The Origin of the Split S_3 EPR Signal in Ca^{2+}-Depleted Photosystem II: Histidine versus Tyrosine†

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Received May 11, 1992; Revised Manuscript Received June 22, 1992

ABSTRACT: The radical formed as the formal S_3 charge storage state in Ca^{2+}-depleted photosystem II and detected as a split EPR signal was previously assigned to an oxidized histidine radical on the basis of its UV spectrum. In a recent paper [Hallahan, B. J., Nugent, J. H. A., Warden, J. T., & Evans, M. C. W. (1992) *Biochemistry 31*, 4562–4573], this assignment was challenged, and it was suggested that the signal arises instead from the well-known tyrosine radical $Tyr_Z^{\bullet}$, the electron carrier between the photooxidized chlorophyll and the Mn cluster. Here, we provide evidence that the measurements of the $Tyr^{\bullet}$, on which the new interpretation was based, are artifactual due to the use of saturating microwave powers. Other than a relaxation-enhancement effect, the formation of the split S_3 signal is accompanied by no change in the $Tyr^{\bullet}$ signal. Although essentially unrelated to the origin of the S_3 radical, several other experimental and interpretational problems in the work of Hallahan et al. (1992) are pointed out and rationalized. For example, the inability of Hallahan et al. (1992) to observe the split S_3 signal in samples containing DCMU or without a chelator, in contrast to our observations, is attributed to a number of technical problems including the incomplete inhibition of the enzyme. We thus conclude that the assignment of the split S_3 signal as $His^{\bullet}$, although not proven, remains the most reasonable on the basis of current data.

Photosystem II (PS-II)[1] catalyzes the light-driven water oxidation resulting in oxygen evolution [see Rutherford (1989) for a review]. A manganese cluster acts both as the active site and as a charge accumulating device [reviewed in Rutherford et al. (1992)]. The enzyme cycle involves five redox states which are denoted S_0–S_4, depending on the number of positive charges stored (Kok et al., 1970). Oxygen is released after formation of the transient S_4 state. The PS-II reaction center contains two polypeptides, D_1 and D_2, which carry the electron-transfer components. Light induces a separation of charges between P_{680} and Ph. This charge separation is stabilized by electron transfer from Ph^- to Q_A and by the reduction of P_{680}^+ by Tyr_Z, the tyrosine 161 of the D_1 polypeptide (Debus et al., 1988b; Metz et al., 1989). Further stabilization of charges is obtained by reduction of $Tyr_Z^{\bullet}$ by the Mn complex and by the oxidation of Q_A^- by the secondary quinone, Q_B. In addition to Tyr_Z, there is a second redox-active tyrosine in PS-II, Tyr_D, the tyrosine 160 of the D_2 polypeptide [Debus et al., 1988a; Vermaas et al., 1988; see also Barry and Babcock (1987)]. In the dark, $Tyr_D^{\bullet}$ slowly oxidizes the S_0 state, but in the other S states $Tyr_D^{\bullet}$ is stable (Styring & Rutherford, 1987). Tyr_D is able to reduce the S_2 and S_3 states (Vass & Styring, 1991, and references cited therein).

Ca^{2+} is an obligatory cofactor in oxygen evolution, and its depletion inhibits the enzyme cycle after formation of the S_3 state (Boussac & Rutherford, 1988a; Boussac et al., 1992) [also reviewed in Debus (1992), Homann (1990), Rutherford et al. (1992), and Yocum (1991)]. Ca^{2+} depletion of PS-II requires the release of the 17- and 23-kDa extrinsic polypeptides [reviewed in Debus (1992), Homann (1990), Rutherford et al. (1992), and Yocum (1991)] and light since the S_3 state was shown to be the most susceptible S state to Ca^{2+} depletion (Boussac & Rutherford, 1988b). The illumination of Ca^{2+}-depleted PS-II induces the formation of a 164 G wide EPR signal after reconstitution with the extrinsic polypeptides (Boussac et al., 1989) or a 130 G wide signal in the absence of the extrinsic polypeptides (Boussac et al., 1990b). Addition of citrate or EGTA in the Ca^{2+}-depleted S_3 state results in the stabilization of the S_2 state formed after deactivation of the S_3 state. This stable S_2 state is also characterized by a modified multiline EPR signal (Boussac et al., 1989, 1990a,b). A similar modified multiline signal was also reported by Ono and Inoue (1990) and Sivaraja et al. (1989) under different conditions. The S_3 EPR signal was better resolved in flash experiments and was shown to be split as well as broadened (Boussac et al., 1990c). The signal was proposed to originate from the oxidation of an amino acid interacting magnetically with the Mn cluster (Boussac et al., 1989, 1990c). Absorbance changes corresponding to the S_2 to S_3 transition in Ca^{2+}-depleted PS-II were measured in the UV region, and it was found that its spectrum was similar to that of oxidized histidine in vitro. It was thus proposed that histidine was oxidized in the Ca^{2+}-depleted S_3 state (Boussac et al., 1990c).

The latter proposal was recently challenged, and it was instead proposed that the S_3 EPR signal arises from the $Tyr_Z^{\bullet}$ (Hallahan et al., 1992). We will show in this report that there is no experimental evidence to support this proposal.

MATERIALS AND METHODS

EPR spectroscopy at helium temperature was done on a Bruker ER200D spectrometer equipped with an Oxford Instrument cryostat and interfaced with a microwave frequency meter, HP 5350B, and a Bruker ER035M NMR gaussmeter. Ca^{2+} depletion, EGTA treatment, and polypeptide reconstitution was done as in Boussac et al. (1989). Illumination of the samples was done with a 800-W projector through

† A.B. and A.W.R. are supported by the CNRS.

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[1] Abbreviations: PS-II, photosystem II; EPR, electron paramagnetic resonance; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PPBQ, *p*-phenylbenzoquinone; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; P_{680}, reaction center chlorophyll of PS-II; Tyr_D, the tyrosine 160 of the D_2 polypeptide, a side-path electron donor; Tyr_Z, the tyrosine 161 of the D_1 polypeptide, the electron donor to P_{680}; D_1 and D_2, polypeptides of the PS-II reaction center.

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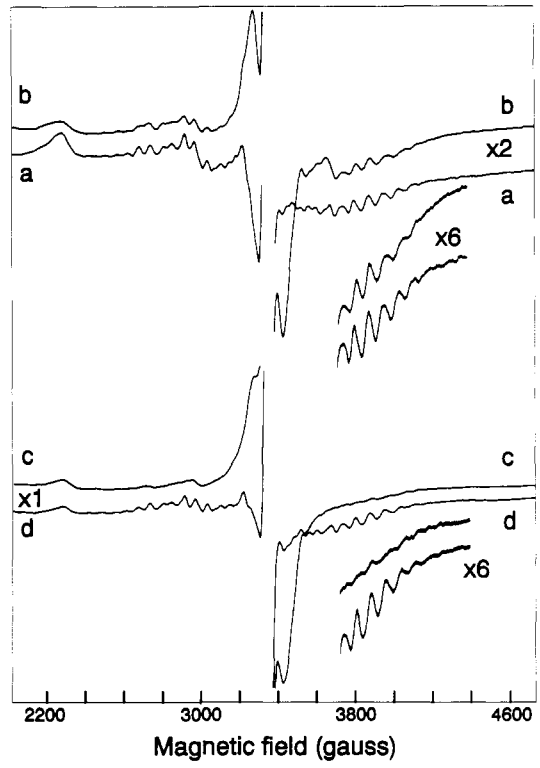

FIGURE 1: EPR spectra of the S_2 and S_3 states in Ca^{2+}-depleted PS-II with DCMU or PPBQ. Spectrum a corresponds to a dark-adapted preparation of NaCl-washed/EGTA-treated/polypeptide-reconstituted PS-II membranes. Spectrum b was recorded after illumination for 2 min at 0 °C in the presence of 2×10^{-4} M DCMU. Spectrum c was recorded after illumination for 2 min at 0 °C in the presence of 0.5 mM PPBQ. After the recording of spectrum c, the sample was thawed and dark-adapted for 45 min at 0 °C, and then spectrum d was recorded. Instrument settings: temperature, 10 K; modulation amplitude, 22 G; microwave power, 20 mW; microwave frequency, 9.4 GHz; modulation frequency, 100 kHz. The center parts of the spectra corresponding to the Tyr• region were deleted. The signal gain for spectra c and d was two times smaller than for spectra a and b. The insets show the spectra at a comparably higher gain (×6) in both cases. DCMU and PPBQ were dissolved in dimethyl sulfoxide. The Chl concentration was about 6 mg/mL.

water and infrared filters in a nonsilvered dewar filled with ethanol cooled to 0 °C with liquid nitrogen. After illumination, the samples were rapidly cooled (<2 s) to 200 K and then to 77 K.

RESULTS AND DISCUSSION

The proposal (Hallahan et al., 1992) that the S_3 signal arose from Tyr_Z• was based solely on the observation at 50 K of an increase in the characteristic Tyr radical signal(s) which correlated with the presence of the S_3 signal. The amplitude of this increased Tyr• matched the extent of the S_3 signal under various conditions (Hallahan et al., 1992).

To test the validity of these observations, we measured EPR spectra in the spectral region corresponding to that of Tyr_Z• in the S_2 and S_3 states in Ca^{2+}-depleted membranes. At the same time, and in the same samples, we monitored the stable S_2 Mn multiline signal and the S_3 signals themselves. Figure 1 shows the stable multiline signal (S_2) in dark-adapted Ca^{2+}-depleted membranes (spectrum a). Illumination of an identical sample, but in the presence of PPBQ, at 0 °C resulted in the loss of the multiline signal and formation of the S_3 signal (spectrum c). After the recording of spectrum c, the sample was thawed and dark-adapted for ≈45 min in the dark at 0 °C, leading to the loss of the S_3 signal and the reappearance of the stable multiline signal (spectrum d) as previously reported (Boussac et al., 1989).

Figure 2 shows the Tyr radical signal recorded at 10 K (Figure 2A,C), a temperature which allows the observation of the S_2 and S_3 signals when present and also at 50 K (Figure 2B,D). At both temperatures, the spectra were recorded in nonsaturating conditions (microwave power = 0.5 μW, Figure 2A,B) and in saturating conditions (microwave power = 2 mW, Figure 2C,D).

The results in Figure 2A,B show no variation in the amplitude of the Tyr• signal for S_2 and S_3 at 10 and 50 K in nonsaturating conditions. A small shift in the hyperfine peaks occurs in the 10 K spectrum (Figure 2A), but it does not correspond to a change in the number of spins as measured by the double integral (the level of uncertainty is estimated as ±5% for nonsaturating powers; see Figure 3B). We thus conclude that when S_3 is present, it is not accompanied by any increase in the number of spins contributing to the Tyr• signal and that in the presence of S_2 and S_3 only Tyr_D• contributes to the well-known Tyr• signal. The relaxation study at 10 and 50 K in the S_2 and S_3 states was also done in salt-washed PS-II lacking the 17- and 23-kDa polypeptides but treated with 10 mM EGTA to stabilize the S_2 state (Boussac et al., 1990b). Results identical to those shown in Figure 3 were obtained except that an additional radical (corresponding to ≈0.1 spin per reaction center), attributed to an oxidized chlorophyll, was found after the illumination (not shown).

How can we rationalize the seemingly contradictory observations in Hallahan et al. (1992)? In that paper, the increase in signal size is demonstrated and monitored at microwave powers which are saturating for Tyr_D•. Figure 2C,D shows that, under similar conditions, we indeed see a 25% increase in the amplitude of the Tyr• signal when S_3 is present. Since there is no increase in the number of spins contributing to the Tyr• signal at nonsaturating powers, it seems clear that the increased signal amplitude under saturating conditions is due to an S_3-induced enhancement of the relaxation of Tyr_D•.

We had previously monitored the Tyr• signal at 15 K in the presence and absence of S_3 and had seen no increase in its amplitude at nonsaturating powers (Boussac et al., 1989). We have repeated the saturation studies at 10 and 50 K using a 12.5-kHz field modulation to minimize rapid passage effects and using the double integral of the measured signal (Figure 3A,B). The results show that little change in the estimated $P_{1/2}$ occurs but that there is a marked change in the "homogeneity factor" (i.e., the slope of the line through the points at high power) resulting in the observed S_3-induced increase in signal amplitude of the Tyr_D• at saturating powers. We have scrutinized our earlier data (Boussac et al., 1989), and similar phenomena were observed, i.e., a change in the homogeneity factor resulting in an increase in the amplitude of Tyr_D• in the S_3 sample at some saturating microwave powers (data not shown).

We have replotted the saturation data presented in Figure 5 of Hallahan et al. (1992) for Tyr_D• and the S_3-induced increase in this signal using the relative signal amplitudes given at 1 mW, a value given for both signals at the same power. This treatment results in the amplitude of the Tyr_D• signal at 5 μW being the same as that of the S_3-induced increase at 1 mW; i.e., it corresponds well to the data presented in Figure 5A of Hallahan et al. (1992) and thus gives us confidence in our quantification of the data of Figure 5B in Hallahan et al. (1992). From the quantitative replots of Hallahan's data, it is clear that the size of the S_3-induced signal ("Tyr_Z•") at the lowest recorded powers is negligable compared to the Tyr_D• signal (i.e., 3.6% of the Tyr_D• signal at 5 μW)

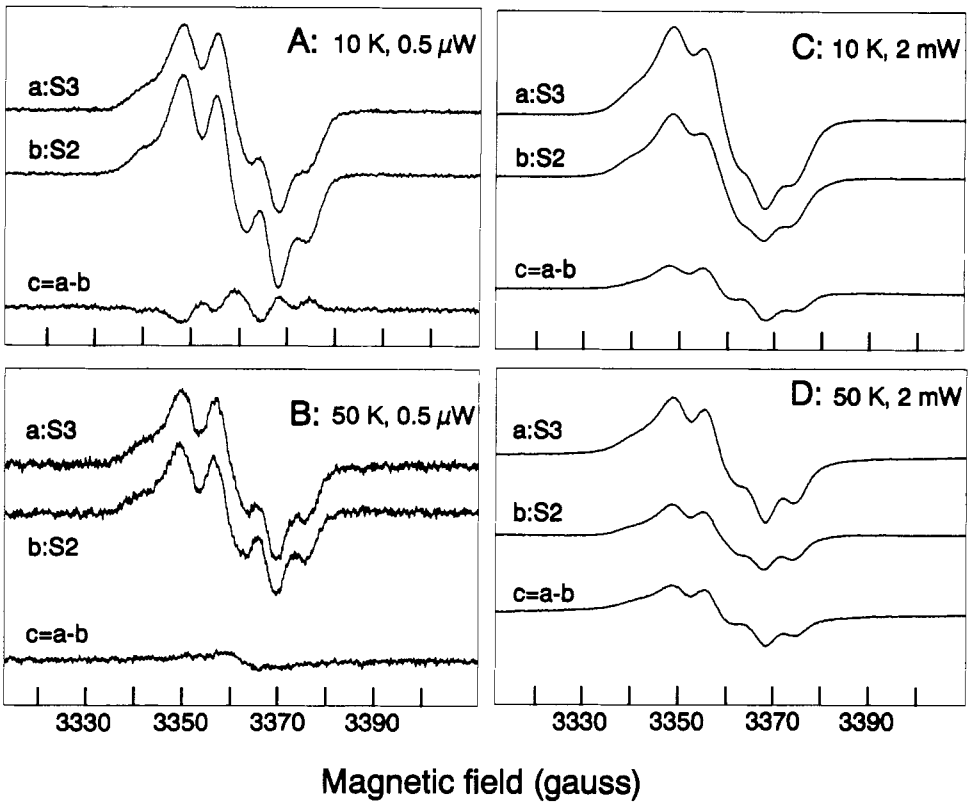

FIGURE 2: EPR spectra of the $g = 2$ region in the S_2 and S_3 states in Ca^{2+}-depleted PS-II. All the spectra were recorded on the same sample. Spectra a (in panels A–D) were recorded after the pretreatment as described in Figure 1c, i.e., in the S_3 state. Spectra b were recorded after the pretreatment as described in Figure 1d, i.e., in the S_2 state. The microwave power and temperature are indicated. In each case spectra c correspond to the difference spectra a – b. Instrument settings: modulation amplitude, 2.8 G; microwave frequency, 9.4 GHz; modulation frequency, 100 kHz.

[data not shown but see Figure 5 of Hallahan et al. (1992)].

The demonstration that the increase in the signal amplitude of the Tyr• radical is due to an S_3-induced relaxation enhancement of Tyr_D• fully explains all the correlations between the two signals reported in Hallahan et al. (1992) (e.g., matching decays at room and low temperature etc.). It also eliminates the inherently paradoxical notion (Hallahan et al., 1992) that the same component (suggested to be Tyr_Z•) could give rise to a fast relaxing split signal (S_3) and a virtually unbroadened free radical signal at the same time.

The change in the homogeneity factor, when going from S_2 to S_3, could reflect a spacial change in the relaxer; i.e., in S_2, the dominant relaxer is the isolated Mn cluster, while in S_3, the relaxer is the Mn cluster and the magnetically coupled radical. The lack of a detectable $P_{1/2}$ change between S_2 and S_3 may indicate that the distance between the Tyr_D• and the relaxer does not decrease upon oxidation of the S_3 radical, i.e., that the S_3 radical is not between the Mn and Tyr_D•.

In Hallahan et al. (1992), some of the other observations, although not used in the arguments on the origin of the S_3 signal, were nevertheless in contradiction with earlier reports or with our unpublished observations. In particular, it was reported that the S_3 signal could not be observed in the presence of DCMU, or at pH 7.5, or in the absence of citrate/EGTA (i.e., in the absence of the modified Mn multiline signal). In what follows, we attempt to rationalize these descrepancies.

Figure 1 (spectra a and b) shows that the S_3 signal can be formed to 50% in the presence of DCMU. We have observed that the longer the illumination time at 0 °C, the smaller the S_3 signal produced. This is attributed to a certain probability for the S_3 state to be reduced by electrons other than those coming from Q_A^-. This leads to the trapping of the $S_2Q_A^-$ state. In Hallahan et al. (1992) this phenomenon was compounded by the following problems: (1) The authors preilluminate the sample prior to DCMU addition. They contend that the sample contains at least 50% Q_B. It is known that dark adaption of a sample under these conditions (i.e., a fully reduced plastoquinone pool) can lead to about 50% of stable Q_B^- (Rutherford et al., 1982). Addition of DCMU results in a quantitative conversion of Q_B^- to Q_A^- (Velthuys & Amesz, 1974). Thus it is predicted that a significant proportion of the centers would be inactive in the conditions used in Hallahan et al. (1992). (2) The membranes used in Hallahan et al. (1992) were far from being homogeneous in the stable S_2 state. Figure 1 shows a typical stable S_2 multiline signal. The stable multiline signal, the baseline noise level, and the oxidized cyt b_{559} signal should be compared to those in Figure 1 in Hallahan et al. (1992). If, in Hallahan et al. (1992) a significant number, or even the majority of centers, were in S_1, as is implied by this comparison, then in the presence of DCMU, these centers would not have reached S_3 upon illumination. (3) In Hallahan et al. (1992) it seems that signficant fractions of O_2-evolving activity remained in the treated samples (up to 25%); i.e., Ca^{2+} depletion is incomplete. In the presence of DCMU no S_3 signal would be expected in the active centers. Since, Ca^{2+} depletion occurs mainly in the S_3 state in salt-washed membranes (Boussac & Rutherford, 1988b), illumination in the absence of DCMU could result in Ca^{2+} loss and thus formation of the S_3 signal.

In Figure 1b, it seems clear that the S_3 signal formed in the presence of DCMU is better resolved than that formed in the presence of PPBQ (Figure 1c). This is similar to the result seen in the S_3 signal formed with flash illumination (Boussac et al., 1990c). Previously, the better resolution seen in the flash-induced signal was explained by assuming that a fraction of the centers lacked the 17- and 23-kDa polypeptides. Such

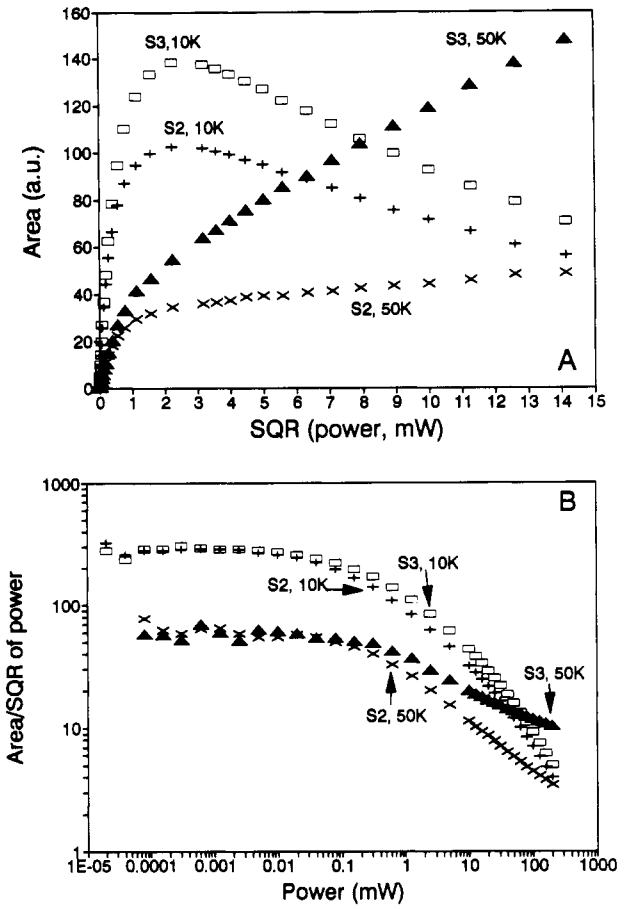

FIGURE 3: Relaxation study of $Tyr_D^{\bullet}$ at 10 and 50 K in the S_2 and S_3 states in Ca^{2+}-depleted PS-II. Panel A shows a plot of the area of the $Tyr_D^{\bullet}$ signal versus the square root of the microwave power. Panel B shows the same data plotted as the standard relationship: log (area/square root of power) versus log (microwave power). The temperature used was either 10 or 50 K as marked, and the S state was either S_2 or S_3 as marked. EPR spectra were obtained using a modulation frequency of 12.5 kHz. We note that essentially similar results are obtained when the amplitude of the signal measured at a given field value value is used instead of the area of the signal. However, due to changes in the signal shape at high power, we consider the use of the area of the signal to be more accurate. In addition, the use of the area of the signal considerably improves the signal to noise ratio.

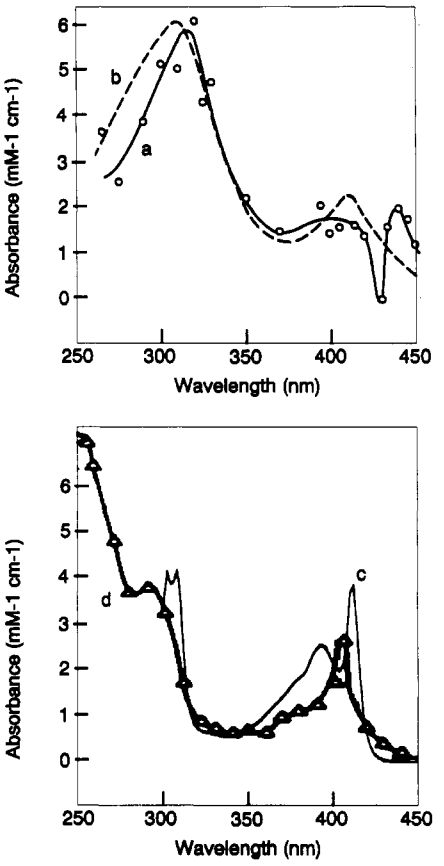

FIGURE 4: Comparison of UV spectra of His$^{\bullet}$ and Tyr$^{\bullet}$ formed in vitro with free radicals formed in vivo. Spectrum a: absorbance change of the S_2 to S_3 transition in NaCl-washed/EGTA-treated/polypeptide-reconstituted PS-II. This spectrum was redrawn from Boussac et al. (1990c). Spectrum b: absorbance change of the His$^{\bullet}$/His transition in vitro formed by pulse radiolysis. This spectrum was redrawn from Rao et al. (1975). Spectrum c: absorbance change of the Tyr$^{\bullet}$/Tyr transition in ribonucleotide reductase. This spectrum was redrawn from Bollinger et al. (1991). Spectrum d: absorbance change of the Tyr$^{\bullet}$/Tyr transition obtained in vitro by UV irradiation. This spectrum was redrawn from Bent and Hayon (1975).

centers are known to give a narrower split S_3 signal (Boussac et al., 1990b), and S_3 in these centers was suggested to be more difficult to trap. For the S_3 signal obtained with DCMU, a related explanation seems valid. Reduction of S_3 by exogenous electrons, resulting in the trapping of the $S_2Q_A^-$ state when DCMU is present, is likely to be favored in centers lacking the 17- and 23-kDa polypeptides. Thus the S_3 signal showing the greater splitting, i.e., in centers which have the 17- and 23-kDa polypeptides present, is favored under these conditions.

In contrast to the report in Hallahan et al. (1992), we have observed the S_3 signal in the pH range 4–7.5 (not shown). However, with short incubation times (5 min) at pH 7.5 the signal is slightly diminished (the signal was ≈65% of that recorded at lower pH values). With longer incubations [as in Hallahan et al. (1992)], it was found that the Mn cluster (and hence the split S_3 signal) was lost.

We have shown previously that the split S_3 signal can be formed in centers lacking the modified Mn multiline signal (Boussac et al., 1990b). Hallahan et al. (1992) were unable to reproduce this result and claimed that high concentrations of EGTA or citrate (and thus the presence of the modified multiline signal) were necessary before the split S_3 signal could be observed. We rationalize this result in the knowledge that the material used for this experiment in Hallahan et al. (1992) was only weakly inhibited (40% activity) and thus contained significant amounts of Ca^{2+}.

As discussed above, our earlier EPR data indicated that the S_3 state in inhibited PS-II consisted of an organic free radical interacting with the Mn cluster. The chemical identity of the radical was suggested to be His$^{\bullet}$ from its UV spectrum. In Hallahan et al. (1992) the assignment of the UV spectrum was criticized on the grounds that the spectrum is similar to the (Tyr$^{\bullet}$) radical in ribonucleotide reductase reported by Bollinger et al. (1991). In Figure 4, the His$^{\bullet}$ and Tyr$^{\bullet}$ spectra in vitro are compared to the ribonucleotide reductase spectrum of Bollinger et al. (1991) and to the spectrum of the S_2 to S_3 transition. The original assignment of the former to Tyr$^{\bullet}$ and the latter to His$^{\bullet}$ seems evident.[2]

In conclusion, the present work shows that there is no evidence for the assignment of the split S_3 signal to $Tyr_Z^{\bullet}$.

[2] We note that, for Tyr$^{\bullet}$, the UV spectrum of the radical formed by radiolysis is complicated by the presence of an extra band at 330 mm resulting from an intermediate state (Land & Ebert, 1967; Bensasson et al., 1983). If heterogeneity also occurs in the His$^{\bullet}$ formed in this way, it does not seem to result in such major changes in its spectrum (Rao et al., 1975). It would, however, be useful to determine the His$^{\bullet}$ spectrum generated by UV irradiation in order to clarify this point.

Although as yet not proven, the assignment of the split S_3 signal to His$^+$ remains the most reasonable interpretation of the available data.

ACKNOWLEDGMENT

We acknowledge B. J. Hallahan, J. H. A. Nugent, J. T. Warden, and M. C. W. Evans for providing us their manuscript before publication.

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