G. F., & Kell, D. B. (1984) *Biochim. Biophys. Acta 768*, 257-292.
Zoratti, M., Pietrobon, D., & Azzone, G. F. (1982) *Eur. J. Biochem. 126*, 443-451.
Zoratti, M., Petronilli, V., & Azzone, G. F. (1986a) *Biochim. Biophys. Acta 851*, 123-135.
Zoratti, M., Favaron, M., Pietrobon, D., & Azzone, G. F. (1986b) *Biochemistry 25*, 760-767.

Location and Magnetic Relaxation Properties of the Stable Tyrosine Radical in Photosystem II†

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ABSTRACT: Dipolar interactions with neighboring metal ions can cause enhanced spin-lattice relaxation of free radicals. We have applied the theory of dipolar relaxation enhancement and shown that the dependence of the enhanced relaxation on the protein structure surrounding the free radical can be used to obtain distances from the free radical to the protein surface. To test the theoretical predictions, we have examined the effect of added Dy^{3+} complexes on the microwave power saturation of free radicals in two protein complexes of known structure: myoglobin nitroxide and the reaction center from *Rhodobacter sphaeroides*. Three cases have been considered: (1) metal ions bound to a specific site, (2) metal ions bound randomly over the protein surface, and (3) metal ions distributed randomly in solution. Only case 3, which assumes no specific binding, gave good agreement between the distances obtained by using the two model systems. The effect of added Dy^{3+} complexes on the microwave power saturation of signal II_{slow} from photosystem II (PSII) was used to determine the location of the stable tyrosine radical giving rise to signal II_{slow}. Assuming that the surface of a membrane-bound protein can be approximated as planar, we have obtained distances from the tyrosine radical to the membrane surface in thylakoids, in PSII membranes, and in Tris-washed PSII membranes. The distances we have determined are in good agreement with those predicted on the basis of a structural homology between the D1 and D2 subunits of PSII and the structurally characterized L and M subunits of the reaction center from purple non-sulfur bacteria. We have also examined the temperature dependence of the microwave power at half-saturation ($P_{1/2}$) of signal II_{slow} from 4 to 200 K in dark-adapted PSII membranes. Above 70 K, the $P_{1/2}$ increases as $T^{2.5}$, which is consistent with a Raman relaxation mechanism. But between 10 and 70 K, the $P_{1/2}$ is nearly independent of temperature. Such temperature independence of the $P_{1/2}$ is highly unusual.

The EPR[1] signal II_{slow} species, D^+, was the first radical observed from PSII (Commoner et al., 1956) and is known to be associated with the electron-donor side of the photosystem. Recent studies have identified D^+ as a tyrosine cation radical (Barry & Babcock, 1987), and site-specific mutagenesis has indicated it to be Tyr-160 of the D2 polypeptide from the PSII reaction center core (Debus et al., 1988). Signal II_{slow} is called "slow" because it is stable on the order of days at 0 °C. D is oxidized in the light, via the S_2 and S_3 states of the O_2-evolving complex (Babcock & Sauer, 1973). These S states refer to the model of Kok et al. (1970) postulating five intermediate oxidation states (S states S_0-S_4) of the O_2-evolving complex which store the equivalents to oxidize H_2O to O_2. Evidence that D^+ oxidizes the S_0 state to S_1 in the dark has led to speculation that the role of D^+ is to maintain oxidizing equivalents that prevent PSII from being trapped in the dark in the possibly unstable S_0 state (Styring & Rutherford, 1987).

Signal II_{slow} has a line width of ~19 G and a g value of 2.0047 ± 0.0002 (Kohl, 1972). The signal exhibits partially resolved hyperfine structure attributed to protons (O'Malley et al., 1984). There have been a number of studies on the decay kinetics of signal II_{slow} and great interest in identifying the molecule giving rise to the signal. Nevertheless, many aspects of the magnetic properties of this free radical, including the temperature dependence of the spin relaxation rates, had not been explored until recently. In order to characterize some of the magnetic properties of D^+, we have studied the temperature dependence of the microwave power saturation of signal II_{slow} between 4 and 200 K in dark-adapted thylakoids, PSII membranes, and Tris-treated PSII membranes (which lack three extrinsic polypeptides and Mn).

Likewise, the location of D^+ in the PSII reaction center has not been established. To obtain distance information, we have used the effect of added Dy^{3+} complexes on the microwave power saturation of signal II_{slow}. Bloembergen (1949) and Abragam (1955) developed the theory for the enhanced relaxation caused by dipolar interactions with rare earth ions. Changes in relaxation and line widths have since been used

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[1] Abbreviations: $(BChl)_2^+$, bacterial chlorophyll dimer; chl, chlorophyll; D^+, tyrosine radical in photosystem II giving rise to EPR signal II_{slow}; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; Fe-Q_A, iron-quinone electron acceptor of photosystem II; HEDTA, *N*-(hydroxyethyl)ethylenediaminetriacetic acid; kDa, kilodalton(s); MbNO, myoglobin nitroxide; MES, 2-(*N*-morpholino)-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PSII, photosystem II; *Rb. sphaeroides*, *Rhodobacter sphaeroides*; Tris, tris(hydroxymethyl)aminomethane.

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to examine the accessibility of paramagnetic sites in proteins to rare earth ions, such as Gd^{3+} and Dy^{3+}, added to solution. Hyde and Rao (1978) extended this technique by examining the effects of rare earth ions on free radicals in a quantitative way. We have applied Hyde and Rao's (1978) theory to the case where added rare earth ions are acting on a protein-bound free radical. To test the validity of the theory, we have examined the effect of added Dy^{3+} complexes on the microwave power saturation of free radicals in two proteins of known structure: MbNO and the reaction center of *Rhodobacter sphaeroides*. Based on the results of these model systems, this method has been applied to determine the location of D^+ in PSII.

For our study in PSII, Dy^{3+} was chosen as the exogenous rare earth ion because it is the most potent relaxer and was added as the Dy–EDTA or Dy–HEDTA complex to avoid disrupting the Mn site and to alleviate specific binding effects. Uncomplexed lanthanide ions have been shown to disrupt O_2 evolution (Zitkus et al., 1987) and release extrinsic polypeptides and Mn (Ghanotakis et al., 1985). Our results for the negatively charged Dy–EDTA complex and the neutral Dy–HEDTA complex were compared to determine whether electrostatic interactions influence the approach of the complex to the protein surface. To scale the Dy effect, we used myoglobin nitroxide (MbNO) and the bacterial chlorophyll dimer in *Rb. sphaeroides* as the model systems. The bacterial reaction center is believed to have a high degree of structural homology to PSII and should provide an excellent model for distances to D^+.

Materials and Methods

Spinach thylakoids were prepared from dark-adapted market spinach by the method of Yocum et al. (1981), ending in a pH 6.0, 30% (v/v) ethylene glycol, 20 mM MES, 15 mM NaCl, 1 mM EDTA buffer with 1 mg/mL bovine serum albumin. PSII membranes were obtained by the procedure of Berthold et al. (1981) with the modifications described by Beck et al. (1985). O_2 evolution was measured with a Clark-type O_2 electrode, and rates between 450 and 600 μmol of O_2/(mg of chl·h) were obtained with 250 μM added 2,5-dichloro-*p*-benzoquinone as an electron acceptor. All spinach samples were pelleted and resuspended in pH 6.0, 30% ethylene glycol, 20 mM MES, 15 mM NaCl, 0.05% Triton TX-100 buffer for use in the EPR experiments. All steps were performed in dim green light.

Tris-washed PSII membranes were prepared by incubation of PSII membranes in pH 8.0, 0.8 M Tris-HCl buffer at 0.5 mg of chl/mL for 30 min on ice, in room light. Membranes were pelleted, then resuspended again with the Tris buffer, and then repeatedly pelleted and resuspended in pH 6.0, 30% ethylene glycol, MES–NaOH, NaCl buffer containing 1 mM EDTA to remove the Mn. Residual Mn after Tris treatment was found to be about 10%. Mn was quantitated by treating the samples 1:1 with 1 M HCl for 30 min and then freezing the samples and measuring the EPR signal intensity of the hexaaqua Mn compared to a standard of known Mn concentration.

Sperm whale myoglobin (Sigma) was dissolved to approximately 0.4 mM in pH 7.0, 30% ethylene glycol, 50 mM MOPS buffer and converted to the NO-ligated form with 8 mM sodium dithionite and 2 mM sodium nitrite [modified from Blum et al. (1983)]. Buffers were degassed by bubbling with gaseous N_2, and sample preparation was carried out under a N_2 atmosphere.

Lanthanide-containing buffers for the PSII and thylakoid samples were prepared by chelating $DyCl_3 \cdot 6H_2O$ or $LaCl_3 \cdot 7H_2O$ with an excess (4:5) of EDTA or HEDTA in the sample buffer at pH 6.0. Samples were suspended in the buffer with the appropriate concentration of the chelated lanthanide, pelleted, and resuspended in the same buffer to 2–4 mg of chl/mL. The addition of the chelated lanthanides caused no loss of O_2 evolution activity. Dy–EDTA-containing buffers for the MbNO samples were prepared at pH 7.0. The 0.4 mM MbNO stock solution was then diluted 1:1 with Dy–EDTA-containing buffer to give 0.2 mM MbNO and the desired Dy–EDTA concentration.

Purified *Rb. sphaeroides* reaction centers were helpfully supplied by Dr. Harry Frank and Shahriar Taremi (University of Connecticut, Storrs). The bacterial samples were prepared at pH 7.5 in 30% ethylene glycol, and the reaction center stock solution was diluted to give an optical density of approximately 1.1 at 800 nm and the desired Dy–EDTA concentration.

EPR spectroscopy was performed on a Varian E-9 spectrometer. All of the EPR data presented in this paper were obtained by using a 100-kHz field modulation. Temperature control in the 4–100 K range was achieved with an Oxford ESR-900 liquid helium cryostat. In the 80–220 K range, temperatures were obtained with a liquid N_2 cooled gas flow system controlled by a Lakeshore Cryotronics DRC-34C temperature controller. Temperatures below 40 K were measured by using a silicon diode sensor or a carbon glass resistor in an EPR tube at the sample position. Higher temperatures were measured with a Pt sensor, also in an EPR tube at the sample position. A plot of the intensity of signal II_{slow} versus $(\text{temperature})^{-1}$ followed Curie law behavior between 13 and 220 K; this was used to verify the temperature calibrations. A Cr^{3+}/MgO crystal, which remained at room temperature, was inserted via a port in a modified front plate for a Varian E-231 cavity and was used as a microwave power standard on each scan.

Determination of $P_{1/2}$ Values. Portis (1953) and Castner (1959) have derived expressions for the effect of progressive power saturation on an inhomogeneously broadened resonance. Beinert and Orme-Johnson (1967) reduced these in the limit of inhomogeneously broadened lines to $S\alpha\sqrt{P}/(1 + P/P_{1/2})^{1/2}$, where S is the EPR absorption signal amplitude, P is the applied microwave power, and $P_{1/2}$ is the microwave power at half-saturation. $P_{1/2}$ is proportional to $(H_{1/2})^2$, the microwave magnetic field needed to saturate a spin packet and defined by $(H_{1/2})^2 = 1/\gamma^2 T_1 T_2$. For intermediate cases between perfectly homogeneous and inhomogeneous, the absorption amplitude can be empirically fit to the function:

$$S = K\sqrt{P}/(1 + P/P_{1/2})^{b/2} \quad (1)$$

where b is the inhomogeneity parameter determined by the ratio of the Lorentzian spin packet width to the Gaussian envelope width and K is a proportionality factor. For the EPR derivative amplitude, b varies from 1.0 to 3.0. Following the method of Rupp et al. (1978), the saturation profiles were fit to eq 1 by using a nonlinear least-squares fitting program (Bevington, 1969). Preliminary fits were done by allowing b, $P_{1/2}$, and K to vary simultaneously. For EPR signal II_{slow}, the value of b obtained from these fits was found to increase monotonically with temperature. This is expected when the homogeneous line width becomes larger with increasing temperature. Final fits were done with the value of b specified to ensure that b varied smoothly with temperature. For signal II_{slow}, the line width is largely determined by inhomogeneous broadening arising from unresolved hyperfine interactions.

To determine whether rapid-passage effects were influencing our $P_{1/2}$ values, we compared data obtained for signal II_{slow}

with 100-, 10-, 1.0-, or 0.27-kHz magnetic field modulations at 5.2 and 12.6 K. At these temperatures, eq 1 gave a better fit to the data when the field modulation frequency was lower, perhaps because of small rapid-passage effects at the highest modulation frequencies. But the distances obtained from the effect of Dy–EDTA on $P_{1/2}$ were not significantly different with different modulation frequencies.

Distance Determinations. Theory. Dipolar interactions with neighboring metal sites cause fluctuations in the local field observed by a free radical, dependent on the lifetime of the spin state of the metal ions. Time-dependent perturbation theory can be used to show that this will result in an enhancement of the relaxation of the free radical. Since relaxation mechanisms are additive, this dipolar-induced term is simply added to the intrinsic relaxation of the free radical:

$$(1/T_1)_{\text{measured}} = (1/T_1)_{\text{intrinsic}} + (1/T_1)_{\text{dipole-induced}} \quad (2)$$

Bloembergen (1949) and Abragam (1955) determined equations for the enhancement of the relaxation of a free radical due to the influence of paramagnetic metals in a solid. As Hyde and Rao (1978) discuss, for rare earth ions such as Dy^{3+}, one term of these equations will dominate. So for the effect of one Dy^{3+} ion on the relaxation of a single free radical, the expression of interest is

$$\frac{1}{T_{1j}} = \tfrac{2}{3}J_k(J_k + 1)\left(\frac{2T_{1k}}{1 + \omega_j^2 T_{1k}^2}\right)c_{jk}^2 \quad (3)$$

where $c_{jk}^2 = (9/4)(\gamma_j)^2(\gamma_k)^2(\hbar^2 \sin^2 \theta_r \cos^2 \theta_r)r^{-6}$ and the subscripts j and k refer to the free radical and the Dy^{3+} ion, respectively; r is the distance between the sites; θ_r is the angle between the radial vector and the magnetic field.

The spin–lattice relaxation actually observed for a free radical is a superposition of the relaxation behaviors of all the individual radicals, each interacting with its neighboring metal sites. Using an argument of Kittel and Abrahams (1953), Hyde and Rao (1978) simplified the problem of summing over the contributions of all the individual free radicals, each involving a sum over the interactions with all metal ions:

$$\left(\frac{1}{T_1}\right)_{\text{Dy-induced}} = \sum_j \sum_k \left[\frac{4T_{1k}J_k(J_k + 1)}{3(1 + \omega_j^2 T_{1k}^2)}\right]c_{jk}^2 \quad (4a)$$

Instead, a free radical in an "average" environment is considered, and the sum is taken over all available sites for the Dy complex, whether occupied or not, weighted by the probability (f) that any given site is occupied:

$$\left(\frac{1}{T_1}\right)_{\text{Dy-induced}} = f\sum_i \left[\frac{4T_{1k}J_k(J_k + 1)}{3(1 + \omega_j^2 T_{1k}^2)}\right]c_{jk}^2 \quad (4b)$$

The probability (f) is proportional to the concentration of metal ion. It is assumed that all metal spin–lattice relaxation times are the same. All of the constant terms in the expression can be included outside the summation. Since all orientations of the radial vector with the applied field are equally probable, the angular dependence is also a constant contribution, and the dipolar-induced relaxation is proportional to the sum over all sites:

$$(1/T_1)_{\text{Dy-induced}} \propto [\text{Dy}]\sum_i r^{-6} \quad (5)$$

It is the calculation of this sum that determines the distance dependence of the dipolar interaction.

For a system with a constant T_2, changes in $1/T_1$ can be followed by measuring the $P_{1/2}$ value. Therefore, from eq 2:

$$(P_{1/2})_{\text{measured}} = (P_{1/2})_{\text{intrinsic}} + (P_{1/2})_{\text{Dy-induced}} \quad (6)$$

The Dy-induced $P_{1/2}$ term will be proportional to the Dy-induced $1/T_1$. If the $P_{1/2}$ terms in the presence and in the absence of Dy^{3+} are measured, the Dy-induced $P_{1/2}$ can be determined from the difference. If the change in $P_{1/2}$ caused by a given concentration of Dy^{3+} is designated $\Delta P_{1/2}$, we can write

$$\Delta P_{1/2} = C\Delta[\text{Dy}](\sum_i r^{-6}) \quad (7)$$

where the constant C represents the collection of terms that are expected to remain much the same for all dipolar interactions between free radicals and Dy^{3+} ions.

The effect of added Dy^{3+} on the spin–lattice relaxation of the free radical is measured as a change in $P_{1/2}$ with Dy^{3+} concentration: $\Delta P_{1/2}/\Delta[\text{Dy}]$. In order to determine the distance dependence of this effect, it is necessary to calculate $\sum_i r^{-6}$ over all possible sites for the Dy^{3+} ion. When Dy–EDTA is added to samples, three scenarios can be imagined.

(1) The first possibility is that the Dy–EDTA is binding to a specific site on the protein and that only the Dy bound to this site is contributing to the enhanced relaxation. In this case there is only one possible binding site, $i = 1$, and an r^{-6} dependence results: $\Delta P_{1/2}/\Delta[\text{Dy}] = Cr^{-6}$. It is unlikely that this is the appropriate treatment for the samples considered in this paper. If there were tight binding to a single site, the site would fill rapidly, giving saturation of the effect with added Dy^{3+}. For the samples in this study, a linear increase of $P_{1/2}$ with [Dy] was observed up to 20 mM Dy–EDTA, the highest concentration used. This would argue for such a low affinity that the "binding site" would have a concentration similar to that of the random sites in solution.

(2) The Dy–EDTA could be bound nonspecifically to the surface of the protein. This is a more likely possibility. Dy^{3+} chelated by EDTA has two remaining coordination sites, usually occupied by water. The Dy–EDTA complex could bind to the protein surface by ligating to surface carboxyl groups. Likewise, the EDTA complex could form hydrogen bonds with surface groups. To determine a distance dependence in this case, it is necessary to evaluate $\sum_i r^{-6}$ over all possible sites, i, on the surface of the protein. If the distribution of the sites for Dy–EDTA is envisioned as continuous, this sum can be treated as a surface integral of the form

$$\sum_i r^{-6} = \int_0^{2\pi} d\theta \int_0^{\pi} (r^{-6})r^2 \sin \phi \, d\phi \quad (8a)$$

where the dependence of the radial distance r on θ and ϕ is determined by the structure of the protein under consideration.

(3) The final possibility is that the Dy–EDTA remains dispersed randomly in solution. For the high concentrations of Dy–EDTA used here, this is the most likely case. This requires that we sum for the distance dependence over all space not excluded by the protein. The sum over a continuum of sites yields an integral of the form

$$\sum_i r^{-6} = \int_0^{2\pi} d\theta \int_0^{\pi} \sin \phi \, d\phi \int_{r(\theta,\phi)}^{\infty} (r^{-6})r^2 \, dr \quad (8b)$$

Again, $r(\theta,\phi)$ is determined by the structure of the protein.

Distance Determinations. Calculations. In order to obtain the appropriate distance dependence, both the distribution of the interacting Dy^{3+} ions and the structure of the protein surrounding the free radical must be considered. Three possible modes for the Dy–EDTA distribution are discussed above: binding to a single site, binding randomly over the protein surface, and dispersed in solution. We have studied the Dy-induced relaxation of free radicals in both water-soluble

proteins (MbNO) and membrane-bound proteins (PSII and the reaction center of *Rb. sphaeroides*).

If Dy–EDTA binding at a single site is the only significant contribution to the change in $P_{1/2}$, then $\sum_i r^{-6} = k^{-6}$, where k is the distance from the binding site to the free radical. This will hold true regardless of the particular protein structure. This distance dependence can be used to model effects in systems where there is a known binding site for Dy^{3+}. Blum et al. (1980, 1983) have chosen to use an r^{-6} dependence to model the effect of Dy^{3+} complexes on the microwave power saturation behavior of a variety of paramagnetic sites. But an r^{-6} dependence is not actually appropriate, because they explicitly assume that there is no unique binding site and postulate that the Dy^{3+} complex is binding to multiple sites over the protein surface (Blum et al., 1980, 1981, 1983). We found no evidence that the Dy–EDTA complex was binding at a specific site on MbNO, *Rb. sphaeroides* reaction center, or PSII.

Soluble Proteins: *MbNO.* On the basis of crystallographic data (Watson, 1969), we have approximated MbNO as an oblate spheroid with the heme Fe offset from the center of the spheroid by m (Å) along one of the major axes and by n (Å) along the minor axis.

(*a*) *Surface Binding of Dy–EDTA.* If Dy–EDTA is binding to the surface, $\sum_i r^{-6}$ is approximated by the integral in eq 8a. For this model, we define the radial distance:

$$r = [\mu n \cos\phi + m \sin\theta \sin\phi + [(\mu n \cos\phi + m \sin\theta \sin\phi)^2 + F(\phi)(a^2 - m^2 - \mu n^2)]^{1/2}]/F(\phi) \quad (9)$$

where $\mu = a^2/b^2$, a is the half-width of the major axes, b is the half-width of the minor axis, and $F(\phi) = 1 + (\mu - 1) \times \cos^2\phi$. If m and n are considerably smaller than a and b, the integral can be solved to a good approximation as

$$\sum_i r^{-6} = \frac{2\pi a^2}{(a^2 - m^2 - \mu n^2)^4} \times [(a^2 - 2m^2 - 2\mu n^2)[2 + \tfrac{4}{3}(\mu - 1) + \tfrac{2}{5}(\mu - 1)^2] + 8\mu^2 n^2[\tfrac{2}{3} + \tfrac{2}{5}(\mu - 1)] + 4m^2[2 + \tfrac{2}{3}(\mu - 1)]] \quad (10)$$

(*b*) *Random Distribution of Dy–EDTA.* If Dy–EDTA is distributed randomly in the frozen solution, the same definition for radial distance, eq 9, is used in the integral (eq 8b). Again, an approximate solution is obtained:

$$\sum_i r^{-6} = \frac{\pi a^3}{3(a^2 - m^2 - \mu n^2)^3}\left[\left[\frac{1}{2} - \frac{3(m^2 + \mu n^2)}{4a^2}\right]\left(2\mu^{3/2} + 3\mu^{1/2} + \frac{3}{2\sqrt{\mu - 1}} \ln X\right) + \frac{9m^2\mu^{1/2}}{2a^2} + \frac{9m^2}{4a^2\sqrt{\mu - 1}} \ln X + \frac{18\mu^2 n^2 - 9m^2}{2a^2}\left[\frac{\mu^{3/2}}{2(\mu - 1)} - \frac{\mu^{1/2}}{4(\mu - 1)} - \frac{1}{8(\mu - 1)^{3/2}} \ln X\right]\right] \quad (11)$$

where $X = [\sqrt{\mu} + (\mu - 1)^{1/2}]^2$.

These results for $\sum_i r^{-6}$ are completely general and can be applied to any model protein that can be approximated as a spheroid, if it contains a free radical site located so that m and n are small compared to a and b. But because of the complex dependence on the structural parameters, this dependence is not useful for obtaining distances in uncharacterized water-soluble proteins.

Membrane Proteins: *PSII.* D^+ in PSII is part of a large membrane-bound protein complex. In thylakoids, only the outer side of the membrane is exposed to Dy–EDTA. If it is assumed that the outer membrane surface can be approximated as an infinitely large plane, the radial distance is simply $r = k_1 \cos\phi$, where k_1 is the distance from D^+ to the surface of the plane. In PSII membranes, both membrane surfaces are exposed. If both surfaces are treated as planar, $r_1 = k_1 \cos\phi$ and $r_2 = k_2 \cos\phi$, where k_2 is the distance to the plane approximating the inner membrane surface.

(*a*) *Surface Binding of Dy–EDTA.* For samples, such as thylakoids, where only one side of the membrane is exposed, the solution to the integral (eq 8a) is straightforward and yields

$$\sum_i r^{-6} = (2\pi/5)k_1^{-4} \quad (12a)$$

For samples, such as PSII membranes, where both membrane surfaces are exposed

$$\sum_i r^{-6} = (2\pi/5)(k_1^{-4} + k_2^{-4}) \quad (12b)$$

(*b*) *Random Distribution of Dy–EDTA.* If Dy–EDTA is dispersed in solution, the integral (eq 8b) for thylakoids uses the same simple expression as above for r. This results in a concise solution:

$$\sum_i r^{-6} = (\pi/6)k_1^{-3} \quad (13a)$$

In PSII membranes, the enhanced relaxation is a function of the distance from D^+ both to the outer membrane surface (k_1) and to the inner membrane surface (k_2):

$$\sum_i r^{-6} = (\pi/6)(k_1^{-3} + k_2^{-3}) \quad (13b)$$

When the surface of a large protein or a membrane-bound protein complex can be approximated as an infinite plane, $\sum_i r^{-6}$ can be expressed as a simple function of the distance from the free radical to the surfaces of the protein. If the membrane can be sealed to limit the approach to only one surface, the expression for $\sum_i r^{-6}$ contains only one variable, k_1. And having determined k_1, using eq 12b or 13b, as appropriate, will allow us to solve for k_2. This approach can be generally applied to free radicals in suitable membrane proteins.

Membrane Proteins: *Rb. sphaeroides.* We have modeled the bacterial reaction center from the structure presented by Yeates et al. (1987). The L and M subunits are approximated as an elliptical cylinder embedded in the membrane, with the H subunit as a hemisphere protruding on one surface.

(*a*) *Surface Binding of Dy–EDTA.* Dy–EDTA binding to the membrane surface for the bacterial reaction center is approximated in the same way as for PSII membranes, except for the surface of the H subunit. The radial component over the hemisphere of radius k_3 is described as $r_2 = k_2' \cos\phi + [k_2'^2 \cos^2\phi - (k_2^2 - k_3^2)]^{1/2}$, where $k_2' = k_2 - w$ (w is the distance from the Dy^{3+} to the surface, occupied by the EDTA complex). The resulting expression is

$$\sum_i r^{-6} = (2\pi/5)(k_1^{-4} + k_2^{-4}\cos^5\phi_2) + (\pi/40)[(1 - \cos^5\phi_2)/\cos^5\phi_2]k_2'^{-4} \quad (14)$$

where ϕ_2 is the angle between the axis of the cylinder and the radial vector connecting the site of the radical and the junction where the hemisphere connects to the plane.

(*b*) *Random Distribution of Dy–EDTA.* If the Dy–EDTA remains in solution, the bacterial reaction center is still approximated as above, and integrating over all space not excluded by the planes approximating the membranes and L and M subunits, or by the hemisphere of the H subunit, results in an expression:

$$\sum_i r^{-6} = (\pi/6)(k_1^{-3} + k_2^{-3}\cos^4\phi_2) + (\pi/48)[(1 - \cos^4\phi_2)/\cos^4\phi_2]k_2'^{-3} \quad (15)$$

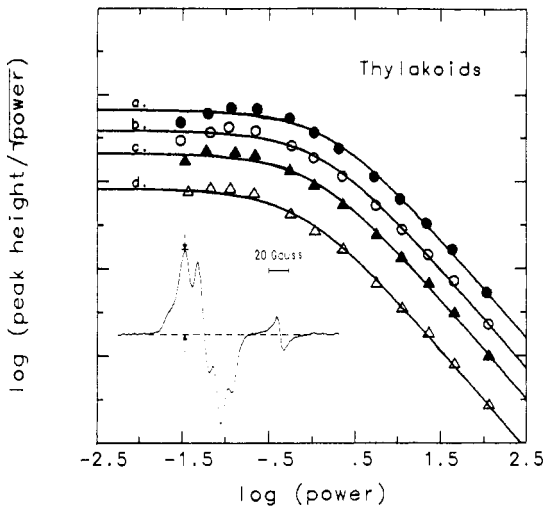

FIGURE 1: Continuous microwave power saturation of EPR signal II_{slow} in dark-adapted thylakoids. The concentrations of Dy–EDTA added were (a) 20 mM, (b) 15 mM, (c) 10 mM, and (d) 0 mM. The curves are offset on the y axis by an arbitrary amount. Conditions: temperature, 14.0 K; field modulation width, 4.0 G. Inset: EPR signal II_{slow} in dark-adapted PSII membranes. The intensity of signal II_{slow} was measured as the peak to base line height as indicated by the arrows. The scan was centered at 3250 G with a width of 100 G. The signal at 3288 G is from the Cr^{3+}/MgO crystal. The intensity of the Cr^{3+} signal was measured as the peak-to-peak height.

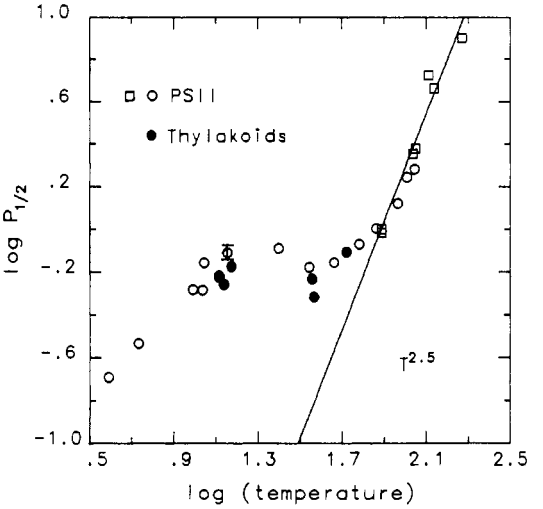

FIGURE 2: Temperature dependence of the $P_{1/2}$ of signal II_{slow} in dark-adapted PSII membranes (○, □) and thylakoids (●). The circles represent $P_{1/2}$ (mW) data taken with a liquid He cryostat; the open squares are $P_{1/2}$ data taken with a liquid N_2 cooled system. The data taken with the liquid N_2 cooled system were normalized to the liquid He $P_{1/2}$ values to adjust for changes in the microwave cavity Q factor caused by the different temperature control equipment. The slope of the higher temperature data indicates a $T^{2.5}$ temperature dependence. The error bar at 14 K for the PSII membrane data indicates the standard deviation for this $P_{1/2}$ determination, which is the average of five measurements on different PSII preparations. Conditions are described in Figure 1.

The equations for the sum over the radial vector dependence, $\sum_i r^{-6}$, are applicable to any protein that can be approximated with the same geometric forms. With the appropriate equations for $\sum_i r^{-6}$ from a model system, the proportionality constant, C, can be determined by plotting the change in the $P_{1/2}$ value versus the change in the concentration of Dy–EDTA to obtain the slope, $\Delta P_{1/2}/\Delta[Dy]$:

$$C = \frac{(\Delta P_{1/2})/(\Delta[Dy])}{\sum_i r^{-6}} \qquad (16)$$

The constant C should be roughly the same for any free radical interacting with Dy^{3+} at the same temperature, but the values are determined by factors (such as the cavity Q) that will vary with the instrumentation. Data for the model system and the unknown sample should be measured on the same spectrometer. This value for C can then be applied to $\Delta P_{1/2}/\Delta[Dy]$ data for an uncharacterized system, and a value for $\sum_i r^{-6}$ can be determined. If $\sum_i r^{-6}$ can be expressed as a simple function of the distance from the free radical to the surface of the protein, this distance of closest approach can be determined.

RESULTS

Temperature Dependence of Signal II_{slow} Relaxation. The signal intensity, S, of EPR signal II_{slow} was measured as the peak to base line height of the lowest field peak (inset to Figure 1). Figure 1 shows typical plots of the continuous microwave power saturation behavior for signal II_{slow} from thylakoids. Data are shown as log $(S/\sqrt{P})$ vs log P, where P is the microwave power. In this presentation, log $(S/\sqrt{P})$ remains constant in the limit that $P \ll P_{1/2}$. In the $P \gg P_{1/2}$ limit, log $(S/\sqrt{P})$ exhibits a linear dependence on log P, having slope $m = -0.5b$.

The microwave power saturation for signal II_{slow} from dark-adapted PSII membranes was measured over the range 4–200 K (Figure 2). Also included in Figure 2 are data for signal II_{slow} from thylakoids. Between 13 and 40 K, the $P_{1/2}$ of signal II_{slow} from PSII membranes appeared to be only slightly higher than that from thylakoids. In the high-temperature region, $T > 70$ K, the $P_{1/2}$ of signal II_{slow} from PSII membranes exhibited a $T^{2.5}$ temperature dependence. This is consistent with a Raman relaxation mechanism and is similar to the $T^{2.0}$ dependence reported for the $P_{1/2}$ of ultraviolet-induced tyrosine radicals (Sahlin et al., 1987). But below 70 K, an unusual temperature dependence was observed. Between 40 and 70 K, the $P_{1/2}$ continued to decrease but no longer followed a $T^{2.5}$ dependence. Between 40 and 10 K, the $P_{1/2}$ was nearly constant and actually appeared to increase slightly with decreasing temperature. Below about 10 K, the $P_{1/2}$ again dropped with decreasing temperature. This behavior points to the existence of an unidentified, additional relaxation mechanism for signal II_{slow}. The unknown mechanism is most effective above 13 K, although it becomes less significant above 40 K where the temperature-dependent Raman relaxation process is becoming dominant.

One explanation for this phenomenon is a dipolar interaction between D^+ and a bound paramagnet in the protein. Several paramagnetic metal sites are known to be associated with PSII: the four Mn of the oxygen-evolving center, the heme Fe of cytochrome b_{559}, and the Fe^{2+} of the $Fe–Q_A$ electron acceptor. Of these, cytochrome b_{559} can be immediately eliminated, as only a small amount of the paramagnetic, oxidized form is present in untreated, dark-adapted samples.

To determine if the relief from microwave power saturation below 70 K was caused by a dipolar interaction of D^+ with the Mn site, a study of the saturation was done for signal II_{slow} from Tris-washed membranes over the range 8–180 K (data not shown). The removal of Mn and the extrinsic 17-, 23-, and 33-kDa polypeptides by Tris treatment did decrease the microwave power necessary to saturate signal II_{slow} but did not eliminate the unusual temperature dependence of the $P_{1/2}$ observed below 70 K. The unknown relaxation mechanism cannot, therefore, be assigned to a dipolar interaction with the Mn. The decrease in $P_{1/2}$ of signal II_{slow} upon Tris washing may indicate the loss of a weak magnetic interaction between D^+ and the S_1 state of the Mn complex. It is, however, equally likely that the change in behavior in the Tris–PSII membranes

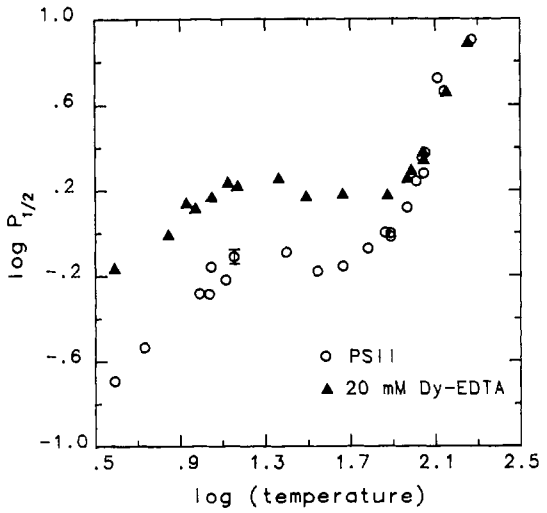

FIGURE 3: Temperature dependence of the $P_{1/2}$ for signal II_{slow} in PSII membranes containing 20 mM Dy–EDTA (▲) compared with the $P_{1/2}$ from untreated PSII membranes (○). The measurements were made as in Figure 2.

is due to a structural change which alters the effectiveness of the existing relaxation pathways. A structural change of this type might also explain the small difference between the $P_{1/2}$ of signal II_{slow} from PSII membranes and the $P_{1/2}$ of signal II_{slow} from thylakoids.

The remaining known metal site is the Fe^{2+} associated with Q_A on the electron-acceptor side of PSII. Recent work (Zimmermann & Rutherford, 1986; Petrouleas & Diner, 1987) has shown that, in the presence of phenyl-*p*-benzoquinone, it is possible to oxidize the Fe^{2+} of the Fe–Q_A to Fe^{3+}, with an estimated yield of about 50% (Petrouleas & Diner, 1987). We found that the $P_{1/2}$ of signal II_{slow} was unchanged when the Fe was oxidized by the procedure of Petrouleas and Diner (1987).

If none of the known paramagnetic sites in dark-adapted PSII membranes are involved, our data may indicate the presence of another, as yet undetected, paramagnet associated with PSII. Alternately, it is possible that the unusual temperature dependence reflects a coupling of signal II_{slow} spin–lattice relaxation or phase memory to a temperature-dependent molecular motion as has been reported in anthracene-d_{10} (Brown, 1979).

The temperature dependence of the $P_{1/2}$ for MbNO was also studied. Two forms of MbNO exist in frozen samples (Yonetani et al., 1972), giving distinct EPR spectra (Morse & Chan, 1980). Measuring the signal intensity at the $g \approx 2.0$ peak, where there is considerable intensity from both forms, and at the low-field shoulder ($g \approx 2.1$), which is dominated by a single form, resulted in slightly different values for $P_{1/2}$. However, the temperature dependences of the $P_{1/2}$ values for the two forms were the same over the temperature range measured. Between 10 and 60 K, both sets of $P_{1/2}$ values showed a $T^{2.4}$ dependence (data not shown), in keeping with a Raman relaxation process. Both forms also show the same concentration dependence for the effect of added Dy on $P_{1/2}$.

Effect of Dy–EDTA on Signal II_{slow}. Figure 3 shows the effect of 20 mM Dy–EDTA on the microwave power saturation of signal II_{slow} over the temperature range 4–200 K. Dipolar interactions between Dy and D^+ provide relief from power saturation for signal II_{slow} at low temperatures, but above 100 K this enhancement is no longer a significant addition compared with the intrinsic relaxation. The neutral Dy–HEDTA complex caused the same effect on the microwave power saturation as the negatively charged Dy–EDTA, indicating that charge repulsions or attractions do not affect the approach of Dy–EDTA to the membrane.

In order to confirm that the effect of Dy–EDTA on the $P_{1/2}$ of signal II_{slow} was due only to magnetic interactions, 20 mM of the diamagnetic complex La–EDTA was added to PSII membranes. No significant difference in the $P_{1/2}$ of signal II_{slow} was observed between untreated PSII membranes and PSII membranes with 20 mM La–EDTA added.

PSII-enriched preparations are thought to consist of membrane sheets surrounded by an annulus of detergent (Dunahay et al., 1984). In these PSII preparations, the Dy–EDTA complex has access to both sides of the membrane. In order to measure the distance to D^+ from the outer membrane surface, the effect of Dy–EDTA was examined with intact thylakoids. The intactness of the thylakoids toward the Dy complex was verified by addition of Dy^{3+}. Intact right-side-out thylakoids show very little inhibition when rare earth ions are added to the O_2 evolution assay medium, even at 2.5 mM (Zitkus et al., 1987). But PSII membranes are completely inactivated. Our results showed that addition of 1.0 mM Dy^{3+} to PSII membranes reduced the O_2 evolution activity to about 10%. At the same concentration, thylakoids retained 99% of their activity. It was assumed that the impermeability of the thylakoids toward Dy^{3+} indicated that they were intact toward the larger Dy–EDTA and Dy–HEDTA complexes. Addition of the Dy^{3+} as the Dy–EDTA or Dy–HEDTA complexes caused no inhibition of O_2 evolution, even in PSII membranes incubated for up to several hours in the dark with 20 mM Dy–EDTA.

The plots of the change in $P_{1/2}$ ($\Delta P_{1/2}$) versus Dy–EDTA concentration for MbNO and for the *Rb. sphaeroides* chlorophyll dimer are shown in Figure 4. The data for $\Delta P_{1/2}$ versus Δ[Dy–EDTA] for signal II_{slow} from thylakoids, PSII membranes, and Tris-washed PSII membranes are presented in Figure 5. The data for PSII membranes (Figure 5b) and Tris–PSII membranes (Figure 5c) are the averages of three concentration dependence studies, and the error bars indicate the standard deviations of the $\Delta P_{1/2}$ values determined for 10 mM Dy–EDTA, which are representative of the errors for all concentrations. The data for the PSII membranes (Figure 5b) included points from the Dy–HEDTA results, which exhibited the same behavior as Dy–EDTA.

To determine which assumption for the distribution of Dy–EDTA is most appropriate for this experiment, MbNO was used as a model system to obtain distances to the $(BChl)_2^+$ in *Rb. sphaeroides* reaction centers. Distances were calculated by assuming each of the possible models for Dy–EDTA distribution. These values can then be compared with the known distance from the X-ray crystal structure.

One-Site Binding. If the Dy–EDTA is interacting from a single binding site, C is calculated with $\Delta P_{1/2}/\Delta$[Dy] data for MbNO by using eq 16, where $\sum_i r^{-6} = k^{-6}$. We have chosen to assume that the Dy^{3+} coordinates directly with a group on the protein surface at a site near the closest approach. A distance of k = 8.3 Å from the MbNO heme Fe to the closest point on the surface of the spheroid was used. With this value for C, the same equations were used to determine k^{-6} in the bacterial reaction center, where k was the distance from $(BChl)_2^+$ to a binding site on the protein surface.

Surface Binding. For the assumption that Dy–EDTA is associated randomly over the surface of the MbNO, $\sum_i r^{-6}$ is calculated from eq 10. To obtain values for the parameters, we have used crystallographic data for MbNO (Watson, 1969) and lanthanide–EDTA complexes (Hoard et al., 1965). Assuming that Dy–EDTA binds via hydrogen-bonding interac-

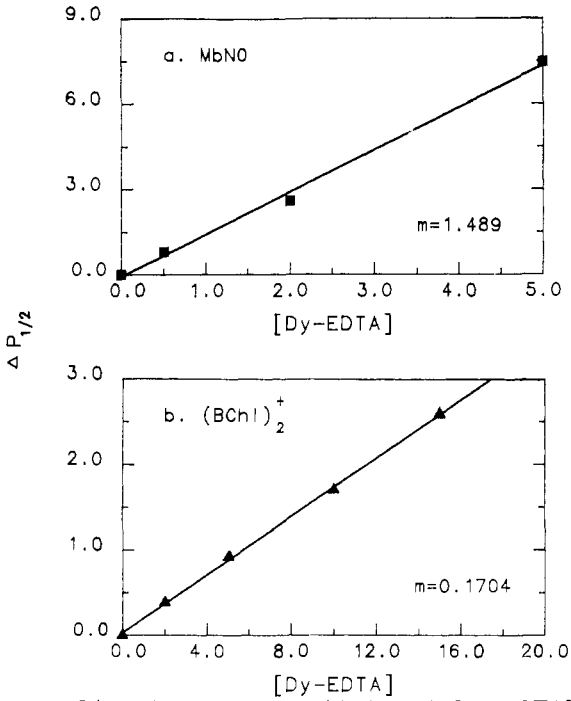

FIGURE 4: Change in the $P_{1/2}$ value with change in [Dy-EDTA] for (a) MbNO and (b) $(BChl)_2^+$ from *Rb. sphaeroides* reaction centers. $\Delta P_{1/2}$ (mW) is the difference between the $P_{1/2}$ in samples with Dy-EDTA and without Dy-EDTA. The slopes of the linear fits, m, are given in units of mW/mM.

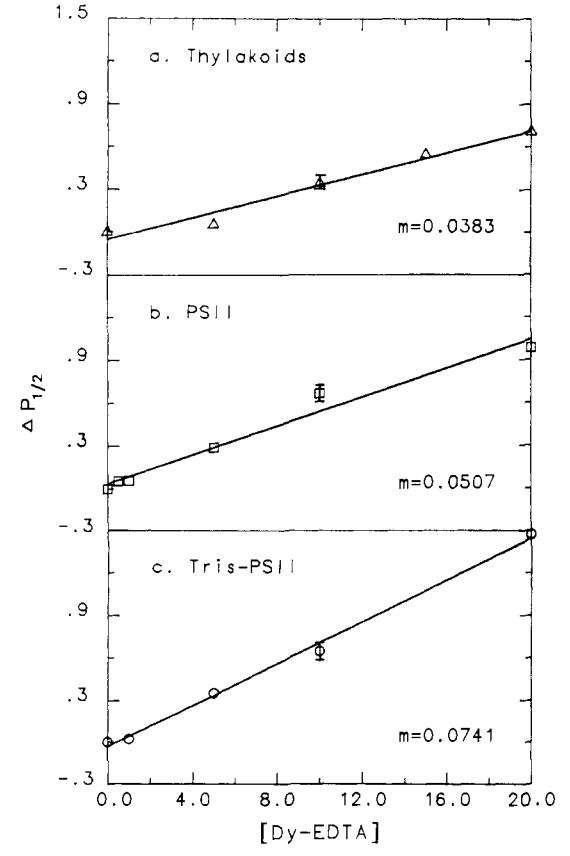

FIGURE 5: Change in the $P_{1/2}$ value with change in [Dy-EDTA] for signal II_{slow} in (a) thylakoids, (b) PSII membranes, and (c) Tris-washed PSII membranes. Each data point is the average of two to three measurements. The error bars indicate the standard deviations of $\Delta P_{1/2}$ from the average of three measurements. The slopes of the linear fits, m, are given in units of mW/mM.

tions, we have approximated the Dy-EDTA complex, with the hydrogen bond, as a sphere of radius 6.1 Å. Adding this distance to the parameters for MbNO, we have obtained a = (20.0 + 6.1) Å, b = (13.5 + 6.1) Å, m = 8.0 Å, and n = 4.5 Å. The resulting value for $\sum_i r^{-6} = 1.09 \times 10^{-4}$. By use of the $\Delta P_{1/2}/\Delta$[Dy] data for MbNO with eq 16, $C = 1.37 \times 10^4$.

Table I: Distance from $(BChl)_2^+$ to *Rb. sphaeroides* Reaction Center Surface

sample	$\Delta P_{1/2}/\Delta$[Dy] (mW/mM)	distance (Å) Dy-EDTA distribution: one site	surface	random
MbNO	1.49 ± 0.07			
$(BChl)_2^+$	0.170 ± 0.003	11.9 ± 0.1	11.8 ± 0.2	14.5 ± 0.2

Table II: Distance from D^+ to PSII Surface

sample	$\Delta P_{1/2}/\Delta$[Dy] (mW/mM)		distance (Å) model: MbNO	$(BChl)_2^+$
thylakoids	0.038 ± 0.004	outer side	26.9 ± 1	26.2 ± 1
PSII	0.051 ± 0.004	inner side	42.0 ± 6	40.9 ± 6
Tris-PSII	0.074 ± 0.003	inner side	27.7 ± 1	26.9 ± 1

We have approximated the L and M subunits of the *Rb. sphaeroides* reaction center as an elliptical cylinder with a height of 50 Å, based on X-ray crystallographic data and interpretation by Yeates et al. (1987). The H subunit was assumed to be a hemisphere of radius 25 Å bound to one face. The value for C, calculated from the data from MbNO, was used with the $(BChl)_2^+$ data and eq 14 and 16 to obtain k_1. The distance from $(BChl)_2^+$ to the protein surface is presented in Table I as $(k_1 - 6.1)$.

Random Distribution of Dy-EDTA. If Dy-EDTA is interacting from random positions in the frozen solution, the structural parameters include a radius for the Dy-EDTA with a van der Waals exclusion and a van der Waals distance from the surface of the protein. This added distance was assumed to be approximately 6.1 Å, so the values for the parameters were the same as those given above. $\sum_i r^{-6}$ for MbNO was calculated with eq 11. This was used to calculate a value of $C = 2.98 \times 10^3$. Equations 15 and 16 were used with data for $(BChl)_2^+$ and this C to obtain k_1. The distance from $(BChl)_2^+$ to the protein surface is presented in Table I as $(k_1 - 6.1)$.

Based on X-ray crystallographic data and interpretation by Yeates et al. (1987), the chlorophyll dimer was assumed to be approximately 14 Å from one face of the cylinder. Comparison of the distances calculated under the different models for the distribution of Dy-EDTA shows that both the single-site and surface-binding models cause a significant underestimate of this distance. As expected, the assumption that Dy-EDTA remains dispersed in solution provides the best model for the dipolar interaction between Dy^{3+} and $(BChl)_2^+$.

The assumption that Dy-EDTA is randomly dispersed was used to calculate the distances included in Table II with both MbNO and *Rb. sphaeroides* as model systems. In all cases, C was calculated from $\sum_i r^{-6}$ by using eq 16. C_{MbNO} was obtained with the values a = 26.1 Å, b = 19.6 Å, m = 8.0 Å, and n = 4.5 Å and equation 11. C_{BChl} was calculated from the values k_1 = 20.1 Å, k_2 = 42.1 Å, k_2' = 36.0 Å, and ϕ_2 = 35.9°, which were used in eq 15 to obtain $\sum_i r^{-6}$. Distances to D^+ in thylakoids were calculated by using these values for C, the data for $\Delta P_{1/2}/\Delta$[Dy], and eq 13a to obtain k_1. The distance from D^+ to the outer membrane surface reported in Table II is $(k_1 - 6.1)$ Å. This distance k_1 can then be used in eq 13b to obtain k_2 for the $\Delta P_{1/2}/\Delta$[Dy] data from PSII membranes and Tris-PSII membranes. The distance from D^+ to the inner membrane surface is $(k_2 - 6.1)$ Å.

Uncertainties are included in Tables I and II based on errors in the linear fits of the $\Delta P_{1/2}$ vs Δ[Dy–EDTA] data used for each calculation. This can provide a measure of the precision of the values, but not necessarily of the accuracy. A more reasonable guideline for the accuracy of the values is comparison between distances obtained with the different model systems, which agree to within ±2 Å. Distances obtained in photosystem II from the two model systems are in good agreement, although the small, soluble MbNO and the large, membrane-bound reaction center are extremely dissimilar. This is supportive of the general applicability of this method.

Discussion

Quantitating magnetic interactions can provide a great deal of insight on the locations of intrinsic paramagnets or metal ion binding sites in proteins. It is a particularly attractive method for a system like PSII, which has so many paramagnetic sites essential for function. The enhancement of the spin–lattice relaxation of a free radical by a paramagnetic metal ion has been extensively treated (Bloembergen, 1949; Abragam, 1955; Hyde & Rao, 1978). But the use of exogenous metal ions to determine distances on the basis of the relaxation of intrinsic paramagnets, even in the simple case of a free radical, requires that we consider the relaxation as the sum of all the dipolar interactions. By considering instead a single radical in an "average" environment, we have derived analytical expressions for the effect of added Dy–EDTA on the relaxation rate of the radical.

Three possible models for the distribution of Dy–EDTA have been discussed. It seems unlikely in these systems that the enhancement in relaxation is due to Dy–EDTA bound in a single site. Not surprisingly, the values in Table I show that this model does not give very good distance estimates in the bacterial reaction center. It has been suggested that Dy–EDTA associates with a large number of sites, randomly dispersed over the protein surface. This could occur by hydrogen bonding, electrostatic interactions, or coordination to the Dy ion. However, no difference was observed in the magnitude of the Dy effect in PSII or Tris-washed PSII membranes when the neutral Dy–HEDTA complex was used rather than the negatively charged Dy–EDTA. This suggests that electrostatic interactions, at least, are not leading to surface binding. Further, the distances obtained by assuming binding of Dy–EDTA to the protein surface are smaller than expected. Considering the linear dependence of $\Delta P_{1/2}$ up to high concentrations of added Dy–EDTA in our experiments, the most likely situation is that the Dy–EDTA is randomly distributed in the solution.

Several researchers have attempted to use the dipolar interaction between Dy^{3+} and paramagnetic sites to obtain distances. But they have generally treated the effect as if Dy^{3+} were binding to a single site, even where there is evidence to the contrary. Blum et al. (1980, 1981, 1983) used an r^{-6} dependence even when they had assumed that the Dy^{3+} complex was associating with the surface of the protein, or when there was no evidence of association at all. This resulted in unreasonable results when water-soluble proteins were used to model distances in membrane-bound proteins (Ohnishi et al., 1982; Prince, 1983).

Itoh et al. (1987) have reported a previous attempt to use the dipolar interaction between Dy^{3+} and D^+ to obtain distances in PSII. They used the method of Blum et al. (1983) for calibrating the Dy effect and were unable to get reasonable estimates. Itoh et al. (1987) also reported only small effects using the Dy–EDTA complex and chose to use the uncomplexed Dy^{3+} ion, attributing the small effect of Dy–EDTA to repulsion of the negatively charged complex by the protein surface. Our results show no difference between the negatively charged Dy–EDTA complex and neutral Dy–HEDTA. Ghanotakis et al. (1985), Zitkus et al. (1987), and our own results have shown that addition of uncomplexed lanthanide ions, including Dy^{3+}, to PSII membranes causes loss of O_2 evolution. Ghanotakis et al. (1985) have proposed that lanthanides bind specifically and replace Ca^{2+} near the water oxidation site.

When used appropriately, the dipolar interaction between added metals and paramagnetic sites in proteins can provide useful distance information. In the case where a specific binding site for the metal exists, the distance dependence of the interaction is simply r^{-6}. But even when there is no specific binding site, dipolar interaction with Dy–EDTA dispersed in solution can be used to obtain distances. And because it does not involve binding interactions, no information on the location of specific binding sites is necessary. The theory can also be adapted to proteins of a wide variety of structures. The complex dependence on the structural parameters would make it difficult to obtain distance information from the dipolar interactions in a small, soluble protein like MbNO. But globular, water-soluble proteins can serve as model systems. And in large membrane-bound proteins, if the surface can be approximated as a large plane, the distance dependence is quite simple. These are the types of systems most difficult to probe by other methods.

The temperature dependence of the microwave power saturation of signal II_{slow} also contains important information on the environment of D^+. Three clear temperature regions emerge from the study, each region apparently dominated by a different relaxation mechanism. The $T^{2.5}$ dependence observed above 70 K is reasonable for an isolated tyrosine radical relaxing via a Raman mechanism. Sahlin et al. (1987) studied the temperature dependence of the microwave power saturation for ultraviolet-induced tyrosine free radicals in a frozen solution and found a $T^{2.0}$ dependence. Below 70 K, the $P_{1/2}$ of signal II_{slow} exhibits an unusual temperature dependence. It would be expected that the microwave power saturation between 20 and 70 K would continue to show the same dependence found at high temperatures; this has been observed for the ultraviolet-induced tyrosine radical (Sahlin et al., 1987). Instead, the $P_{1/2}$ for signal II_{slow} in this region shows very little change with temperature, with the relaxation remaining about as effective as at 70 K. An additional mechanism for relaxation must be coming into play.

One possibility for an additional relaxation mechanism would be a magnetic interaction with a paramagnetic metal in PSII. This type of interaction has been observed in ribonucleotide reductase (Sahlin et al., 1987). Above 30 K, the tyrosine radical in ribonucleotide reductase exhibits a faster relaxation with a much stronger temperature dependence than the $T^{2.0}$ dependence observed at low temperatures. This has been attributed to magnetic interactions of the free radical with the iron center. Other researchers have already proposed that the Mn site is close enough to D^+ to affect signal II_{slow} relaxation (de Groot et al., 1986; Styring & Rutherford, 1988). Our results show that there is, indeed, a small decrease in the $P_{1/2}$ of signal II_{slow} upon removal of the Mn and the extrinsic polypeptides, but the unusual temperature dependence is not eliminated. High-spin Fe^{2+} is also known to be an effective relaxer. But a comparison of the $P_{1/2}$ of signal II_{slow} when the iron associated with Q_A is in the Fe^{2+} versus the Fe^{3+} state did not show any change. If the Mn and iron–quinone sites are not involved, the presence of another paramagnet asso-

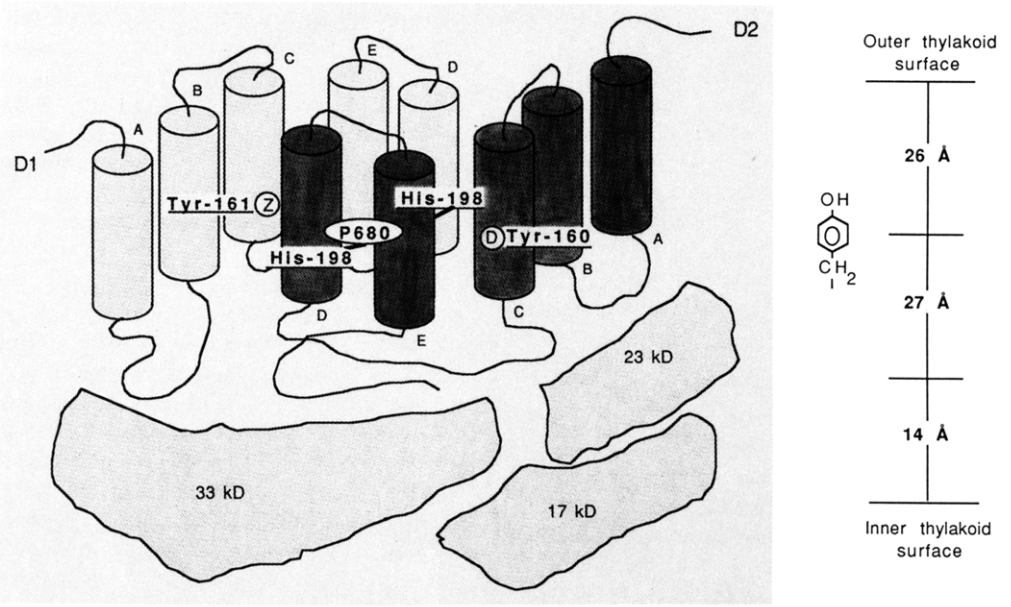

FIGURE 6: Model for the location of the stable tyrosine radical, D^+, in PSII. The folding of the D1 and D2 subunits is adapted from the proposal of Trebst and Draber (1986) and is based on an analogy of these polypeptides and the L and M polypeptides of the reaction center from purple non-sulfur bacteria (Michel & Deisenhofer, 1986). This diagram includes the extrinsic polypeptides (33, 23, and 17 kDa) that bind to the inner membrane surface. The cylinders represent the membrane-spanning helices of the D1 and D2 polypeptides. They are labeled A–E by analogy to the nomenclature of Deisenhofer et al. (1985) for the L and M subunits of the bacterial reaction center. D^+ has been identified as Tyr-160 of the C helix of the D2 subunit; Z, the electron donor to P680, has been proposed to be Tyr-161 of the C helix of the D1 subunit (Debus et al., 1987). P680, the primary electron donor of PSII, is thought to be bound to His-198 from D1 and to His-198 from D2. The distances on the right indicate the following: the distance to D^+ from the outer surface of PSII, 26 Å; the distance to D^+ from the inner surface of polypeptide-depleted PSII, 27 Å; and the width of the bound extrinsic polypeptides, approximately 14 Å.

ciated with PSII may be indicated. However, alternative relaxation pathways can also be imagined. Although the cause of the unusual temperature dependence of the microwave power saturation of signal II_{slow} has not yet been identified, the phenomenon may provide an additional clue to the environment of this stable tyrosine radical and to the structure of the photosystem.

The results from using the effect of Dy–EDTA on signal II_{slow} power saturation to obtain distance estimates are presented in Table II. With intact thylakoids, only the outer surface of the vesicle is exposed to the Dy complex. For this case, a distance of about 26 Å was obtained. In PSII membranes, both membrane surfaces are exposed. The approach to the inner membrane surface is at a considerably greater distance, about 41 Å. For Tris-washed PSII membranes, not only are both membrane surfaces exposed, but three extrinsic polypeptides and the Mn of the O_2-evolving center are removed from the inner surface of the membrane. We found that Tris washing causes a measurable decrease in the distance to D^+ from the membrane surface. This would mean that the extrinsic 17-, 23-, and 33-kDa polypeptides represent a thickness of 14 ● 2 Å. Our results agree well with the thickness of 10–20 Å for the extrinsic polypeptides reported from freeze–etch electron micrographs (Staehelin et al., 1987). Following removal of the extrinsic polypeptides, a distance of 27 Å was found from the inner face of the reaction center. The signal II_{slow} tyrosine radical appears to be buried near the middle of the PSII reaction center.

Mutagenesis experiments have shown that Tyr-160 in the D2 subunit gives rise to EPR signal II_{slow} (Debus et al., 1987). Comparisons of the likely membrane-spanning regions of D2 with the structure of the M subunit of the bacterial reaction center have led to speculation that Tyr-160 is located near the inner membrane surface (Trebst & Draber, 1986; Barber, 1987). However, orientation studies on signal II_{slow} show that the tyrosine must be oriented perpendicularly to the membrane (O'Malley et al., 1984). This means that the tyrosine ring can point either toward the surface or toward the center of the membrane from its position in the polypeptide chain. So the tyrosine is probably pointing farther into the membrane than its position in the sequence might suggest. Also, the D2 polypeptide has substantial hydrophilic regions connecting the membrane-spanning regions, and these may block approach to D^+ even if Tyr-160 is not very deeply embedded in the lipid membrane.

The distance information obtained from our experiments indicates that D^+ is quite inaccessible. This is consistent with the stability of D^+, and with its inertness toward nonlipophilic reducing agents. An increased access of D^+ from the inner membrane surface following Tris washing is consistent with the increased lability of signal II_{slow} in Tris-washed membranes in the presence of reductant.

Figure 6 summarizes the distances obtained from our study of the dipolar interaction between exogenous Dy–EDTA and D^+. The distance from the outer membrane surface to D^+ was measured at 26 Å. In the absence of the 17-, 23-, and 33-kDa polypeptides, a distance of 27 Å was determined from the inner membrane surface. The height of the extrinsic polypeptide unit removed by Tris was found to be about 14 Å. These distances result in a membrane thickness, in the absence of extrinsic polypeptides, of about 53 Å. Deisenhofer et al. (1985) showed by X-ray structure analysis that the reaction center complex from *Rhodopseudomonas viridis* has a thickness of approximately 50 Å. Our results for the polypeptide-depleted PSII reaction center are very similar, in support of the proposed structural homology between the bacterial photosystem and PSII.

Added in Proof

Electron microscopy of purified PSII complexes from the thermophilic cyanobacterium *Synechococcus sp.* has led to a model in which the transmembrane dimension of PSII without the extrinsic polypeptides is 56 Å (Dekker et al., 1989), in good

agreement with our estimate of 53 Å.

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REFERENCES

Abragam, A. (1955) *Phys. Rev. 98*, 1729–1735.
Babcock, G. T., & Sauer, K. (1973) *Biochim. Biophys. Acta 325*, 483–503.
Barber, J. (1987) *Trends Biochem. Sci. 12*, 321–326.
Barry, B. A., & Babcock, G. T. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7099–7103.
Beck, W. F., de Paula, J. C., & Brudvig, G. W. (1985) *Biochemistry 24*, 3035–3043.
Beinert, H., & Orme-Johnson, W. H. (1967) in *Magnetic Resonance in Biological Systems* (Ehrenberg, A., Malmström, B. G., & Vänngård, T., Eds.) pp 221–247, Pergamon Press, Oxford.
Berthold, D. A., Babcock, G. T., & Yocum, C. F. (1981) *FEBS Lett. 134*, 231–234.
Bevington, P. R. (1969) *Data Reduction and Error Analysis for the Physical Sciences*, McGraw-Hill, New York.
Bloembergen, N. (1949) *Physica 15*, 386–426.
Blum, H., Leigh, J. S., & Ohnishi, T. (1980) *Biochim. Biophys. Acta 626*, 31–40.
Blum, H., Cusanovich, M. A., Sweeney, W. V., & Ohnishi, T. (1981) *J. Biol. Chem. 256*, 2199–2206.
Blum, H., Bowyer, J. R., Cusanovich, M. A., Waring, A. J., & Ohnishi, T. (1983) *Biochim. Biophys. Acta 748*, 418–428.
Brown, I. M. (1979) in *Time Domain Electron Spin Resonance* (Kevan, L., & Schwartz, R., Eds.) pp 195–229, Wiley, New York.
Castner, T. G. (1959) *Phys. Rev. 115*, 1506–1515.
Commoner, B., Heise, J. J., & Townsend, J. (1956) *Proc. Natl. Acad. Sci. U.S.A. 42*, 710–718.
Debus, R. J., Barry, B. A., Babcock, G. T., & McIntosh, L. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 427–430.
de Groot, A., Plijter, J. J., Evelo, R., Babcock, G. T., & Hoff, A. J. (1986) *Biochim. Biophys. Acta 848*, 8–15.
Deisenhofer, J., Epp, O., Miki, K., Huber, R., & Michel, H. (1985) *Nature (London) 318*, 618–624.
Dekker, J. P., Boekema, E. J., Witt, H. T., & Rögner, M. (1989) *Biochim. Biophys. Acta* (in press).
Dunahay, T. G., Staehelin, L. A., Seibert, M., Ogilvie, P. D., & Berg, S. P. (1984) *Biochim. Biophys. Acta 764*, 179–193.
Ghanotakis, D. F., Babcock, G. T., & Yocum, C. F. (1985) *Biochim. Biophys. Acta 809*, 173–180.
Hoard, J. L., Lee, B., & Lind, M. D. (1965) *J. Am. Chem. Soc. 87*, 1612–1613.
Hyde, J. S., & Rao, K. V. S. (1978) *J. Magn. Reson. 29*, 509–516.
Itoh, S., Isogai, Y., Tang, X.-S., & Satoh, K. (1987) in *Progress in Photosynthesis Research* (Biggens, J., Ed.) Vol. I, pp 483–486, Martinus Nijhoff Publishers, Dordrecht.
Kittel, C., & Abrahams, E. (1953) *Phys. Rev. 90*, 238–239.
Kohl, D. H. (1972) in *Biological Applications of Electron Spin Resonance* (Swartz, H. M., Bolton, J. R., & Borg, D. C., Eds.) pp 213–264, Wiley-Interscience, New York.
Kok, B., Forbush, B., & McGloin, M. (1970) *Photochem. Photobiol. 11*, 457–475.
Michel, H., & Deisenhofer, J. (1986) in *Encyclopedia of Plant Physiology III: Photosynthesis* (Staehelin, L. A., & Arntzen, C. J., Eds.) Vol. 19, pp 371–381, Springer Verlag, Berlin.
Morse, R. H., & Chan, S. I. (1980) *J. Biol. Chem. 255*, 7876–7882.
Ohnishi, T., Blum, H., Harmon, H. J., & Hompo, T. (1982) in *Electron Transport and Oxygen Utilization* (Ho, C., Ed.) pp 387–393, Elsevier, Amsterdam.
O'Malley, P. J., Babcock, G. T., & Prince, R. C. (1984) *Biochim. Biophys. Acta 766*, 283–288.
Petrouleas, V., & Diner, B. A. (1987) *Biochim. Biophys. Acta 893*, 126–137.
Portis, A. M. (1953) *Phys. Rev. 91*, 1071–1079.
Prince, R. C. (1983) *Biochim. Biophys. Acta 723*, 133–138.
Rupp, H., Rao, K. K., Hall, D. O., & Cammack, R. (1978) *Biochim. Biophys. Acta 537*, 255–269.
Sahlin, M., Petersson, L., Gräslund, A., Ehrenberg, A., Sjöberg, B.-M., & Thelander, L. (1987) *Biochemistry 26*, 5541–5548.
Staehelin, L. A., DeWit, M., & Seibert, M. (1987) in *Progress in Photosynthesis Research* (Biggens, J., Ed.) Vol. II, pp 297–300, Martinus Nijhoff Publishers, Dordrecht.
Styring, S., & Rutherford, A. W. (1987) *Biochemistry 26*, 2401–2405.
Styring, S., & Rutherford, A. W. (1988) *Biochemistry 27*, 4915–4923.
Trebst, A., & Draber, W., (1986) *Photosynth. Res. 10*, 381–392.
Watson, H. C. (1969) *Prog. Stereochem. 4*, 299–333.
Yeates, T. O., Komiya, H., Rees, D. C., Allen, J. P., & Feher, G. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6438–6442.
Yocum, C. F., Yerkes, C. T., Blankenship, R. E., Sharp, R. R., & Babcock, G. T. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7507–7511.
Yonetani, T., Yamamoto, H., Erman, J. E., Leigh, J. S., & Reed, G. H. (1972) *J. Biol. Chem. 247*, 2447–2455.
Zimmermann, J.-L., & Rutherford, A. W. (1986) *Biochim. Biophys. Acta 851*, 416–423.
Zitkus, M., Hierholzer, P. D., Ivey, S., & Berg, S. P. (1987) in *Progress in Photosynthesis Research* (Biggens, J., Ed.) Vol. II, pp 321–324, Martinus Nijhoff Publishers, Dordrecht.