

Dithionite elicits, in the presence of cyanide at pH 6.5, a full size $g = 1.98$ signal. Although no redox titrations were carried out, the fact that the signal vanishes above ca. +30 mV and is a maximum at −390 mV places the reduction potential in the range 0 mV > E_m > −350 mV, consistent with the midpoint potential of Q_A in untreated BBY preparations from spinach (van Mieghem et al., 1989). This provides additional support for the earlier assignment of the $g = 1.98$ signal to the $Q_A^-Fe^{2+}$ state.

The g = 2.0045 Signal Results from Q_A^- Uncoupled from the Iron. Reduction of Q_A with dithionite has the advantage that it eliminates the signals from oxidized donor side components in the $g = 2$ EPR region. This allows the detection of a new signal at $g = 2.0045$ and $\Delta H = 9.5$ G. The signal shows a dependence on pH, [CN^-], and time complementary to the $g = 1.98$ signal. It is accordingly assigned to the $Q_A^-Fe^{2+}$ state modified by the high pH, high cyanide treatment. The $g = 2.0045$ signal shows behavior typical of an uncoupled free radical, implying that under the conditions of production of this signal, the magnetic interaction of Q_A^- with the iron has vanished. A signal with practically the same g-value and width ($g = 2.0044$ and $\Delta H = 9.2$ G) has been observed by Klimov et al. (1980) after removal of the Fe^{2+} and has been attributed to the plastosemiquinone, Q_A^-. A similar signal ($g = 2.0045$ and $\Delta H = 8$ G) has also been observed in the reaction centers of the purple photosynthetic bacteria after loss of the iron (Loach & Hall, 1972; Feher et al., 1972; Prince et al., 1977) or replacement with diamagnetic Zn^{2+} (Debus et al., 1986). In the present case, the reversibility of the high pH cyanide effects and the Mössbauer data (see below) indicates that the disruption of the magnetic interaction with the iron is due to the conversion of the latter to a low-spin state under these conditions (see below).

Mössbauer Parameters Indicate Conversion of the Iron to Low Spin at High pH, High Cyanide Concentration. The Mössbauer results show that at pH 8.1 and 300 mM NaCN (conditions that elicit a maximum $g = 2.0045$ signal) about 80% of the Fe^{2+} doublet converts to a new doublet, with parameters IS(Fe) = 0.26 mm/s and QS = 0.36 mm/s. These values fall into the general range of values reported for low-spin iron complexes (Greenwood & Gibb, 1971). There is also some overlap with the range of values for high-spin Fe^{3+} complexes, but this possibility is very unlikely for the following reasons. High-spin dilute Fe^{3+} complexes show pronounced relaxation effects at low temperatures, and no such effects are observed in the present case. Also, no new EPR signals attributable to high-spin Fe^{3+} are detected in the EPR spectrum under the same conditions (high pH, high cyanide concentration, absence of dithionite). Of the low-spin complexes, 6- or 5-cyano complexes can be excluded as they are characterized by very small isomer shifts (–0.15 to +0.06 mm/s at 80 K) (Greenwood & Gibb, 1971). The present isomer shift value agrees well with the values of cyano-substituted omplexes with nitrogen donor ligands (Greenwood & Gibb, 1971). Furthermore, on the basis of the small quadrupole splitting value and, also, the absence of low-spin Fe^{3+} resonances in the EPR spectra, an Fe^{3+} ($S = 1/2$) state can be excluded. We conclude, therefore, that $Fe^{2+}(S = 2)$ under these conditions has been converted to $Fe^{2+}(S = 0)$.

Iron Remains in Place during the High pH Cyanide Treatment. The experiment showing reversible formation of the $Q_A^-Fe^{2+}$ $g = 1.98$ signal following the high pH conversion to the $g = 2.0045$ form also shows that the iron is not dissociated during the high pH cyanide treatment. Alternative support and insights are provided by the Mössbauer results. If the iron were to dissociate from its histidine–protein ligands, cyanide would occupy all of its coordination positions and the Mössbauer parameters would be similar to those of 5- or 6-cyano complexes. Apparently, this is not the case here, and the observation that the Mössbauer parameters are similar to those of low-spin Fe^{2+} complexes with nitrogen donor ligands would suggest that the iron preserves its histidine ligands. Actually, good simulations of the isomer shift value are obtained if we use the empirical partial isomer shift scale for low-spin Fe^{2+} complexes of Bancroft et al. (1970) and assume a $(CN)_2(imidazole)_4$ or $(CN)_3(imidazole)_3$ ligand configuration. Corrections for the different isomer shift reference and temperature of observation are required, but these happen to approximately cancel each other out. It is also notable that the iron Mössbauer absorption area is recovered quantitatively after centrifugation of the dilute cyanide-treated sample. A dissociated complex would have been lost to the supernatant.

Site of Binding and Number of Bound Cyanides. It was argued in our earlier study (Koulougliotis et al., 1993) that the site of binding of cyanide is the iron itself. This conclusion was based mainly on the competition of cyanide with NO. As development of the $g = 1.98$ signal required about an order of magnitude higher cyanide concentration than that required to displace NO, we suggested that binding of a second cyanide was required for the conversion of the $Q_A^-Fe^{2+}$ signal to the $g = 1.98$ form (Koulougliotis et al., 1993). Whether the second cyanide binds on the iron or closer to Q_A is not clear. The conversion of the $g = 1.98$ to the free semiquinone $g = 2.0045$ signal with increasing CN^- concentration (Figure 7) suggests that a third cyanide binds under these conditions. As the change of the EPR semiquinone signal is the result of drastic changes on the iron ligand field (conversion from high to low spin; Figure 8), it is most likely that the new cyanide binds to the iron itself.

There is a precedent for binding of more than one cyanide in iron proteins. In protocatechuate 3,4-dioxygenase, cyanide binds to the Fe^{3+} in two steps (Whittaker & Lipscomb, 1984). Binding of the first cyanide retains the high-spin state of the iron, but it somewhat affects the ligand field parameters of the iron. Binding of the second cyanide converts the Fe^{3+} to low spin. In transferrin, three cyanides have been postulated to bind in competition with carbonate at the C-terminal Fe^{3+}, converting the iron to low spin (Swope et al., 1988). In the case of PSII, earlier studies indicated that the iron has at least two labile coordination positions. NO and formate (both known to displace bicarbonate) bind simultaneously (Diner & Petrouleas, 1990), and recent studies with a number of carboxylate anions suggest that under certain conditions some of these anions can bind as bidentate ligands to the iron (Petrouleas et al., 1994). It is likely that these two positions can be occupied successively by two cyanides. Whether a third cyanide can also bind by displacing one of the four postulated histidine ligands to the iron is not proven at present, but would be consistent with the comparison made earlier of the present Mössbauer parameters with synthetic cyanide complexes.

Active Species for Binding and the Dissociation Constants. The plot of Figure 7 shows that the active species responsible for conversion of the $g = 1.98$ to $g = 2.0045$ signal is CN^-. It is likely that this is also the species responsible for the development of the $g = 1.98$ signal and the competition with NO (Koulougliotis et al., 1993). The approximate K_d's for all of these effects (in reverse order, see also the Results section) are 10–20 μM (pH 6.5), 0.1–0.2 mM (pH 6.5), and 1.2 mM. The first K_d is the lowest that has been observed for the binding of ligands to the non-heme iron. Compare, for example, the $K_d \sim 40\ \mu M$ for binding of HCO_3^-/CO_2 (Stemler & Murphy, 1983; Blubaugh & Govindjee, 1988) and the K_d for binding of NO: 30 μM in chloroplasts and 250 μM in BBY membranes (Petrouleas & Diner, 1990; Diner & Petrouleas, 1990).

We also note that, of all the molecules competing with bicarbonate for binding to the iron, cyanide is the only one that can displace bicarbonate efficiently at high pH. It is known that the K_d of HCO_3^-/CO_2 binding decreases with increasing pH (Stemler & Govindjee, 1973). As a result, NO binding to the iron in competition with bicarbonate is significantly inhibited at high pH (Petrouleas & Diner, 1990; Diner & Petrouleas, 1990), and a number of carboxylate anions (including formate), which compete efficiently with bicarbonate at pH 6.5 or lower, show poor binding at pH ≥7.5 (Deligiannakis et al., 1994; Petrouleas et al., 1994). In the case of cyanide, the complementary pH and time dependence of the $g = 1.98$ and $g = 2.0045$ signals, the Mössbauer experiment, and the comparison of the absorption area of the $g = 2.0045$ signal with Y_D^+ all indicate that the $g = 2.0045$ signal at high pH and 300 mM cyanide concentration represents ≥70–80% of the centers.

Future Studies. The conversion of the iron to a diamagnetic form by the cyanide treatment can have a number of useful applications in the study of PSII. ENDOR studies of Q_A^- should now be possible in the absence of magnetic coupling of Q_A^- with the iron. Informative ENDOR spectra were obtained in the purple photosynthetic bacteria following extraction of the iron or its replacement with diamagnetic Zn^{2+} (Lubitz et al., 1985). Preliminary experiments with G. C. Dismukes and M. Zheng of BBY membranes treated with cyanide indeed show that the ENDOR experiment is feasible.

It would also be interesting to study the effects of the cyanide treatment on the iron redox properties, the Fe^{3+} EPR spectra, and the Q_A/Q_B electron transfer rate. The latter has been studied in chloroplasts in relation to the cyanide-induced g = 1.98 $Q_A^-Fe^{2+}$ signal (Koulougliotis et al., 1993), but the conditions were not optimal for the development of the g = 2.0045 signal. Another application is in the study of the relaxation properties of the PSII donor side tyrosine radicals. The selective conversion of the iron to low spin can eliminate the contribution of the Fe^{2+} to the relaxation properties. Careful screening of possible adverse effects of the high pH, high cyanide treatment to the donor side components is, however, required.

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