Electron Spin Resonance of Chlorophyll and the Origin of Signal I in Photosynthesis

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Communicated by S. I. Weissman, January 7, 1971

ABSTRACT A comparison has been made between Signal I, the photo-electron spin resonance signal associated with the primary light conversion act in photosynthesis, and free-radical signals generated in various chlorophyll species \textit{in vitro}. The esr signals obtained from chlorophyll monomer, (Chl-L)+, chlorophyll dimer, (Chl)L, and chlorophyll oligomer, (Chl)L+, are broader than Signal I, whereas the chlorophyll–water adduct, (Chl-H\textsubscript{2}O),+, gives a signal very much narrower than Signal I. The unusually narrow signal from (Chl-H\textsubscript{2}O),+ has been ascribed to spin migration, or to unpaired spin delocalization over a large number of chlorophyll molecules. The linewidth of Signal I can be accounted for by a similar delocalization process. A theoretical relationship between the esr linewidth and the number of chlorophyll molecules, N, over which an unpaired spin is delocalized, takes the form \(\Delta H_N = \frac{1}{\sqrt{N}} \cdot \Delta H_N^0\), where \(\Delta H_N^0\) is the linewidth of monomer (Chl-L)+. This relationship for \(N = 2\) accounts well for the line widths of Signal I in green algae, blue-green algae, and photosynthetic bacteria in both the 'H- and 'H-forms. The linewidth of Signal I (as well as the optical properties of reaction-center chlorophyll) are consistent with unpaired spin delocalization over an entity containing two chlorophyll molecules, (Chl-H\textsubscript{2}O-Clh)+.

In 1956, Commoner and coworkers (1) discovered that electron spin resonance (esr) signals could be produced in intact photosynthetic organisms or chloroplast preparations by irradiation with light. The most prominent of the photo-esr signals (Signal I) is rapidly reversible, has the free electron \(g\)-value 2.0025, a peak-to-peak linewidth (\(\Delta H\)) of about 7.5 g (plants) or about 9.5 g (bacteria), a Gaussian line shape, and no hyperfine structure. Subsequent esr studies (2-5) have been reviewed by Weaver (2) on chloroplast or chromatophore preparations, (3-6), active-center preparations (7-19), organisms of unusual isotopic composition (20, 21), and on in \textit{vitro} chlorophyll systems (21-31) have made it probable that Signal I arises by the photooxidation of special chlorophyll molecules located in the photosynthetic reaction center, and that this is the chlorophyll responsible for the long-wavelength absorption associated with the reaction center (32) (P700, plants; or P870, bacteria). It has further been suggested (21) that Signal I is due to the formation of Chl+ or BChl+; however, Signal I is much narrower than the esr signals recorded from Chl+ or BChl+. To explain the unusual esr and spectral properties of reaction-center chlorophyll, chlorophyll aggregation of an unspecified nature (33), chlorophyll–l lipid and chlorophyll–protein interactions (34), or perturbations in the chlorophyll \(\pi\) system caused by unspecified changes in the environment (24) have been suggested. None of these interpretations, however, account for both the optical and the esr properties of active-center chlorophyll.

Chlorophyll species important in esr

Recent investigations by infrared and nuclear magnetic resonance (35) spectroscopy have characterized and differentiated several in \textit{vitro} chlorophyll species. Clearly, it is essential that in \textit{vitro} esr observations be interpreted in terms of the chlorophyll species actually present. Chlorophyll is able to act both as electron donor and electron acceptor in charge-transfer complexes. The central Mg atom of chlorophyll is coordinatively unsaturated when it has the coordination number 4, and at least one of the Mg axial positions must always be occupied by an electron donor group. In the absence of other nucleophiles, the ketone C=C function in Ring V of one chlorophyll molecule serves as donor to the Mg atom of another, forming chlorophyll dimers, (Chl), or oligomers, (Chl)n. Extraneous nucleophiles (bases) can compete for the coordination site at Mg, with disruption of the chlorophyll–chlorophyll interactions, to form chlorophyll–ligand adducts, (Chl-L). The nature of the nucleophile determines whether the chlorophyll–nucleophile adduct is monomeric or polymeric. Bifunctional ligands, such as dioxane, pyrazine, 1,4-diazobicyclo(2.2.2)octane, and, in particular, water can cross-link chlorophyll molecules or chlorophyll dimers by coordination to Mg to form large (chlorophyll–nucleophile) micelles (to be published). The chlorophyll species present in a particular experiment are very sensitive to temperature, adventitious nucleophiles such as water, and solvent, factors not always taken into account in previous work.

EXPERIMENTAL METHODS

Chlorophyll samples were dried, prior to solution preparation, by codistillation with CCl\textsubscript{4} (36). The solvents used for \textit{in vitro} measurements were first dried over Linde 3A molecular sieve and degassed under reduced pressure. Solutions were prepared and oxidant was added in a nitrogen-filled dry box or on the vacuum line.

Irradiations were performed with a 150-W Varian Eimac lamp. Infrared components were removed by 2 cm of water and 2 dichroic infrared-rejecting filters. All irradiations were by red light with a Corning 2404 sharp cut-off filter. All \textit{in vitro} measurements were made in 4-mm quartz esr tubes at \(-170^\circ\mathrm{C}\); \textit{in vivo} measurements were made at room temperature on concentrated slurries of cells held in a Varian water cell (V-4548).

Abbreviations: Chl, chlorophyll; Chl-L, chlorophyll monomer; esr, electron spin resonance; BChl, bacteriochlorophyll.
Table 1. Line widths (ΔH) of in vitro chlorophyll \textit{esr} signals

<table>
<thead>
<tr>
<th>System*</th>
<th>Solvent</th>
<th>λ\text{max} (nm)</th>
<th>Oxidant</th>
<th>ΔH (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[H]Chl a</td>
<td>†</td>
<td>663</td>
<td>FeCl₃ or I₂</td>
<td>9.3 ± 0.3†</td>
</tr>
<tr>
<td>[H]Chl a</td>
<td>†</td>
<td>663</td>
<td>FeCl₃ or I₂</td>
<td>3.8 ± 0.2†</td>
</tr>
<tr>
<td>[HI]BChl</td>
<td>‡</td>
<td>773</td>
<td>I₂</td>
<td>12.8 ± 0.5‡</td>
</tr>
<tr>
<td>[HI]BChl</td>
<td>§</td>
<td>773</td>
<td>I₂</td>
<td>5.4 ± 0.2‡</td>
</tr>
<tr>
<td>(Chl₂)</td>
<td>CCl₄</td>
<td>665,678</td>
<td>I₂</td>
<td>9.0 ± 0.5</td>
</tr>
<tr>
<td>(Chl₂)</td>
<td>Film</td>
<td>665,678</td>
<td>I₂</td>
<td>10.0 ± 0.5</td>
</tr>
<tr>
<td>(Chl₂)</td>
<td>CH₃OH</td>
<td>665,678</td>
<td>I₂</td>
<td>9.0 ± 0.5</td>
</tr>
<tr>
<td>(Chl₂)</td>
<td>CH₃OH</td>
<td>665,678</td>
<td>O₂</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>(Chl-H₂O)₂</td>
<td>Film</td>
<td>743</td>
<td>O₂</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>(Chl-H₂O)₂</td>
<td>Film</td>
<td>743</td>
<td>I₂</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>(Chl-H₂O)₂</td>
<td>Film</td>
<td>743</td>
<td>Light</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>(Chl-H₂O)₂</td>
<td>Film</td>
<td>743</td>
<td>Light</td>
<td>0.8 ± 0.2</td>
</tr>
</tbody>
</table>

* [HI], ordinary hydrogen; [HI], fully deuterated.
† Solvent: CH₃OH or CH₃OH–CH₂Cl₂. Concentration of Chl and oxidant, 10⁻⁴ to 10⁻² M.
‡ g-Values, 2.00025 ± 0.00002. Spectra recorded at liquid-N₂ temperature.
§ Solvent: CH₃OH–glycerol (1:1, v/v).
¶ McElroy, Feher, and Mauzerall (21).

Esr spectra were recorded on a Varian V-4500 spectrometer with 100-KHz modulation, maximum amplitude of 1/4 ΔH² in a TE₂₀⁻ mode cavity. Maximum microwave power was 5 mW; intentional saturation produced no significant broadening. Before and after recording spectra, the Fieldial and field-frequency stabilizer were calibrated with potassium peroxylamine disulfonate (as = 13.0 ± 0.07 g). Line shapes were checked by the method given in the Varian epr operations manual. In all in \textit{vivo} experiments involving line shape analysis, the dark base line was removed by repetitive light-dark subtraction cycles using a Varian C-1024 computer of average transients.

RESULTS

Esr of monomer chlorophyll

The monomeric chlorophyll species, Chl-Lₐ or Chl-L₂, are present in polar, electron-donor solvents such as pyridine, tetrahydrofuran, acetone, etc. (37). Chlorophyll free radicals can be obtained from (Chl-L) species only by chemical or electrochemical (24) oxidation. The signal shape varies only slightly with solvent, (L), oxidant, or temperature. Our values for ([HI]Chl-L)⁺⁺ and ([HI]Chl-L)⁺⁺ obtained by chemical oxidation are listed in Table 1 and agree well with those of Borg et al. (24), who have established the very important point that the esr signal observed in oxidized chlorophyll is really due to a \textit{z}-cation radical of chlorophyll \textit{a}. The deuterium isotope effect measured here on the signals from monomer chlorophyll \textit{a} corresponds closely to that observed in bacteriochlorophyll by McElroy \textit{et al.} (21), and in Mg octaethylporphyrin by Borg \textit{et al.} (24).

Esr of chlorophyll dimer and oligomer

Chlorophyll dimer, Chl₂, is present in nonpolar solvents with good solvation characteristics for the ring (CCl₄, benzene), whereas chlorophyll oligomers, (Chl₂)ₙ, are present in aliphatic hydrocarbon solvents (with poor solvation characteristics) such as octane, where \( n > 10 \) (38). Again, chlorophyll free-radical signals can be obtained from (Chl₂) or (Chl₂)ₙ only by chemical oxidation. Both (Chl₂)⁺⁺ and (Chl₂)⁺⁺ have esr signals, including values determined for these species in solution (Table 1), that are at least 9–10 g wide, very similar to that of (Chl-L)⁺⁺.

Esr of (Chl-H₂O)₂

The (Chl-H₂O)₂ species exists in aliphatic hydrocarbon solvents. It consists of a large, ordered array of chlorophyll molecules, held together by water coordinated to the Mg atom of one chlorophyll molecule and hydrogen-bonded simultaneously to the keto and the carboxethoxy ester carbonyl functions of another chlorophyll; repetition of this interaction results in large entities of colloidal dimensions (29, 39). This is the only chlorophyll species known to yield a reversible photosensitive signal that is sufficiently intense to be readily detected. The unusually narrow line width of this signal (~1 g or less, Table 1) has been attributed to migration of the unpaired spin over the entire aggregate. When the unpaired spin is delocalized over a sufficiently large number of chlorophyll molecules, or at a sufficiently rapid rate, the esr signal collapses to a very narrow line (29, 30).

The (Chl-H₂O)₂ species absorbs in the red at 743 nm and is the most red-shifted of any chlorophyll \textit{a} species so far prepared in vitro. In the (Chl₂)ₙ species, where C=O–Mg interactions are involved, the macrocycle planes cannot be made parallel (40), and the red-shift is small (λ\text{max} = 680 nm). The large red-shift of (Chl-H₂O)₂ thus reflects a major structural difference, between this species and (Chl₂)ₙ, in the way the chlorophyll molecules are arrayed relative to each other.

Although there is no evidence that (Chl-H₂O)₂ is an important species in nature, hydrated bacteriochlorophyll species absorbing near 865 nm appear to be present in bacteria. From visible absorption spectroscopy, we believe the (Chl₂)ₙ species absorbing at 860 nm probably is the form in which antenna chlorophyll occurs in nature (to be published).

Esr of photosynthetic organisms and preparations

Table 2 lists values for Signal I observed in various organisms and chloroplast or chromatophore preparations. The esr parameters are highly reproducible, and only slight variations are observed in different cultures of the same organisms, or in successive measurements on the same culture.

From Tables 1 and 2, the linewidth of Signal I in photosynthetic preparations is seen to be significantly narrower than the linewidth observed for (Chl-L)⁺⁺. The difference in ΔH is much larger than can be accounted for by any reasonable experimental error. Thus, all the defined chlorophyll species listed in Table 1 absorb light at wavelengths either much shorter or much longer than the P₇₀₀ chlorophyll in the photosynthetic reaction center, and they all have esr signals either much broader or much narrower than Signal I.

Spin delocalization and esr linewidth

We have noted that the narrow photo-esr of (Chl-H₂O)₂ can be accounted for by a process of spin migration that effectively delocalizes the unpaired spin over a large number of chlorophyll molecules. We propose to reconcile the in \textit{vivo} and in \textit{vivo} esr signals by a similar process.

Consider an esr spectrum whose second moment (Δµ²) arises primarily from unresolved isotropic and anisotropic hyperfine interactions. These assumptions are reasonable for a large \( \tau \)
system with a symmetrical Gaussian line shape (20, 41), particularly in view of the narrow symmetrical signals of the deuterated systems. We want to calculate the second moment of pairs of such molecules when the unpaired electron is shared equally between the members of the pairs (42, 43). First, we calculate the second moment of noninteracting molecules with a fixed orientation, k, relative to the external magnetic field. The monomer second moment for this orientation, k, is thus,
\[
\langle \Delta \omega^2 \rangle_k = \sum_i^n I_{ik} a_{ik}^2,
\]
where
\[
\sum_i^n a_{ik} I_{ik} = 0, \quad \sum_i^n I_{ik} = 1,
\]
and \(n\) is the number of "stick" resonances located at fields \(a_{ik}\) with intensities \(I_{ik}\). This treatment neglects line broadening from intermolecular interactions. Now, consider pairs of molecules in the same orientation, k, such that the unpaired electron-spin density is shared equally between the two members of the pair. Using the McConnell relation (44), we assume the unpaired spin density is halved at every site, neglecting additional interactions between the molecules. This assumption has been verified for aromatic hydrocarbon-dimer cations (42, 43). Of such units, \(n^2\) types of pairs exist. With the above spin-density assumption, the pair is resonant at \((a_{ik} + a_{jk})/2\), with intensity \(I_{ik} I_{jk}\). (For convenience, we treat the i j pair separately from the j i pair.) Thus, the second moment for spins delocalized over pairs of molecules is
\[
\langle \Delta \omega^2 \rangle_k = \sum_i^n I_{ik} I_{jk} \left( \frac{a_{ik} + a_{jk}}{2} \right)^2
\]
\[
= \frac{1}{2} \sum_i^n I_{ik} a_{ik}^2 = \frac{1}{2} \langle \Delta \omega^2 \rangle_k.
\]
The cross terms vanish because of symmetry. It follows that the total second moment, including all orientations, is \(\langle \Delta \omega^2 \rangle = 1/2 \langle \Delta \omega^2 \rangle_k\).

Thus, for Gaussian lines, or for any line shape for which \(\langle \Delta \omega^2 \rangle = C \cdot \Delta H^2\), we have
\[
\Delta H_i = \frac{1}{\sqrt{2}} \Delta H_i.
\]
This treatment can be generalized to give
\[
\Delta H_N = \frac{1}{\sqrt{N}} \Delta H_M
\]
where \(\Delta H_M\) is the linewidth of \((\text{Chl} \cdot \text{L})^+\)-monomer and \(\Delta H_N\) is the linewidth when the unpaired spin is delocalized over N molecules. Eqs. (1) and (2) are consistent with the results of Hanna and McConnell for large linear polynye radicals (41).

**Origin of Signal I**

If we proceed on the assumption that the narrowing of the \(\text{in vivo}\) esr signals is due to a process of spin delocalization, Eq. (2) permits an estimate of the number of chlorophyll molecules involved in the delocalization. By inspection, it can be seen that the \(\text{in vivo}\) signals of Table 2 are related to \(\Delta H\) of \((\text{Chl} \cdot \text{L})^+\) and \((\text{BChl} \cdot \text{L})^+\) (values in Table 1) by the factor \(1/\sqrt{2}\), i.e., in Eq. (2), \(N = 2\). The linewidth of Signal I is very well accounted for by the assumption that this esr signal arises in an entity \((\text{Chl} \cdot \text{H}_2\text{O} \cdot \text{Chl})^+\). Linewidths calculated by Eq. (1) never differ from the observed values by more than 10\%, and are always slightly narrower than those observed \(\text{in vivo}\) (last column, Table 2). The differences between observed and calculated linewidths may well arise from the simplifying assumption of "stick" spectra in the derivative. Linewidths calculated on the assumption that the unpaired spin is delocalized over two properly positioned chlorophyll molecules accounts equally well for the esr signals observed in green algae, blue-green algae, photosynthetic bacteria, and active-center preparations.

The \((\text{Chl} \cdot \text{H}_2\text{O})_n\) entity, where \(n\) is very large, absorbs at 743 nm. Kasha–McCrae (45) considerations predict that an aggregate of size 2, \((\text{Chl} \cdot \text{H}_2\text{O} \cdot \text{Chl})\), will absorb near 700 nm, based on the 743-nm maximum of \((\text{Chl} \cdot \text{H}_2\text{O})_n\) and 663-nm maximum of the monomer \((\text{Chl} \cdot \text{L})\). Both the esr and the optical spectra of active-center chlorophyll appear to be compatible with our model. It may be noted that this analysis holds for any \((\text{Chl} \cdot \text{X} \cdot \text{Chl})^+\), where the necessary geometrical relationship between the chlorophyll molecules is enforced (either by the ligand X or even by some structural matrix that maintains the chlorophyll molecules in the proper position relative to each other). At this time, we know of no ligand other than water that has the necessary properties, but the

**Table 2. Observed and calculated line widths (\(\Delta H\)) of esr Signal I in plants and bacteria**

<table>
<thead>
<tr>
<th>Organisms*</th>
<th>Observed†</th>
<th>Calculated‡</th>
<th>R$\dagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td>[H]Synecho-&lt;br&gt;chus lividus</td>
<td>7.1 ± 0.2</td>
<td>6.6 ± 0.3</td>
<td>1.08 ± 0.06</td>
</tr>
<tr>
<td>[H]S. lividus</td>
<td>2.95 ± 0.1</td>
<td>2.7 ± 0.1</td>
<td>1.10 ± 0.05</td>
</tr>
<tr>
<td>[H]Chlorella&lt;br&gt;vulgaris</td>
<td>7.0 ± 0.2</td>
<td>6.6 ± 0.3</td>
<td>1.06 ± 0.05</td>
</tr>
<tr>
<td>[H]C. vulgaris</td>
<td>2.7 ± 0.1</td>
<td>2.7 ± 0.1</td>
<td>1.00 ± 0.05</td>
</tr>
<tr>
<td>[H]Scenedesmus&lt;br&gt;obliquus</td>
<td>7.1 ± 0.2</td>
<td>6.6 ± 0.3</td>
<td>1.08 ± 0.06</td>
</tr>
<tr>
<td>[H]S. obliquus</td>
<td>2.7 ± 0.1</td>
<td>2.7 ± 0.1</td>
<td>1.00 ± 0.05</td>
</tr>
<tr>
<td>[H]HP700</td>
<td>7.0 ± 0.2</td>
<td>6.6 ± 0.3</td>
<td>1.06 ± 0.05</td>
</tr>
<tr>
<td>[H]Rhodo-&lt;br&gt;pseudomonas&lt;br&gt;epihedrion</td>
<td>9.6 ± 0.2 (15)**</td>
<td>9.1 ± 0.4</td>
<td>1.06 ± 0.05</td>
</tr>
</tbody>
</table>

*Whole cells, except as otherwise indicated.
†This work, unless a reference is given.
‡Calculated from Eq. (1), using monomer chlorophyll values from Table 1: [H]Chl a, 9.3 ± 0.3 g; [H]Chl a, 3.8 ± 0.2 g; [H]BChl, 12.8 ± 0.5 g; [H]BChl, 5.4 ± 0.2 g. All line shapes are Gaussian.
§Ratio of \(\Delta H_{in vitro}/(\Delta H_{in vivo}/\sqrt{2})\); the \(\Delta H_{in vivo}\) values are in footnote †.
¶Measured on an active center preparation furnished by L. F. Vernon.
**Chromatophore or reaction-center preparations.
possibility that there are such ligands in nature cannot be excluded. We emphasize the basic difference between the chlorophyll dimer, (Chl), which is the species present in a CCl₄ or benzene solution, and the entity (Chl·H₂O·Chl). The optical and esr properties of reaction-center chlorophyll cannot be accounted for in terms of a chlorophyll dimer formed by keto C==O—Mg interactions. As there are so many ways in which chlorophyll can be “aggregated”, this term should be used only when the mode of aggregation is specified.

The model of the photosynthetic unit that emerges from this analysis has the following features: The bulk of the chlorophyll is considered to be present as the oligomers, (Chl)n, absorbing near 680 nm; this is the light-gathering or antenna chlorophyll. The active center, where charge separation occurs (30), is (Chl·H₂O·Chl), and the observed unpaired spin is largely confined to this entity. The structures [(Chl)n·Chl·H₂O·Chl(Chl)n] or [(Chl)n·Chl·H₂O·Chl] are both compatible with the esr and optical data. In the latter structure, the terminal chlorophyll is available for coordination at Mg to a ligand that could be involved in electron transport. The entity (Chl·H₂O·Chl) makes two chlorophyll molecules available for electron transport so that removal and replacement of the electron can be simultaneous. A highly water-free environment is required to maintain the integrity of the chlorophyll oligomer. The model suggested here from esr data is very similar to one we have suggested from infrared and visible absorption spectroscopy (46).

This work was performed under the auspices of the U.S. Atomic Energy Commission.