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OF CHLOROPLASTS

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Blackwell, M.F.

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Mary Frances Blackwell
(Ph.D. thesis)

September 1982

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Electron Paramagnetic Resonance Studies
of Triplet States in Photosystem I of Chloroplasts

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by

Mary Frances Blackwell
Lawrence Berkeley Laboratory
University of California
Berkeley, California 94720

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ABSTRACT

The work presented in this thesis is divided into two categories of investigation: experimental studies of chlorophyll triplet states in chloroplasts and photosystem I subchloroplasts by means of electron paramagnetic resonance (EPR) spectroscopy, and theoretical studies of triplet state photokinetics.

Experimental studies presented in Chapters Three through Five concern the mechanism of primary photochemistry in photosystem I. In Chapter Three, we present evidence that charge recombination between $P700^+$ and A^- populates the triplet state of P700. In Chapter Four, we show that when X is not reduced prior to measurement, A^- does not always transfer its electron to X, but instead undergoes charge recombination with $P700^+$. We show that this observation is consistent either with heterogeneity of photosystem I reaction centers, or with the existence of a branch point in photosystem I primary photochemistry. In Chapters Four and Five we present evidence for the existence of a chlorophyll antenna species which is not integral to the photosystem I reaction center, but which can transfer photoexcitation to P700.

The theoretical analysis of triplet photokinetics presented in Chapter Two starts with a quantum mechanical derivation of the quantum yields for triplet spin sublevel population. The rate equations descriptive of triplet

photokinetics are shown to contain the quantum yields explicitly. We demonstrate that the spin lattice relaxation rates between the spin sublevels are time dependent as a result of the metastability of the triplet state. Explicit account of the time dependence of the spin lattice relaxation rates yields simple solutions of the triplet photokinetic rate equations which are exact in the high temperature limit.

The theoretical development is continued in Chapter Five, where we derive expressions for the photokinetics of triplet EPR signals. Our theoretical analysis yields a new triplet EPR signal polarization mechanism, making it the first analysis which can fully describe EPR signal polarization dynamics.

In Chapter Five, we present a final experimental and theoretical analysis of P700 and antenna triplet photokinetics. We show that the theoretical analysis presented in Chapters Two and Five is successful in describing the photokinetics of both the P700 and antenna triplet EPR signals.

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ABBREVIATIONS

<u>a</u>	absorption
A	earliest known acceptor in photosystem I
ADP	adenosine diphosphate
ATP	adenosine triphosphate
CIDEP	chemically induced electron polarization
<u>e</u>	emission
EDTA	ethylene diamine tetracetic acid
EPR	electron paramagnetic resonance
NAD(P)	nicotinamide adenine dinucleotide (phosphate)
P700	primary donor in photosystem I
P860	primary donor in purple bacteria
PMS	phenazine methosulfate
p ^R	radical pair triplet state in bacteria
RP	radical pair
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TLC	thin layer chromatography
X	secondary acceptor in photosystem I

SYMBOLS

h	Planck's constant
H_0	magnetic field
I	intensity
J	exchange interaction
k	Boltzmann's constant
k_i	triplet spin sublevel population rate
K_i	triplet spin sublevel depopulation rate
K_{AV}	average triplet spin sublevel decay rate, $1/3 \sum_i K_i$
$\underline{M}$	matrix
N_E	number of excitations
N_{iB}	Boltzmann population of triplet sublevel i
N_i	number of molecules in state T_i
N_T	total triplet population, $\sum_i N_i$
S^n	singlet level n
S_+	EPR signal for the transition $T_0 \rightarrow T_1$
S_-	EPR signal for the transition $T_{-1} \rightarrow T_0$
T^m	triplet level m
T_i	triplet spin sublevel i
$\vec{v}$	vector
$W_{1(2)}$	$\Delta M_S = 1(2)$ spin lattice relaxation rate
W_{ij}	spin lattice relaxation rate from T_i to T_j
α	spin $+1/2$
β	spin $-1/2$
ϕ_i	triplet spin sublevel population quantum yield
ϕ_T	total triplet quantum yield, $\sum_i \phi_i$

CHAPTER ONE

INTRODUCTION TO PHOTOSYNTHESIS AND PRIMARY EVENTS
IN PHOTOSYSTEM I1. Why We Need to Understand Photosynthesis

Photosynthetic organisms are the only self-sufficient living things on earth: they need not eat other creatures in order to live. Plants create themselves from fairly simple molecules available in the environment by simply basking in the sunlight. Not only are plants the first food in the food chain, but they also provide almost all the oxygen in the air which animals must breathe in order to live. Thus the very existence of all other life forms, including humanity, depends ultimately and crucially on plants. A fundamental human fear is that society will be limited by the capacity of plants to provide enough energy to support our growth. One of the greatest evils within human society is the spectre of mass starvation, which is occurring most of the time somewhere on earth. The 'energy crisis' is really no different from what has always been the most crucial issue facing any organism: finding a secure source of energy to exploit for existence and growth. Yet despite the efforts of technology, we have failed to come up with a source of food which would bypass the need to eat other living things, or of energy which is more convenient and abundant (i.e. "economical") than fossil fuel - ultimately plants, again. There is an elegant simplicity in Calvin's [1] suggestion that we simply grow and burn

high fuel-yield plants as a source of energy: our most sophisticated solar engineering is no match for the engineering of even the simplest photosynthetic prokaryotes.

Obviously, it is of more than academic interest to ask: How do plants do it? Can we do it too? The hope is that by understanding the principles of photosynthesis, we can imitate plants, and thus free ourselves from our animal-like dependence on other living creatures.

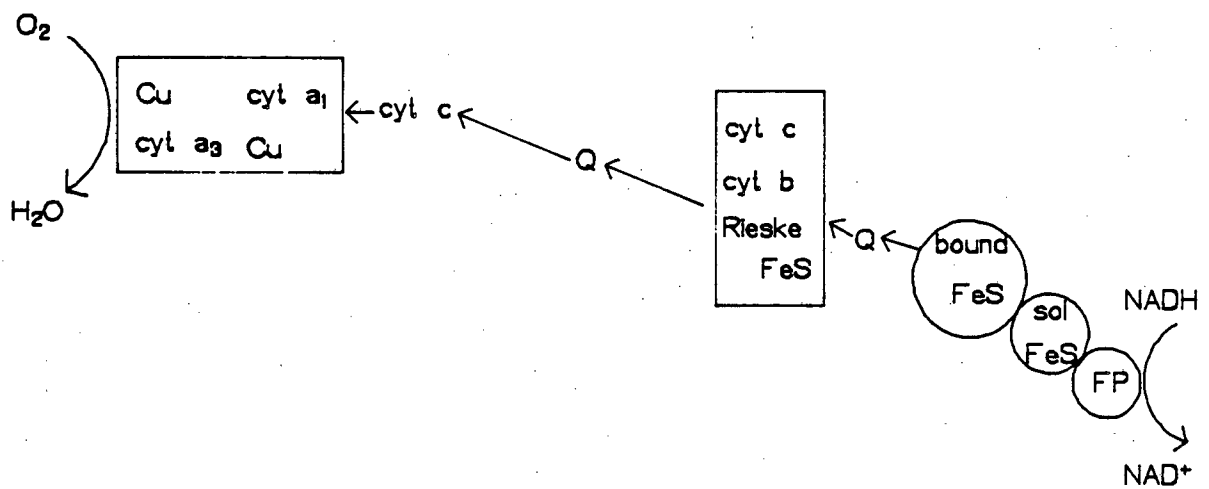
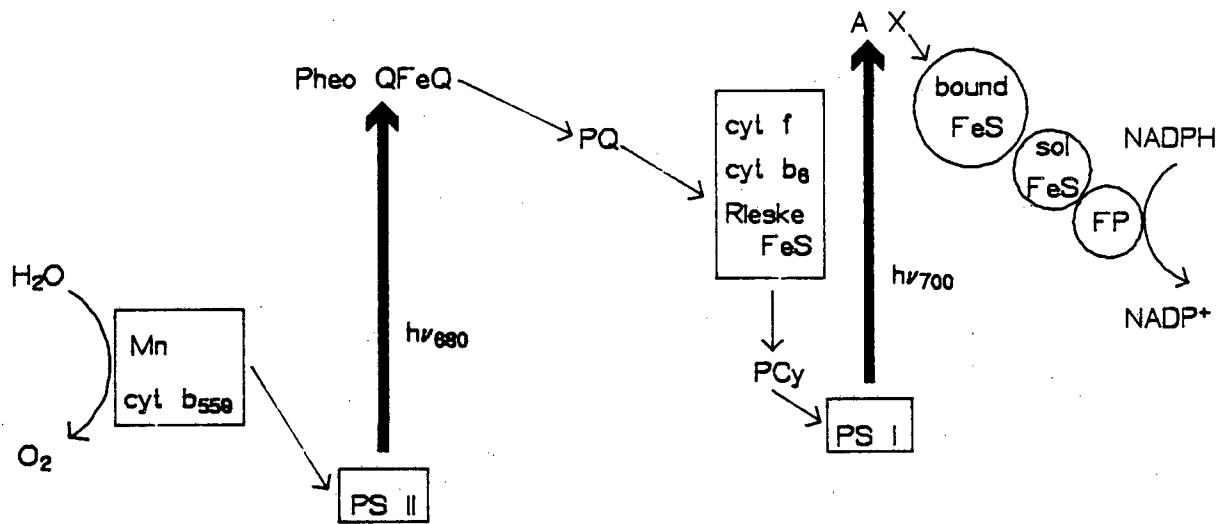
The ultimate human creation would have to be the genetic engineering of photosynthetic people: no doubt these would soon realize that they need do nothing more with their lives but find a niche where they could securely bask in the sun. And so human technology would end where life on earth started: with the creation of plants.

2. Basic Principles of Photosynthesis

We have a rough sketch now of the basic principles of photosynthesis: all further work will fill in the details without causing any fundamental changes in the design. All living things get energy for life by means of membrane-bound electron/proton transport chains. A specific environmental substrate, which we discuss below, initiates the transport of electrons and protons by means of the chain; the transport occurs across a membrane which separates aqueous spaces, thus causing a local or global trans-membrane potential which is usable free energy. In higher plants and animals, the transported electron itself ultimately reduces NADP or NAD, which in turn provide reducing equivalents for reactions in the synthesis of cellular constituents; the transmembrane potential is stored in a third phosphate bond when ADP is phosphorylated and becomes ATP. This phosphate bond energy is used whenever cellular reactions are energetically unfavorable (i.e.

nonspontaneous).

In eukaryotic cells, the most fundamental difference between the respiratory organelle (i.e. mitochondrion) and the photosynthetic one (i.e. chloroplast) is that the latter is green: as we shall now discuss, the constitution of the respiratory and photosynthetic transport chains are otherwise remarkably similar. So we come to the question: why are plants green? The answer is twofold. The first part involves the composition of the transport chains. The respiratory chain of mitochondria is represented by the lower scheme in Figure 1, whereas the photosynthetic chain is represented by the upper scheme. We note that both chains span the free energy difference between the $\text{H}_2\text{O} - \text{O}_2$ couple and the $\text{NADPH} - \text{NADP}^+$ or $\text{NADH} - \text{NAD}^+$ couple. Both chains contain similar groups of electron-proton transferases: a NAD(P)H oxidase group which contains iron sulfur proteins and a flavoprotein; a quinone-cytochrome-Rieske iron sulfur protein oxidoreductase; and a metal-cytochrome group involved with the oxidation or reduction of water (we note that the metal-cytochrome group is much less well characterized in chloroplasts). The most notable difference between the respiratory chain and the photosynthetic chain is that the latter requires two energy inputs which are the light reactions of photosystem I and photosystem II. That is, the charge transport chain in plants contains large assemblies of chlorophyll molecules organized in specific ways by bonding within membrane proteins. The chlorophylls associated with each photosystem harvest sunlight and transfer it to a 'special pair' of chlorophylls, called the primary donor, which is then able to donate an electron to the first acceptor in the charge transport chain. Light-induced oxidation of chlorophyll and reduction of its partner in the reaction center is thus the initiating (primary) event in the photosynthetic light



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Figure 1

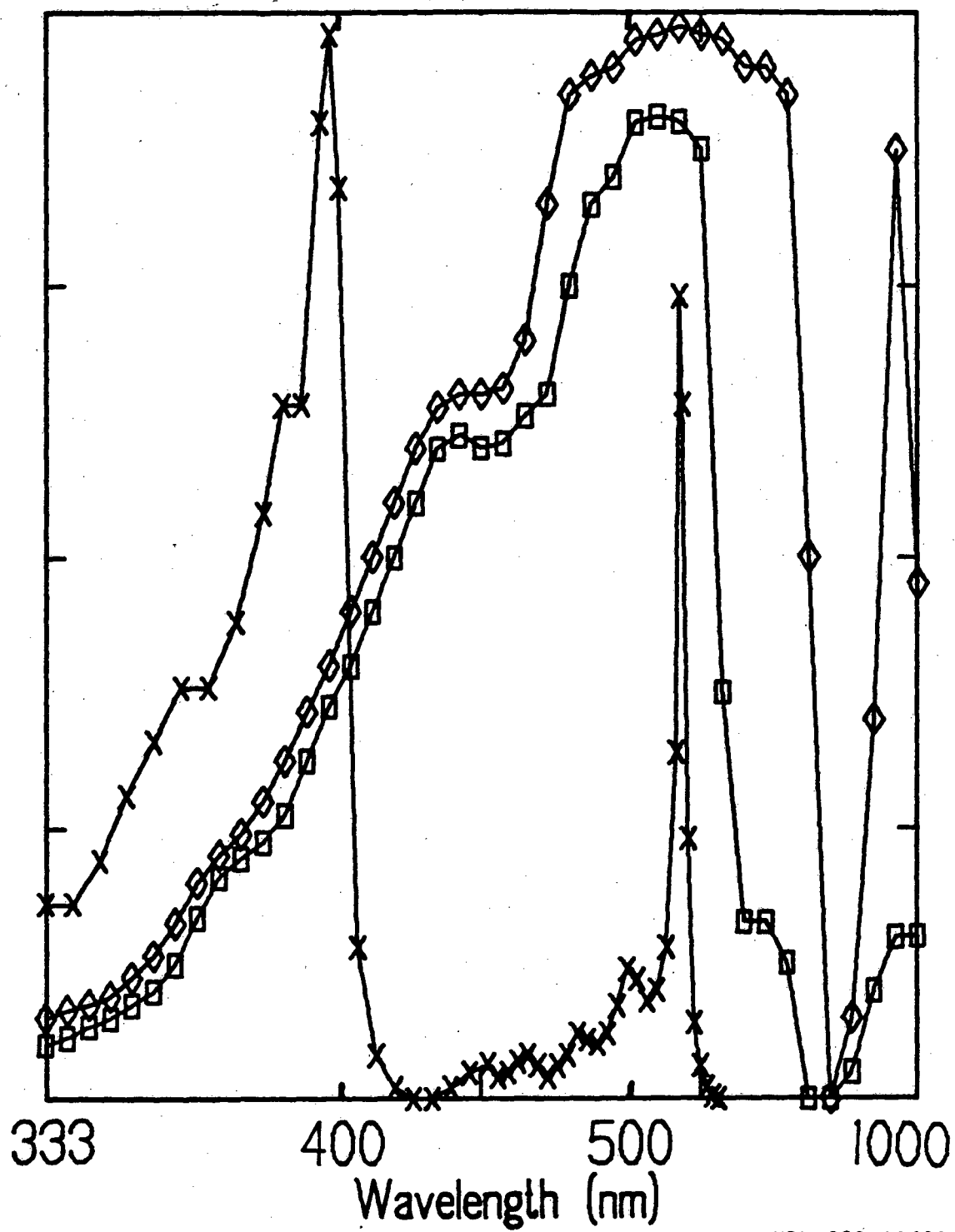
Schematic representations of the electron transport chains of chloroplasts (top scheme) and of mitochondria (lower scheme). Both chains span the free energy difference between the $\text{H}_2\text{O} - \text{O}_2$ and the $\text{NAD(P)}^+ - \text{NAD(P)H}$ redox couples. The light arrows in Figure 1 indicate the direction of electron flow through the chains. In mitochondria, the free energy differences between the two redox couples is negative, corresponding to a spontaneous flow of electrons from NADH to O_2 . In chloroplasts, the free energy difference between the two redox couples is positive, corresponding to a nonspontaneous flow of electrons from H_2O to NADP^+ . The energy which drives the flow of electrons in the photosynthetic chain is provided by sunlight, which is absorbed by chlorophylls and accessory pigments associated with photosystems I and II. This photoexcitation is transferred to special chlorophyll molecules in the reaction center which are able to undergo charge separation, represented by heavy arrows in Figure 1. Figure 1 illustrates the similarities in composition of the two chains. Both contain: a NAD(P)H oxidase group which contains a flavoprotein and both soluble and membrane-bound iron sulfur proteins; a quinone-cytochrome-Rieske iron sulfur protein oxidoreductase group; and a metal-cytochrome group involved with the oxidation or reduction of water.

Legend

FeS = Iron sulfur protein
 FP = flavoprotein
 cyt = cytochrome
 Q = quinone
 PQ = plastoquinone
 Pheo = pheophytin
 PS = photosystem

reaction and is represented by the heavy arrows in Figure 1. Thus plants utilize sunlight rather than oxygen as their fundamental energy source. For higher animals, the ultimate initiating substrate is O_2 . The first part of the answer to the question, why are plants green?, is thus that the light reactions of photosynthesis are necessary, because the oxidation of water to yield O_2 is not a spontaneous process. Moreover, the O_2 released by photosynthesis is the energy source for respiration.

Why are the assemblies of chlorophyll in the charge transport chain so large, that is, why are there more than simply the two reaction center chlorophylls which undergo oxidation? Figure 2 shows the spectrum of sunlight which reaches the surface of the earth without being absorbed by the atmosphere ($\diamond$) and the spectrum of light which is absorbed by the leaf of a plant ($\square$) [2]. There is remarkable agreement between these two spectra: the plant can absorb all the sunlight available to it. The absorption spectrum of chlorophyll in vitro, shown in Figure 2 (X) does not match the spectrum of sunlight very well, nor does the absorption of any single chlorophyll species in the leaf [3]. The second part of the answer to the question, why are plants green?, is thus that plants mimic the spectrum of sunlight. In order to do so, plants need more than a single kind of chlorophyll; it needs chlorophyll in different protein environments with different absorption spectra, plus some "accessory" pigments, such as carotenoids, which absorb where chlorophyll does not. These assemblies of pigments are called light-harvesting and antenna proteins [3]. Of course, if these species could not transfer their excitation to the reaction center, the light absorbed by them could not be converted to usable free energy. The details of the mechanism of energy transport from sunlight to the reaction center via light-harvesting and antenna pigments are therefore



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Figure 2

The spectra of solar energy incident on the Earth's surface ($\diamond$) [2], of light absorbed by a fig leaf ($\square$) [2], and of light absorbed by chlorophyll a in vitro ($\times$).

crucial for the understanding of the efficiency of photosynthesis. However, we mention these aspects of the harvesting of sunlight in plants here, only to put the reaction center in its proper context, and to do more than that takes us beyond the scope of this thesis.

The broader focus of the work presented in this thesis has been the constitution of the photosynthetic electron transport chain. That is, we are addressing the very specific questions: what is the mechanism of primary charge separation? How many early electron acceptors are there, and what are their chemical identities?

3. Composition of Photosynthetic Reaction Centers

The composition of the electron transport chain and the details of the light reaction in the simplest photosynthetic organisms, the prokaryotic purple bacteria, is fairly well established in detail [4]. Although each organism has to some extent its own special assembly of pigments and its own membrane morphology in order to establish an evolutionary niche for itself on earth, all purple bacteria share the same fundamental reaction center design. The primary donor is a "special pair" of bacteriochlorophylls called P870 in most organisms [5]; the earliest acceptor may be one of a second pair of bacteriochlorophylls [6]; the second acceptor is one of a pair of bacteriopheophytins [7]; and the next two acceptors are in a complex of two quinones with a single iron atom [8], called the iron-quinone complex. All of these components are housed within a reaction center protein complex which weighs roughly 100 kilograms per mole, and which is ubiquitous among the purple photosynthetic bacteria [4].

The reaction center composition of a second large group of simple photosynthetic prokaryotes, the green bacteria, has only recently been

studied [9]. This reaction center, which may also be ubiquitous among the green bacteria, also has a 'special pair' of bacteriochlorophyll molecules which functions as the primary electron donor. The earliest electron acceptors to be observed, which may not turn out to be the most primary acceptors, appear to be iron-sulfur proteins.

It has long been known [3] that the higher plants and algae differ from the photosynthetic prokaryotes in that they contain not one but two photosystems, denoted photosystem I and photosystem II. The two prokaryotic reaction centers we have discussed above are likely to be the evolutionary precursors of the two photosystems in higher plants. Recent evidence [10] overwhelmingly suggests that photosystem II is analogous to the purple bacterial reaction center: its donor is likely to be a chlorophyll monomer [10], pheophytin is known to function as an early acceptor [11] and it has been demonstrated to contain an iron-quinone complex [12]. Using the detailed knowledge about the purple bacterial reaction center, workers have been able to suggest experiments to perform on photosystem II which have so far given results affirming its analogy to the purple bacterial light reaction. The situation is reversed in the case of the analogy between photosystem I and the green bacterial reaction center: we know far more about the former than the latter, but the recent experiments [9] confirm the analogy so far.

The exact nature of the coupling between photosystem I and photosystem II in plants has been called into question lately, owing to recent advances in the understanding of chloroplast membrane organization [13,14] and the stoichiometry between photosystem I and photosystem II [15]. Whereas "the Z scheme" [16], requires a 1 : 1 stoichiometry between photosystem I and photosystem II, recent experiments fail to confirm this stoichiometry

[15,17]. Moreover, it is supposed [13] that oxygen evolution, which is associated with photosystem II, occurs chiefly in the granal thylakoids, whereas photosystem I appears to have a uniform density throughout the chloroplast lamellae [14], or to be located only in unstacked regions [17]. Therefore, there is some justification to study the two light reactions separately, as has in fact so far been done somewhat out of necessity, and to reserve questions about the exact nature of their coupling to one another for future investigations.

We henceforth confine ourselves to the primary reactions in photosystem I.

4. The Photosystem I Reaction Center

The physical structure of the photosystem I reaction center unit has been extensively studied during the last decade [18], and is evidently far more complicated than the purple bacterial reaction center. In contrast to the latter's small subunit housing six chromophores and the secondary acceptor, the most highly simplified photosystem I reaction centers isolated to date have contained about 20 chlorophylls [18], including P700. There have been reports of procedures for isolating reaction centers with 6 [19] or 10 [20] chlorophylls per P700, but there is reason to believe [18, 20] that these reaction centers are protein complexes of the same size as those which contain more chlorophylls, but from which chlorophyll molecules have been stripped. The excess chlorophylls in the reaction center protein complex almost certainly function as antenna for the photosystem I light reaction. The reaction center chlorophyll protein complex has been reported to have a molecular weight of between 70 and 200 kilograms per mole [18,20]. Most workers seem to think that the reaction center chlorophyll

protein contains only chlorophyll, i.e. that it does not contain any of the iron-containing species which function as early electron acceptors [18,20]. These are evidently contained in smaller subunits weighing from 12 to 40 kilograms per mole, which are also observed during sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS - PAGE) of highly purified photosystem I reaction centers [20]. Because the iron-containing species are known to be denatured by harsh detergents such as SDS [20], their presence in the reaction center chlorophyll protein complex as well as their relationship to the smaller subunits remain to be determined. All of these characteristics together suggest that the photosystem I reaction center is more complicated and differentiated than that of the purple bacteria and hence of photosystem II.

Workers for the most part have given up the idea that a reaction center as simple as the one isolable from purple bacteria will ever be isolated from higher plants and algae, i.e. that we will find a simple reaction center 'core' which contains only P700 and the early acceptors, like the one obtained from purple bacteria.

5. The Primary Reactions in Photosystem I

The current model of the photosystem I reaction center may be represented as $P700 \ A \ X \ Fd_B \ Fd_A$. We shall be discussing each of these species somewhat in detail. In order to facilitate what is necessarily a complicated presentation of data, we have compiled it all in one place, namely in Table 1. Items under discussion from Table 1 appear in parentheses within the following text.

Table 1

A summary of the photochemical reactions which have been reported for photosystem I reaction centers in various redox states. References to work which support the assignment of a rate to a particular process appear in brackets.

I. Dark State $P700\ A\ X\ Fd_B\ Fd_A$

Reaction #	Temp.	Light Reaction
		?
IA	room	$P700\ A\ X\ Fd_B\ Fd_A \longrightarrow P700^+ A^- X\ Fd_B\ Fd_A$ 200 ps [30]
IB	room	$P700\ A\ X\ Fd_B\ Fd_A \longrightarrow P700^+ A\ X^- Fd_B\ Fd_A$ <100 ns [25]
IC	room	$P700\ A\ X\ Fd_B\ Fd_A \longrightarrow P700^+ A\ X\ Fd_B\ Fd_A^-$
ID		30 ms [26a]
		?
IE	10 K	$P700\ A\ X\ Fd_B\ Fd_A \longrightarrow P700^+ A\ X\ Fd_B\ Fd_A^-$
IF	10 K	irreversible [22]

Table 1 (cont'd)

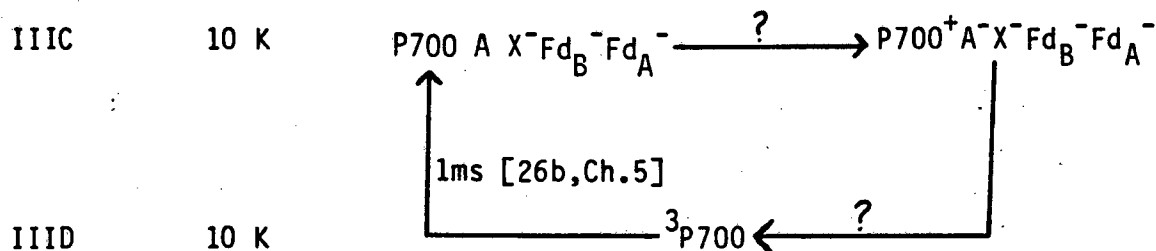
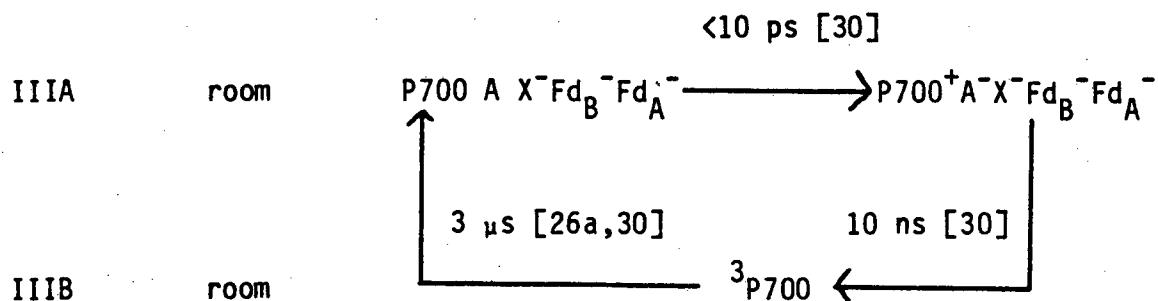
II. Dark State $P700^+ A^- X^- Fd_B^- Fd_A^-$

Reaction #	Temp.	Light Reaction
IIA	room	$P700^+ A^- X^- Fd_B^- Fd_A^- \xrightarrow{?} P700^+ A^- X^- Fd_B^- Fd_A^-$
IIB	room	$P700^+ A^- X^- Fd_B^- Fd_A^- \xrightarrow{10 \text{ ns [30]}} P700^+ A^- X^- Fd_B^- Fd_A^-$
IIC	room	$P700^+ A^- X^- Fd_B^- Fd_A^- \xrightarrow{10 \text{ ns [30]}} P700^+ A^- X^- Fd_B^- Fd_A^-$
IID	room	$P700^+ A^- X^- Fd_B^- Fd_A^- \xrightarrow{250 \text{ μs [26a]}} P700^+ A^- X^- Fd_B^- Fd_A^-$
IIE	10 K	$P700^+ A^- X^- Fd_B^- Fd_A^- \xrightarrow{?} P700^+ A^- X^- Fd_B^- Fd_A^-$
IIF	10 K	$P700^+ A^- X^- Fd_B^- Fd_A^- \xrightarrow{(1ms [26b, 36, Chs. 4&5])} P700^+ A^- X^- Fd_B^- Fd_A^-$
IIG	10 K	$P700^+ A^- X^- Fd_B^- Fd_A^- \xrightarrow{10 \text{ ns [30]}} P700^+ A^- X^- Fd_B^- Fd_A^-$
IIH	10 K	$P700^+ A^- X^- Fd_B^- Fd_A^- \xrightarrow{120 \text{ ms [29]}} P700^+ A^- X^- Fd_B^- Fd_A^-$

Table 1 (cont'd)

III. Dark State P700 A X⁻Fd_B⁻Fd_A⁻

Reaction #	Temp.	Light Reaction
------------	-------	----------------



The Primary donor, P700, and Secondary Acceptors, Centers A and B

P700 is the 'special pair' of chlorophylls which functions as the primary electron donor in photosystem I [3, cf. ref 21, which presents evidence that P700 is not a dimer]; and Fd_B and Fd_A are iron-sulfur centers [22] which function as secondary electron acceptors. P700 and Centers A and B were observed fairly early for a good reason, which is that it is fairly easy to observe them. The oxidation of the reaction center chlorophyll in the light is accompanied both by changes in its optical absorption spectrum around 700 nm and in the Soret [3] as well as in the near infrared region [23], and by the appearance of electron paramagnetic resonance (EPR) arising from the unpaired electron left on the P700 cation, which has been called Signal I [24]. The reduction of iron-sulfur proteins is also associated with optical absorption changes around 430 nm [25], and with EPR signals observable at temperatures below about 25 K [22].

Another reason that these spectroscopic characteristics can be observed easily is that at room temperature the charge separation between P700 and Center A is long-lived, about 30 ms in isolated reaction centers [26a] (Reaction ID), so that the light-induced absorption changes accompanying this charge separation may be observed under conditions of steady-state measurement at room temperatures. At temperatures below about 100 K, the light-induced charge separation between P700 and Center A is irreversible [22] (Reaction IF), again making it easy to observe light-induced changes.

Component X

Evidence that the transfer of electrons from P700 to Centers A and B involves at least two intermediate acceptors was not so easy to obtain.

McIntosh et al. [27] and Evans et al. [28] observed EPR signals at liquid-helium temperatures from photooxidized P700 and a photoreduced acceptor, denoted X, during continuous illumination of samples in which Centers A and B had been chemically reduced prior to illumination. In samples in this redox state, the EPR signals from $P700^+$ and X^- are not observed in the absence of illumination. Thus it was concluded [27, 28] that the light-induced charge separation between P700 and X is reversible down to temperatures as low as 5 K. Sauer et al. [26b] and Shuvalov et al. [29] observed that photoinduced charge separation decays biphasically in samples in which Centers A and B are reduced but X is not reduced prior to illumination. The slower phase, which was determined to have a halftime of 120 ms at 5 K [29] (250 μ s at room temperature [26a]), was attributed to charge recombination between P700 cation and X anion (Reactions II H and D, respectively). The faster phase, having a halftime of 1 ms at 5 K (3 μ s at room temperature), will be discussed below. In order to assign the 120 ms component of $P700^+$ decay to charge recombination with X^- , Shuvalov et al. [29] measured the decay kinetics of the EPR signal arising from X^- . A halftime of 120 ms at 5 K was obtained from a difficult measurement in which events of 2500 flashes were averaged. However, this decay time is in complete agreement with the halftime observed in the decay of P700 changes [29], allowing the confident assignment of this halftime to the P700 cation/X anion charge recombination (Reaction IIH).

X^- may also be observed in the dark at liquid helium temperatures if samples are illuminated during freezing in the presence of good electron donors prior to EPR measurements. That is, X may be phototrapped in its reduced state so that the reaction centers are prepared in the state $P700 X^- Fd_B^- Fd_A^-$. Figure 3 shows the EPR spectrum which is observed at 10 K

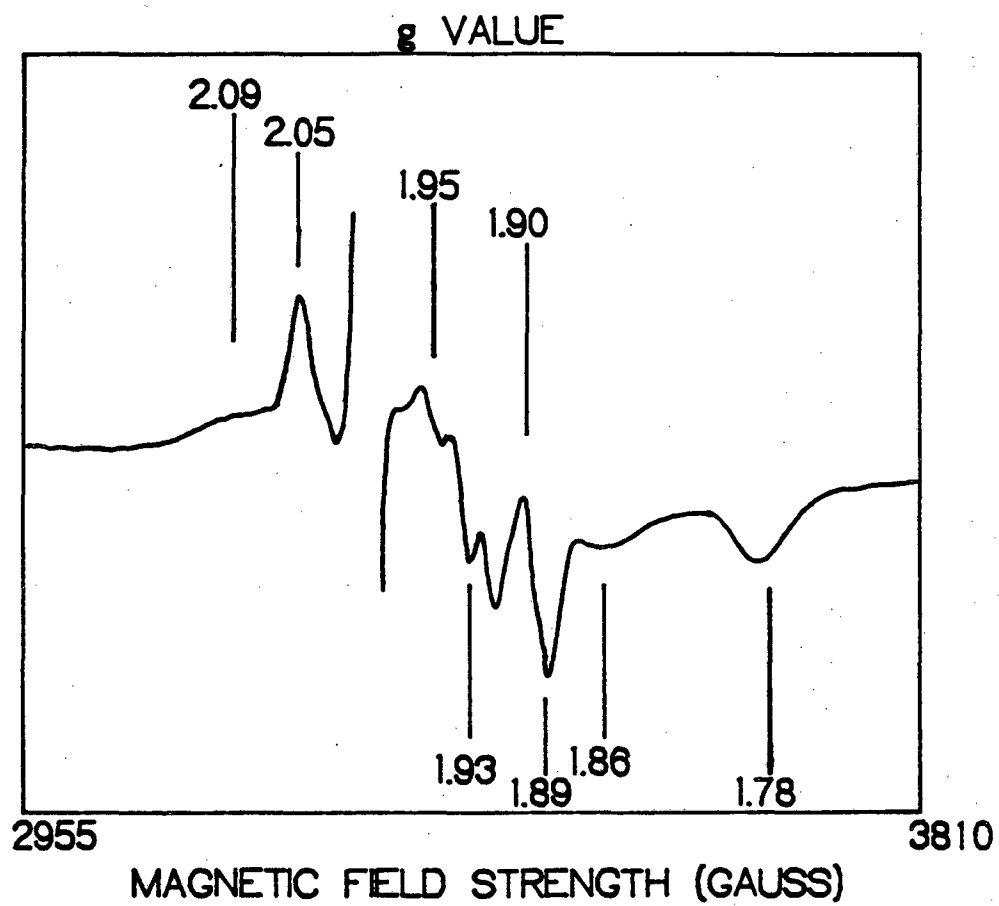


Figure 3

EPR spectrum recorded in the dark at 10 Kelvin in a sample of photosystem I reaction centers which had been prepared in the redox state $P700AX^-Fd_B^-Fd_A^-$ prior to measurement. Assignments of spectral features to Centers A and B and component X are given in Table 2. Spectrometer conditions: 12,500 gain, 20 Gauss modulation amplitude, 100 kHz modulation frequency, 20 mW microwave power, 9.16 GHz microwave frequency, 250 Gauss min^{-1} scan rate.

Table 2

Principle g values assigned to membrane-bound iron sulfur Centers A and B and Component X.

Component	g_z	g_y	g_x
X^-	2.09	1.90	1.78
Fd_B^-	2.05	1.93	1.89
Fd_A^-	2.05	1.95	(1.86) ^a

^anot visible in Figure 3. Feature at $g = 1.86$ in Figure 3 is part of the g_y signal of X^- .

when X , Fd_B and Fd_A are reduced, and Table 2 shows the assignment which has been made for each of the spectral features in Figure 3. The EPR spectrum of X^- indicates that it is an iron-containing species but does not fit easily into any category of spectra from biological iron species [28] and is difficult to observe in practice. The EPR signals of Centers A and B, as well as those of most iron-sulfur proteins, are microwave-power saturated at roughly 30 mW [22] at 10 K, whereas that of X^- is not saturated at 100 mW power [28]. Moreover, EPR signals of most iron-sulfur proteins may be observed up to about 30 K without too much difficulty (i.e., poor signal/noise), whereas X^- may be observed only below about 10 K [28], and then usually with poor signal amplitude. What makes X^- easily observable via EPR is that it has atypical g tensor anisotropy for an iron-sulfur protein: as shown in Figure 3 and Table 2, two of the principal g values are distinct from those arising from the other membrane-bound ferredoxins and are, therefore, not convoluted with the signals from Centers A and B. The saturation characteristics of the X^- EPR signal, as well as its g tensor anisotropy, have suggested to some [28] that the signal may arise from a species similar to the iron-quinone complex of bacteria, which has EPR properties similar to those of X^- , rather than from an anomalous iron-sulfur center.

In the Chapters Three through Five, the EPR spectra of the iron-containing species in photosystem I will be routinely presented as an assay of the redox state of photosystem I.

The Intermediate Acceptor, A

Evidence that X is not the only acceptor was obtained first from studies of the kinetics, in the time regime of 1 μ s to about 10 ms, of

optical absorption changes by Sauer et al. [26a,26b], and of optical and EPR changes by Shuvalov et al. [29,30]. When X has been phototrapped in its reduced state, so that the reaction centers are in the state P700 X⁻ prior to kinetics measurements, both groups observed that P700 is oxidized by a laser flash, and it returns to the ground state during the dark period following the flash with a halftime of 1 ms at 5 K (3 μ s at room temperature)(Reactions III B and D). The oxidation of P700 by a laser flash under these redox conditions thus provided evidence for the existence of an earlier acceptor than X or Centers A and B (Reaction IIA).

Both groups noted the similarity between the decay rates of P700 changes in this redox state and the fast component of the biphasic decay when X is not reduced prior to flash illumination (Reactions II B and F). An explanation for the puzzling observation of biphasic kinetics when Centers A and B are reduced but X is not reduced prior to illumination is suggested by the experiments presented in Chapter 4 of this thesis, and is discussed in detail there.

While these studies provided evidence for the existence of a new acceptor, denoted A, they also raised the issue of the mechanism of recovery of P700⁺ to the ground (singlet) state, i.e. of what physical processes give rise to the 1 ms decay at 5 K (3 μ s at room temperatures) of P700 changes. Before we address this question, it is useful to emphasize what is actually being measured in the kinetics measurements we have discussed so far, and why these measurements are difficult compared to the steady-state measurements mentioned above. A first point is that the assay for charge separation in the kinetics experiments was the observation either of optical absorption changes or of EPR changes arising from the photooxidation of P700. In these experiments one is often forced to make

the assumption that changes observed from P700 can be correlated to changes in its reaction partner, because the latter changes may not themselves be easily observable or, if observable, may not be easily deconvoluted from P700 changes. All that one can say with confidence is that the decay kinetics of P700 observed during optical kinetics experiments reflect the return of P700 to the ground state; they do not say much about any intermediate state that P700 may pass through in addition to the cationic state, so long as the intermediate state has a different absorption spectrum from that of P700. This point has caused some confusion, and has only recently begun to be appreciated [23]. We will address this point in Chapter 3.

A second point is that if significant spectral changes are occurring on a time scale which is much faster than 1 μ s, they must of course be observed with instrumentation capable of higher time resolution than that used in the experiments of Sauer et al. [26a,26b] or that used, at first, by Shuvalov et al. [29,30]. Even before more highly time-resolved experiments had been reported, there was reason to doubt that the 1 ms halftime at 5 K (3 μ s at room temperature) could be attributed to the P700⁺A⁻ radical pair lifetime. The P870-bacteriopheophytin radical pair in bacterial reaction centers was known from optical measurements to have a temperature-independent lifetime of 10 ns [31] under analogous reaction conditions, i.e., when forward photochemistry is blocked and the radical pair lifetime is the charge recombination time rather than a forward electron transfer time. It was thought [32] that a 1 ms halftime for the corresponding reaction in photosystem I, being five orders of magnitude longer, was unreasonable. Subsequent optical experiments in the 1 ps to about 100 ns time regime have confirmed this intuition. Shuvalov et al.

[30] observed at room temperature a biphasic decay of optical changes attributed to $P700^+ A^-$ radical pair formation when reaction centers were prepared in the redox state $P700 X^-$ prior to illumination. They assigned a 10 nsec component at 694 nm to the recovery of changes in the spectrum of A; the remaining change decayed with a halftime of 3 μ s (Reaction III B). These kinetics are compatible with the idea that charge recombination occurs within photosystem I with a halftime of 10 ns, just as in bacteria; the state which charge recombination populates is however not the ground state, otherwise P700 changes would also decay with this halftime. Studies of the temperature dependence of these kinetics have not been reported. Again making the analogy to the bacterial light reaction, where the radical pair (P^R) state populates the triplet state of the primary donor (the P^R triplet) as an intermediate in the backreaction between P870 and bacteriopheophytin, one might predict that the backreaction between P700 and A would populate 3P700 . We [33] obtained the first direct evidence that this is in fact the case.

We come finally to the real focus of this thesis. We observed the light-induced EPR signal from 3P700 in chloroplasts and photosystem I subchloroplasts prepared in the redox state $P700 A X^- Fd_B^- Fd_A^-$. These experiments are presented in Chapter 3 of this thesis. These experiments establish that the triplet state of P700 is at least sometimes populated as an intermediate in the backreaction between $P700^+$ and A^- , and so they suggest, but by no means determine, that the 1 ms (3 μ s) decay of P700 optical and EPR changes may correspond to the lifetime of the P700 triplet state (Reactions III B and D). In order to determine this point, we needed to make direct measurements of the P700 triplet state lifetime. We studied the kinetics of the triplet state of P700 by looking at its EPR signal

kinetics. These results are presented in Chapter 5. However, in order to extract the P700 triplet lifetime from the EPR signal kinetics data, it was first necessary to develop the existing theory of EPR triplet signal dynamics, and this theoretical development is presented in Chapter 2, along with some background material regarding triplet state energetics and dynamics.

The Forward Photochemical Reaction Rates

In order to complete our discussion about Table 1, we need to discuss the forward photochemical reactions represented by Reactions IA,B,C and E, by Reactions II A,C,E and G, and by Reactions III A and C. Very little is known for certain about most of these rates, as is indicated by the number of question marks in Table 1. However, it is fairly clear from the work of Shuvalov et al. [30] that the charge separation between P700 and A occurs in less than 10 ps, and that under conditions in which Centers A and B are not reduced prior to illumination, the optical changes due to the photoreduction of A reverse in 200 ps. This suggests that forward electron transfer to X occurs at that time (Reaction IB). However, when Centers A and B are reduced prior to measurement, the reversal time of changes due to A- slows down to 10 ns, regardless of whether X is reduced prior to measurement [30]. These results suggest that both the forward electron transfer time from A⁻ to X (Reactions IIC and G) and the radical pair P700⁺A⁻ lifetime (Reactions IIB, IIF, IIIB and IIID) each have roughly 10 ns halftimes.

6. The Significance of Chlorophyll Triplet States

Before leaving this general Introduction about photosynthesis, it is

useful to stand back and look at the focus of this thesis in its larger context. Given that photosynthesis itself has, as we showed above, fundamental significance to humanity, what possible meaning do chlorophyll triplet states have within the context of normal photosynthesis, much less in mutilated chloroplasts at 10 Kelvin? The meaning is twofold. First, the triplet state may have a physiological role as a photoprotector. What happens if the reaction center is closed, i.e. cannot initiate charge separation after excitation? The forward photosystem I turnover rate is so efficient that under physiological conditions the reaction center would probably only be closed by very high sunlight intensities. This is a condition which is potentially damaging to the plant, i.e., one which may cause irreversible photodegradation. The plant must have some efficient means of dissipating excess light energy. One way would be fluorescence, but it has long been known [34] that photosystem I does not fluoresce significantly at room temperature. Another way the plant can avoid irreversible photodamage to chlorophyll is if the latter can undergo a transition from the photoexcited singlet states to triplet states. Not only is this a way for chlorophyll to dissipate energy it has absorbed, but it is photoprotected while it is in the triplet state, because the triplet state has insignificant absorption in the regions where sunlight has its highest intensities [2]. Because the absorption of light is very fast (on the order of 10^{-15} s) and the population of the triplet state by intersystem crossing is also fast (1-10 ns)[35], the long triplet lifetime (.1-1 ms [36, Chapter Five]) makes it possible for chlorophyll to spend much of its time in a state in which it is transparent to sunlight. We can use our understanding of photoprotection in plants to advance solar energy technology, for which a major stumbling block is irreversible

photoprocesses.

Secondly, aside from their possible physiological importance, triplet states are important as an assay for charge separation. Again, because the P700 triplet is so long-lived, from the observation of it using a standard EPR spectrometer, we can deduce the occurrence of charge - separation and charge - recombination processes that occur on a timescale (1 - 10 ns) which is much faster than the limiting instrument response time (about 50 ns with a cavity Q of 5000). If we try to remove various acceptors or to reduce them, we can say with confidence that we have not fully reduced the primary acceptor as long as we see the P700 radical pair triplet. We used this argument in our interpretation of the results in Chapter 4. Indeed, it would seem possible to use this method to deduce the number of acceptors which mediate the transfer of electrons from P700 to X and Centers A and B, which was in fact the initial motivation behind the work presented in Chapter Four of this thesis. However, this brings us to what has turned out to be the serious limitation of the P700 triplet EPR experiments, namely that, as Dutton said with regard to the bacterial P^R EPR triplet which he helped to discover [37], "We always see it"[38]. This statement means that the P700 triplet populated by a back reaction between P700 and A would not be distinguishable from that populated by any backreaction between P700 and an acceptor preceding A, assuming that these backreactions both occur on a timescale much faster than the EPR response time. Thus the observation of the triplet state of P700 alone will never prove the existence of an acceptor earlier than A; for this we also need to establish the redox state of A prior to illumination. This was attempted for some of the work presented in Chapter 4, which unfortunately gave ambiguous or negative results.

We are however confident to depart from this general Introduction with the statement that the experiments presented in this thesis constitute a nearly complete description of the characteristics of triplet states in photosystem I.

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CHAPTER TWO

TRIPLET STATE EPR SIGNAL DYNAMICS

1. Introduction

For a system of molecules such as chlorophylls for which the ground state is a singlet state, the occupation of triplet states is normally a dynamic process. Population of triplet states occurs when the system is perturbed by chemical or photochemical processes and, in the case where these do not result in irreversible chemistry, the system always returns eventually to the ground singlet state. Triplet state dynamics are thus determined by theoretical analyses of the triplet state population and depopulation mechanisms, and by experimental studies of the corresponding rates. The purpose of this chapter is to describe the theory of triplet state energetics and dynamics, and to describe the triplet state EPR signal.

We shall start out at a fundamental level, by giving a quantum mechanical description of the triplet state.

2. The Quantum Mechanical Origin of Singlet and Triplet Electronic States

The division of the states of e.g. chlorophyll molecules into two manifolds, the singlet and the triplet, arises from a super selection rule which is the basis of the division of elementary particles into fermions and bosons. Singlets and triplets are two-particle states, that is, the simplest case of Fermi statistics.

A super selection rule for two indistinguishable particles may be stated thus: the two-particle state must be either symmetric or antisymmetric to the exchange of the two particles. A mathematical representation of the rule starts with the definition of an operator $\underline{X}$ which exchanges the coordinates of the two particles, i.e.

$$(1) \quad \underline{X} \psi(1,2) = \psi(2,1)$$

where $\psi(1,2)$ is the two-particle state. Then, because $\underline{X}$ commutes with the two-particle Hamiltonian, $\underline{H}(1,2)$, i.e. because,

$$(2) \quad [\underline{X}, \underline{H}(1,2)] = 0$$

it is clear that $\psi(1,2)$ must also be an eigenstate of $\underline{X}$, i.e. that

$$(3) \quad \underline{X} \psi(1,2) = \lambda \psi(1,2)$$

and, finally, that $\lambda = \pm 1$, because

$$(4) \quad \underline{X}^2 \psi(1,2) = \lambda^2 \psi(1,2) = \psi(1,2).$$

We now include the parity symmetry explicitly by writing the wavefunction as $\psi_{\pm}(1,2)$, where

$$(5) \quad \underline{X} \psi_{\pm}(1,2) = \pm \psi_{\pm}(1,2)$$

The super selection rule is then that the two-particle state vector must

either be symmetric ($\lambda = +1$, "Bosons") or antisymmetric ($\lambda = -1$, "Fermions") to the exchange of the two particle coordinates, and can never be a superposition of symmetric and antisymmetric.

Thus the two-electron system is described by an antisymmetric state vector, $\psi_{-}(1,2)$. To see in detail the implications of the state vector antisymmetry, we shall first assume that a two-particle state vector may be written as the product of general spatial and the spin state vectors, $\psi_{\pm}(r_1 r_2)$ and $\chi_{\pm}(s_1 s_2)$, respectively. This means that we may only look at systems describable by a Hamiltonian that does not contain any operators that couple the spatial coordinates to the spin coordinates. After we look at such systems, we shall add the spin-orbit coupling and see how it contributes to triplet state dynamics. In the absence of spin-orbit coupling the antisymmetry of the $\psi_{-}(1,2)$ to the exchange of the two electrons requires that either the spatial or the spin wavefunction be antisymmetric, but not both. Thus we write $\psi_{-}(1,2)$ in the form

$$(6) \quad \psi_{-}(1,2) = \psi_{\pm}(r_1 r_2) \chi_{\mp}(s_1 s_2)$$

where (-) implies antisymmetry and (+) implies symmetry to exchange. Two-particle states described by a symmetric spatial function and an antisymmetric spin function are singlet states, whereas those described by antisymmetric spatial functions and symmetric spin functions are triplet states.

We now write the spatial function $\psi_{\pm}(r_1 r_2)$ in terms of single particle spatial vectors, ϕ_a and ϕ_b , in the following way,

$$(7) \quad \psi_{\pm}(r_1 r_2) = [\phi_a(r_1)\phi_b(r_2) \pm \phi_a(r_2)\phi_b(r_1)]$$

Only now are we prepared to make sense of triplet state energetics. First of all, it is clear that the antisymmetric linear combination in Eq. 7, i.e., the triplet spatial function, corresponds to what are generally called antibonding states, whereas the symmetric, singlet spatial function corresponds to bonding states. Now, we already know that bonding states and antibonding states have different energies. However, in order to show that they do, we consider only the orbital part of $\underline{H}(1,2)$ and denote it $\underline{H}(r_1 r_2)$. Then, because

$$(8) \quad \iint \psi_{\pm}(1,2) \underline{H}(r_1 r_2) \psi_{\pm}^*(r_1 r_2) dr_1 dr_2 = \iint \psi_{\pm}(r_1 r_2) \underline{H}(r_1 r_2) \psi_{\pm}^*(r_1 r_2) dr_1 dr_2$$

we can see that the singlet and triplet states are separable into two manifolds. Secondly, we know that these two manifolds will indeed have their own energetics, because we may write Eq. 8 in the form,

$$(9) \quad \iint \psi_{\pm}(r_1 r_2) \underline{H}(r_1 r_2) \psi_{\pm}^*(r_1 r_2) dr_1 dr_2 = E_1^{ab} + E_2^{ab} + K^{ab} \pm J^{ab}$$

where the $E_i^{ab} = (E_i^a + E_i^b)/2$, K^{ab} is the coulomb interaction energy, and J^{ab} is the exchange interaction energy. Thus the energy difference between $\psi_+(r_1 r_2)$ and $\psi_-(r_1 r_2)$ is $2J^{ab}$.

Finally, we note from Eq. 7 that the spatial function will vanish for the triplet state in which two electrons have identical spatial functions, i.e., for the case that $\phi_a = \phi_b$. That is, there is no triplet state T^0 which has the antisymmetric spatial function that corresponds to the symmetric spatial function of the ground singlet state S^0 .

From now on, we shall use a more convenient notation in which we write,

$$(10) \quad \psi_-(r_1 r_2) \chi_+(s_1 s_2) = |T^m\rangle$$

and $\psi_+(r_1 r_2) \chi_-(s_1 s_2) = |S^n\rangle$

where m and n are energy indices.

3. Singlet-Triplet Interconversion Dynamics

In the previous section, we assumed that the state vector could be written as the product of the space and spin vectors, as in Eq. 5. It is clear in that case that radiative transitions between singlet and triplet states are strictly forbidden, because the transition probabilities are given by

$$(11) \quad P(S^n \rightarrow T_i^m) = |\langle S^n | \underline{e}_i | T_i^m \rangle|^2$$

Eq. 11 is zero because, according to Eq. 8, the singlet and triplet spin states factor out and their dot product is zero.

However, in most organic molecules, there is a small amount of coupling between the spin and the orbital angular momenta, represented by the spin-orbit coupling Hamiltonian, so that the state vector may no longer be written as the product of the space and spin vectors. Rather, we write the states of the total Hamiltonian, $\underline{H}$, which now includes spin-orbit coupling, as

$$(12) \quad |S^\mu\rangle = \sum_n |S^n\rangle \langle S^n | S^\mu \rangle + \sum_m |T^m\rangle \langle T^m | S^\mu \rangle$$

$$|T^v\rangle = \sum_n |S^n\rangle \langle S^n | T^v \rangle + \sum_m |T^m\rangle \langle T^m | T^v \rangle$$

Although $\underline{H}$ is diagonal in the sense that

$$(13) \quad \langle S^\mu | \underline{H} | S^\mu \rangle = E_S^\mu \delta_{\mu', \mu}$$

and

$$\langle T^v | \underline{H} | T^v \rangle = E_T^v \delta_{v', v}$$

the S^μ and T^v manifolds are no longer separable, because

$$\langle S^\mu | \underline{H} | T^v \rangle \neq 0$$

We can also see by inspection of Eq. 12 that

$$(14) \quad \langle S^\mu | \underline{e}_r | T_i^v \rangle \neq 0$$

In summary, then, spin-orbit coupling perturbs the system so that radiative transitions are allowed between the perturbed quasi-stationary states of $\underline{H}$. Eqs. 12 and 14 have been used [1] to estimate the radiative lifetime of the lowest lying triplet state, T^1 , which can return to the ground state S^0 by emitting a photon. That is, the radiative lifetime of T^1 has been related to the matrix element of the dipole operator, $\underline{e}_r$, in Eq. 14.

For our purposes, it is equally important to understand the non-radiative intersystem crossing and internal conversion processes which populate S^1 and T^1 . The intersystem crossing process is generally represented phenomenologically by rate equations of the form

$$(15) \quad \frac{dN_i^1}{dt} = k_i N_S^1$$

$$\frac{dN_S^1}{dt} = -\sum_i k_i N_S^1$$

where N_i^1 is the population of T_i^1 and N_S^1 is the population of S^1 .

We now wish to justify these phenomenological rate equations by giving them a quantum mechanical interpretation. We know that we may write the time dependence of the state vector in terms of complex energies when there are irreversible processes like internal conversion, radiative decay and intersystem crossing going on, e.g. that we may write

$$(16) \quad \hat{E}_S^n = E_S^n + \frac{1}{2}i\Gamma^n$$

Suppose that the system is photoexcited from the ground state S^0 to one of the eigenstates S^n which has the complex energy $\hat{E}_S^n$. Then consider the case that Γ^n is Γ_{IC}^n , the internal conversion rate. The probability that the system is still in state S^n at time t is given by

$$(17) \quad |\langle S^n | t \rangle|^2 = |\langle S^n | 0 \rangle|^2 \exp(-\Gamma_{IC}^n t)$$

By agreeing to look only after times much longer than any of the $(\Gamma_{IC}^n)^{-1}$, we guarantee that we may describe the system by

$$(18) \quad |t\rangle = |S^1\rangle \exp(-i\hat{E}_S^1 t)$$

where the complex part of $\hat{E}_S^1$ is yet to be specified. We can write Eq. 18 because of Kasha's rule [2], which states that the emitting level of a given spin multiplicity is the lowest excited level of that multiplicity. Kasha's rule thus implies that the Γ_{IC}^n are much larger than the corresponding radiative rates.

We now write

$$(19) \quad \hat{E}_S^1 = E_S^1 + \frac{1}{2} i k_S^1$$

where $k_S^1 = \sum_n \sum_i k_i^n + k_D$. k_D is the sum of radiative and non-radiative decay rates from S^1 to S^0 , and the k_i^n are intersystem crossing rates to sublevel T_i^n . Then the probability that the system is in state S^1 at time t is given by

$$(20) \quad |\langle S^1 | t \rangle|^2 = \exp(-k_S^1 t)$$

In order to satisfy Eq. 15, the probability that the system is in the state T_i^n at time t must be given by

$$(21) \quad |\langle T_i^n | t \rangle|^2 = \frac{k_i^n}{k_S^1} [1 - \exp(-k_S^1 t)]$$

If we now define the quantum yield of intersystem crossing to level T_i^n as

$$(22) \quad \phi_i^n = \frac{k_i^n}{k_S^1}$$

we obtain

$$(23) \quad |\langle T_i^n | t \rangle|^2 = \phi_i^n (1 - \exp(-k_S^1 t))$$

The populations of the T_i^n for which $n \neq 1$ will undergo rapid internal conversion to T_i^1 by Kasha's rule. We assume that internal conversion conserves M_S , so that the probability that the system is in triplet sublevel i of T^1 at times t which are much longer than the internal

conversion times, is given by

$$(24) \quad |\langle T_i^1 | t \rangle|^2 = \phi_i (1 - \exp(-k_S^1 t))$$

where

$$(25) \quad \phi_i = \sum_n \phi_i^n$$

Our final result is therefore that a derivation of Eq. 15 from quantum mechanics results in expressions for the populations of the triplet spin sublevels which include the quantum yields explicitly. We shall use Eqs. 24 and 25 later on in Eq. 51, where we set up the rate equations for the magnetic resonance dynamics of T^1 .

However, the origin of the k_i in quantum mechanics is still rather a mystery. We may suppose that, just as the radiative decay rates correspond to the matrix elements of the transition dipole operator, e_r , the k_i correspond to the matrix elements of an intersystem crossing Hamiltonian. This is indeed the case [3].

The matrix elements of the intersystem crossing Hamiltonian given in [3] are extremely complicated, and so we shall not write them out here. We note however that they are functions of the position and momentum coordinates, and do not contain any dependence on the angular momenta [3]. Therefore, they do not see an externally applied magnetic field, that is

$$(26) \quad [H_{ISC}, H_{ZEE}] = 0$$

where $\underline{H}_{ISC}$ is the intersystem crossing Hamiltonian, and $\underline{H}_{ZEE}$ is the Zeeman interaction Hamiltonian given by

$$(27) \quad \underline{H}_{ZEE} = g_1 \beta \underline{S}_{1Z} |\vec{H}_0| + g_2 \beta \underline{S}_{2Z} |\vec{H}_0|$$

$\vec{H}_0$ is the external field, and Z is the direction of $\vec{H}_0$. Eq. 27 assumes that the two spins are isotropic. The implication of Eq. 26 is that the intersystem crossing processes will populate the zero field spin sublevels, regardless of whether we are detecting the triplet state via EPR, i.e. in an externally applied magnetic field. We shall see the significance of Eq. 26 following a discussion of triplet spin sublevel energetics, which we present next.

4. Triplet Spin Sublevel Energetics

To the Hamiltonian of Eq. 13, we must now add the dipolar interaction Hamiltonian, $\underline{H}_{DIP}$, which removes the degeneracy of the triplet spin sublevels at zero field. Pure singlet and triplet spin states are states of the coupled spin angular momentum operator, $\underline{S}$, where

$$(28) \quad \underline{S} = \underline{S}_1 + \underline{S}_2$$

The coupled spin states, $|S M_S\rangle$, are eigenstates of $\underline{S}^2$ and $\underline{S}_Z$, i.e.

$$\underline{S}^2 |S M_S\rangle = S^2 |S M_S\rangle$$

(29)

$$\underline{S}_Z |S M_S\rangle = M_S |S M_S\rangle$$

where $S = S_1 \pm S_2 = 1$ or 0 . The state $S = 1$ is threefold degenerate in M_S , i.e. M_S has three values, $M_S = 1, 0$ and -1 , whereas the state $S = 0$ is nondegenerate, with $M_S = 0$. The threefold degenerate state $S = 1$ is of course the triplet state, whereas the nondegenerate state is the singlet state. In terms of the notation we shall be using, and in terms of the individual spins, α and β , the triplet sublevels in Eq. 29 are given by

$$\begin{aligned} |1\ 1\rangle &= \alpha\alpha = T_1 \\ (30) \quad |1\ 0\rangle &= 2^{-1/2}(\alpha\beta + \beta\alpha) = T_0 \end{aligned}$$

$$|1\ -1\rangle = \beta\beta = T_{-1}$$

whereas the singlet spin state is

$$|0\ 0\rangle = 2^{-1/2}(\alpha\beta - \beta\alpha) = S$$

We note that these spin vectors have the proper symmetries with respect to the exchange of the electrons that is required by Eq. 6 above.

The dipolar Hamiltonian, $\underline{H}_{DIP}$, removes the degeneracy of the three triplet spin sublevels in Eq. 30, that is, results in the construction of three nondegenerate zero field spin sublevels, T_X , T_Y and T_Z . The form and action of $\underline{H}_{DIP}$ has been extensively described elsewhere [4,5], and so we

shall give only a cursory description here. $\underline{H}_{DIP}$ is an anisotropic operator of the form

$$(31) \quad \underline{H}_{DIP} = -X\underline{S}_X^2 - Y\underline{S}_Y^2 - Z\underline{S}_Z^2$$

where X, Y and Z are given by averages over the electronic wavefunction of the operators

$$(32) \quad X = g^2 \beta^2 \left\langle \frac{r^2 - 3x^2}{r^5} \right\rangle; \quad Y = g^2 \beta^2 \left\langle \frac{r^2 - 3y^2}{r^5} \right\rangle; \quad Z = g^2 \beta^2 \left\langle \frac{r^2 - 3z^2}{r^5} \right\rangle$$

where $r = r_1 - r_2$, and where $g = (g_1 + g_2)/2$. Clearly, $X + Y + Z = 0$, so that the system is overdetermined, and may be described in terms of two parameters, D and E , such that

$$(33) \quad \begin{aligned} D &= \frac{1}{2}(X + Y) - Z \\ E &= -\frac{1}{2}(X - Y) \end{aligned}$$

which are called the zero field splitting parameters. Then we define the states T_X , T_Y and T_Z , by

$$(34) \quad \underline{H}_{DIP}|T_X\rangle = X|T_X\rangle; \quad \underline{H}_{DIP}|T_Y\rangle = Y|T_Y\rangle; \quad \underline{H}_{DIP}|T_Z\rangle = Z|T_Z\rangle$$

Eqs. 33 and 34 yield the energy level diagram given in Figure 1 for the zero field spin state energetics.

5. The Triplet State EPR Spectrum

If we now apply an external magnetic field, we must add to the above

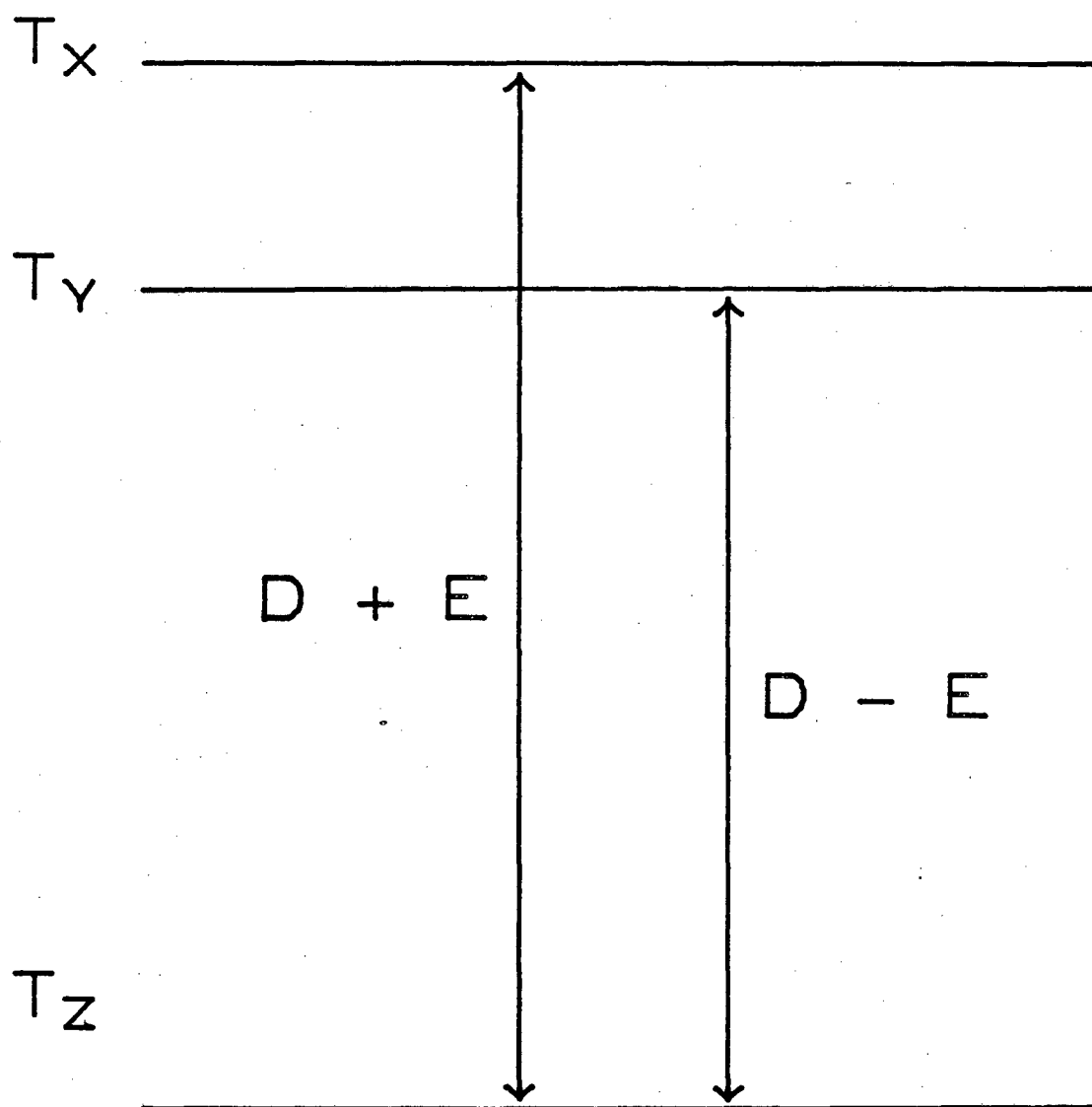
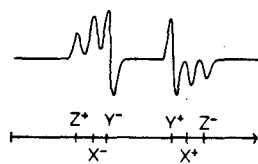
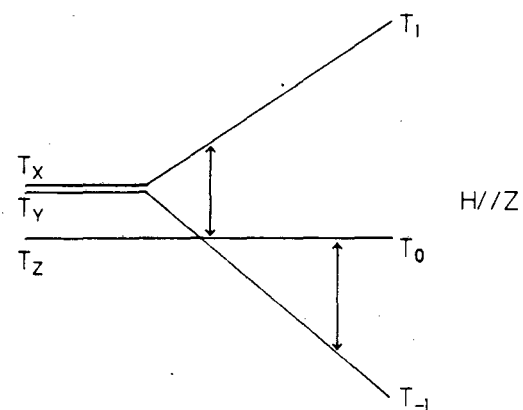
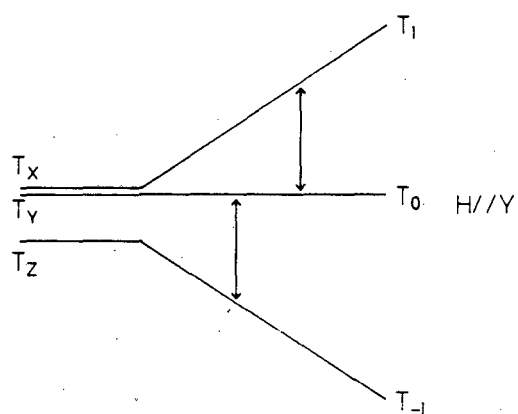
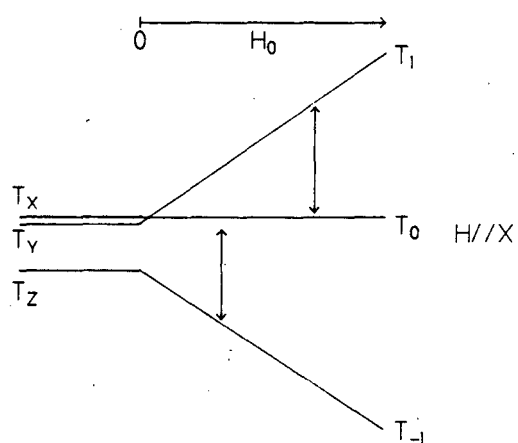


Figure 1

The zero field triplet spin sublevel energy diagram.

the Zeeman Hamiltonian of Eq. 27. Recall that the commutation properties of the spin angular momenta are represented as $[\underline{S}_i, \underline{S}_j] = i\epsilon_{ijk}\underline{S}_k$, where $\epsilon_{ijk} = 1$ for cyclic permutations, and $\epsilon_{ijk} = -1$ for anticyclic permutations. Then it is clear that $\underline{H}_{DIP}$ and $\underline{H}_{ZEE}$ do not commute, and therefore, that they cannot have simultaneous eigenstates. It is also clear from Eqs. 27 and 29 that the vectors of Eq. 30 are eigenstates of $\underline{H}_{ZEE}$ if we continue to assume that $g_1 = g_2 = g$, as we did when we wrote Eqs. 31 and 32. In the case of most organic molecules, the Zeeman interaction energies at X-band (corresponding to $\vec{H}_0 = 3400$ Gauss) are $10 - 10^2$ times as large as the dipolar splittings. Thus the high field eigenstates can usually be represented by the triplet spin vectors of Eq. 30, and the dipolar splittings are present in the EPR spectrum as an inhomogeneous broadening.

Figure 2 shows the dependence of the triplet spin states on the external magnetic field strength. The $\Delta M_S = 1$ EPR transitions are indicated by arrows in Figure 2: the arrows are all the same length because the EPR spectrometer operates at a fixed microwave frequency and the magnetic field is swept. We see that the magnetic field at which resonance will occur depends upon the angle that the magnetic field $\vec{H}_0$ makes with the molecular axis system defined by $\underline{H}_{DIP}$. We shall always be looking at systems which give rise to a powder spectrum in the dipolar interaction, and so we have included in Figure 2 the triplet state powder spectrum which one would observe in a spectrometer which uses field modulation for signal detection. Field modulation produces a first derivative of the microwave absorption spectrum [6]. As shown in Figure 2, extrema in the first derivative occur when $\vec{H}_0$ is parallel to the X and Z molecular axes, whereas the first derivative crosses zero when $\vec{H}_0$ is parallel to the Y molecular axis.



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Figure 2

A schematic representation of the dependence of the triplet spin sublevels on the strength of an applied magnetic field. T_X , T_Y and T_Z are the zero field eigenstates of the dipolar interaction Hamiltonian; T_1 , T_0 and T_{-1} are the eigenstates of the Zeeman interaction Hamiltonian; and $\vec{H}_0$ is the applied magnetic field. The three diagrams from top to bottom represent the energy levels when the external field $\vec{H}_0$ is oriented along the X, Y and Z molecular axes, respectively. The arrows indicate where $\Delta M_S = 1$ EPR transitions would take place in an EPR spectrometer which operates at a fixed microwave frequency and in which the field is swept. The lowest figure shows the EPR powder spectrum obtained when the triplet sublevels are populated according to the Boltzmann law, and when the signal is detected by means of magnetic field modulation. $X\pm$, $Y\pm$ and $Z\pm$ indicate the positions of these transitions with respect to the alignment of $\vec{H}_0$ along the molecular axes.

The zero field splitting parameters, $|D|$ and $|E|$ are calculated from experimental data by use of the following relations

$$\begin{aligned} H_Z^+ - H_Z^- &= 2|D| \\ (35) \quad H_X^+ - H_X^- &= |D| + 3|E| \\ H_Y^+ - H_Y^- &= |D| - 3|E| \end{aligned}$$

where $H_{i\pm}$ is the magnetic field position of the extrema or zero crossings indicated in Figure 2. These readings from spectral data give the parameters in Gauss. The parameters in cm^{-1} are usually reported, and are obtained by use of the conversion factor, $1 \text{ Gauss} = 0.93482 \times 10^{-4} \text{ cm}^{-1}$.

6. EPR Polarization

The powder spectrum shown in Figure 2 is the first derivative of the EPR absorption. That is, it corresponds to a Boltzmann distribution of the population of the triplet spin sublevels. As we mentioned in the Introduction to this chapter, the population of the triplet state is dynamic. Thus, the selectivity of population and depopulation rates, k_i and K_i , respectively, often results in polarized EPR signals, which reflect a non-Boltzmann population of the triplet spin sublevels. That is, one of the $\Delta M_S = 1$ transitions of the pair for each orientation of $\vec{H}_0$ is observed to be in emission and the other in absorption. We shall see this more clearly later, when we give triplet state kinetics a thorough examination. However, we will now describe the two population mechanisms which can result in EPR polarization.

The Intersystem Crossing Mechanism

The first mechanism we shall look at is the intersystem crossing mechanism, which we discussed in detail above. We said that intersystem crossing always populates the zero field spin sublevels, and we now know that this means the levels T_X , T_Y and T_Z . Thus the k_i correspond here to k_X , k_Y and k_Z . In terms of these zero field rates, the rates we shall observe at high field, i.e. k_1 , k_0 and k_{-1} , are

$$(36) \quad k_1 = \frac{1}{2}(k_i + k_j) = k_{-1} ; k_0 = k_k$$

when $\vec{H}_0$ is parallel to the k molecular axis. Here the i, j, k are permutations of X, Y and Z . We note that Eq. 36 results from the fact that

$$(37) \quad T_{\pm 1} = \frac{1}{2}(T_i \pm i T_j); \quad T_0 = T_k \quad \text{when } \vec{H}_0 \text{ is parallel to } k$$

If we assume the k_i 's to be all different, then the quantum yields of Eq. 24 will also be all different. The polarization of the triplet state EPR signals observed under the six possible permutations of $\phi_i, \phi_j > \phi_k$ are listed in Table 1.

We note that the EPR signals may also be polarized by selective depopulation of the triplet spin sublevels, as we shall see later on. The relations between the depopulation rates, K_i , and the highfield rates, K_1 , K_0 and K_{-1} are given by Eq. 36 if we let $k \rightarrow K$.

The Radical Pair Mechanism

We note that two polarization patterns which are not included in Table 1 are ae ae and its opposite, ea ea. It is easy to see by a

Table 1

Initial polarizations of triplet state EPR signals induced by selective populations of the zero field triplet spin sublevels. $X_{\pm}, Y_{\pm}$ and $Z_{\pm}$ indicate the positions of the high field and low field, respectively, $\Delta M_S = 1$ transitions when the external magnetic field is oriented along the X, Y or Z molecular axis (see Figure 2). e denotes the emission and a denotes the absorption of microwaves.

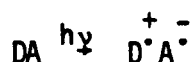
Z^-	X^-	Y^-	Y^+	X^+	Z^+	relative quantum yield
e	e	e	a	a	a	$\phi_X, \phi_Y > \phi_Z$
a	a	a	e	e	e	$\phi_X, \phi_Y < \phi_Z$
a	a	e	a	e	e	$\phi_Y, \phi_Z > \phi_X$
e	e	a	e	a	a	$\phi_Y, \phi_Z < \phi_X$
e	a	e	a	e	a	$\phi_X, \phi_Z > \phi_Y$
a	e	a	e	a	e	$\phi_X, \phi_Z < \phi_Y$

consideration of Figure 2 that these patterns are "high field" patterns, i.e. that ae ae corresponds to the case $\phi_0 > \phi_1$, ϕ_{-1} and ea ea corresponds to $\phi_0 < \phi_1$, ϕ_{-1} . Eq. 36 is inconsistent with either of these two patterns, so that neither of these patterns can result from intersystem crossing.

However, the polarization pattern ae ae is consistent with a mechanism that populates the T_0 level in excess of its population of the T_1 and T_{-1} levels. The radical pair mechanism does just that, and so the polarization pattern ae ae is called the radical pair polarization pattern. A triplet state EPR signal with this polarization pattern is called a radical pair polarized triplet signal.

The radical pair mechanism was first invoked by Levanon and Norris [7] to explain the observation by Dutton et al. [8] of a triplet EPR signal in photosynthetic purple bacteria which had the radical pair polarization pattern. The quantum mechanical description of this effect is, however, contained in the CIDEP (Chemically Induced Dynamic Electron Polarization) literature, where it was first elucidated by Adrian [9].

The model is as follows. A system consisting of an electron donor and an electron acceptor undergoes charge-separation following photoexcitation, which is represented as



The system is supposed to start out in a pure singlet spin state. However, this state is not an eigenstate of the radical pair spin Hamiltonian, H_{RP} . The radical pair spin Hamiltonian is given by

$$(38) \quad H_{RP} = g_1 \beta \hat{H}_0 |S_{1Z} + g_2 \beta \hat{H}_0 |S_{2Z} - J(2\mathbf{S}_1 \cdot \mathbf{S}_2 + \frac{1}{2})$$

For simplicity, we will neglect the hyperfine interaction, which leads to spin polarization at zero field.

The states of $\underline{H}_{RP}$ are given by the expressions

$$(39) \quad \underline{H}_{RP} |\pm^{RP}\rangle = \pm \omega |\pm^{RP}\rangle$$

where

$$\omega = (J^2 + \delta^2)^{1/2} \quad \text{and} \quad \delta = gH_0(g_1 - g_2)$$

and where

$$(40) \quad \begin{aligned} |+\text{RP}\rangle &= \left(\frac{\omega + J}{2\omega}\right)^{1/2} |S^{\text{RP}}\rangle + \left(\frac{\omega - J}{2\omega}\right)^{1/2} |T_0^{\text{RP}}\rangle \\ |-\text{RP}\rangle &= \left(\frac{\omega + J}{2\omega}\right)^{1/2} |T_0^{\text{RP}}\rangle - \left(\frac{\omega - J}{2\omega}\right)^{1/2} |S^{\text{RP}}\rangle \end{aligned}$$

Eqs. 39 and 40 assume that in an external magnetic field, the T_1 and T_{-1} states are not close enough to the singlet state for there to be any mixing between them. In the radical pair, the S - T_0 splitting, $2J$, is small enough that the coefficients in Eq. 40 may be large.

The Adrian [9] analysis considers that at time t , the state of the system is given by

$$(41) \quad |t\rangle = \exp(-i\omega t) |+\text{RP}\rangle \langle +\text{RP}| S^{\text{RP}}\rangle + \exp(i\omega t) |-\text{RP}\rangle \langle -\text{RP}| S^{\text{RP}}\rangle$$

so that the probability that the system is in state T_0 at time t is given

by

$$(42) \quad |\langle T_0^{RP} | t \rangle|^2 = \delta^2 \omega^{-2} \sin^2 \omega t$$

Clearly, the preparation of the system in a non-stationary state gives rise to quantum mechanical mixing between S and T_0 .

However, Eq. 42 predicts an oscillation of infinite duration, and for our purposes, we need the radical pair to undergo irreversible transitions to the molecular T_0 state of the donor, which we denote T_0^D . We can try to define complex energies as in Eq. 16, such that

$$(43) \quad \begin{aligned} \hat{E}_+ &= \omega + \frac{1}{2}i\Gamma_+ \\ \hat{E}_- &= -\omega + \frac{1}{2}i\Gamma_- \end{aligned}$$

where the Γ_+ and Γ_- are related to matrix elements of the radical pair recombination Hamiltonian, H_{RC} , i.e.

$$(44) \quad \Gamma_+ = |\langle + | H_{RC} | T_0^D \rangle|^2$$

$$\Gamma_- = |\langle - | H_{RC} | T_0^D \rangle|^2$$

Then Eq. 41 becomes

$$(45) \quad |t\rangle = \exp(-i\hat{E}_+ t) | +^{RP} \rangle \langle +^{RP} | S^{RP} \rangle + \exp(-i\hat{E}_- t) | -^{RP} \rangle \langle -^{RP} | S^{RP} \rangle$$

so that Eq. 42 becomes

$$(46) \quad |\langle T_0^{RP} | t \rangle|^2 = \frac{\delta^2}{2\omega^2} [\exp(-r_+ t) + \exp(-r_- t) - 2(\cos(2\omega t) \exp(-\frac{1}{2}(r_+ + r_-)t))]$$

The triplet population in Eq. 46 will be significant only when $\omega = \delta$, i.e., when $J < \delta$, in which case $r_+ = r_-$. In that case, we will obtain

$$(47) \quad |\langle T_0^{RP} | t \rangle|^2 = \frac{\delta^2}{\omega^2} \sin^2(\omega t) \exp(-rt)$$

Because $|\langle T_0^{RP} | t \rangle|^2 + |\langle S^{RP} | t \rangle|^2 + |\langle T_0^D | t \rangle|^2 = 1$, the decay of population in the radical pair triplet state, T_0^{RP} , will correspond to a buildup of population in the T_0^D molecular state of the donor.

Now that we have introduced the theoretical concepts of triplet state energetics and dynamics, we wish to develop a theory of triplet state photokinetics which we can use to analyze experimental results.

6. Theoretical Analysis of EPR Triplet Signal Dynamics

Figure 3 shows the model which has been used [11,12] for the analysis of triplet state photodynamics. In this model, molecules in the ground state, S^0 , are subjected to one of a variety of illumination regimes, such as continuous illumination, light modulation and square wave or laser pulse excitation. These illumination regimes are represented in Figure 3 by the general function $F(I,t)$. Illumination results in photoexcitation to higher singlet states, followed by rapid ($< 10^{-12}$ sec) [13] internal conversion to the lowest excited singlet state, S^1 . Molecules in state S^1 may return directly to the ground state, e.g. by fluorescence, with a rate k_D . They

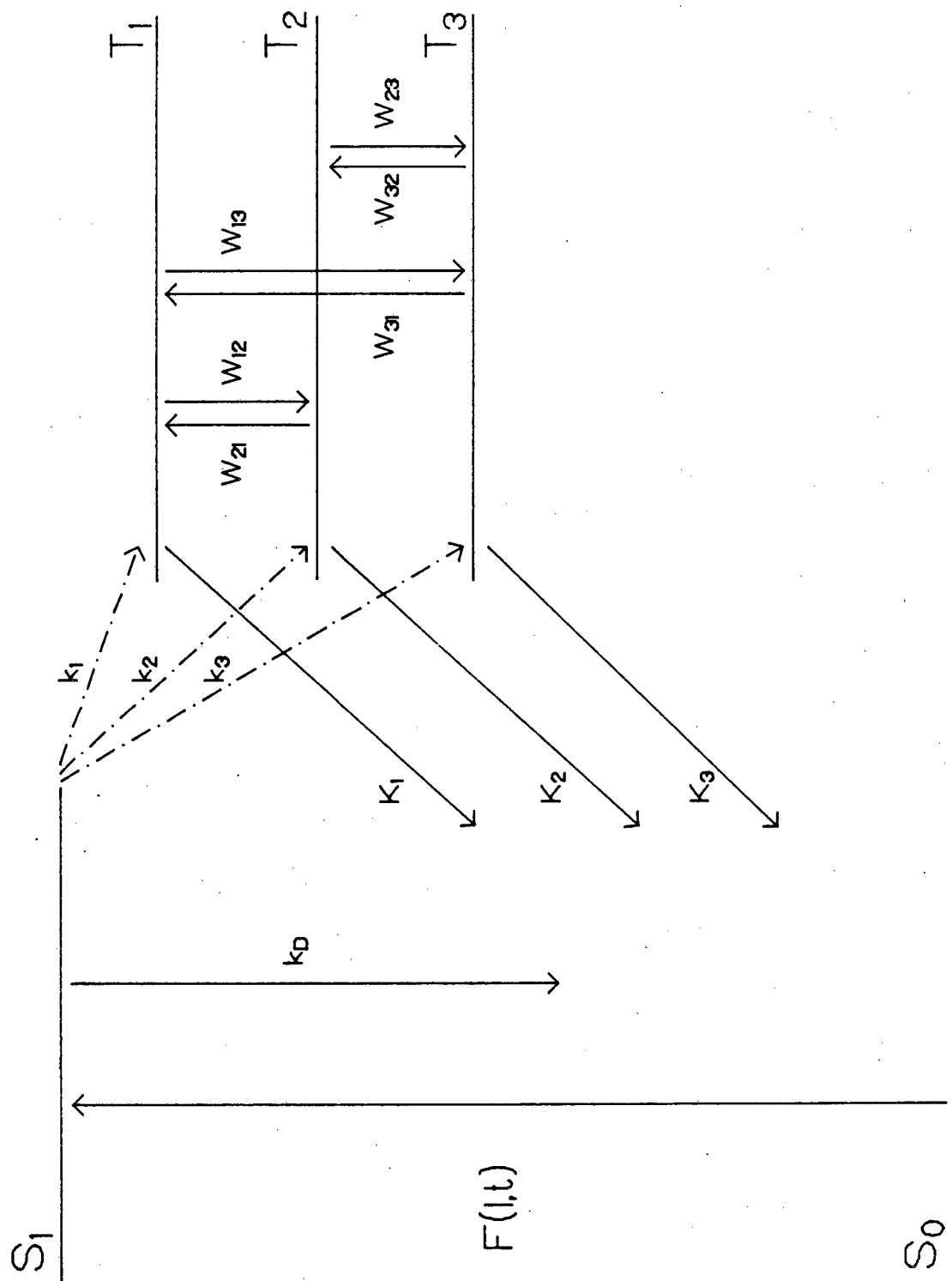


Figure 3

The model used for the analysis of triplet state photokinetics. S^0 is the singlet ground state; S^1 is the first singlet excited state; T^1 is the lowest lying triplet state; the T_i are the spin sublevels of T^1 ; $F(I,t)$ is a function describing the illumination conditions; k_D is the rate of decay of the population of S^1 directly to the ground state; k_i is the rate of population of T_i ; W_{ij} is the rate of spin lattice relaxation from T_i to T_j ; and K_i is the rate at which population in sublevel T_i returns to the ground state.

may also populate the triplet manifold by intersystem crossing [11-13] or radical pair [7-9] mechanisms, which is again followed by rapid internal conversion to the lowest lying triplet state T^1 . These processes result in the selective population of the triplet spin sublevels, T_i , and are represented in Figure 3 by the rates k_i . Because both population mechanisms result in a non-Boltzmann population of the T_i , spin lattice relaxation drives the population of the triplet state toward the Boltzmann population with the rates W_{ij} . Triplet molecules eventually return to S^0 with the sublevel decay rates, K_i .

Triplet State Rate Equations

The dynamics of the triplet state in Figure 3 are described by the vector equation:

$$(51) \quad \frac{d\vec{N}}{dt} = -\underline{K} \vec{N} - \underline{W} \vec{N} + \vec{\Phi} F(I,t) N_S^0$$

where $\vec{N}$ is the vector of triplet spin sublevels,

$$\vec{N} = \begin{bmatrix} N_1 \\ N_2 \\ N_3 \end{bmatrix}$$

$\underline{K}$ is the triplet decay rate matrix,

$$\underline{K} = \begin{bmatrix} K_1 & 0 & 0 \\ 0 & K_2 & 0 \\ 0 & 0 & K_3 \end{bmatrix}$$

$\underline{W}$ is the spin lattice relaxation rate matrix,

$$\underline{W} = \begin{bmatrix} W_{12} + W_{13} & -W_{21} & -W_{31} \\ -W_{21} & W_{21} + W_{23} & -W_{32} \\ -W_{13} & -W_{23} & W_{31} + W_{32} \end{bmatrix}$$

and $\vec{\phi}$ is the quantum yield vector we obtained in Eq. 24 above,

$$\vec{\phi} = \begin{bmatrix} \phi_1 \\ \phi_2 \\ \phi_3 \end{bmatrix} ;$$

where ϕ_i are defined in Eq. 25 above. We are assuming here that we can look at times greatly in excess of $(k_S^1)^{-1}$ in Eq. 24, so that ϕ_i is the initial population of T_i . N_S^0 is the number of spins in state S^0 ; and $F(I,t)$ is the function describing the conditions of illumination. For simplicity, we shall consider the case in which,

$$(52) \quad F(I,t)N_S^0 = N_E \delta(t - 0)$$

That is, we shall consider that $F(I,t)$ is a delta function laser flash delivered at time zero, which excites a number, N_E , of singlet molecules. Under these conditions, we expect there to be no dependence of the rates in $\underline{K}$ and $\underline{W}$ on the excitation light intensity [14].

Analysis of the Time Dependence of the Spin Lattice Relaxation Rates

We now wish to show that the spin lattice relaxation part, $\underline{W}$, is time dependent. The elements of the vector $\underline{WN}$ are of the form,

$$(53) \quad (\underline{W} \vec{N})_i = W_{ij}N_j - W_{ji}N_j + W_{ik}N_i - W_{ki}N_k$$

As usual, we may write the vector $\underline{W} \vec{N}$ in two equivalent ways. The first way, which is analogous to the method used in [11], is to make the usual definition of the Boltzmann population difference between two levels [15], i.e.,

$$(54) \quad N_{iB} - N_{jB} = \frac{(W_{ij} - W_{ji})}{(W_{ij} + W_{ji})} (N_i + N_j)$$

and then to write the elements of $\underline{W} \vec{N}$ in terms of

$$(55) \quad W_{ij}N_i - W_{ji}N_j = \frac{1}{2}(W_{ij} + W_{ji})(N_i - N_{iB} - (N_j - N_{jB}))$$

We now define spin lattice relaxation rate constants as follows,

$$(56a) \quad W_1 = \frac{1}{2}(W_{12} + W_{21}) = \frac{1}{2}(W_{32} + W_{23})$$

$$(56b) \quad W_2 = \frac{1}{2}(W_{13} + W_{31})$$

where W_1 is the $\Delta M_S = 1$ rate constant and W_2 is the $\Delta M_S = 2$ rate constant. It is possible to make a strict definition of a single rate constant for the two $\Delta M_S = 1$ relaxation processes, as we did in Eq. 56a, only in the case that the energy differences $E_1 - E_2$ and $E_2 - E_3$ are equal to one another. We discuss this in the Appendix. In terms of W_1 and W_2 , $\underline{W} \vec{N}$ is,

$$(57) \quad \underline{W} \vec{N} = \underline{W}_S(\vec{N} - \vec{N}_B)$$

where $\vec{N}_B$ is the vector of Boltzmann populations,

$$\vec{\hat{N}}_B = \begin{bmatrix} N_{1B} \\ N_{2B} \\ N_{3B} \end{bmatrix}$$

and where $\underline{W}_S$ is a symmetrical matrix of the form,

$$\underline{W}_S = \begin{bmatrix} W_1 + W_2 & -W_1 & -W_2 \\ -W_1 & 2W_1 & -W_1 \\ -W_2 & -W_1 & W_1 + W_2 \end{bmatrix}$$

It is clear from Eqs. 55 - 57 that $\vec{\hat{N}}_B$ is a time dependent vector, and therefore that the rate matrix, $\underline{W}$, in Eq. 51 is time dependent. However, the time dependence of $\vec{\hat{N}}_B$ was not taken into account in [11].

The second way, which is analogous to the method used in [12], is to define a parameter, α_{ij} , such that,

$$(58) \alpha_{ij} = \frac{(W_{ij} - W_{ji})}{(W_{ij} + W_{ji})} = \frac{(N_{iB} - N_{jB})}{(N_i + N_j)} \quad ; \text{ where } \alpha_{ij} = -\alpha_{ji}$$

and then to write the elements of $\underline{W} \vec{\hat{N}}$ in terms of,

$$(59) W_{ij}N_i - W_{ji}N_j = \frac{1}{2}(W_{ij} + W_{ji})(N_i(1 + \alpha_{ji}) - N_j(1 + \alpha_{ij}))$$

Thus, in terms of the parameters α_{ij} , $\underline{W} \vec{\hat{N}}$ is of the form,

$$(60) \quad \underline{W} \vec{\hat{N}} = (\underline{W}_S + \underline{W}_{NS})\vec{\hat{N}}$$

where $\underline{W}_S$ is defined in Eq. 57, and where $\underline{W}_{NS}$ is a non-symmetrical matrix of the form,

$$\underline{W}_{NS} = \begin{bmatrix} \alpha_{12}W_1 + \alpha_{13}W_2 & \alpha_{12}W_1 & \alpha_{13}W_2 \\ \alpha_{21}W_1 & (\alpha_{23} + \alpha_{21})W_1 & \alpha_{23}W_1 \\ \alpha_{31}W_2 & \alpha_{32}W_1 & \alpha_{32}W_1 + \alpha_{31}W_2 \end{bmatrix}$$

We see by inspection of Eq. 58 that the α_{ij} are time dependent, i.e. that $\underline{W}_{NS}$ is time dependent. However, the time dependence of the α_{ij} was not taken into account in [12].

Solution of the Rate Equations

Eq. 51 is easier to solve if we use Eq. 57 for $\underline{W}\hat{N}$. Thus, substituting Eq. 57 into Eq. 1, we obtain,

$$(61) \quad \frac{d\hat{N}}{dt} = -\underline{K}\hat{N} - \underline{W}_S(\hat{N} - \hat{N}_B) + \hat{\Phi} N_E \delta(t - 0)$$

Eq. 61 is a non-homogeneous linear equation of the form,

$$(62) \quad L(\hat{N}) = \underline{W}_S\hat{N}_B + \hat{\Phi} N_E \delta(t - 0)$$

According to the superposition principle [16], the solutions of Eq. 62 are of the form,

$$(63) \quad \hat{N} = \hat{N}_1 + \hat{N}_2$$

where $\hat{N}_1$ solves,

$$L(\hat{N}_1) = \hat{\Phi} N_E \delta(t - 0)$$

that is,

$$(64) \quad \vec{N}_1(t) = \exp((-W_S + K)t)(\vec{\Phi}N_E + \vec{C})$$

where $\vec{C}$ is a constant vector of integration; and $\vec{N}_2$ is any solution such that,

$$L(\vec{N}_2) = W_S \vec{N}_B$$

One such $\vec{N}_2$ is $\vec{N}_B$ itself. Thus, to solve Eq. 61, we first find the particular solution of Eq. 61 when $\vec{N} = \vec{N}_B$, i.e. we solve,

$$(65) \quad \frac{d\vec{N}_B}{dt} = -K \vec{N}_B$$

The solution of Eq. 65 is of the form,

$$(66) \quad \vec{N}_B(t) = \exp(-Kt)\vec{N}_B(0)$$

After substituting Eqs. 64 and 66 into Eq. 63, we easily obtain the general solution of Eq. 61, which is,

$$(67) \quad \vec{N}(t) = \exp(-Kt)\vec{N}_B(0) + \exp(-(K + W_S)t)(\vec{\Phi}N_E - \vec{N}_B(0))$$

where we have used $\vec{N}(0) = \vec{\Phi}N_E$. We compare Eq. 67 with the solutions one would obtain if Eq. 61 were integrated under the assumption that $\vec{N}_B =$ constant, i.e., with

$$(67') \quad \vec{N}(t) = \frac{W_S \vec{N}_B}{W_S + K} + (\vec{\Phi}N_E - \frac{W_S \vec{N}_B}{W_S + K}) \exp(-K + W_S)t$$

Eq. 67' contains a constant additive term $\propto N_B$. Thus Eq. 67' is clearly

incorrect at long times, because we expect there to be no triplet signal when there is no population in T^1 .

As usual, explicit expressions are obtained from Eq. 67 by diagonalizing the matrices in the exponentials. This is where we can see that the current analysis has definite additional advantages over previous ones [2,3], in that: (1) no approximations except the high temperature approximation (see Appendix) have been made in the derivation of Eq. 67; and, (2) $\underline{K}$ is already diagonal, and $\underline{K} + \underline{W}_S$ is easily diagonalized exactly, so that no approximations need to be made to derive final expressions for Eq. 67. Even so, in what follows, we shall assume that we are looking at triplet state EPR signal kinetics: that is, we shall assume that the T_i are the high-field triplet eigenstates T_1 , T_0 and T_{-1} . We shall also assume, as in Eq. 36, that the population rates $k_1 = k_{-1}$ and the decay rates $K_1 = K_{-1}$, which is a good assumption in high magnetic fields [2,3].

The diagonalization procedure for the EPR case is as follows. Two successive transformations are applied to $\underline{K} + \underline{W}_S$, i.e., we first diagonalize the matrix $\underline{W}_S$ by means of a unitary operator, $\underline{U}$, so that,

$$(68) \quad \underline{U}^{-1}(\underline{K} + \underline{W}_S) \underline{U} = \begin{bmatrix} K_1 + W_1 + 2W_2 & 0 & 0 \\ 0 & K_{AV} - \frac{1}{3}(K_1 - K_0) + 3W_1 & \frac{\sqrt{2}}{3}(K_1 - K_0) \\ 0 & \frac{\sqrt{2}}{3}(K_1 - K_0) & K_{AV} \end{bmatrix}$$

where,

$$\underline{U} = \begin{bmatrix} \frac{1}{\sqrt{2}} & \frac{1}{\sqrt{6}} & \frac{1}{\sqrt{3}} \\ 0 & \frac{-2}{\sqrt{6}} & \frac{1}{\sqrt{3}} \\ \frac{-1}{\sqrt{2}} & \frac{1}{\sqrt{6}} & \frac{1}{\sqrt{3}} \end{bmatrix} ;$$

and,

$$K_{AV} = \frac{1}{3} (2K_1 + K_0)$$

Then we diagonalize Eq. 69 by means of the transformation, $\underline{D}$, i.e.,

$$(69) \quad \underline{D}^{-1} \underline{U}^{-1} (\underline{K} + \underline{W}_S) \underline{U} \underline{D} = \underline{\Lambda}$$

where,

$$\underline{\Lambda} = \begin{bmatrix} \lambda_1 & 0 & 0 \\ 0 & \lambda_+ & 0 \\ 0 & 0 & \lambda_- \end{bmatrix} ;$$

$$\underline{D} = \begin{bmatrix} 1 & 0 & 0 \\ 0 & \cos\theta & -\frac{1}{4} \sin\theta \\ 0 & \sin\theta & \cos\theta \end{bmatrix} ;$$

and where,

$$\lambda_1 = K_1 + W_1 + \sqrt{W_2^2}$$

$$\lambda_{\pm} = K_{AV} + \frac{1}{2}(3W_1 - \frac{1}{3}(K_1 - K_0)) \pm \frac{1}{2}[(3W_1 - \frac{1}{3}(K_1 - K_0))^2 + \frac{2}{9}(K_1 - K_0)^2]^{1/2}$$

and where,

$$\tan\theta = \frac{2\sqrt{2} \epsilon}{(1 - \epsilon) + [(1 - \epsilon)^2 + 2\epsilon^2]^{1/2}}$$

where we have introduced the small parameter, ϵ , which is defined as,

$$\epsilon = \frac{(K_1 - K_0)}{9W_1}$$

ϵ is typically of the order 0.1 or less [1].

Magnetic resonance $\Delta M_S = 1$ signals are proportional to the population differences $N_1 - N_2$ and $N_2 - N_3$; that is,

$$(70) \quad S_+ = -(N_1 - N_0)$$

$$S_- = -(N_0 - N_{-1})$$

From Eqs. 67 - 70, it is easy to write expressions for the EPR signal amplitudes as functions of time, as we shall do in Chapter Five.

Conclusion

Probably the most obvious limiting case of EPR triplet signal kinetics is the one we have studied by means of EPR kinetics measurements in green plants: namely, the case where a polarized signal is observed to decay to the Boltzmann signal with a spin lattice relaxation rate, followed by the

decay of the Boltzmann signal to zero with a rate equal to the reciprocal of the triplet state lifetime. However, it is precisely this case which cannot be described if we do not take into account the time dependence of the α_{ij} (Eq. 58) or of $\vec{N}_B$ (Eq. 57). That is, as shown above in Eq. 67', if $\vec{N}_B$ or the α_{ij} are assumed to be constants, integration of Eq. 61 will give solutions containing a constant additive term that will never decay, i.e. will give the incorrect behavior at long times. On the other hand, as shown above in Eq. 67, including the time dependence of $\vec{N}_B$ not only yields the proper long-time behavior, but yields simpler solutions as a bonus.

8. Signal Dynamics Under More General Illumination Conditions

In this section we shall discuss the solutions of Eq. 51 when $F(I,t)$ is not a δ - function laser pulse. For simplicity, we shall rewrite Eq. 51 in the form of Eq. 61, i.e.

$$(71) \quad \frac{d\vec{N}}{dt} + \underline{K}\vec{N} + \underline{W}_S\vec{N} + \vec{\phi}F(I,t)(1 - N_T) = \underline{W}_S\vec{N}_B$$

where $N_T = \sum_i N_i$, and we have used $N_S^0 + N_T = 1$; $\vec{N}$, $\underline{K}$, and $\vec{\phi}$ are as defined in Eq. 51, and $\underline{W}_S$ and $\vec{N}_B$ are as defined in Eq. 57.

Eq. 71 is again of the form given by Eq. 62, and so we need only solve for $\vec{N}_1$ in Eq. 63 under more general conditions of illumination; that is, the treatment of $\vec{N}_2$ is clearly unaltered in Eq. 71. Thus we proceed as if $\underline{W}_S\vec{N}_B = 0$ in Eq. 71 and we solve Eq. 71 for $\vec{N}_1$.

We shall divide our discussion of Eq. 71 into two cases: First, the case in which the light intensity in $F(I,t)$ is far from causing a saturation of the signal amplitude, in which case we may say

$$(72) \quad N_T \ll 1$$

in Eq. 71, which is the case that has usually been considered [11,12]; and second, the case in which the light intensities are saturating, in which case we cannot make the approximation of Eq. 72.

In either case we rewrite the light intensity term in Eq. 71 as

$$(73) \quad \vec{\phi} F(I, t) (1 - N_T) = F(I, t) (\vec{\phi} - \underline{\phi} N_T)$$

where

$$\underline{\phi} = \begin{bmatrix} \phi_1 & \phi_1 & \phi_1 \\ \phi_2 & \phi_2 & \phi_2 \\ \phi_3 & \phi_3 & \phi_3 \end{bmatrix}$$

The point here is that the inclusion of N_T in Eq. 73 will add another rate matrix to $\underline{K} + \underline{W}_S$ in Eq. 71 under conditions near saturating light intensities. We note that in the case where the system is far from light saturation,

$$(73') \quad F(I, t) (\vec{\phi} - \underline{\phi} \vec{N}_1) = F(I, t) \vec{\phi}$$

The solutions of Eq. 71 are of the form

$$(74) \quad \vec{N}_1(t) = \underline{U} \int \underline{U}^{-1} F(I, t) dt \vec{\phi} + \underline{U} \vec{C}$$

where

$$\underline{U} = \exp(-(\underline{K} + \underline{W}_S)t + \underline{\phi} \int F(I, t) dt)$$

and $\vec{C}$ is a constant vector of integration. We may write the time dependence in terms of a unitary operator which is the exponential of the integral of $F(I, t)$ only because the time dependent matrix $\underline{\phi} F(I, t)$ is the product of a

time independent matrix and a scalar function of time. This $\phi F(I,t)$ commutes with itself at different times. As usual, our ability to find analytical expressions for Eq. 74 depends crucially upon our ability to find a form for $\underline{U}$, i.e. to deal with $\exp(\phi \int F(I,t) dt)$. We now consider three commonly used illumination regimes.

Continuous Illumination

In the case that we turn on a cw lamp abruptly at time zero, $F(I,t)$ for $t > 0$ is a constant, $c(I)$. If the intensity is far from saturating, then the solutions in Eq. 74 are of the form

$$(75) \quad \hat{N}_1(t) = c(I)(1 - \exp(-(\underline{K} + \underline{W}_S)t)\hat{\phi}$$

In the case of light intensities near saturation, the solutions are of the form

$$(75') \quad \hat{N}_1(t) = c(I)(1 - \exp(-(\underline{K} + \underline{W}_S + \underline{\phi})t)\hat{\phi}$$

Evidently, $c(I)$ is the excitation rate constant at intensity I . When the intensity is far from saturation, Eq. 75 says that the signal risetime will be determined only by the kinetic constants in $\underline{K} + \underline{W}_S$, whereas in the case of light intensities near saturation, the risetime of the signal can be faster than in the case that the intensity is far from saturation, because the excitation rate matrix enters the exponential explicitly.

Periodic Waveform

In the case of light modulation, we assume a square or trapezoidal waveform for $F(I,t)$ which may be represented roughly by a Fourier series of

the form

$$(76) \quad F(I,t) = \frac{1}{2}c(I) \left(1 + \sum_{n \text{ odd}} \frac{1}{n!} \sin(n\omega_c)t \right)$$

where ω_c is the chopping frequency. When the light intensity is far from saturating, the solutions in Eq. 74 are of the form

$$(77) \quad \vec{N}_1(t) = \frac{\vec{\phi}}{2(\underline{K} + \underline{W}_S)} + \frac{1}{2} \sum_{n \text{ odd}} \frac{1}{n!} \left(\frac{\sin(n\omega_c t - \vec{\psi}_n) + \exp(-(\underline{K} + \underline{W}_S)t) \sin \vec{\psi}_n}{((\underline{K} + \underline{W}_S)^2 + n^2 \omega_c^2)^{1/2}} \right) \vec{\phi}$$

where we have used the definitions

$$(78) \quad \sin \vec{\psi}_n = \frac{n\omega_c}{((\underline{K} + \underline{W}_S)^2 + n^2 \omega_c^2)^{1/2}}$$

$$\cos \vec{\psi}_n = \frac{\underline{K} + \underline{W}_S}{((\underline{K} + \underline{W}_S)^2 + n^2 \omega_c^2)^{1/2}}$$

A lockin detector will amplify only those parts of $\vec{N}_1(t)$ which have significant Fourier components at the fundamental frequency, ω_c , so that the signal amplitudes will depend upon

$$(79) \quad \vec{N}_1(t) = \frac{1}{2} \frac{\sin(\omega_c t - \vec{\psi})}{((\underline{K} + \underline{W}_S)^2 + \omega_c^2)^{1/2}}$$

According to Eq. 78, the phase shift, $\vec{\psi}$, is a vector quantity related to

the kinetic constants of Eq. 71 by the expression

$$(80) \quad \tan \vec{\psi} = \frac{\omega_c}{\underline{K} + \underline{W}_S}$$

In the case that the light intensity is close to saturating, $\underline{U}$ of Eq. 74 becomes an exponential whose argument is a Fourier series. It is not possible in this case to derive exact analytical expressions for the solutions of Eq. 74.

To summarize the results of this section, we point out that under conditions where the light intensity is far from saturating, we may always write the solutions in Eq. 74 in the form

$$(81) \quad \vec{N}_1(t) = G(\underline{K} + \underline{W}_S, t) \vec{\phi} N_E$$

for an arbitrary light intensity function $F(I, t)$ and initial conditions $\vec{N}_1(0) = \vec{\phi} N_E$, where

$$G(\underline{K} + \underline{W}_S, t) = \exp(-(\underline{K} + \underline{W}_S)t) [1 - \int \exp((\underline{K} + \underline{W}_S)t) F(I, t) dt]$$

Explicit solutions are found by means of the relation

$$(82) \quad G(\underline{K} + \underline{W}_S, t) = \underline{D} \underline{U} G(\underline{A}, t) \underline{U}^{-1} \underline{D}^{-1}$$

where $\underline{D}$, $\underline{U}$ and $\underline{A}$ are given above in Eqs. 68 and 69.

In the case of signals for which the light is near saturating and where $\exp(\underline{\phi} F(I, t) dt)$ is analytical (e.g. $F(I, t) = c(I) \delta(t - 0)$ and $F(I, t) = c(I)$), we may write

$$(83) \quad \hat{N}_1(T) = G'(\underline{K} + \underline{W}_S + c(I)\underline{\phi}, t)^{\dagger} N_E$$

The diagonalization in this case is straightforward, i.e.

$$(84) \quad G'(\underline{K} + \underline{W}_S + c(I)\underline{\phi}, t) = \underline{D}' \underline{U} G(\underline{\Lambda}, t) \underline{U}^{-1} \underline{D}'^{-1}$$

where $\underline{U}$ is as in Eq. 68, and $\underline{D}'$ is given by

$$\underline{D}' = \begin{bmatrix} 1 & 0 & 0 \\ 0 & \cos\theta' & \gamma \sin\theta' \\ 0 & -\sin\theta' & \cos\theta' \end{bmatrix}$$

where

$$\tan\theta' = \frac{\sqrt{2}}{3} \left(\frac{K_1 - K_0}{K_T + A_T - \lambda_+} \right); \quad \gamma = \frac{1}{4} \left(\frac{K_1 - K_0 + 3(A_1 - A_0)}{K_1 - K_0} \right)$$

and $\underline{\Lambda}'$ is as in Eq. 19, but with

$$\begin{aligned} \lambda_{\pm} &= K_T + \frac{1}{2}(3W_1 + A_T - \frac{1}{3}(K_1 - K_0)) \\ &\pm \frac{1}{2}[(3W_1 - A_T - \frac{1}{3}(K_1 - K_0))^2 + \frac{2}{9}(K_1 - K_0)(K_1 - K_0 + 3(A_1 - A_0))]^{1/2} \end{aligned}$$

where $A_i = c(I)\phi_i$ and $A_T = \sum_i A_i$.

The addition of the Boltzmann populations to the above entails the substitution of the solutions for N_i into Eq. 63, followed by a careful adjustment of the initial conditions.

We shall now end our discussion of the mathematics presented in this section with a description of how Eqs. 75 - 80 were useful to us in the

interpretation of experimental results.—As we shall see in detail in the following chapters, we observed two distinct chlorophyll triplet species during light modulation experiments performed on photosystem I reaction centers. What is relevant to the discussion here is an observation which puzzled us for a long time, namely, that although the rates in the matrices $\underline{K}$ and $\underline{W}_S$ for the two triplet species were nearly identical, one of the triplet signals exhibited no measurable phase shift during light modulation experiments, whereas the other was phase shifted up to 60° at $\omega_c = 33$ Hz. The analysis which we have just presented explains this observation, because we determined that the triplet with a zero phase shift was light saturated at the intensities we routinely used, whereas the triplet with the 60° phase shift was not saturated. Even though we cannot present analytical solutions for the sublevel populations under conditions of saturating light during light modulation experiments, we can guess from Eq. 75 that the rising edge of the signal for square wave modulation will be sharper than the falling edge (in the latter case, $c(I)_\phi$ in Eq. 75 is zero). That is, if the excitation rates $c(I)$ become larger than the rates in $\underline{K}$, then the observed phase shift will be smaller than the one in Eq. 80 which does not contain $c(I)$. Thus phase shift measurements on saturated signals will underestimate the kinetic constants. This is the reason that phase shift measurements are of limited utility.

Appendix

On the Anisotropy of Spin Lattice Relaxation

We and others [11,12,14] have assumed that $\Delta M_S = 1$ spin lattice relaxation rate constants can be extracted as in Eq. 56a above, i.e. that we may write

$$(A1) \quad W_{12} + W_{21} = W_{23} + W_{32}$$

However, as we shall discuss here, Eq. A1 is strictly valid only in the case that there are no zero field splittings, or, if there are zero field splittings, only if the high temperature approximation is valid.

If there are no zero field splittings, then we know that

$$(A2) \quad \frac{W_{12}}{W_{21}} = \frac{W_{23}}{W_{32}} = \exp(\Delta E/kT)$$

where $\Delta E = E_1 - E_2 = E_2 - E_3$. Clearly Eq. A2 means that we may write

$$(A3) \quad W_{12} + W_{21} = W_{21}(\exp(\Delta E/kT) + 1) = W_{23} + W_{32}$$

If we consider that there are zero field splittings, ϵ , which shift the energy levels so that

$$(A4) \quad E_1 - E_2 = \Delta E - \epsilon$$

$$E_2 - E_3 = \Delta E + \epsilon$$

then we may write new rates, w_{ij}' , such that

$$(A5) \quad \frac{w_{12}'}{w_{21}'} = \exp(-\epsilon/kT) \frac{w_{12}}{w_{21}}$$

$$\frac{w_{23}'}{w_{32}'} = \exp(+\epsilon/kT) \frac{w_{21}}{w_{12}}$$

which means that

$$(A6) \quad w_{12}' = \exp(-\epsilon/kT) w_{12}$$

$$w_{21}' = \exp(+\epsilon/kT) w_{21}$$

$$w_{23}' = \exp(+\epsilon/kT) w_{12}$$

$$w_{32}' = \exp(-\epsilon/kT) w_{21}$$

We now define two different $\Delta M_S = 1$ spin lattice relaxation rates, w_1 and w_3 , as follows

$$(A7) \quad w_1 = \frac{1}{2}(w_{12}' + w_{21}') = w_{21}[\exp(-(\Delta E + \frac{1}{2}\epsilon)/kT) + \exp(\epsilon/2kT)]$$

$$w_3 = \frac{1}{2}(w_{23}' + w_{32}') = w_{21}[\exp(-(\Delta E - \frac{1}{2}\epsilon)/kT) + \exp(-\epsilon/2kT)]$$

Clearly w_1 and w_3 are not exactly equal. However, in the high temperature approximation, we may write

$$(A8) \quad \exp(-(\Delta E \pm \epsilon)/kT) \approx 1 - (2\Delta E \pm \epsilon)/2kT$$

$$\exp(\pm \epsilon/2kT) = 1 \pm \epsilon/2kT$$

Substituting Eq. A8 into Eq. A7, one can easily see that $W_1 = W_3$ in the high temperature limit. This limit obtains in most cases.

In the case where the anisotropy in the spin lattice relaxation rates cannot be neglected, $\underline{W}_S$ in Eq. 57 above becomes

$$(A9) \quad \underline{W}_S' = \begin{bmatrix} W_1 + W_2 & -W_1 & -W_2 \\ -W_1 & W_1 + W_3 & -W_3 \\ -W_2 & -W_3 & W_2 + W_3 \end{bmatrix}$$

and the preference is still to solve Eq. 61. However, $\underline{W}_S'$ cannot be diagonalized exactly. Under the transformations $\underline{U}$ and $\underline{D}'$, $\underline{W}_S$ becomes

$$(A10) \quad (\underline{D}')^{-1} \underline{U}^{-1} \underline{W}_S' \underline{U} \underline{D}' = \underline{A}' + \frac{3}{2}(W_1 - W_3) \begin{bmatrix} 0 & \cos\theta' & \frac{1}{4}\sin\theta' \\ \cos\theta' & 0 & 0 \\ -\sin\theta' & 0 & 0 \end{bmatrix}$$

where $\underline{A}'$, $\underline{D}'$, $\cos\theta'$ and $\sin\theta'$ in Eq. A10 are given by Eq. 68 and 69 if one substitutes $(W_1 + W_3)/2$ for W_1 wherever it appears in Eq. 68 and 69.

Eq. A10 may be diagonalized to a very good approximation if one neglects the off-diagonal elements $\propto \sin\theta'$. Including these would introduce corrections of the order $(W_1 - W_3)\sin^2\theta$, and we note that $\sin^2\theta = \epsilon^2$ in Eq. 69. These are very small corrections, indeed.

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CHAPTER THREE

TRIPLET STATES IN PHOTOSYSTEM I OF SPINACH CHLOROPLASTS AND
DIGITONIN SUBCHLOROPLAST PARTICLES1. Introduction

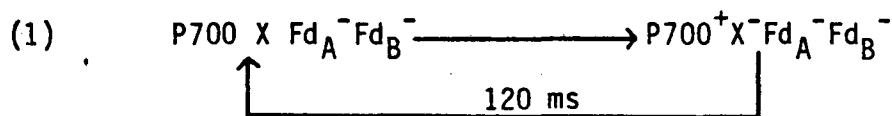
One of the reasons that photosynthesis is such an efficient process is that the movement of electrons from the photoexcited primary electron donor to secondary acceptors is a very rapid process. For example, in the case of photosystem I, it is now fairly well established that donated electrons reach component X in about 200 ps at room temperature [1]. However, the very rapidity of forward photochemistry has made the determination of the number and identities of early acceptors very difficult. If the secondary acceptors are chemically reduced or photochemically trapped in their reduced states prior to photoexcitation, then the normal process of forward photochemistry is prohibited, and the system is forced to return to the ground state by some other process. In purple bacteria, it was observed [2] that this process involves radical pair charge recombination which populates the primary donor triplet state. Based on our discussion in Chapter Two, we know that this means that we should look for a polarized triplet state EPR spectrum with a polarization pattern diagnostic of a radical pair population mechanism. This is in fact was observed, first by Dutton et al. [2].

Ever since the radical pair - polarized triplet state of the bacterial primary donor was observed to be populated as an intermediate of charge

recombination in purple bacteria, it has been obvious that the triplet state of P700 should be looked for as an intermediate of charge recombination in photosystem I of green plants. Thus several groups [3,4] reported attempts to observe a radical pair polarized triplet from P700. Leigh and Dutton [3] reported the observation of light-induced triplet signals at 10 Kelvin in whole chloroplasts which had been treated with sodium dithionite so as to reduce iron sulfur centers A and B. The experiment they did was simply to scan the EPR spectrum during continuous illumination of the sample, without removing "DC" EPR signals arising from the iron sulfur proteins by means of light-chopping and phase sensitive detection. Thus, light-induced signals were observed, but these were convoluted with iron sulfur protein signals over roughly two-thirds of the triplet signal spectral range. Neither zero field splitting parameters nor a polarization pattern could be confidently assigned. Subsequently, Uphaus et al. [4] reported a chlorophyll a EPR triplet signal which they observed in chloroplasts at liquid helium temperatures during light modulation and phase sensitive detection at 1 kHz. Based on the polarization pattern of the triplet EPR signal it was determined that the triplet was populated by an intersystem crossing mechanism rather than by the radical pair mechanism [4].

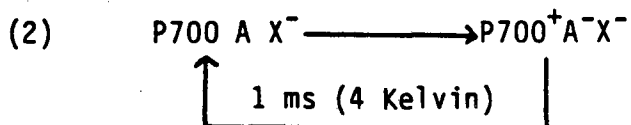
Subsequent to these first attempts to see a radical pair polarized signal in photosystem I, and just prior to our own attempts to do so, two crucial facts were reported which seemed to account for the earlier failures. The first was reported by Shuvalov et al. [5], who observed that the lifetime of the light-induced $P700^+X^-$ radical pair is 120 ms at 10 Kelvin. This suggested that during illumination at 10 Kelvin of chloroplasts which have been treated with dithionite, one will observe the

process



We hypothesized that the $\text{P700}^+ \text{X}^-$ radical pair lifetime is long enough, corresponding to a rate of roughly 10 Hz, that any transient EPR-detectable species arising from process (1) would have negligible Fourier components at the 1 kHz chopping frequency used by Uphaus et al. [4], which would explain why they had failed to observe any such transient species.

The second crucial fact was reported by Sauer et al. [6], who obtained the first evidence that X is not the earliest electron acceptor in photosystem I. As discussed in Chapter One, Sauer et al. [6] observed that when X had been phototrapped in its reduced state prior to measurement, illumination results in photobleaching of the P700 optical absorption spectrum, followed by a 1 ms recovery of the photobleaching at liquid helium temperature. They proposed the following model to explain their observation,



That is, the simplest proposal seemed to be that the 1 ms halftime corresponded to the lifetime of the radical pair $\text{P700}^+ \text{A}^-$. Based on the model of Sauer et al. [6], we hypothesized that the lifetime of the $\text{P700}^+ \text{A}^-$ radical pair was short enough, corresponding to a rate of about 1 kHz, that any population of $^3\text{P700}$ occurring as a result of process (2) might be detectable during light modulation experiments at chopping frequencies of $10 - 10^3$ Hz. Further, our belief that some population of the triplet state

of P700 was occurring on a 1 ms timescale was confirmed by a report of Shuvalov [7] that a component of delayed luminescence with a halftime of about 0.5 ms could be observed in photosystem I reaction center preparations. Shuvalov assigned this luminescence to primary charge recombination, and the 1 ms halftime to the lifetime of the triplet state of P700 [7].

The above reasoning motivated us to do the experiments which are presented in this chapter. The experiments successfully demonstrate that the above hypotheses are for the most part valid; i.e. that by carefully controlling the redox state of photosystem I in chloroplasts and digitonin photosystem I subchloroplasts, we can observe a polarized triplet state EPR signal arising from radical pair recombination in green plants. However, as we shall mention in the Conclusion of this chapter, and discuss in Chapters 4 and 5, the experimental results presented here have led us to a new model of primary events in photosystem I which is very different from the model represented by processes (1) and (2).

2. Materials and Methods

Chloroplasts were isolated from market spinach in 0.4 M sucrose/10 mM NaCl/10 mM Tris buffer, pH 8.0/10 μ M EDTA and collected by centrifugation at 5000Xg for 5 min.

To prepare digitonin photosystem I subchloroplast particles, we resuspended chloroplasts to a chlorophyll concentration of 0.3-0.4 mg ml⁻¹ in 50 mM Tris buffer (pH 8.0) containing 10 mM Mg⁺² to assure clean fractionation of photosystem I from photosystem II [8]. Digitonin was added to the chloroplast suspension as a 10% (wt./vol.) solution to give 0.5% (wt./vol.) digitonin. After incubation for 2 hr at 4°C, the detergent

incubation mixture was centrifuged for 0.5 hr at 30,000Xg. The supernatant contained photosystem I particles with the characteristics: chlorophyll a /chlorophyll b = 5.6 and chlorophyll/P700 = 175.

The supernatant was concentrated for EPR studies by precipitation with protamine sulfate, as described by Nelson et al. [9].

Assays: Chlorophyll was assayed by the method of Arnon [10]. P700 was assayed as follows: a few grains of potassium ferricyanide were added to about 4 ml of sample which had an optical density of roughly unity at the chlorophyll λ_{max} . The solution was allowed to incubate for 15 - 20 minutes at 4°C. This step assured that when the measurement of P700 chemical difference was made there would be no interference at around 670 nm from the time-dependent oxidation of bulk chlorophyll by potassium ferricyanide, because the latter change stops occurring within 15 - 20 minutes. Just before measurement of the absorption difference at 700 nm, a few grains of sodium ascorbate were added to the reference cuvette. P700 concentration was determined from the maximum ferricyanide - ascorbate difference at around 700 nm, assuming Ke's differential extinction coefficient of $64 \text{ mM}^{-1} \text{ cm}^{-1}$ [12].

EPR Sample Preparation

Treatment with Dithionite: Chloroplast or digitonin photosystem I pellets were degassed under reduced pressure and mixed with a solution of sodium dithionite in 100 mM glycine buffer (pH 10) under a nitrogen atmosphere to give a final concentration of 12 mM dithionite.

Treatment with Ferricyanide: Chloroplast or digitonin photosystem I pellets were mixed with $\text{K}_3\text{Fe}(\text{CN})_6$ in 50 mM Tris buffer (pH 8) to give 1 mM ferricyanide.

The treated pellets were then combined with an equal volume of ethylene

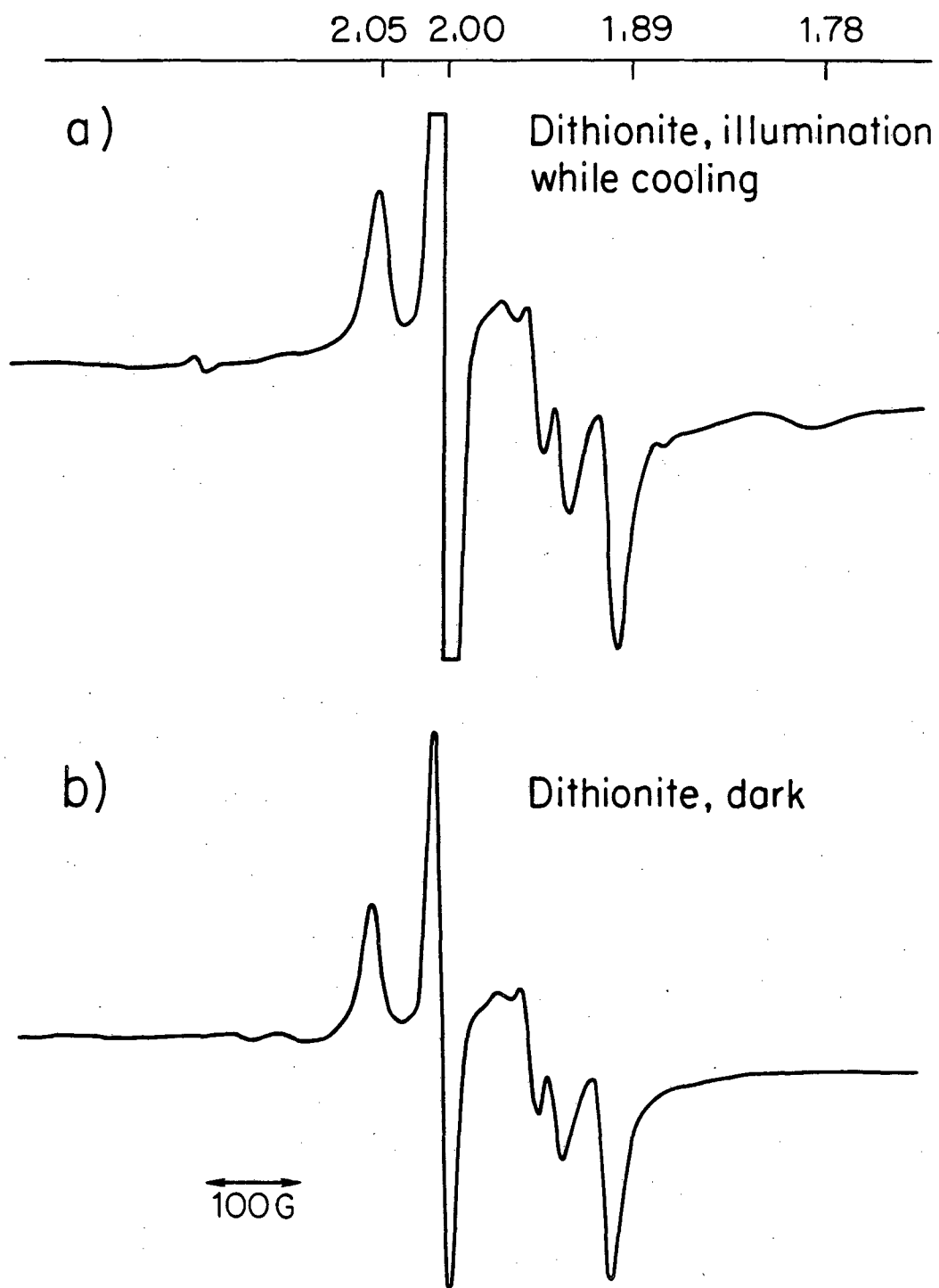
glycol, sealed in EPR tubes, and stored at 77 K. Final chlorophyll concentration of the samples was about 2 mg ml^{-1} . Samples which were illuminated during cooling were illuminated by a tungsten lamp operating at an intensity of 1000 Wm^{-2} for approximately 3 min and frozen during illumination to dry ice/ acetone temperature before they were stored at 77 Kelvin.

EPR Measurements: EPR spectra were recorded on a Varian E-109 spectrometer operating at X band. All measurements utilized 100 kHz field modulation and a TM cavity that was fitted with an optical transmission flange. Light modulation experiments were carried out at 33 Hz. The output of the EPR spectrometer was fed into a Princeton Applied Research (PAR) model 210 selective amplifier tuned to 33 Hz and then to a PAR model 220 lockin amplifier referenced to the chopper. The light source for light modulation experiments was an Oriel xenon lamp operating at 1000 W. The beam was filtered by 5 cm of water in pyrex. An Air Products Helitran cryostat was used for liquid helium temperature EPR measurements.

3. Results

EPR triplet state spectra were recorded for samples prepared in three different redox conditions:

(A). Dithionite, pH 10, frozen under illumination. Chloroplasts or digitonin photosystem I particles that were frozen under illumination in the presence of dithionite gave the dark EPR spectrum shown in Figure 1A. In the light-modulated triplet state EPR spectra for chloroplasts (Figure 2A) and digitonin photosystem I particles (Figure 3A) prepared in this redox state one major triplet, denoted Triplet I, was observed. Triplet I



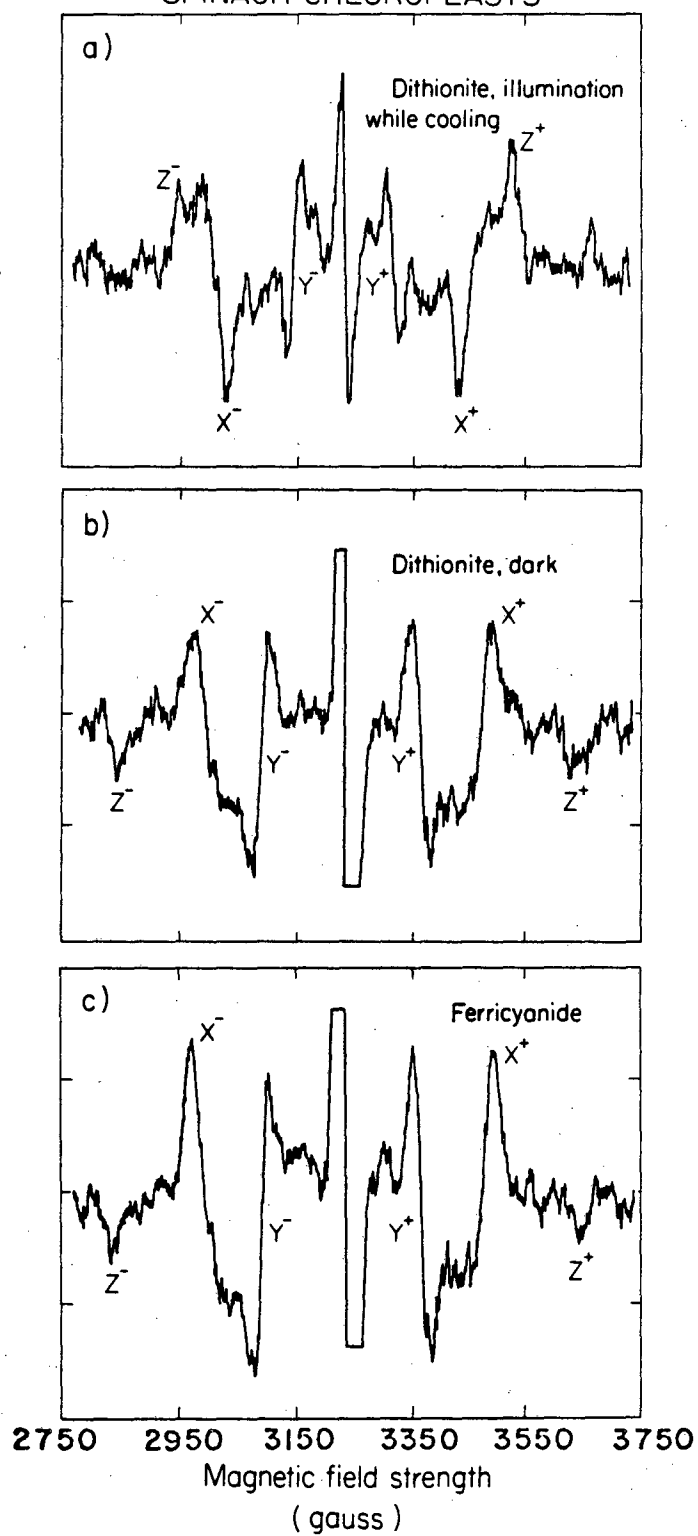
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Figure 1

EPR spectra of samples (A) and (B) recorded in the dark at 10 Kelvin.

Spinach chloroplasts were: (A) Frozen under illumination in the presence of 12 mM sodium dithionite in glycine buffer at pH = 10; and (B) same as (A), but frozen in the dark. EPR spectrometer conditions: gain, 2500; microwave power, 10 mW; modulation amplitude, 16 Gauss; microwave frequency, 9.079 GHz; scan rate, 250 Gauss min⁻¹.

SPINACH CHLOROPLASTS



XBL794-4765

Figure 2

EPR triplets observed during 33 Hz light modulation experiments in samples of chloroplasts prepared in redox states (A) - (C). Conditions (A) and (B) are as described in Figure 1. Figure 2C was recorded from a sample of spinach chloroplasts that had been frozen in the dark in the presence of 1 mM potassium ferricyanide. Spectrometer conditions: gain, 80; microwave power, 1 mW; modulation amplitude, 16 Gauss; microwave frequency, 9.075 GHz; sweep time, 1 hour.

DIGITONIN PSI PARTICLES

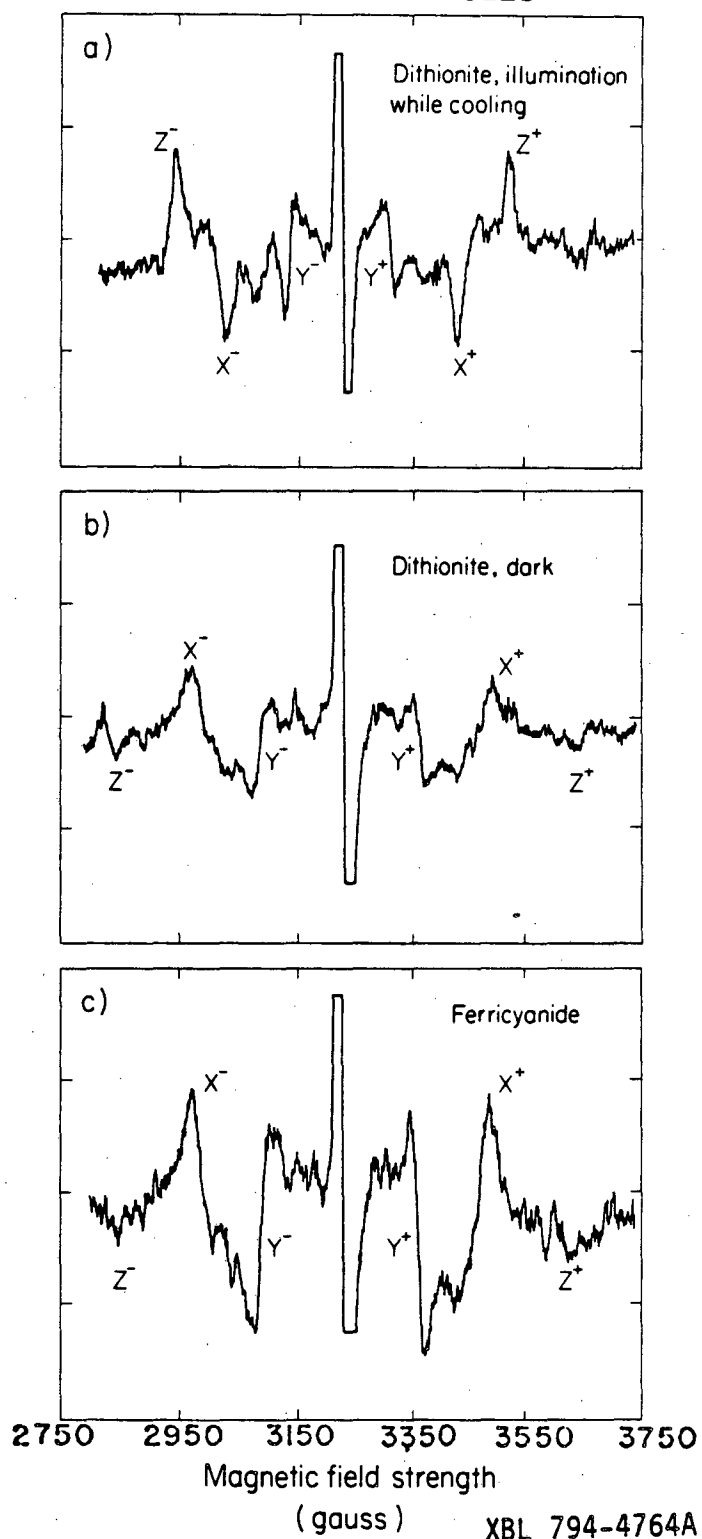


Figure 3

EPR triplets observed during 33 Hz light modulation experiments in samples of digitonin photosystem I particles prepared in redox states (A) - (C), as described in Figure 2. Spectrometer conditions are as described in Figure 2, except that the gain used for (A) and (B) was 20.

has the spin polarization pattern ae ae, where a denotes a signal in absorption and e denotes a signal in emission. The zero field splitting parameters of triplet I are $|D| = 0.0278 \pm 0.0009 \text{ cm}^{-1}$ and $|E| = 0.0039 \pm 0.0009 \text{ cm}^{-1}$ (Table 1).

(B). Dithionite, pH 10, frozen in the dark. Chloroplasts and digitonin photosystem I particles that were frozen dark in the presence of dithionite gave the dark EPR spectrum shown in Figure 1B. Figures 2B and 3B show the light-induced triplet signals obtained in this redox state from chloroplasts and digitonin photosystem I particles, respectively. One major triplet state, denoted Triplet II, was observed. Triplet II has the spin polarization pattern ae eae and zero field splitting parameters $|D| = 0.0383 \pm 0.0013 \text{ cm}^{-1}$ and $|E| = 0.0040 \pm 0.0013 \text{ cm}^{-1}$ (Table 1). We made this assignment as follows. The innermost signals were assigned to the Y^+ and Y^- transitions because of their characteristic shapes. The other major features, labeled X^+ and X^- , were used with the assignments of Y^+ and Y^- and the relation (See Eq. 35 of Chapter Two),

$$H_X^+ - H_X^- + H_Y^+ - H_Y^- = H_Z^+ - H_Z^-$$

to calculate the positions of the third transitions, Z^+ and Z^- . The calculated value agrees with small features that appear reproducibly in the spectra shown in Figures 2 and 3.

(C). Ferricyanide, pH 8. Figures 2C and 3C show the light-induced triplet EPR signals obtained in this redox state for chloroplasts and digitonin photosystem I particles, respectively. Once again we observe Triplet II as the major species, as described above in (B).

Table 1

Zero field splitting parameters and electron spin polarization patterns of observed triplet state EPR signals.

Triplet Species	D	E	Polarization ^c Pattern
I ^a	0.0278	0.0039	<u>ae</u> <u>ae</u>
II ^b	0.0383	0.0040	<u>ea</u> <u>ea</u>

^aError limits $\pm 0.0009 \text{ cm}^{-1}$.

^bError limits $\pm 0.0013 \text{ cm}^{-1}$.

^ca = absorption; e = emission.

4. Discussion

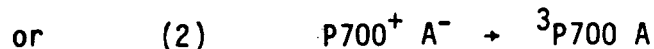
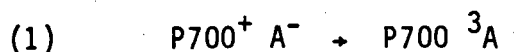
On the Assignment of Triplet I to $^3\text{P700}$

Figure 1A shows that samples of chloroplasts or digitonin photosystem I subchloroplasts that have been frozen under illumination in the presence of dithionite are prepared in the redox state $\text{P700 A X}^-\text{Fd}_\text{B}^-\text{Fd}_\text{A}^-$. In light modulation experiments on samples of chloroplasts prepared in this redox state, Figure 2A shows that we observe Triplet I convoluted with a small amount of Triplet II. The polarization of Triplet I shown in Table 1 is aee aae; i.e. is the radical pair polarization pattern discussed in Chapter Two. Because this polarization pattern is inconsistent with an intersystem crossing mechanism and is consistent with a radical pair population mechanism, we conclude that Triplet I is populated as a product of a radical pair recombination process. The $|D|$ value of Triplet I is equal within experimental error to that observed for chlorophyll a monomer observed in vitro and in vivo [4].

We also observe Triplet I in Figure 3A, i.e. in a photosystem I subchloroplast preparation, which suggests that Triplet I is integral to Photosystem I. Based on the polarization and zero field splittings of Triplet I, and also based upon the fact that it is observed to be integral to photosystem I, it seems plausible to argue that Triplet I is the photosystem I analogue of the purple bacterial P^R state which we discussed in the Introduction. That is, it seems plausible to make the assignment that Triplet I is $^3\text{P700}$.

The argument is that Triplet I seems to arise from a chlorophyll a species in Photosystem I which undergoes reversible light-induced charge separation to a radical pair state. There are really only two candidates:

P700 and A. Thus we are almost certainly looking at one of the following processes during light modulation experiments,



Processes (1) and (2) would be accompanied by very different optical changes, and the optical data of Shuvalov et al.[1] supports process (2).

The assignment of Triplet I to $\ ^3P700$ is also suggested by the dependence of Triplet I on the redox state of photosystem I evident in Figures 2 and 3. Figure 1B shows that samples which have been frozen in the dark in the presence of dithionite are prepared in the redox state $P700 A \times Fd_B^- Fd_A^-$. Illumination of samples in this redox state results in the formation of $P700^+ X^-$ radical pairs. As we argued in the Introduction, the 120 ms lifetime of the $P700^+ X^-$ radical pair is slow enough compared to the 33 Hz chopper frequency that there will be a steady state population of reactions centers in the state $P700^+ X^-$ during 33 Hz experiments. If Triplet I arises from P700, then we expect that the amplitude of Triplet I should be less in this redox state, corresponding to the portion of the population in which P700 is oxidized. Figures 2B and 3B show that the amplitude of Triplet I is attenuated or absent, which is consistent with the assignment of Triplet I to $\ ^3P700$. The preceding argument is supported by the fact that we do not observe Triplet I in Figures 2C and 3C, which are the EPR spectra of samples in which P700 has been chemically oxidized with ferricyanide prior to illumination.

The only difficulty with the assignment of Triplet I to $\ ^3P700$ arises from the fact that we would expect that the $|D|$ value of $\ ^3P700$ would be

smaller than the $|D|$ value of chlorophyll a monomer, for the following reason. The $|D|$ value of the primary donor triplet state, P^R , in bacterial reaction centers is 20% smaller than the $|D|$ value of bacteriochlorophyll a measured in vitro [12]. This result has meshed nicely with other results suggesting that the primary donor, P870, is a bacteriochlorophyll a dimer [12]. That is, the 20% reduction in the $P^R |D|$ value is thought to reflect a greater delocalization of triplet excitation in the dimer, when compared to the monomer. Because P700 has been thought by most workers [13] to be a chlorophyll a dimer, we expected to observe a similar reduction in the $|D|$ value of 3P700 . However, as we have become more confident that Triplet I is indeed 3P700 , we have become less confident that the zero field splitting parameters can give a simple indication of the chlorophyll a aggregation state. Other workers have recently reported evidence in support of the assignment of P700 to a chlorophyll a monomer [14]. This point is discussed in greater detail in Chapter Four.

On the Identity of Triplet II

Two features of Triplet II in Figures 2 and 3 merit attention. The first is that triplet II appears to depend on the redox state of photosystem I and the second is that the zero field splitting parameters of triplet II do not correspond to the parameters measured for any species of chlorophyll or of pheophytin in vivo or in vitro [4]. We shall argue that Triplet II arises from a β - carotene species which is sensitive to the redox state of Photosystem I.

The polarization pattern of Triplet II given in Table 1 is one of those which we know to be consistent with an intersystem crossing mechanism. The fact that it is observed both in chloroplasts and in

digitonin photosystem I subchloroplasts, and has the same redox dependence in both, suggests that it is integral to photosystem I. Thus, although it is populated by intersystem crossing, which means that it is not involved with primary charge separation, its redox dependence suggests that it has a role as a chromophore in photosystem I. That is, it does not appear to be present in Figures 2 and 3 merely as a contaminating signal.

The redox dependence of Triplet II is consistent with the idea that it is associated with reaction centers in which P700 is oxidized. Triplet II has a large amplitude in Figures 2B and 3B in which, as we argued above, there is a significant steady state population of photooxidized P700 during 33 Hz light modulation experiments. Triplet II is even larger in Figures 2C and 3C, in which P700 has been completely oxidized by ferricyanide. The amplitude of Triplet II is largely attenuated in Figures 2A and 3A, in which the reaction centers have been prepared in the redox state $P700^+ A^-$ prior to illumination. Our interpretation has been that this loss in amplitude of Triplet II results from the fact that in this redox state, the $P700^+ A^-$ back reaction returns $P700^+$ to the ground state so efficiently that no significant population of oxidized P700 is achieved, which results in the attenuation of Triplet II.

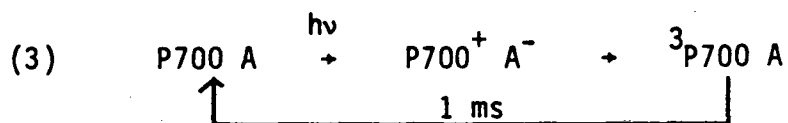
The fact that Triplet II is populated by intersystem crossing, and that its redox dependence is consistent with its association with reaction centers in which P700 is oxidized, suggest that Triplet II has a role as a photoprotector. That is, when P700 is oxidized, it cannot accept excitation from antenna chlorophylls. This is a situation which will occur in the natural world only under conditions of high light intensities, that is, in situations in which the plant can be damaged by light. The data in Figures 2 and 3 suggests that Triplet II may be a species which can

efficiently funnel excitation from photoexcited chlorophylls. This species then undergoes intersystem crossing, and we observe it as Triplet II.

Frank et al. [15] have subsequently reported experiments which are strongly suggestive that Triplet II arises from a carotenoid species, probably β -carotene. They observed a triplet EPR signal for β -carotene in non-ionic detergent micelles. The β -carotene triplet had a $|D|$ value of 0.0333 cm^{-1} , which is very similar to that of Triplet II. It is reasonable to suppose that Triplet II is a carotenoid species, first, because of the similarity of its $|D|$ value to that of β -carotene; and, second, because carotenoids are known to be integral to the purple bacterial light harvesting protein [16], and abundant in plants [17]. Thus, carotenoids will almost certainly prove to be closely associated with the photosystem I antenna system.

5. Conclusion

We did not realize when we reported [18] the above results that they were the first direct evidence that the 1 ms decay component observed by Sauer et al. [6] corresponded to the lifetime of the triplet state of P700 and not to the back reaction between P700^+ and A^- and we did not immediately realize that the observation of $^3\text{P700}$ requires a revision of the model that we presented in the Introduction to this Chapter. The experimental evidence presented in this chapter suggests a new hypothesis: that illumination of photosystem I reaction centers in which forward photochemistry is blocked results in the following process



This should be contrasted with the model we presented in the Introduction

to this chapter, which considered that the 1 ms component corresponded to the P700 radical pair lifetime. It is hard to imagine how we would be able to observe the triplet state of P700 at all if the model given by process (2) in the Introduction were correct. We shall present evidence in Chapter Five that our revised hypothesis was correct, and that the new mechanism represented by process (3) is valid.

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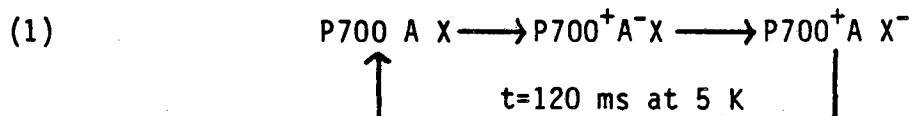
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CHAPTER FOUR

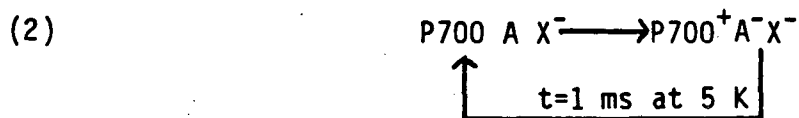
THE DEPENDENCE OF REACTION CENTER AND ANTENNA TRIPLETS
ON THE REDOX STATE OF PHOTOSYSTEM I

1. Introduction

As we argued in the Introduction of Chapter Three, the simplest model suggested by the data of Sauer et al. [1] and of Shuvalov et al. [2] is that when X is not reduced prior to measurement, one observes the process



whereas when X is reduced prior to illumination, one observes the process

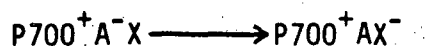


However, this hypothesis now appears to be questionable, for two reasons. First, the mechanism of recovery by process (2) to the ground state P700AX was shown in Chapter Three to involve more than a single step. That is, we have argued that the observation of a radical pair-polarized $^3\text{P700}$ EPR signal constitutes evidence that the triplet state of P700 functions as an intermediate in the $\text{P700}^+\text{A}^-$ back reaction.

Second, the kinetic data are suggestive that photo-reduced A^- does not always transfer its electron to X when X is not reduced prior to photoexcitation. That is, Sauer et al [1] reported that the 1 ms phase was observed regardless of whether X was reduced prior to measurement, and Shuvalov et al. [2] observed the 1 ms phase in samples poised at -625 mV, a potential at which X is not reduced prior to measurement. Thus, under the conditions of these measurements, primary photochemistry would appear to be more complicated than the simple serial transfer of electrons from P700 to A and then to X depicted in process (1).

With these mechanistic questions in mind, we studied the relation between the state of reduction of photosystem I acceptors and the amplitude of the radical pair polarized EPR triplet signal from P700. For these studies we used photosystem I preparations isolated using Triton X-100 detergent. In these preparations we do not observe the carotenoid triplet [3] that we observed in chloroplasts and in digitonin photosystem I subchloroplasts [4]. However, in addition to the radical pair-polarized triplet of P700, in the Triton preparations we now observe an additional triplet species which appears to depend on the redox state of photosystem I.

We observe the radical pair-polarized triplet in samples in which X was not reduced prior to illumination. We believe that this triplet arises when A^- undergoes radical pair recombination with $P700^+$ in preference to forward electron transfer to X. Our results therefore suggest that the processes



and



are in competition when Centers A and B are reduced prior to triplet measurements.

We observe no correlation between the amplitude of the radical pair-polarized triplet and the amplitude of a $g = 2.0$ signal present in the dark in samples which have been frozen under illumination prior to triplet measurements. This $g = 2.0$ signal was attributed by Heathcote et al [5] to A which has been phototrapped in its reduced state. The observation of a radical pair-polarized P700 triplet in samples in which A was known to be fully reduced prior to illumination would constitute evidence for the existence in photosystem I of an earlier acceptor than A. Thus we undertook to study the stoichiometric ratio of the $g = 2.0$ species to P700, and found that many more radicals contributed to this signal than could be accounted for on the basis of photoreduction of A alone. Thus, our results suggest that species other than the primary electron acceptor in photosystem I give rise to the $g = 2.0$ signal.

2. Materials and Methods

Triton X-100 Photosystem I Fraction

A subchloroplast photosystem I fraction was isolated from spinach chloroplasts using Triton X-100 detergent in the presence of mono- or divalent cations as follows. Chloroplasts were isolated from market spinach in 0.4 M sucrose/0.1 M NaCl/ 0.05 M Tris (pH 8) buffer and centrifuged at 7000Xg for 5 min. The pellets were washed in 0.7 mM EDTA to remove any Mn^{+2} EPR signal and centrifuged for 10 min at 15000Xg. The pellets were then resuspended to a chlorophyll concentration of 0.3 to 0.5 mg/ml in 0.05 M

Tris (pH 8)/0.1 M NaCl or 0.01 M MgSO_4 and allowed to incubate 1 hr at 4°C to assure clean fractionation of photosystem I from photosystem II [6]. Triton X-100 was stirred into the chloroplast suspension, to give 1% detergent, and the mixture was incubated in the dark at 4°C for 2-24 hours. The supernatant of a 30 min spin at $30,000\times g$ contained photosystem I complexes with the characteristics: chlorophyll a/chlorophyll b = 5-12, chlorophyll/P700 = 175-200. This photosystem I fraction was concentrated by dialysis against crystalline sucrose before it was used in EPR experiments or subjected to further purification.

Triton X-100 Photosystem I Reaction Centers

Photosystem I reaction centers were isolated from the photosystem I fraction by the procedure of Golbeck et al. [7]. Concentrated photosystem I fraction (25ml) put through a 500 ml Sephacryl S-300 column yielded reaction centers with the characteristics chlorophyll a/chlorophyll b >9, chlorophyll/P700 = 35-65, that eluted with the column void volume. Photosystem I reaction centers were concentrated for EPR studies by dialysis against crystalline sucrose.

Assays

Chlorophyll and P700 were assayed as described in Chapter Three. Carotenoids were assayed qualitatively by thin layer chromatography as described by Barr and Crane [8].

EPR Sample Preparation

Samples of the photosystem I fraction or photosystem I reaction centers were prepared for EPR studies as described in Chapter Three. The final chlorophyll concentrations were about 0.5 or 0.1 mg ml^{-1} , respectively. The samples contained 1 mM phenazine methosulfate (PMS) or neutral red, as noted below.

Samples which were illuminated before and during freezing were cooled to 156 K using a Varian liquid nitrogen temperature controller with a reproducible cooling cycle. Cooling from 273 to 156 K took about 30 s. Samples were illuminated with a Cary High Intensity tungsten lamp using intensities at or below 1000 W m^{-2} . The EPR samples were placed in a cold finger dewar located 40 cm from the lamp, and we did not place any lenses between the sample and the lamp. Photodamage was avoided by keeping the sample temperature below 0°C during illumination and by filtering the light beam through 20 cm of water. We found that at room temperature up to 100% of the chlorophyll was irreversibly photobleached within 60 s of illumination by an unfiltered 1000 W m^{-2} beam from a tungsten lamp.

EPR Measurements

EPR measurements were made as described in Chapter Three, except that we used a TE-102 cavity that was fitted with an optical transmission flange. Light modulation experiments were performed at 33 Hz, as described in Chapter Three, except that a Princeton Applied Research Model HR-8 lock-in amplifier was used for phase sensitive detection, and we did not use a selective amplifier. The light source was a Cary High Intensity tungsten lamp focused onto the optical flange to give 5000 W m^{-2} . A Bell Model 811A digital gaussmeter was used to calibrate the magnetic field. An Air Products Helitran cryostat was used for liquid helium temperature EPR measurements.

3. Results

The EPR spectra of Figures 1-3 were obtained from four identical aliquots of photosystem I reaction centers which were treated so as to produce four different redox states of the photosystem I reaction center,

as follows:

Sample (a): frozen in the dark and then illuminated at 10 K.

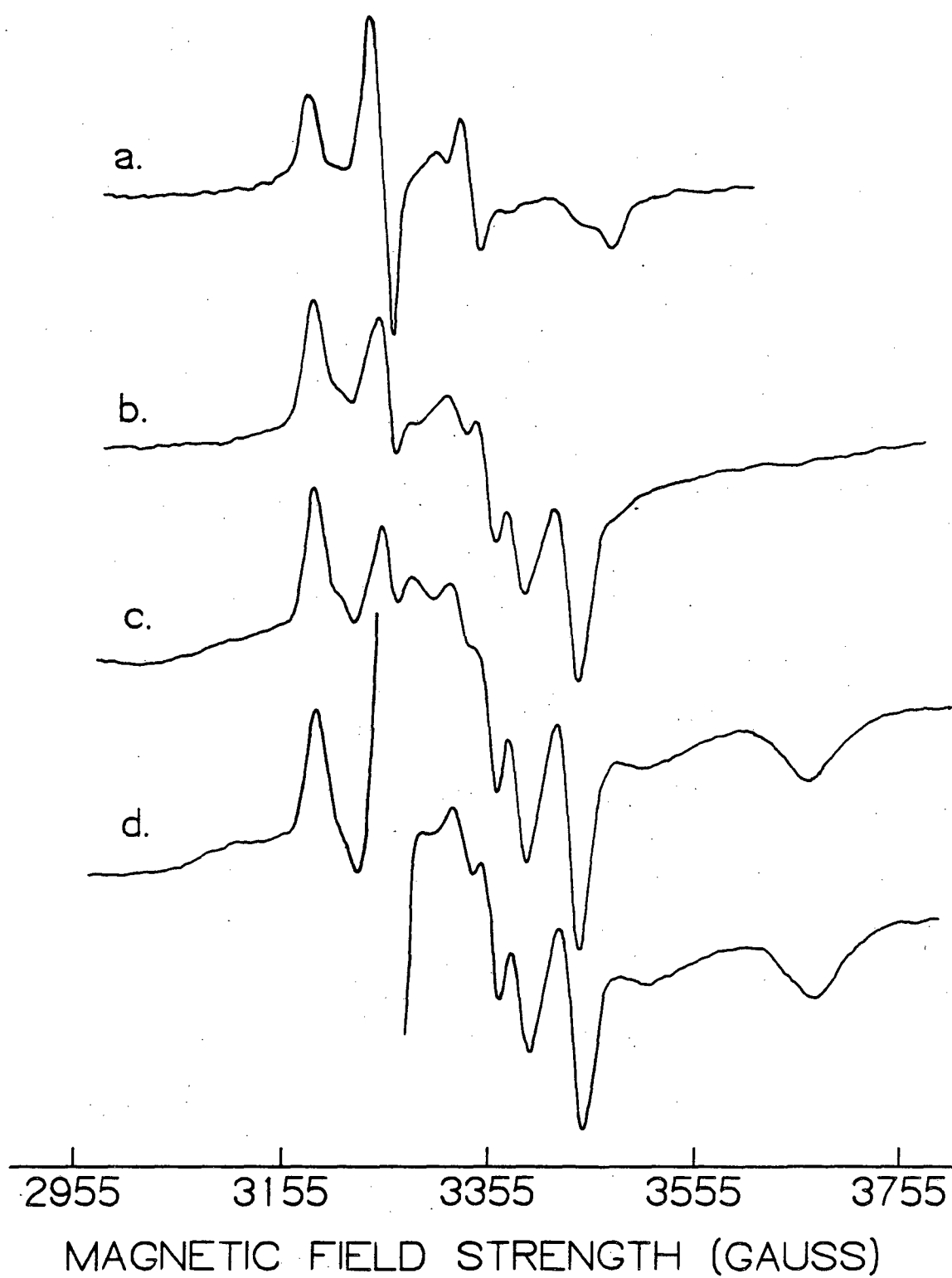
Sample (b): frozen in the dark in the presence of dithionite and PMS.

Sample (c): same as sample (b), but illuminated 60 s at 0°C and then frozen under illumination at low (50 W m^{-2}) light intensity.

Sample (d): same as sample (c), but with higher (310 W m^{-2}) light intensity.

Figures 1 and 2 show the EPR spectra recorded in the dark at 12 K of samples (a)-(d). Illumination of sample (a) at 10 K resulted in the irreversible photooxidation of P700 (Figure 2a) and photoreduction of Center A (Figure 1a), as reported by Bearden and Malkin [9]. In sample (b), ferredoxin centers A and B are reduced by dithionite, but X is not (Figure 1b), and there is not much signal in the $g = 2.0$ region present in the dark (Figure 2b). In sample (c), component X has been phototrapped in its reduced state (Figure 1c), and there is a small signal centered at $g = 2.0$ which has a linewidth of 13.5 Gauss (Figure 2c). In sample (d), component X has been phototrapped in its reduced state, and a much larger 13.5 Gauss wide signal is present at $g = 2.0$ (Figure 2d) than is present in sample (c). We shall frequently refer to this photo-generated signal as 'the $g = 2.0$ signal', to distinguish it from the photoinduced, 8 Gauss wide P700^+ signal in samples like sample (a), which we shall call 'the P700^+ signal'.

Figure 3 shows the EPR triplet spectra observed in samples (a)-(d) during 33 Hz light-modulation experiments. The phase of the lock-in amplifier was calibrated using the P^{R} triplet from a sample of photosynthetic bacteria in which quinones had been removed by sodium dodecyl sulfate treatment [10], and which was kindly provided by W.W. Parson and V.A. Shuvalov. The rise and decay times for the P^{R} state in this



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Figure 1

EPR spectra of samples (a)-(d) recorded in the dark at 10 K. Photosystem I reaction centers were: (a) frozen in the dark in glycine buffer at pH = 10 and then illuminated at 10 K; (b) frozen in the dark in the presence of 25 mM dithionite and 1 mM phenazine methosulfate at pH 10; (c) sample identical to sample (b) which was frozen under low intensity illumination; (d) sample identical to sample (b) which was frozen under higher intensity of illumination. EPR spectrometer conditions: gain, 12,500 (Figure 1a) and 10,000 (Figure 1b-1d); modulation amplitude, 20 Gauss; microwave power, 20 mW; microwave frequency, 9.16 GHz; scan rate, 250 gauss min⁻¹.

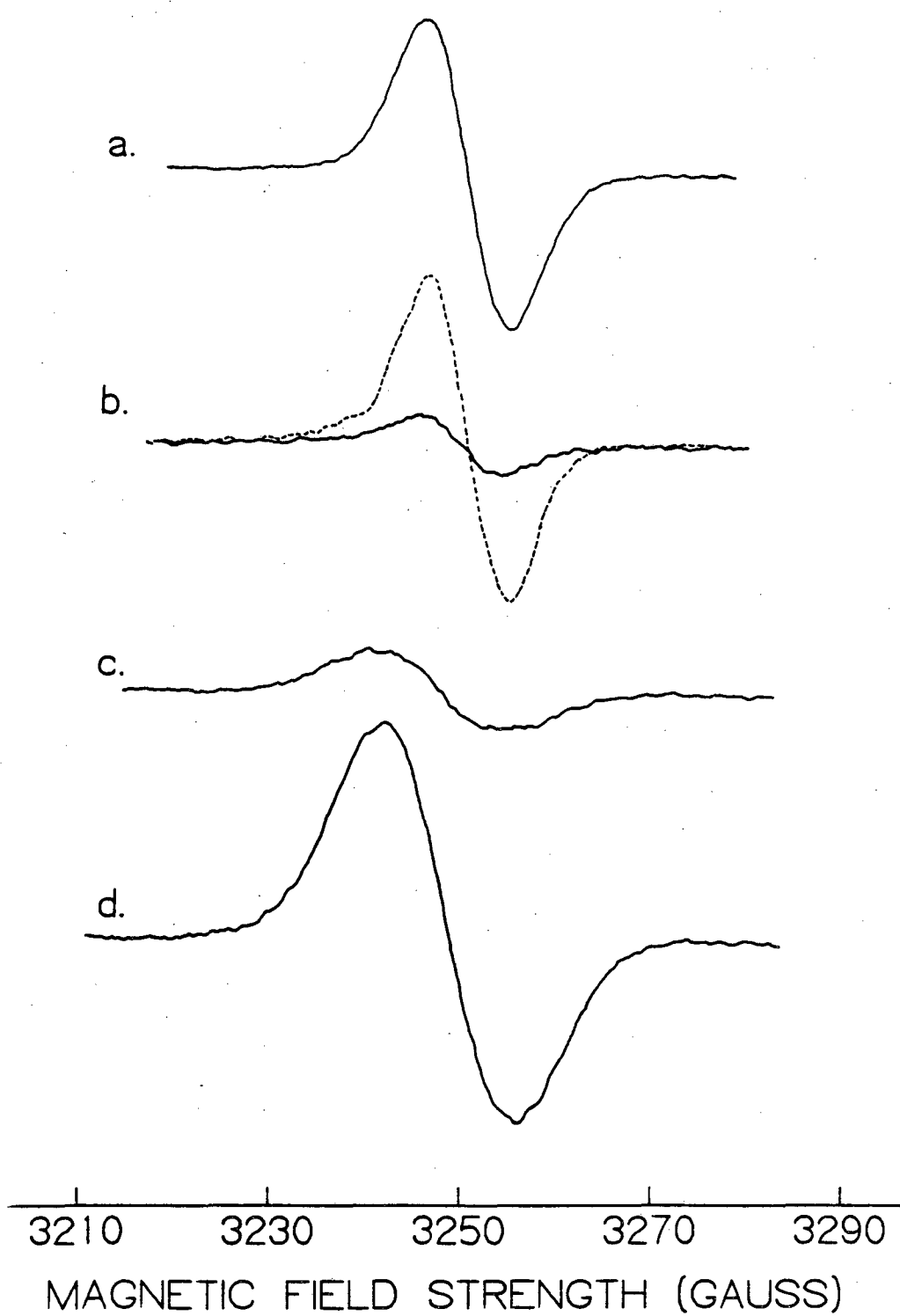
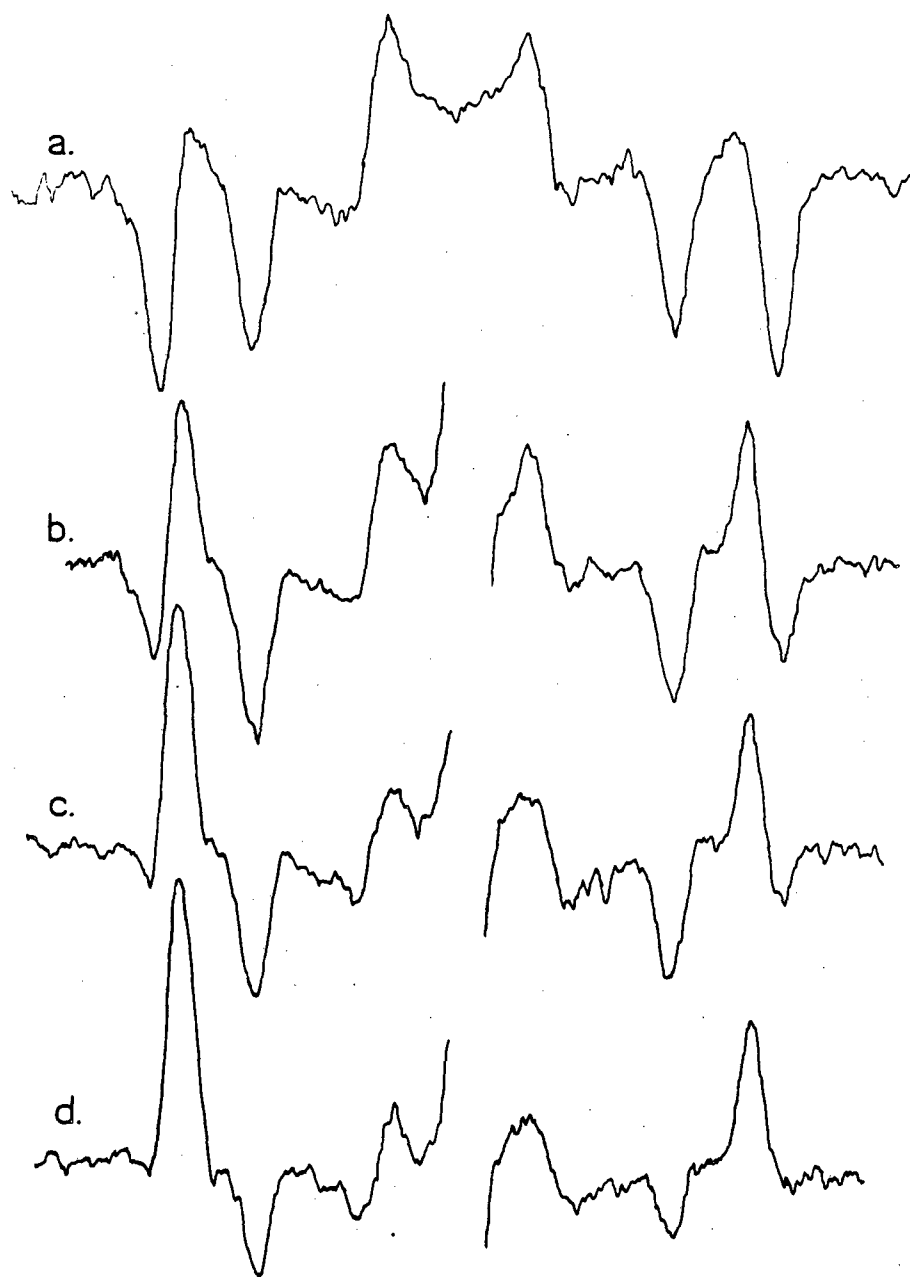


Figure 2

EPR spectra in the $g = 2.0$ region of samples (a)-(d), which are as described in Figure 1. (a) spectrum recorded during illumination of sample (a); (b, solid line) spectrum of sample (b) recorded in the dark before and after illumination; (b, dotted line) spectrum of sample (b) recorded during illumination; (c) and (d) spectra of samples (c) and (d), respectively, recorded in the dark. All spectra were recorded at 10 K. Spectrometer conditions: gain, 20,000; modulation amplitude, 4 Gauss; microwave power, 10 μ W; microwave frequency, 9.16 GHz; scan rate, 25 gauss min^{-1} .



2855 3055 3255 3455 3655
MAGNETIC FIELD STRENGTH (GAUSS)

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Figure 3

Light-modulated EPR triplets observed in samples a-d at zero degrees phase with respect to the maximum of the bacterial P^R triplet amplitude, as described in the text. All spectra were recorded at 10 K. Spectrometer conditions: 33 Hz light modulation; gain, 200; modulation amplitude, 40 Gauss; microwave power, 100 μ W; microwave frequency, 9.16 GHz; scan rate, 63 Gauss/min.

sample are known to be 10 ns and 120 μ s [11], respectively, so this signal is a convenient zero-phase reference for 33 Hz experiments. The triplet spectra in Figure 3 were recorded at zero phase with respect to the maximum amplitude of the P^R triplet. Figures 3a and 3d show the two distinct triplet spectra which we observe in photosystem I reaction centers; the spectra in Figures 3b and 3c are convolutions of these two triplets.

Figure 3d shows the radical pair-polarized triplet spectrum reported in Chapter Three. It has the polarization pattern aee aae and zero field splitting parameters $|D| = 0.0278 \text{ cm}^{-1}$ and $|E| = 0.0038 \text{ cm}^{-1}$. Figure 3a shows the EPR spectrum of a triplet with a polarization pattern eee aaa. This triplet has one of the polarization patterns which we discussed in Chapter Two as being diagnostic of an intersystem crossing population mechanism. Its zero field splitting parameters are $|D| = 0.0301 \text{ cm}^{-1}$ and $|E| = 0.0039 \text{ cm}^{-1}$. The latter $|D|$ value is somewhat larger than that reported for chlorophyll a or chlorophyll b in vitro [12,13] or of the chlorophyll a triplet observed in spinach chloroplasts by Uphaus et al. [12]. However, it seems likely that this triplet arises from a chlorophyll antenna species, and so we shall refer to it as "the antenna triplet" for clarity. Table 1 gives the zero field splitting parameters of the radical pair-polarized and antenna triplets as well as those reported elsewhere [4,12,13] for chlorophyll and pheophytin triplets.

The results presented in Figures 1-3 typify those observed in measurements on eight similar sets of samples of photosystem I reaction centers. The results from five sets of the photosystem I fraction were essentially the same. We never observed the carotenoid triplet which was observed in whole chloroplasts and in digitonin photosystem I subchloroplasts, which we discussed in Chapter Three, despite the fact

that we found substantial amounts of carotenoid in the photosystem I fraction by TLC analysis.

The $g = 2.0$ signal in Figure 2d has the same characteristics as the signal that Heathcote et al. [5] observed in Triton X-100 reaction centers which had been frozen under intense illumination. They attributed this signal to A which had been phototrapped in its reduced state. Figure 4 shows the results of experiments intended to correlate the number of spins represented by the $g = 2.0$ signal with the total reaction center concentration and with the amplitude of light-inducible radical pair-polarized triplet. Figure 4 shows the results of three separate trials. In each trial, a set of EPR samples was prepared from identical aliquots of photosystem I reaction centers or the photosystem I fraction in 0.1 M glycine buffer at pH 10. Several aliquots in each set were treated with dithionite and PMS and were then illuminated before and during freezing, each with a different light intensity, in order to vary the amplitude of the phototrapped $g = 2.0$ signal. (We found that the $g=2.0$ signals photogenerated in sets of samples containing PMS and dithionite had the same amplitudes as those in otherwise identical sets of samples which did not contain PMS.) Each set of samples included a sample which did not contain dithionite or PMS, so that P700 could be irreversibly photooxidized at low temperatures, e.g. sample (a) above. The number of $P700^+$ spins in the latter sample was assumed to be equimolar with the total reaction center concentration. The $P700^+$ signal in the latter sample, and the $g = 2.0$ signals in the other samples in the set were recorded in the dark at 100 K at non-saturating microwave powers. Under these conditions, the integrated areas of the signals are a direct measure of the number of spins, N , giving rise to the $g = 2.0$ signal and of the number of spins

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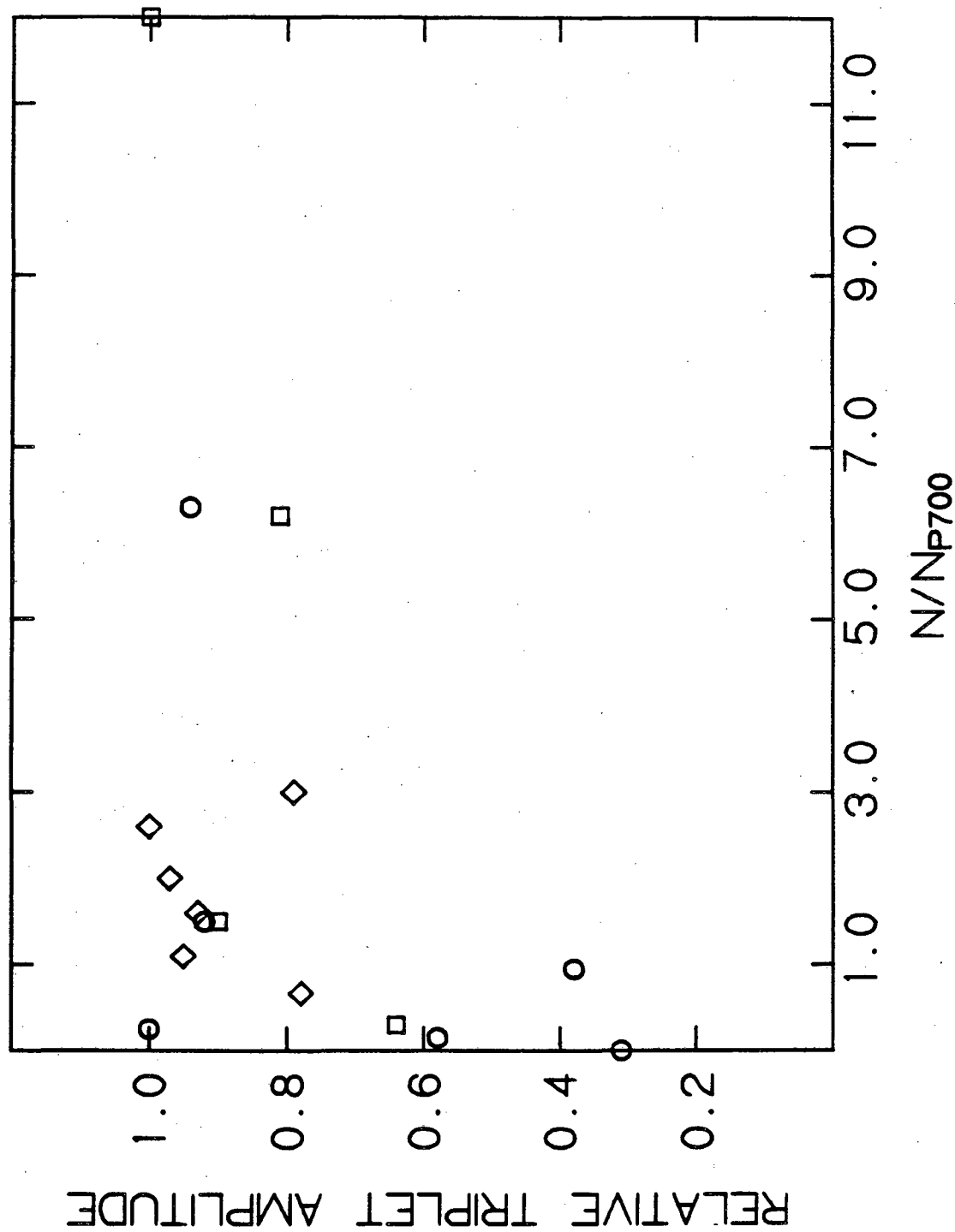


Figure 4

Plot of the ratio N/N_{P700} vs. the amplitude of the radical pair-polarized triplet, where N is the number of spins represented by the $g = 2.0$ signals (e.g. Figure 2d) which are photo-generated by freezing aliquots of reaction centers in the presence of dithionite and phenazine methosulfate during illumination of varying intensity, and N_{P700} is the number of spins represented by the $P700^+$ signal (e.g. Figure 2a) in an identical reaction center aliquot which does not contain dithionite or phenazine methosulfate but has been illuminated at 77 K. (○), (□) and (◇) represent three different sets of measurements. See Results section for details. Conditions for triplet measurements are as in Figure 3, except that 11 Hz light modulation frequency was used. The $g = 2.0$ EPR signal measurements were made at 77 K and 10 μ W microwave power.

giving rise to the $P700^+$ signal, N_{P700} . The ratios N/N_{P700} relate the moles of the $g = 2.0$ species to the moles of P700. EPR triplet spectra were then recorded using an 11 Hz chopper, a frequency at which the antenna triplet makes a minimal contribution to the observed triplet spectra. The amplitude of the low field Z peak of the radical pair-polarized triplet was used in Figure 4 to represent the triplet amplitude. Figure 4 shows that up to 12 spins per reaction center may be photo-generated in the $g = 2.0$ signal without having any apparent effect on the amplitude of light-inducible radical pair-polarized triplet.

4. Discussion

On The Zero Field Splitting Parameters of the Radical Pair-Polarized and Antenna Triplets

In bacterial systems, the $|D|$ value of the P^R triplet is 20% smaller than that of bacteriochlorophyll monomer [14]. This effect is well accounted for by the argument that it results from a delocalization of the P^R triplet over two bacteriochlorophyll molecules in the reaction center special pair [13,14]. The evidence presented here and elsewhere [4,12] suggests that such a simple relation does not hold between observed zero field splitting parameters and the aggregation state of chlorophylls in vivo or in vitro. Table 1 allows comparison of the zero field splitting parameters of the radical pair-polarized and antenna triplets with those previously reported [12,13] for chlorophyll and pheophytin triplets. Although P700 has long been thought to be a dimer [15], the radical pair-polarized triplet $|D|$ value is not significantly different from that of chlorophyll a monomer. This is consistent with the observation of Uphaus et al. [12] that the $|D|$ values of chlorophyll a dimer and chlorophyll b

Table 1
Zero field splitting parameters reported for chlorophyll
and pheophytin triplet states in vivo and in vitro.

<u>Species</u>	<u>Zero Field Splitting Parameters</u>	
	$ D (\text{cm}^{-1})$	$ E (\text{cm}^{-1})$
Radical Pair ^a	0.0278	0.0038
Antenna ^a	0.0301	0.0039
<u>in vitro</u> ^b		
Chlorophyll <u>a</u> monomer	0.0275	0.0036
Chlorophyll <u>b</u> monomer	0.0287	0.0037
Pheophytin <u>a</u>	0.0339	0.0033
<u>in vivo</u>		
Spinach chloroplasts ^b	0.0284	0.0039
Spinach chloroplasts ^c	0.0278	0.0039

^aError limits 0.0002 cm^{-1}

^bFrom ref. [12]

^cFrom ref. [4]

oligomer were within 3% of their respective monomeric values. Indeed, based on their recent findings, Wasielewski et al. [16,17] have argued that P700 is a chlorophyll a monomer rather than a dimer.

The question of the correlation between the zero field splitting parameters and chlorophyll environment is also raised by the chlorophyll antenna $|D|$ value, which is significantly larger than that of either chlorophyll a or chlorophyll b monomer, and smaller than that of pheophytin. We cannot exclude the possibility that the antenna triplet arises from chlorophyll b or pheophytin on the basis of its $|D|$ value. However, it is now generally thought that chlorophyll b and pheophytin do not play a role in photosystem I, so that it is as likely that the antenna triplet arises from chlorophyll a.

On the Redox Dependence of the Antenna Triplet

We mentioned in the Introduction to this Chapter that the antenna triplet appears to depend upon the redox state of photosystem I. Figures 3a-d show clearly that the amplitudes of both the radical pair-polarized triplet and of the antenna triplet depend on the state of reduction of photosystem I acceptors at cryogenic temperatures. The antenna triplet is not observed in samples in which X has been reduced prior to illumination (Figures 3c and 3d); it is observed together with the radical pair-polarized triplet in samples in which centers A and B are reduced but X is not reduced prior to illumination (Figure 3b).

These data are consistent with the assignment of the antenna triplet to an excited antenna species which undergoes intersystem crossing when P700 is unable to accept excitation during the illumination period. That is, we assign the antenna triplet signal to an antenna species which is coupled to

P700 and which can transfer excitation to P700 when the latter is in the ground state. The evidence for this interpretation is that the antenna triplet has its maximum amplitude in sample (a), in which presumably the entire population of reaction centers has undergone irreversible charge separation to the state $P700^+Fd_A^-$, as indicated by the spectra in Figures 1a and 2a. Thus it is plausible to argue that the antenna triplet arises from antenna chlorophyll molecules which are coupled to the reaction center, i.e. able to transfer their excitation to it and therefore sensitive to the state of P700.

We make a similar argument for the observation of the antenna triplet in sample (b). In sample (b), illumination results in charge separation to the state $P700^+X^-$ and so we observed the EPR signals from $P700^+$ (Figure 2b) and X^- (data not shown), as reported by Evans et al. [18]. Figures 2a and 2b show that the amplitude of the light-induced $P700^+$ EPR signal in sample (b) is equal to that observed in sample (a), so that both signals represent the same number of spins. Once again, we argue that the $P700^+$ in sample (a) arises from an irreversible light-induced electron transfer to center A in all of the reaction centers, so that all of the reaction centers in sample (b) could achieve the state $P700^+X^-$ during illumination. The 120 ms [2] lifetime of the state $P700^+X^-$ is slow compared to the 33 Hz chopper period, so there will be a significant steady-state population of reaction centers in the state $P700^+X^-$ during experiments using 33 Hz phase sensitive detection. This steady-state population of reaction centers in which P700 is oxidized during the illumination period would then give rise to the antenna triplet.

In samples (c) and (d), X is reduced prior to measurement. Illumination results in charge separation to the state $P700^+A^-$, which has a

lifetime more than an order of magnitude shorter than the 33 Hz illumination period. In this case, no significant steady-state population of reaction centers in the state $P700^+$ is achieved, and the antenna triplet is not observed.

On the Redox Dependence of the Radical Pair-Polarized Triplet

In sample (b), we observe a convolution of the radical pair-polarized triplet with the antenna triplet. One explanation of this observation is that the state 3P700 is populated as an intermediate in the $P700^+X^-$ back reaction. However, this does not seem likely, because the latter process cannot give rise to a polarized triplet on P700 if the spin states of the $P700^+X^-$ radical pair become thermally relaxed before charge recombination populates 3P700 . The X^- EPR signal is not saturated at 100 mW at 10 K, indicating that its spin-lattice relaxation time is not longer than a few microseconds. The spin-lattice relaxation time of $P700^+$ was reported to be 800 μ s at 10 K [19]. Spin-lattice relaxation will destroy any polarization on the $P700^+X^-$ radical pair within the 120 ms lifetime. Therefore, it seems likely that the radical pair-polarized triplet arises from $P700^+A^-$ radical pair recombination rather than from $P700^+X^-$ pair recombination. This means that the radical pair $P700^+A^-$ sometimes undergoes charge recombination to the state 3P700 in preference to forward electron transfer from A^- to X . Two possible interpretations of this phenomenon would be that there is a heterogeneity in the population of photosystem I reaction centers, or there is a similarity between the rates of the two processes, $P700^+A^- \rightarrow ^3P700$ and $P700^+A^- \rightarrow P700^+X^-$. We shall discuss these two interpretations in the Conclusion of this chapter.

In the case of samples (c) and (d), we observe that the reduction of

the species giving rise to the $g = 2.0$ EPR signal shown in Figure 2d has no apparent effect on the amplitude of the radical pair-polarized triplet. This suggests that the $g = 2.0$ radical is not the primary photosystem I acceptor in its reduced state. If this radical were the fully reduced primary acceptor, then charge separation could not occur and we would not observe the radical pair-polarized triplet. Figure 4 shows that up to 12 spins per P700 may be photo-generated and contribute to the $g = 2.0$ signal without having any apparent effect on the amplitude of the radical pair-polarized triplet. Thus, the results presented in Figure 4 show that there are species other than A^- contributing amplitude to the $g=2.0$ signal. We suggest that photooxidized antenna chlorophyll contributes to this amplitude. However, it is also possible that the $g=2.0$ signal contains a contribution from phototrapped A^- . The observation of the radical pair-polarized triplet in samples in which A was known to be fully reduced would be evidence for the existence of an earlier acceptor than A. Unfortunately, the EPR spectrum of A^- is apparently very similar to that of the other photogenerated species which contribute to the $g=2.0$ signal, and so it may be difficult to establish the state of reduction of A on the basis of these EPR data.

Rutherford and Mullet [20] reported studies on photosystem I reaction centers in which X and Centers A and B had been denatured by treatment with lithium dodecyl sulfate. They observed the radical pair-polarized triplet in samples which contained dithionite and neutral red and were frozen in the dark. After these samples had been thawed and refrozen during illumination, the $g = 2.0$ signal attributed to A^- was present and the radical pair-polarized triplet amplitude was smaller, suggesting that charge separation is not occurring when the $g = 2.0$ radical is

phototrapped. After the latter samples had been thawed and refrozen in the dark, the $g = 2.0$ signal was smaller, and the original amplitude of the radical pair-polarized triplet was restored. Our results (data not shown) with photosystem I reaction centers that have been treated with urea and ferricyanide by the method of Golbeck et al [7] to remove iron from X and Centers A and B are in agreement with the results of Rutherford and Mullet [20]. These observations can be understood if A or an even earlier acceptor is easier to phototrap in the reduced state in preparations in which X and Centers A and B are not present. When these secondary acceptors are active, they must be reduced before earlier acceptors can be photoreduced, and the latter must then be reduced in close proximity to three or four negatively charged sites.

5. Conclusion

On the possibility of a branch point in Photosystem I

The observation of the antenna triplet and of the full extent of $P700^+$ steady-state photooxidation in sample (b) are suggestive that the reaction centers are able to be completely converted to the state $P700^+X^-$ during continuous illumination. On the other hand, the observation of the radical pair-polarized triplet signal from P700 in sample (b) is suggestive that every time a reaction center is excited, it has some probability that A^- will not transfer its electron to X but will instead undergo radical pair recombination to 3P700 . These two apparently conflicting observations may be rationalized in the following way. The lifetime of the state $P700^+X^-$ (120 ms) is much longer than the lifetime of the state 3P700 (1 ms), so that the reaction centers are in the former state most of the time during continuous illumination. This could be why we observe the full extent of

significant fraction of the photosystem I population, P700 and A would not be connected to the secondary acceptors, X and Centers A and B. This interpretation is consistent with the observation of the radical pair polarized $^3\text{P700}$ EPR signal when X is not reduced prior to illumination and also with the observations of biphasic P700 optical kinetics [1,2]. This interpretation is also consistent with the observation that the light-induced P700^+ EPR signal arising from the $\text{P700}^+\text{X}^-$ charge separation has the same amplitude as the P700^+ EPR signal observed in the redox state $\text{P700}^+\text{Fd}_\text{A}^-$: if a certain fraction of the reaction centers are not connected to X and Centers A and B, then the irreversible charge separation between P700 and Center A cannot occur in that fraction of the reaction center population. Thus the P700^+ EPR signal would have the same amplitude in both the $\text{P700}^+\text{X}^-$ and the $\text{P700}^+\text{Fd}_\text{A}^-$ charge separations. A very simple experiment would settle this question. It is to compare the amplitude of the EPR signal from P700^+ in a ferricyanide treated sample to the amplitude of the EPR signal from P700^+ arising from the $\text{P700}^+\text{Fd}_\text{A}^-$ charge separation at temperatures below about 100 Kelvin, when the latter charge separation is irreversible. If the two signal amplitudes are equal, then we know that all of the reaction centers can undergo the $\text{P700}^+\text{Fd}_\text{A}^-$ charge separation, and are therefore connected to the secondary acceptors, and that there is no heterogeneity in the photosystem I reaction center population. If the two signal amplitudes are not equal, then we know that P700 in a fraction of the reaction centers cannot undergo charge separation with Center A, and thus may not be connected to the secondary acceptors. To our knowledge, this experiment has not been done yet.

If there is such a heterogeneity in the photosystem I reaction center population, then we would like to know its physiological significance. It

is possible that heterogeneity could arise as an artifact of detergent treatment. However, if the heterogeneity is physiological, then we might speculate that a certain fraction of photosystem I is connected to X and Centers A and B and to photosystem II, so that it is functional for whole chain electron transport from water to NADP^+ . The remaining fraction would not be connected to X and Centers A and B but would be coupled to the quinone-cytochrome-Rieske FeS oxidoreductase (see Figure 1 of Chapter One) and would undergo cyclic electron transport.

Finally, we need to mention the results reported by Shuvalov et al. [23] which are suggestive that a photokinetic branch point or heterogeneity exists within photosystem I at physiological temperatures. In room temperature kinetics measurements, Shuvalov et al. [23] observed a biphasic decay of optical changes attributed to $\text{P700}^+\text{A}^-$ radical pair formation under conditions where Centers A and B were reduced prior to measurement. They assigned a 10 nsec component at 694 nm to the recovery of changes in the spectrum of A due to radical pair recombination to a triplet state, presumably $^3\text{P700}$. They also observed a 3 μs component which they attributed to the $^3\text{P700}$ lifetime. However, they noted that the amplitude and decay rate of the 10 ns component were the same regardless of whether X was reduced by exogenous donors and background illumination during the kinetics measurements. This result again suggests a similarity between the two rate constants for the processes of $\text{P700}^+\text{A}^-$ pair recombination and forward electron transfer from A^- to X, or a heterogeneity in the photosystem I reaction center population. If the observations of Shuvalov et al. [23] result from a branch point in photosystem I primary photochemistry, then it would appear that when Centers A and B are reduced, the forward electron

transfer time from A^- to X is on the order of 10 ns, which is 50 times slower than the 200 ps forward transfer time when Centers A and B are not reduced prior to illumination [23]. Clearly this means that the reduction of Centers A and B affects the kinetics of primary events in photosystem I, and that the kinetics involving P700, A and X occurring during primary photochemistry under physiological conditions will need to be studied in some other way, such as by selectively removing Centers A and B from the reaction center. So far, however, any treatment which removes Centers A and B also removes X [7,20].

Whether there is, on the one hand, a heterogeneity within the photosystem I reaction center population, or, on the other hand, a branch point in photosystem I primary reaction kinetics, remains an open question and the subject for future investigations.

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CHAPTER FIVE

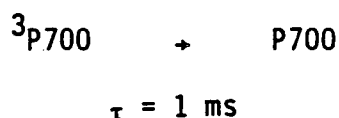
ANTENNA AND RADICAL PAIR TRIPLET STATE KINETICS

1. Introduction

There is no way to improve upon the deduction by Shuvalov back in 1976 [1], which predated all the work presented here, that primary radical pair recombination within photosystem I not only populates the triplet state of P700, but that the lifetime of the triplet state of P700 is on the order of 1 ms. Shuvalov's evidence was the recombination luminescence data mentioned in the Introduction to Chapter Three. We note that Shuvalov's deduction predated the first direct evidence for the existence of the intermediate acceptor A, which was not generally available until more than three years later [2]. The work that we will present in this chapter will only prove that Shuvalov was right.

The clear picture that has emerged from Chapters Three and Four is that our original working hypothesis was incorrect: this was that the 1 ms component of reversal of P700 optical changes corresponds to the $P700^+A^-$ radical pair lifetime. This ties into what we said in Chapter One, i.e. that intermediate states which P700 passes through before it returns to the ground state will not be distinguishable by optical absorption spectroscopy at 700 nm so long as the intermediate states of P700 all involve bleaching at 700 nm. One state that will not affect the bleaching at 700 nm is the state 3P700 , because it has as small an extinction coefficient at 700 nm as does P700 cation [3]. Thus we suspect that the observation of the polarized triplet state EPR spectrum of P700 is evidence for the assignment

of the 1 ms component of optical recovery at 700 nm to the process,



This would be a more satisfactory explanation of the observations of Sauer et al. [2] because it has never been thought plausible that the lifetime of the $\text{P700}^+\text{A}^-$ radical pair should be as long as 1 ms [4].

In this chapter, we report studies of the kinetics of triplet EPR signals observed in Triton X-100 photosystem I reaction center preparations. These studies were complicated by the convolution of ${}^3\text{P700}$ EPR signals with those from the antenna chlorophyll triplet reported in Chapter Four. Because the antenna chlorophyll triplet arises from 40-200 chlorophylls per P700 in the preparations that we have studied, the amplitude of the antenna chlorophyll EPR signal has a very different dependence upon the excitation light intensity than does the amplitude of the P700 triplet signal. Thus we studied the light intensity dependence of the amplitudes of both the antenna and the radical pair polarized P700 triplet. At low light intensities, we observe mainly the triplet state of P700 and not the antenna triplet state in samples which have been prepared in the redox state $\text{P700 A X}^-\text{Fd}_\text{B}^-\text{Fd}_\text{A}^-$ prior to illumination. However, the signal/noise for the triplet signal from P700 is too poor for us to be able to determine the complete magnetic field dependence of the triplet decay rates, and their dependence on the microwave power level. This means that we cannot give a good number for the lifetime of ${}^3\text{P700}$. On the other hand, from the study of the dependence of the antenna triplet signal amplitude on the light intensity, we are able to demonstrate that the antenna triplet

signal arises from a species which is able to donate excitation to P700. We are also able to successfully demonstrate the validity of the theory presented in Chapter Two, because it can account for our data, whereas the previous analyses [6,7] cannot.

2. Theoretical Section

We now wish to derive explicit expressions for the EPR signal amplitudes as a function of time, $S_+(t)$ and $S_-(t)$, given in Eq. 70 of Chapter Two. According to Eqs. 67-70 of Chapter Two,

$$(1) \quad \hat{N}(t) = \exp(-\underline{K}t) \hat{N}_B(0) + \underline{U} \underline{D} \exp(-\underline{\Delta}t) \underline{D}^{-1} \underline{U}^{-1} (\phi N_E - \hat{N}_B(0))$$

where $\underline{K}$, $\hat{N}_B(0)$, $\underline{U}$, $\underline{D}$, $\underline{\Delta}$, and $\hat{\phi}$ are as defined in Chapter Two. We now define

$$(2) \quad \hat{N}_B(0) = \hat{\phi} \phi_T N_E$$

where

$$\phi_T = \sum_i \phi_i$$

$$\hat{\phi} = \begin{bmatrix} f_1 \\ f_0 \\ f_{-1} \end{bmatrix}$$

and where

$$\frac{f_i}{f_j} = \exp(-\Delta E_{ij}/kT)$$

and $\sum_i f_i = 1$. We also define the polarization vector, $\hat{P}$, as

$$\vec{P} = \begin{bmatrix} P_1 \\ P_0 \\ P_{-1} \end{bmatrix}$$

where

$$(3) \quad P_i = (\phi_i - f_i \phi_T) N_E$$

and clearly, $\sum_i P_i = 0$.

Then the signal amplitudes $S_+(t)$ and $S_-(t)$ in Eq. 70 of Chapter Two are given by the exact expressions

$$\begin{aligned} (4) \quad S_{\pm}(t) = & \frac{1}{2} N_E \phi_T (f_1 - f_{-1}) \exp(-\lambda_1 t) \\ & \mp \frac{1}{2} N_E ((P_1 + P_{-1} - 2P_0) \frac{\cos^2 \theta}{\det D}) \exp(-\lambda_+ t) \\ & \mp \frac{1}{8} N_E ((P_1 + P_{-1} - 2P_0) \frac{\sin^2 \theta}{\det D}) \exp(-\lambda_- t) \\ & \mp \phi_T N_E (f_{\pm 1} \exp(-K_1 t) - f_0 \exp(-K_0 t)) \end{aligned}$$

where $\det D = \cos^2 \theta + \sin^2 \theta / 4$. Clearly it would be very useful to simplify Eq. 4. Fortunately, we can make some good approximations. In most systems, it is possible to assume that the spin lattice relaxation rates are at least ten times as fast as the triplet sublevel decay rates [5,6]. This approximation is often good at 10 Kelvin, and is even better at higher temperatures. The first consequence of making this assumption is that we may write a small parameter, ϵ , as in Eq. 69 of Chapter Two, where

$$\epsilon = (K_1 - K_0) / 9W_1 < 10^{-1}$$

Thus, because $\sin \theta \approx \epsilon$, we may immediately neglect the decay rate λ_- in Eq.

4, whose amplitude is proportional to $\sin^2\theta$. We may also write $\cos^2\theta = 1$ and $\det D = 1$, and we may think of the remaining rates as

$$\lambda_1 = W_1 + 2W_2$$

$$\lambda_+ = 3W_1$$

By assuming that $W_1, W_2 \gg K_i$ we are neglecting the off-diagonal elements in Eq. 68 of Chapter Two. Thus, the assumption that $W_1, W_2 \gg K_i$ allows us to simplify Eq. 4 to

$$(5) \quad S_{\pm}(t) = \pm N_E \{ (\phi_0 - \phi_1) \exp(-\lambda_+ t) + \phi_T (f_0 \exp(-K_0 t) - f_{\pm 1} \exp(-K_1 t)) \\ - \frac{1}{2} \phi_T [(f_0 - f_{\pm 1}) (\exp(-\lambda_1 t) + \exp(-\lambda_+ t)) \\ + (f_0 - f_{\mp 1}) (\exp(-\lambda_1 t) - \exp(-\lambda_+ t))] \}$$

Equation 5 is still rather complicated, so it is worthwhile to pause here and to consider what signal kinetics governed by Eq. 5 would look like. A second result of the assumption that $W_1, W_2 \gg K_i$ is that the rates λ_i and λ_+ are separable from the K_i in the sense that they represent processes which occur on very different timescales. Let us see what Eq. 5 looks like at times much less than $1/K_i$, that is, before the triplet sublevel populations have begun to decay significantly to the ground state. On such a time scale, we may say $\exp(-K_i t) \approx 1$, so that Eq. 5 becomes

$$(5') \quad S_{\pm}(t) = \pm N_E [(\phi_0 - \phi_1) \exp(-\lambda_+ t) \\ + \frac{1}{2} \phi_T (f_0 - f_{\pm 1}) (2 - \exp(-\lambda_1 t) - \exp(-\lambda_+ t)) \\ - \frac{1}{2} \phi_T (f_0 - f_{\mp 1}) (\exp(-\lambda_1 t) - \exp(-\lambda_+ t))]]$$

According to Eq. 5', $S_{\pm} = \pm N_E (\phi_0 - \phi_1)$ at time zero. Eq. 5' shows that the initial polarized signal amplitude $\pm N_E (\phi_0 - \phi_1)$ will decay to zero at a rate λ_+ and that spin lattice relaxation will cause biphasic signal

kinetics with the rates λ_1 and λ_+ . If λ_1 and λ_+ are very different from one another, Eq. 5' implies that $S_{\pm}$ can have complicated behavior at early times. However, Eq. 5' also implies that no matter how different λ_1 and λ_+ are with respect to one another, as long as both are much greater than the K_i , the signals $S_{\pm}$ will approach their thermal equilibrium values, $\pm \phi_T N_E (f_0 - f_{\pm 1})$, before the sublevel populations begin to decay to the ground state.

If we now look at times much longer than the time it takes for the system to reach thermal equilibrium, then according to Eq. 5, the decay of the sublevel populations to the ground state gives rise to kinetics in the signals $S_{\pm}$ which is of the form $\pm(f_0 \exp(-K_0 t) - f_{\pm 1} \exp(-K_1 t))$, which are biphasic if K_1 and K_0 are different. We shall show in the next section that the decay of the sublevel populations to the ground state can give rise to EPR polarization if K_1 and K_0 are different. Thus there appear to be two distinct mechanisms of EPR signal polarization: first, the initial polarization, $\pm N_E (\phi_0 - \phi_1)$, which decays to zero with the rate λ_+ ; and, second, the polarization caused by selective decay of the sublevel populations to the ground state. We shall discuss these two polarization mechanisms in the next section.

Before doing so, however, it is useful to compare the solutions presented here in Eqs. 4 and 5 to those obtained previously by Levanon and Vega [7] and by Winscom [6]. Because they did not take the time dependence of the spin lattice relaxation rates into account, neither analysis gives rise to term $\pm(f_0 \exp(-K_0 t) - f_{\pm 1} \exp(-K_1 t))$ in Eqs. 4 and 5. On the other hand, the solutions obtained by Levanon and Vega [7] contain crossterms proportional to $\cos \theta \sin \theta$. These terms vanish exactly in our analysis, because when explicit account is made of the triplet quantum yields, and when the Boltzmann populations at time zero are defined as in Eq. 2, these

terms are multiplied by $P_{1-} + P_{0-} + P_{-1}$, which is exactly zero. However, the crossterms $\cos\phi\sin\phi$ in the Levanon and Vega analysis were the only terms capable of explaining the case where an absorptively polarized signal turns over into emission before decaying to zero. In our case, this phenomenon will be explained by the term $\pm(f_0\exp(-K_0t) - f_{\pm 1}\exp(-K_1t))$, as we shall describe in the next section. Thus, we believe that the analysis we have developed and are presenting here and in Chapter Two is the first analysis which is capable of describing the case of an absorptively polarized signal which turns over into emission before decaying to zero. It is also the first analysis which is capable of describing a polarized signal which decays to the Boltzmann difference before the triplet relaxes to the ground state.

Triplet EPR Signal Polarization

Eq. 5 suggests that there are two distinct mechanisms resulting in a non-Boltzmann population of the triplet spin sublevels which may be observed in an EPR experiment.

The first polarization mechanism is represented by the first term in Eq. 5, which we shall call $S_{I\pm}(t)$, where

$$(6) \quad S_{I\pm}(t) = \pm N_E(\phi_0 - \phi_1)\exp(-\lambda_+t)$$

$S_{I\pm}(t)$ has the value $\pm N_E(\phi_0 - \phi_1)$ at time zero, and it decays to zero with the spin lattice relaxation rate $\lambda_+ \approx 3W_1$. The amplitude of the EPR signals $S_{\pm}$ at time zero is the initial sublevel population difference, and this population difference which results from a selectivity in the triplet sublevel population rates, i.e. differences between the k_i . We shall refer

to EPR polarization resulting from this mechanism as 'initial polarization'.

It will be convenient to write the initial signal polarizations in terms of polarization ratios, $P_{I\pm}(t)$, such that

$$(6') \quad P_{I\pm}(t) = \frac{S_{I\pm}(t)}{\phi_T N_E (f_0 - f_{\pm 1})}$$

$P_{I\pm}(t)$ is a convenient quantity because it gives the signal $S_{I\pm}(t)$ scaled to the Boltzmann population difference at thermal equilibrium.

The second polarization mechanism is represented by the second term in Eq. 5, which we shall call $S_{D\pm}(t)$, where

$$(7) \quad S_{D\pm}(t) = \pm \phi_T N_E (f_0 \exp(-K_0 t) - f_{\pm 1} \exp(-K_1 t))$$

If we assume as above that $K_i \ll \lambda_+$, λ_1 , and if we observe the EPR signal at times which are much longer than the spin lattice relaxation times, then the signal $S_{D\pm}(t)$ starts out with the amplitude $(f_0 - f_{\pm 1})\phi_T N_E$, which is the amplitude of the triplet state EPR signal at thermal equilibrium.

According to Eq. 7, if $K_0 \neq K_1$, it will be possible to observe that $S_{D\pm}(t)$ deviates from a simple monophasic decay of the thermal equilibrium signal to zero. When $K_0 \neq K_1$, we will observe a biphasic decay of two components with opposite signs, $\pm f_0 \exp(-K_0 t)$ and $\mp f_{\pm 1} \exp(-K_1 t)$. We shall refer to EPR polarization which results from the second mechanism as 'decay polarization'.

These two polarization mechanisms result in three polarization cases, which we shall now describe in detail.

A. The case of decay polarization when there is zero initial polarization

If the system is observed not to be polarized at time zero, either because the spin sublevels have not been selectively populated, or because spin lattice relaxation has resulted in a Boltzmann population of the spin sublevels at a time much less than the EPR spectrometer response time, then it is still possible to observe EPR polarization by the mechanism of decay polarization if $K_0 \neq K_1$. The decay polarization ratio may be defined as in Eq. 6', i.e.

$$(8) \quad P_{D\pm}(t) = \frac{S_{D\pm}(t)}{\frac{1}{2} N_E (f_0 - f_{\pm 1})} = \frac{f_0}{f_0 - f_{\pm 1}} \exp(-K_0 t) - \frac{f_{\pm 1}}{f_0 - f_{\pm 1}} \exp(-K_1 t)$$

As mentioned above regarding Eq. 6', the value of $P_{D\pm}(t)$ gives the signal $S_{D\pm}(t)$ in units of the Boltzmann population difference.

We now wish to study what the decay polarization looks like by means of computer simulations, and we shall rewrite Eq. 8 in a form convenient for computations. According to Eq. 2

$$(9) \quad \frac{f_{\pm 1}}{f_0} = \exp(-(E_{\pm 1} - E_0)/kT)$$

Thus we expect both the decay polarization $S_{D\pm}(t)$ and the decay polarization ratio $P_{D\pm}(t)$ to depend upon temperature. To include this dependence explicitly in Eq. 8, we rewrite Eq. 9 as

$$(10) \quad \frac{f_{\pm 1}}{f_0} = 1 - \frac{(E_{\pm 1} - E_0)}{kT} + \frac{(E_{\pm 1} - E_0)^2}{k^2 T^2} - \dots$$

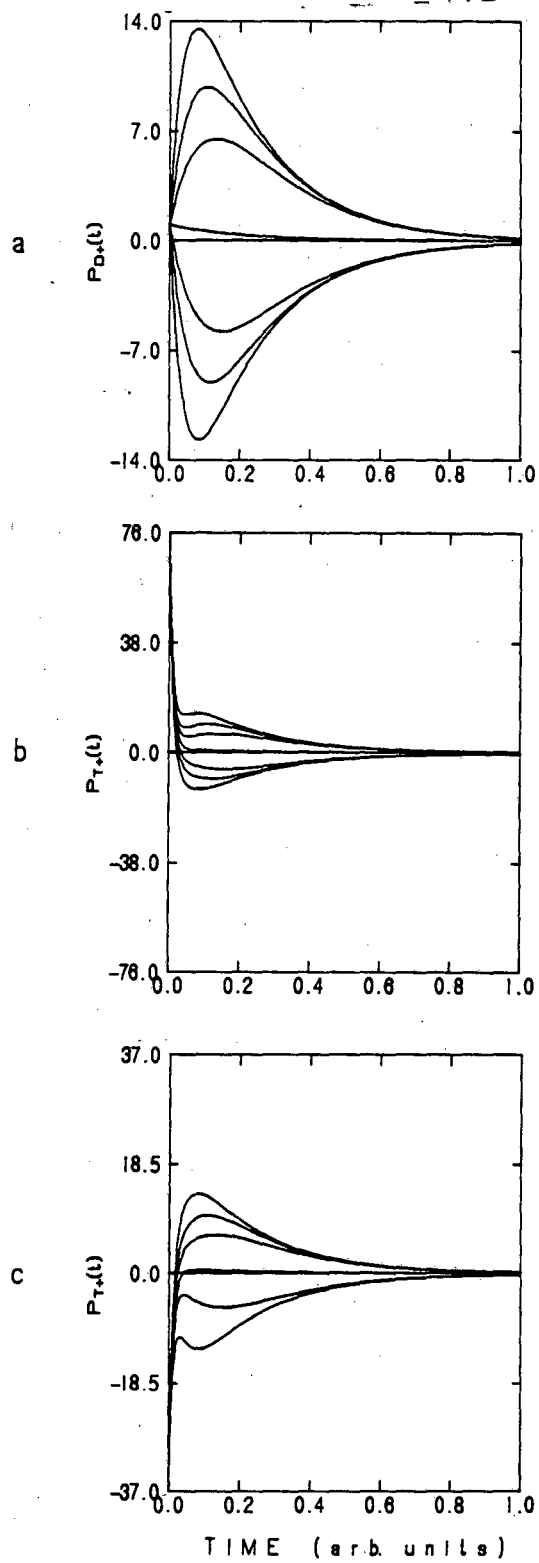
At X band, $(E_{\pm 1} - E_0)/kT = \pm 0.44/\text{Kelvin}$. We shall confine ourselves to temperatures above about 10 Kelvin, drop terms of the order $(0.44/\text{Kelvin})^2$,

and write Eq. 8 as

$$(11) \quad P_{D\pm}(t) = (\pm 0.44/\text{Kelvin})^{-1} [\exp(-K_0 t) - (1 \pm 0.44/\text{Kelvin}) \exp(-K_1 t)]$$

Figures 1A and 2A simulate the time dependence of the decay polarization ratio in Eq. 11 at a temperature of 10 Kelvin. The simulated kinetic traces in Figure 1A from top to bottom show a range of cases in which polarization would be observed owing to the difference between K_1 and K_0 , in the range between $5K_0 < K_1$ and $K_1 < .2K_0$, where the slower of the two decays has been held constant. When K_1 and K_0 are within factors of 5, 3 and 2 of one another, Figure 1A shows that the signal can achieve decay polarization amplitudes of 14, 9, and 6.5 times the Boltzmann signal amplitude at 10 Kelvin, respectively. Figure 2A shows similar series of traces for the range between $.84K_0 < K_1$ and $K_1 < 1.2K_0$. Figure 2A shows that when K_1 and K_0 are within 20% of each other, it is possible to observe a decay polarization amplitude which is two times as large as the EPR signal amplitude at thermal equilibrium.

It is evident from Eq. 11 and from Figures 1A and 2A that the amplitude of the polarization arising as a result of the decay polarization mechanism depends upon both the difference between K_1 and K_0 and the temperature, and we shall now briefly discuss these two dependences. We can see by inspection of Eq. 8 that, for the transition between T_0 and T_1 , the decay polarization ratio will have limiting values of $f_0/(f_0 - f_1)$ and $-f_1/(f_0 - f_1)$ for the limiting cases $K_0 \gg K_1$ and $K_1 \gg K_0$, respectively. According to Eq. 11, the limiting amplitudes of the decay-polarized EPR signals at 10 Kelvin are roughly 23 times as large as the thermal



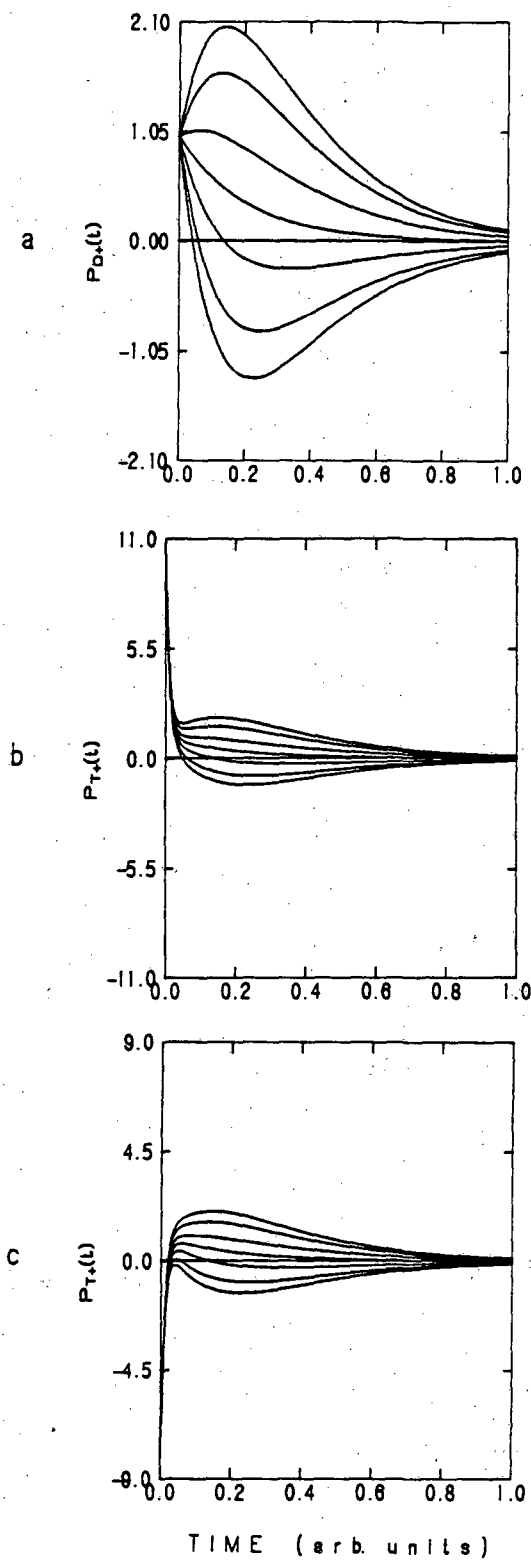
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Figure 1

(a). Computer simulations of the decay polarization ratio $P_{D+}(t)$ as a function of time at 10 Kelvin. The traces from top to bottom represent ratios between the depopulation rates K_1 and K_0 of : $K_0 = 5K_1$, $K_0 = 3K_1$, $K_0 = 2K_1$, $K_0 = K_1$, $K_1 = 2K_0$, $K_1 = 3K_0$, and $K_1 = 5K_0$, respectively. In each case, the slower of the two decays has been held constant. Because the ratio of the amplitude of the polarized signal to the amplitude of the signal at thermal equilibrium at 10 Kelvin is being plotted, the vertical axis is in units of the Boltzmann population difference at 10 Kelvin.

(b). Computer simulations of the total polarization ratio $P_{T+}(t)$ as a function of time at 10 Kelvin when there is both initial polarization and decay polarization. All conditions are as in Figure 1a, except that the signals start out at time zero with an initially absorptive, polarized amplitude which is ten times as large as the amplitude of the thermal equilibrium signal at 10 Kelvin. The middle trace, i.e. the one for the case that $K_1 = K_0$, shows the case of initial polarization when there is zero decay polarization.

(c). Computer simulations of the total polarization ratio $P_{T+}(t)$, as in Figure 1b, except that the signals start out at time zero with an initially emissive, polarized amplitude which is ten times as large as the amplitude of the thermal equilibrium signal at 10 Kelvin.



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Figure 2

(a). Computer simulations of the decay polarization ratio $P_{D+}(t)$ as a function of time at 10 Kelvin. The traces from top to bottom represent ratios between the depopulation rates K_1 and K_0 of: $K_0 = 1.2K_1$, $K_0 = 1.14K_1$, $K_0 = 1.06K_1$, $K_0 = K_1$, $K_1 = 1.06K_0$, $K_1 = 1.14K_0$, and $K_1 = 1.2K_0$, respectively. In each case, the decay time of the slower of the two components has been held constant. Because the ratio of the polarized signal amplitude to the signal amplitude at thermal equilibrium is being plotted, the vertical axis is in units of the Boltzmann population difference at 10 Kelvin.

(b). Computer simulations of the total polarization ratio $P_{T+}(t)$ as a function of time at 10 Kelvin when there is both initial polarization and decay polarization. All conditions are as in Figure 2a, except that the signals start out at time zero with an initially absorptive, polarized amplitude which is ten times as large as the amplitude of the thermal equilibrium signal at 10 Kelvin. The middle trace, i.e. the one for the case that $K_1 = K_0$, shows the case of initial polarization when there is zero decay polarization.

(c). Computer simulations of the total polarization ratio $P_{T+}(t)$, as in Figure 2b, except that the signals start out at time zero with an initially emissive, polarized amplitude which is ten times as large as the amplitude of the thermal equilibrium signal at 10 Kelvin.

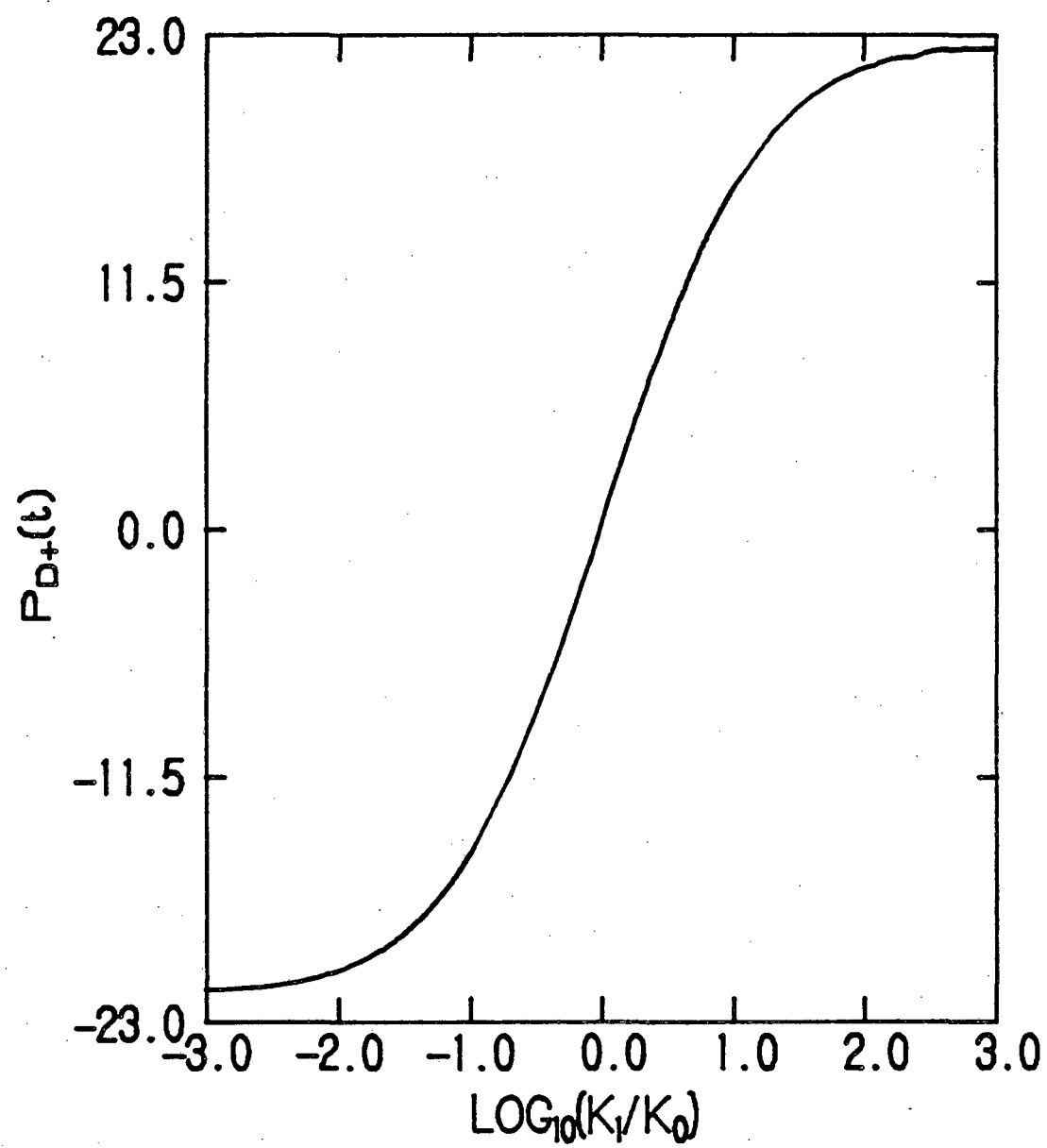
equilibrium EPR signal amplitude. Figure 3 is a plot of the decay polarization ratio at 10 Kelvin vs. $\log_{10}(K_1/K_0)$. Figure 3 shows that the decay polarization ratio changes rapidly in the region where K_1 and K_0 are similar, and makes an asymptotic approach to the limiting values, -23 and 23, when K_1 and K_0 are more than an order of magnitude different. We may conclude from Figure 3 that the decay polarization can make an important contribution to the EPR signal photokinetics observed at 10 Kelvin.

According to Eq. 11, the limiting values of the decay polarization ratio will be roughly linear functions of the temperature, above about 10 Kelvin. For example, the limiting value of the decay polarization at 273 Kelvin corresponds to a population difference which is 620 times as large as the Boltzmann population difference when K_1 and K_0 are different by a factor of ten. Clearly the contribution of decay polarization to observed EPR signal kinetics can become more important at high temperatures than the contribution of initial polarization.

In summary, the decay polarization mechanism can give rise to sizeable polarizations of triplet state EPR signals even when the signals are not initially polarized by selective population of the triplet sublevels, or when spin lattice relaxation is so rapid that the initial polarization decays to zero before it can be detected. The amplitude of the decay polarization depends both upon the selectivity in the depopulation rates, K_1 and K_0 , and upon the temperature.

B. Initial polarization case with zero decay polarization

We next consider the complementary case of case A, namely, the case in which the triplet spin sublevels are selectively populated, and therefore, initially polarized, but in which there is no selectivity in the



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Figure 3

A plot of the polarization ratio vs. $\log_{10}(K_1/K_0)$ at 10 Kelvin. The maximum amplitudes of the polarization ratio in traces like those presented in Figures 1a and 2a were computed. During the computation, K_1 was held constant, and K_0 was varied from $K_0 = 0.001K_1$ to $K_0 = 1000K_1$. Figure 3 is a plot of 300 calculated polarization maxima.

depopulation rates. In this case, if the spin lattice relaxation rates are well-separated from the rate of triplet decay to the ground state, then the EPR signals will decay at the rate $\approx 3W_1$ to the Boltzmann population difference, and will then decay to zero at the triplet decay rate.

Simulations of the time dependence of the polarization ratios for the case of initial polarization with zero decay polarization will be presented in the next section.

As we mentioned above and in Chapter Two, this case is the simplest case of EPR triplet signal kinetics, yet it cannot be described by previous analyses [6,7].

C. Combination of initial polarization and decay polarization

The most complicated, and probably most common, case is a combination of cases A and B, namely, the case in which there is both observable initial polarization and observable decay polarization. This is the case which is encountered in the experimental section of this chapter.

In this case, we may write the total polarization ratio, $P_{T\pm}(t)$, as

$$(11) \quad P_{T\pm}(t) = P_{I\pm}(t) + P_{D\pm}(t)$$

where $P_{I\pm}(t)$ is defined in Eq. 6' and $P_{D\pm}(t)$ is defined in Eq. 8.

Figures 1B,C and 2B,C show simulations of the time dependence of $P_{T\pm}(t)$ for a range of cases in which there is a combination of initial polarization and decay polarization which correspond to the decay polarization cases in Figures 1A and 2A. The middle traces in the sets in Figures 1B,C and 2B,C are the cases in which $K_1 = K_0$ and are thus the cases of initial polarization where there is no decay polarization. Figures 1B

and 2B illustrate the case of a lowfield EPR first derivative signal which starts out polarized in absorption, and Figures 1C and 2C illustrate the case of a lowfield EPR first derivative signal which starts out polarized in emission. When the EPR signals are first derivatives, the case of the highfield signal polarized in emission is obtained from the case of the lowfield signal polarized in emission by a 180° rotation of Figures 1 and 2 about the baseline. The same relation holds between the cases of highfield and lowfield signals polarized in absorption.

It is clear from Figures 1 and 2 that the theoretical analysis which we have presented here and in Chapter Two is able to account for the case of initial polarization when there is zero decay polarization, as well as for the case of initially absorptive signals which overshoot the baseline into emission before decaying to zero. As we mentioned above, neither case can be described by previous analyses [6,7].

Conclusions

Before proceeding to a presentation of experimental results, it is useful to make some general statements about the results of the analysis presented here.

First, if K_1 and K_0 are similar, the observed decay polarization rate is a convolution of K_1 and K_0 . To extract K_1 and K_0 from experimentally observed decays, the two components must of course be deconvoluted. This can be tricky in practice, but it must be done in order for experimentally determined decay rates to have any physical meaning.

Second, if K_1 and K_0 are very different, and if one of them is of the order of the EPR response time and the other is longer, then the kinetic

traces and their field dependences will mimic case C. It would then be possible to mistake a triplet decay time for a spin lattice relaxation time. Although the EPR response time is generally less than about 100 μ s, and the triplet sublevel lifetimes are generally much longer than 100 μ s, this possible confusion should be kept in mind. The polarization mechanism can be determined in this case by a study of the temperature dependence of the signal polarization.

Third, with the exception of rare cases covered by the latter comment, an initially emissive signal is evidence for initial polarization, and an absorptive signal which turns over into emission is evidence for decay polarization.

3. Materials and Methods

Sample Preparation. EPR samples of Triton X-100 photosystem I fraction and reaction centers in the redox states noted below were prepared as described in Chapter Four.

A P700-depleted antenna chlorophyll fraction was prepared as follows. P700 photosystem I reaction centers were eluted from a Sephacryl S-300 column in 50 mM Tris (pH 8)/1% Triton X-100 buffer, as described in Chapter Four. It had a chlorophyll/P700 ratio of 80, and a chlorophyll a to chlorophyll b ratio of 12. This eluate was then loaded onto a DE-11 cellulose column which had been pre-equilibrated in 50 mM Tris (pH 8)/1% Triton X-100 buffer. Roughly half of the chlorophyll protein in the reaction center fraction was tightly bound to the DE-11 column at low salt concentrations, whereas the other half passed through with the column void volume. A ferricyanide-ascorbate P700 assay of the fraction of chlorophyll

protein which passed through the column did not detect any P700. This fraction was then concentrated by dialysis against crystalline sucrose for EPR studies, and we shall refer to it below as "the antenna fraction". The tightly bound protein was recovered by elution of the DE-11 column with 0.5 M NaCl. It contained 95% of the P700 in the original Sephacryl S-300 eluate.

EPR Measurements. The EPR measurements were made with a Varian E-109 spectrometer operating at X band and equipped with a TE 102 cavity fitted with an optical transmission flange and an Air Products Helitran cryostat. All measurements were made at 10 K. Temperature measurements were made with a gold chromel thermocouple. Time resolved EPR signals were detected by means of 1 MHz or 100 kHz field modulation, as specified below. The response time of the system operating at 1 MHz was 2 μ s, and the response time at 100 kHz was measured to be about 300 μ s. The output of the field modulation lock-in amplifier was fed into a Nicolet Explorer IIIA digital oscilloscope using a Model 204 plug-in. The oscilloscope traces were transmitted to a home-built signal averager and then to a Digital VAX 11/780 computer for storage and analysis. Decay constants were obtained by fitting kinetic traces with a nonlinear least squares computer program (ZXSSQ of the IMSL library).

The light source was a Phase-R DL-1400 dye laser operating at 640 nm (rhodamine 101 in methanol) with a 400 ns (FWHM) pulse width. The laser operated at a repetition rate of 2 Hz and energies of 30-50 mJ/pulse.

4. Results

EPR kinetics experiments were performed on samples of Triton X-100

photosystem I fraction prepared in two redox states:

State A: Frozen in the dark at pH 10 (no dithionite) and illuminated briefly at 10 Kelvin. This treatment prepared photosystem I in the state $P700^+A \times Fd_B Fd_A^-$ prior to kinetics measurements.

State B: Frozen under illumination in the presence of 12 mM dithionite at pH 10. This treatment prepared photosystem I in the state $P700AX-Fd_B^- Fd_A^-$ prior to kinetics measurements.

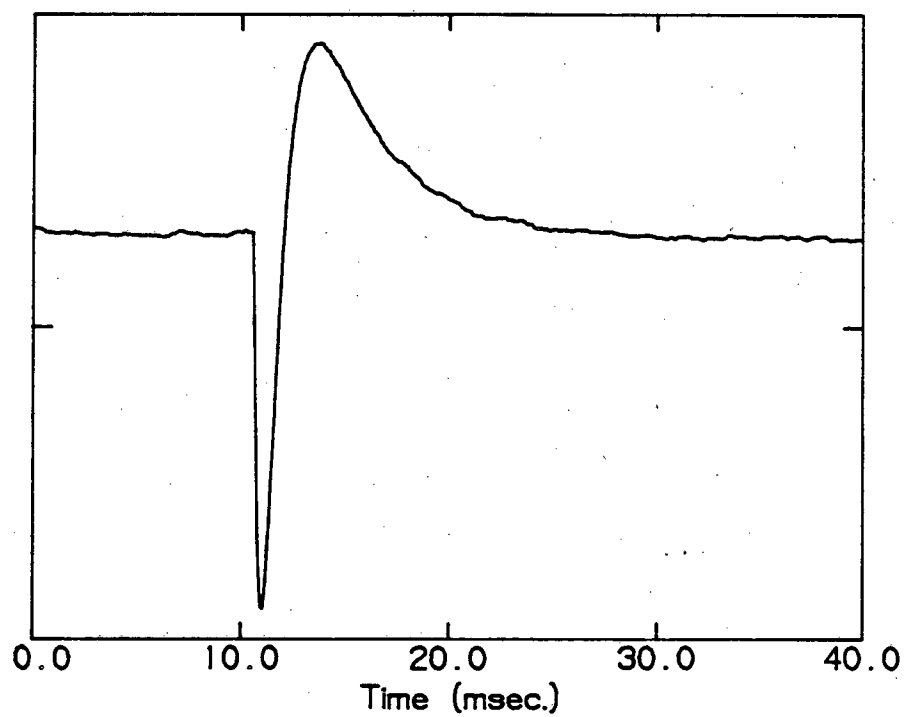
We shall be referring to 'samples in State A' and 'samples and state B' throughout the rest of this chapter.

Results of experiments performed with 100 kHz field modulation

The signal/noise is very much better with the system operating at 100 kHz field modulation, and so we used 100 kHz field modulation for preliminary experiments, in spite of its poorer time resolution.

Figures 4 a and b show the results of flash experiments on samples in States B and A, respectively, with illumination at full laser intensity at a field position of 2985 Gauss. This field position is on the highfield side of the lowfield maximum of the Z peak of the radical pair polarized P700 triplet signal. The signals in both Figures 4 a and b start out in emission and turn over into absorption before decaying to zero. According to the radical pair mechanism presented in Chapter Two, the lowfield Z peak of the radical pair polarized P700 triplet EPR signal must start out in absorption. Thus, the fact that both signals in Figure 4 start out in emission means that we are observing the antenna triplet in both cases. The

a



b

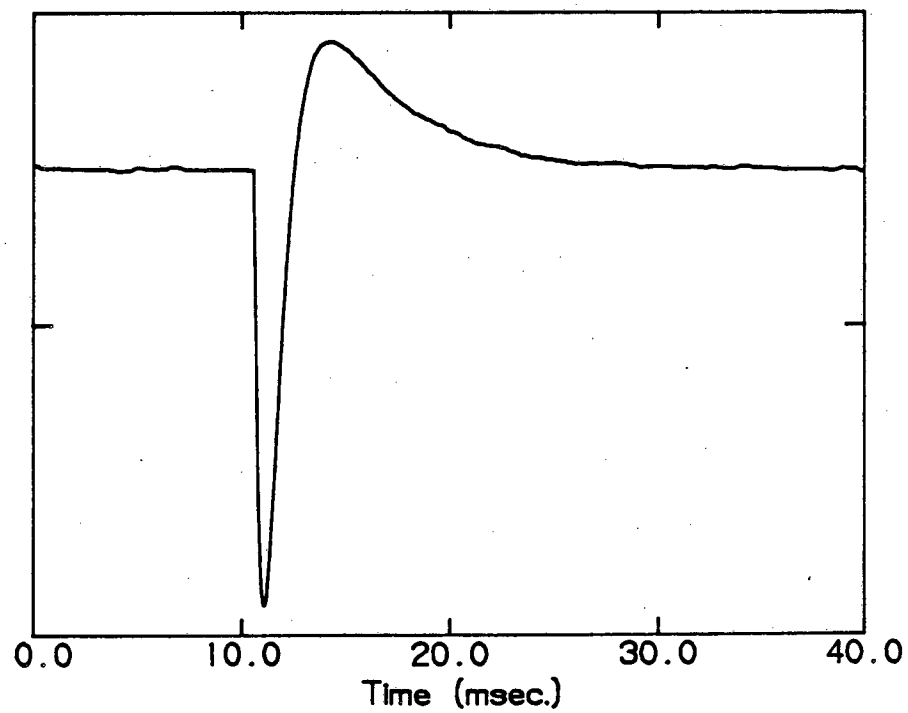


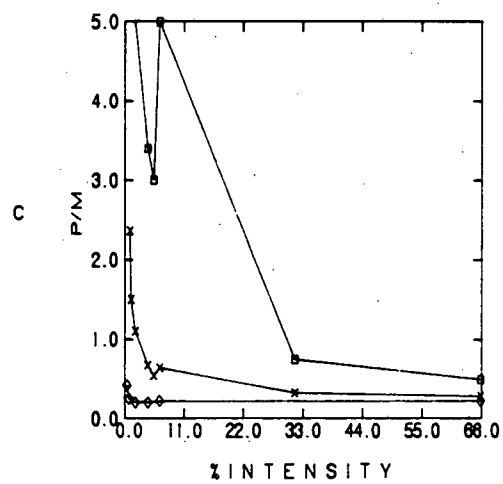
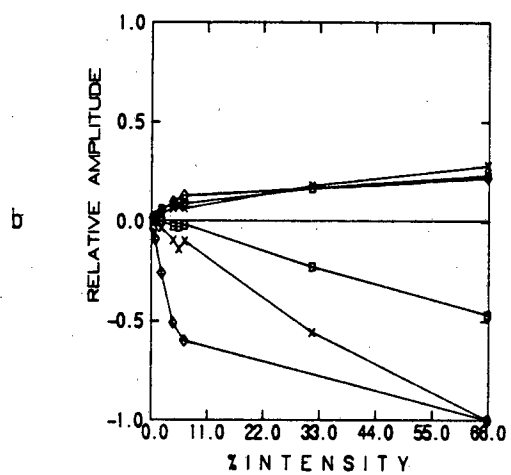
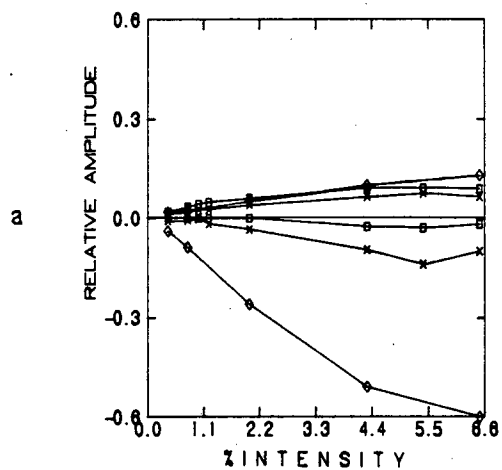
Figure 4

(a). Kinetic trace obtained at 10 Kelvin at a magnetic field strength of 2985 Gauss in a sample of photosystem I reaction centers which were frozen in the dark at pH 10 and were then illuminated at 10 Kelvin. This treatment prepared the sample in the redox state $P700^+AXF_d_BF_d_A^-$ prior to the kinetics measurements. The laser flash was delivered at full light intensity. EPR Spectrometer conditions: 100 kHz field modulation, 350 μ s time constant, 0.1 mW microwave power, 40 Gauss modulation amplitude, and 9.213 GHz microwave frequency. The kinetic trace is the average of 500 events.

(b). Kinetic trace obtained at 10 Kelvin at a magnetic field strength of 2985 Gauss in a sample of photosystem I reaction centers which were frozen under illumination at pH 10 in the presence of 12 mM sodium dithionite. This treatment prepared the sample in the redox state $P700 AX^-F_d_B^-F_d_A^-$ prior to the kinetics measurements. The sample used in Figure 4b was otherwise identical to the sample used in Figure 4a. Other conditions of the measurement are as described in Figure 4a.

ratios of the maximum of absorption (P) to the maximum of emission (M) is measured to be 0.2 in Figure 4b and 0.33 in Figure 4a. The P/M ratio measured from kinetic traces at the lowfield Z peak of the antenna triplet in samples in state A is characteristically 0.2 when the measurements are made using 100 kHz field modulation, and 0.1 when the measurements are made using 1 MHz field modulation. Thus the higher P/M ratio in the sample in state B suggests some contribution from P700.

We reasoned that we were observing mostly antenna triplet in Figure 8B because photoexcitation of the sample at full laser intensity may be sufficient not only to saturate the $^3\text{P700}$ EPR signal, but also to photoexcite a far larger number of antenna chlorophyll molecules than can transfer excitation to the reaction center. The amplitudes of the antenna and the radical pair P700 triplet signals should have very different dependence upon the intensity of the exciting light. To test our reasoning, we performed the experiment presented in Figure 5, in which we varied the intensity of the exciting light by placing neutral density filters in the beam. We have plotted the intensity dependences for signals measured at 2985 Gauss ($\square$) and at 2970 Gauss (X) for a sample in State B, and the intensity dependence for signals measured at 2985 Gauss ($\diamond$) for a sample prepared in State A. 2970 Gauss is near the lowfield emissive maximum of the antenna triplet Z peak, whereas 2985 is near the lowfield absorptive maximum of the radical pair P700 triplet Z peak. These samples were prepared from aliquots of the same photosystem I fraction. The signal amplitudes for intensities between 0.4% and 6.5% are plotted in Figure 5a, and the full intensity dependence of the signal amplitudes is plotted in Figure 5b. The negative amplitudes in Figures 5a and b are the emissive maxima (M) whereas the positive amplitudes are the absorptive maxima (P).



XBL 829-11482

Figure 5

(a). A plot of the light intensity dependence of the amplitudes of EPR signals observed during kinetics experiments. The light intensity was varied between 0.4% and 6.5% of maximum intensity by placing neutral density filters in the laser beam before it entered the EPR cavity.^o Measurements were made at 2985 Gauss magnetic field strength on a sample prepared in the redox state $P700^+Fd_A^-$ prior to illumination ($\diamond$); at 2985 Gauss on an otherwise identical sample prepared in the state $P700X^-$ prior to illumination ($\square$); and at 2970 Gauss on the sample prepared in the state $P700X^-(X)$. Emissive minima observed in kinetic traces like those presented in Figure 4 are plotted as negative amplitudes in Figure 5a, whereas the positive amplitudes in Figure 5a correspond to the absorptive maxima in the kinetic traces. The amplitudes have been normalized to the maximum emissive amplitudes. EPR spectrometer conditions are as described in Figure 4. EPR measurements were made at 10 Kelvin.

(b). A plot of the light intensity dependence of the amplitudes of EPR signals observed during kinetics measurements. The light intensity was varied between 0.4% and 65% of maximum intensity, as described for Figure 5a.

(c). A plot of the normalized amplitudes of the absorptive maxima (P) to the emissive minima (M) vs. the light intensity observed during kinetics measurements, as described for Figures 5a and 5b. ($\diamond$) measurements at 2985 Gauss on the sample in the redox state $P700^+Fd_A^-$; ($\square$) measurements at 2985 Gauss on the sample in the redox state $P700^+X^-$; (X) measurements at 2970 Gauss on the sample in redox state $P700^+X^-$. The uncertainty in the measurement of P/M is responsible for the scatter below 10% of maximum light intensity.

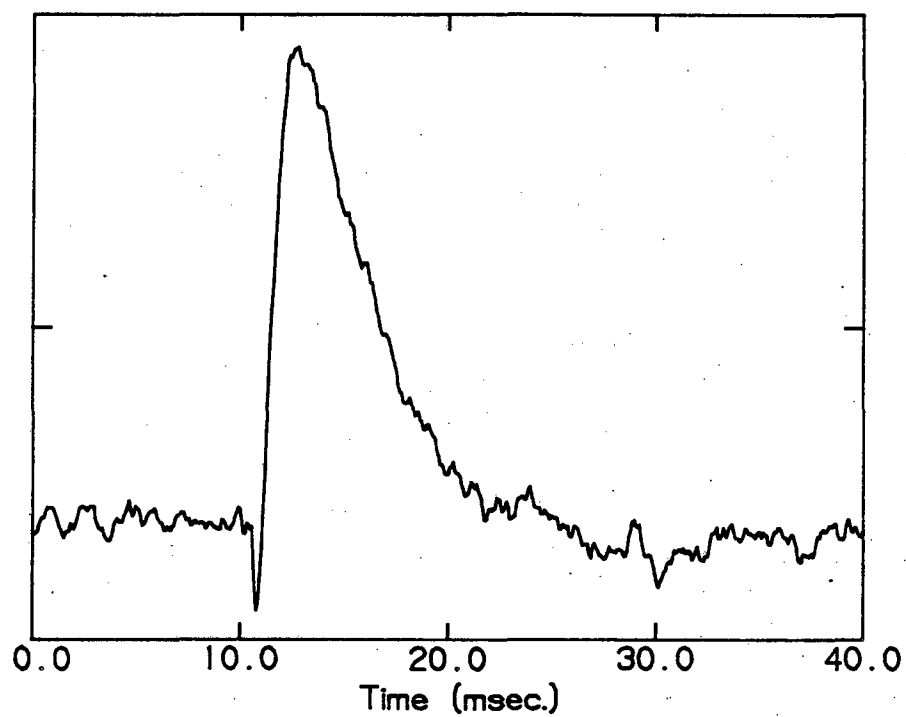
taken from kinetic traces like those shown in Figure 4. The results in Figures 5a and b show that the antenna triplet in the sample in state A has an intensity dependence very different from that of the sample in state B. Figure 5c is a plot of the ratio of the amplitudes of the absorptive maxima (P) to those at the emissive maxima (M) as a function of the excitation light intensity. Figure 5c clearly shows that the ratio P/M is an inverse function of the excitation light intensity in the sample in State B, and is a constant function of the excitation light intensity in the sample in State A.

The results in Figure 5 suggest that we can find a light intensity at which the radical pair P700 triplet signal kinetics is not convoluted with antenna triplet signal kinetics. However, for illustrative purposes, and also for later interpretation of results, we have included Figure 6, which shows the kinetic traces recorded at 6.5% light intensity at 2985 Gauss (Figure 6a) and 2970 Gauss (Figure 6b) for the sample in redox State B. These signals show an obvious convolution of the antenna and radical pair triplet signal kinetics.

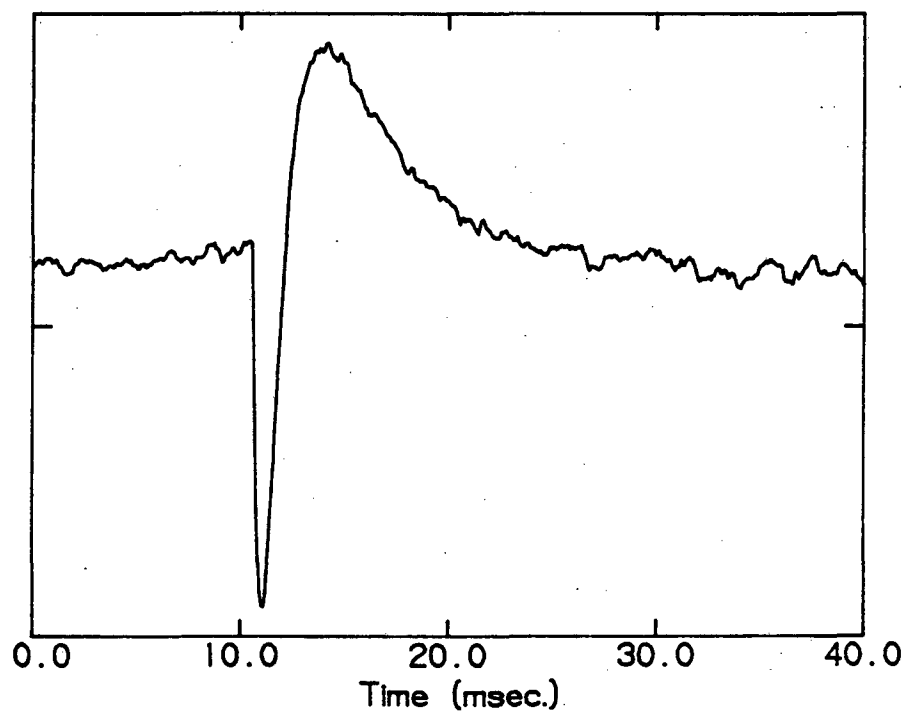
P700 Triplet Lifetime Measurements

Figure 7a shows a kinetic trace obtained from a sample in State B at 2985 Gauss and at 2% excitation light intensity, using 100 kHz field modulation. There is no evident initial emissive signal arising from the antenna triplet. A nonlinear least squares fit to a one-exponential curve gave a value of 1.7 ms for the decay time of the P700 triplet lowfield Z peak. The average of determinations of the decay time of the P700 triplet lowfield Z peak in three different samples prepared in state B was 1.8 ms. Figure 7b shows data collected under the same conditions as in Figure 7a,

a



b

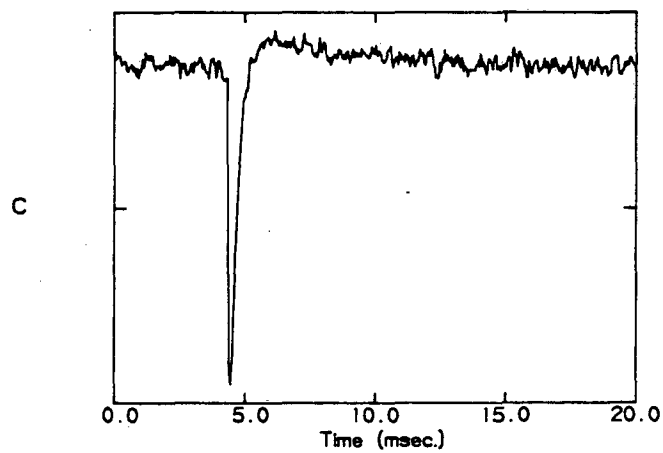
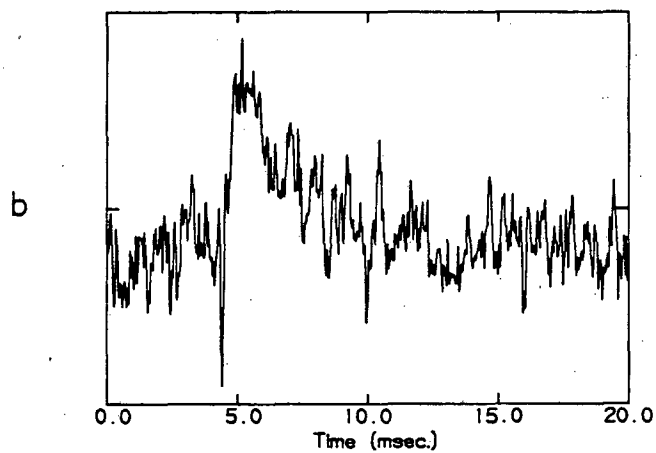
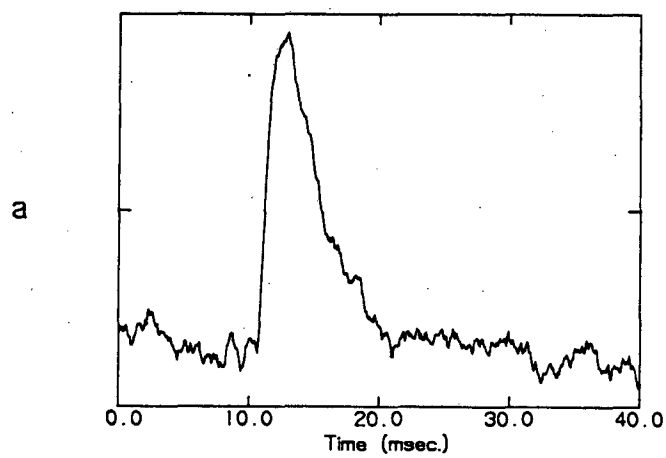


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Figure 6

(a). EPR signal kinetic trace obtained at a magnetic field strength of 2985 Gauss at 6.5% light intensity from a sample of photosystem I reaction centers which were in the redox state $P700^{+}X^{-}$ prior to measurement. Spectrometer conditions are as described in Figure 4. Measurements were made at 10 Kelvin.

(b.) EPR signal kinetic trace obtained at a magnetic field strength of 2970 Gauss at 6.5% light intensity from the same sample used for Figure 6a.



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Figure 7

(a). EPR kinetic trace obtained at a magnetic field strength of 2985 Gauss at 2% light intensity from a sample of photosystem I reaction centers which were in the redox state $P700^{+}X^{-}$ prior to measurement. Spectrometer conditions are as described in Figure 4. EPR measurements were made at 10 Kelvin.

(b). EPR kinetic trace from the sample used in Figure 7a at 2% light intensity using 1 MHz field modulation. Spectrometer conditions: 3.7 Gauss modulation amplitude, 100 μ s time constant, 0.1 mW microwave power, 9.229 GHz microwave frequency. The kinetic trace is the average of 6000 events.

(c). EPR kinetic trace obtained at a magnetic field strength of 2985 Gauss at 2% light intensity from a sample of photosystem I reaction centers which were in the redox state $P700^{+}Fd_A^{-}$ prior to measurement, detected by means of 1 MHz field modulation. Spectrometer conditions are as described in Figure 7b.

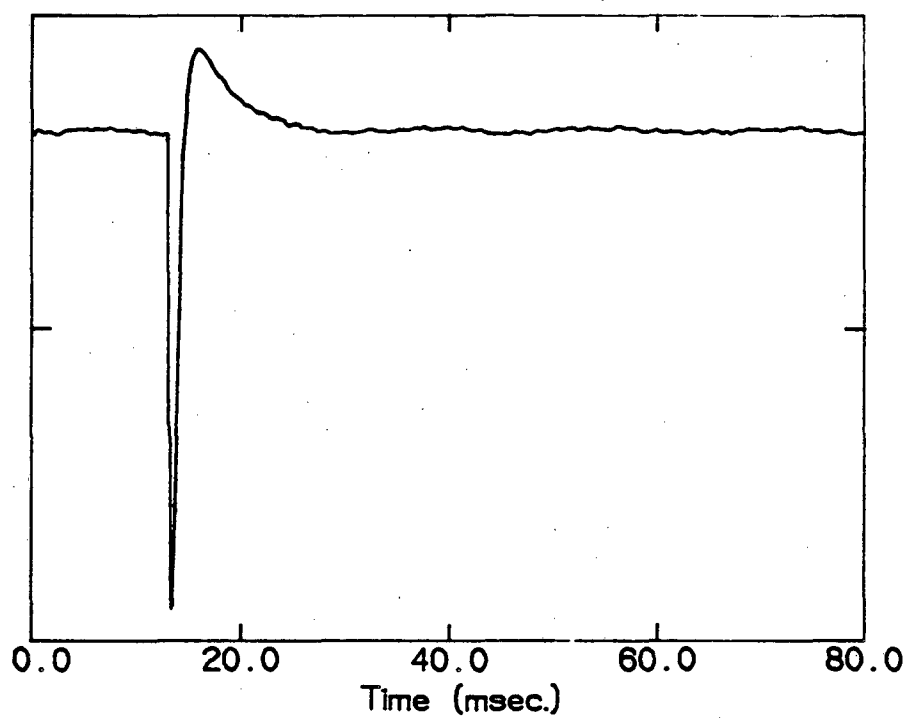
but using 1 MHz field modulation for signal detection. A nonlinear least squares fit of the decay in Figure 7b gave a value of 0.78 ms for the decay time of the P700 triplet lowfield Z peak. The average of determinations of the decay time of the P700 triplet lowfield Z peak in two different samples prepared in state B was 0.9 ms. We note that the 100 kHz response time, 300 μ s, gives rise to an overestimation of the decay times. For purposes of comparison with Figure 7b, we have included Figure 7c, which shows data collected under the same conditions as for Figure 7b, but with an identical photosystem I fraction aliquot which was prepared in State A.

Antenna Triplet Kinetics Measurements

Figures 8a and 8b show the kinetics observed in a sample prepared in State A at the lowfield and highfield antenna Z peaks, respectively, with 100 kHz field modulation and full laser light intensity. The lowfield Z peak starts out in emission and turns over into absorption before decaying to zero, and the highfield Z peak starts out in absorption and turns over into emission before decaying to zero. Clearly this is diagnostic of the case of initial polarization combined with decay polarization which we discussed in the theoretical section.

Figures 9 a-c show the results of EPR kinetics studies on the antenna fraction described in the Materials and Methods section. This fraction contains chlorophyll a proteins, but no detectable P700. During light modulation experiments at 33 Hz, we observed a light-induced triplet EPR signal (data not shown). The polarization pattern of the triplet signal was eee aaa, and its zero field splitting parameters were $|D| = 0.028 \text{ cm}^{-1}$ and $|E| = 0.003 \text{ cm}^{-1}$. Figures 9 a-c show the kinetics measured using 1 MHz field modulation, at the Z, X and Y peaks, respectively.

a



b

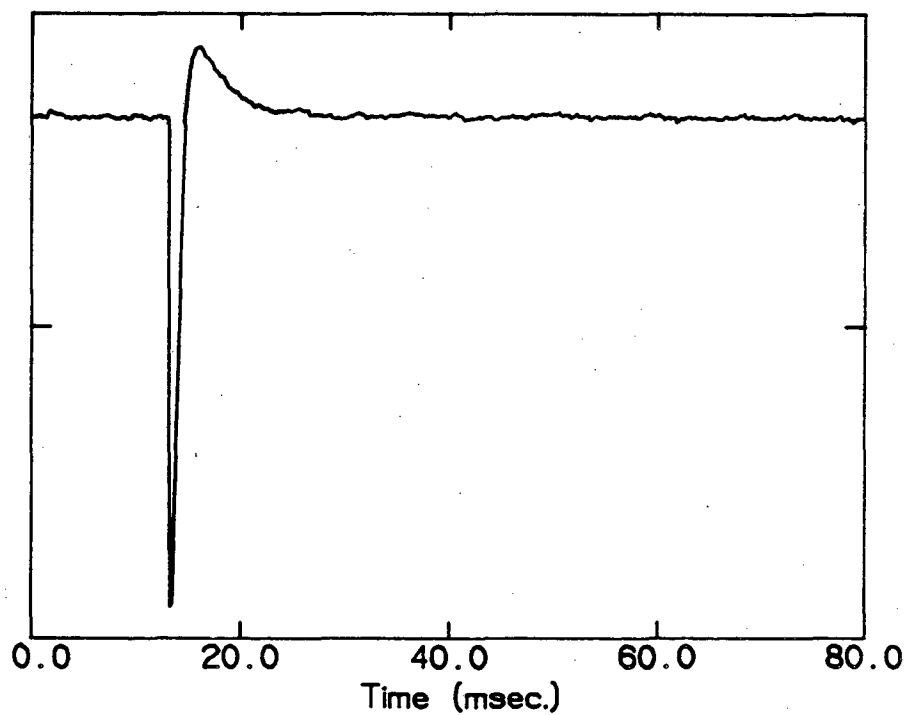
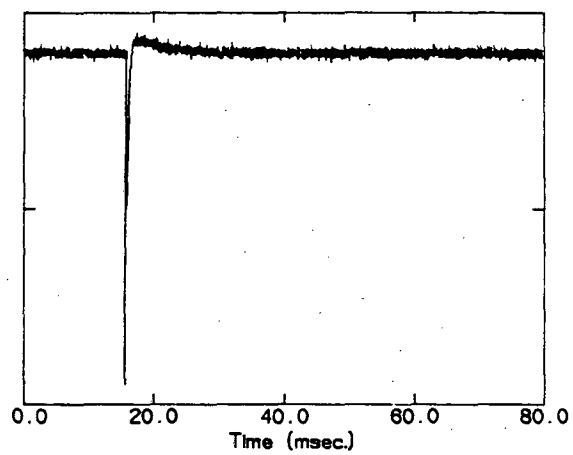


Figure 8

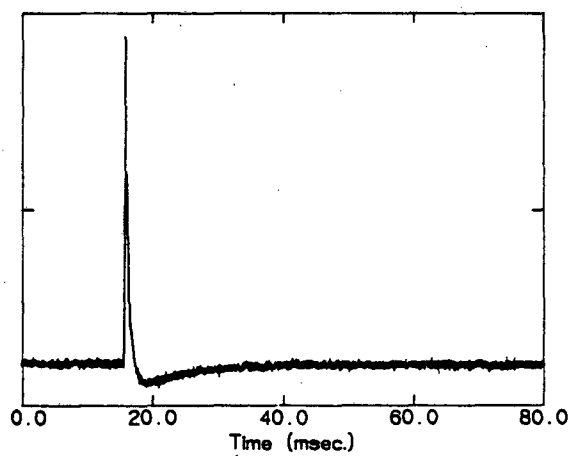
(a). EPR kinetic trace observed at 2950 Gauss in a sample of photosystem I reaction centers which was in the redox state $P700^+Fd_A^-$ prior to measurement. This magnetic field strength corresponds to the lowfield Z peak of the antenna triplet. The signal was detected by means of 100 kHz field modulation. EPR spectrometer conditions are as described for Figure 4, except that the microwave frequency was 9.147 GHz. The measurement was made at 10 Kelvin.

(b). EPR kinetic trace observed at 3560 Gauss in the same sample used in Figure 8a. This magnetic field strength corresponds to the highfield Z peak of the antenna triplet. EPR spectrometer conditions are as described for Figure 8a.

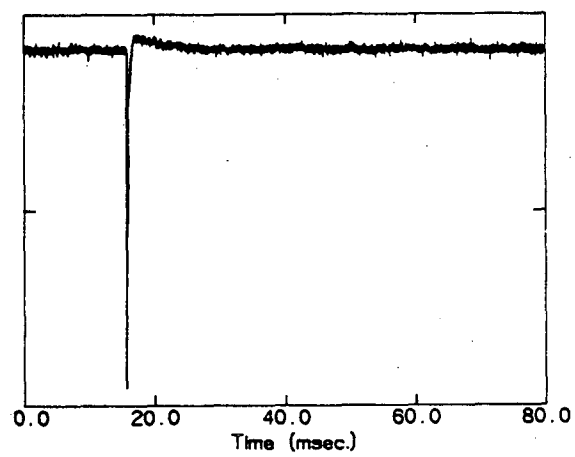
a



b



c



XBL 829-11484

Figure 9

(a). EPR kinetic trace observed at 2950 Gauss in a P700-depleted antenna chlorophyll fraction which was isolated after Triton X-100 treatment of spinach chloroplasts. This magnetic field position corresponds to the lowfield Z peak of the antenna triplet. EPR spectrometer conditions: 1 MHz field modulation, .5 μ s time constant, 3.2 Gauss modulation amplitude, 0.1 mW microwave power, 9.190 GHz microwave frequency, 10 Kelvin, 5000 passes.

(b). EPR kinetic trace observed at 3180 Gauss in a P700-depleted antenna chlorophyll fraction which was isolated after Triton X-100 treatment of spinach chloroplasts. This magnetic field position corresponds to the positive maximum of the lowfield Y peak of the antenna triplet. All other conditions are as described for Figure 9.

(c). EPR kinetic trace observed at 3035 Gauss in a P700-depleted antenna chlorophyll fraction which was isolated after Triton X-100 treatment of spinach chloroplasts. This magnetic field position corresponds to the lowfield X peak of the antenna triplet. All other conditions are as described in Figure 9.

Summary of Results

Three main results emerge from the experimental data which we have presented in this chapter, upon which we shall focus the following discussion. The first result is that in the sample in which X is reduced prior to measurement, the amplitude of the radical pair polarized P700 triplet EPR signal has a light intensity dependence very different from that of the antenna triplet EPR signal amplitude. At low enough light intensities, we observe mainly the P700 triplet signal. We have measured the decay rate of the P700 triplet EPR signal at the lowfield Z peak, and so we shall discuss the lifetime of the triplet state of P700. The second result is that the amplitude of the antenna triplet observed in the sample in which X is reduced prior to kinetics measurements has a very different dependence upon the light intensity than does the antenna triplet observed in the sample in which P700 is oxidized prior to measurement. We shall discuss the implications of this result with regard to the possibility of excitation transfer from the species giving rise to the antenna triplet to P700. The third result is that we observe the antenna triplet signal in a P700-depleted antenna chlorophyll protein preparation. We shall discuss the photokinetics of the antenna triplet and show that the theoretical analysis we presented in this chapter and in Chapter Two is successful in describing the antenna triplet signal photokinetics, as well as those of the P700 triplet signal.

5. Discussion

On the P700 Triplet State Lifetime

As reported in the Results section, the decay time of the lowfield Z peak of the P700 triplet state EPR signal was measured to be 1.8 ms in 100

kHz experiments and 0.9 ms in 1 MHz experiments. Unfortunately, our data do not yield a conclusive number for the P700 triplet state lifetime, for the following reasons.

First, we were not able to analyze kinetic data for the X and Y peaks. The decay observed optically, with which we need to compare our results, will be equal to K_{AV} , where $K_{AV} = (K_X + K_Y + K_Z)/3$. The three zero field sublevel decays rates can therefore be obtained only from a complete field profile of the kinetics. The data presented in the 1 MHz experiments in Figure 7 has very poor signal/noise. It is clear by inspection of Figure 3d in Chapter Four that the signal amplitude at the X and Y peaks is about one fifth as big as the amplitude at the Z peak, with the result that measurements performed at the X and Y peaks have fivefold poorer signal/noise. Thus we could not measure the decay times at the X and Y peaks with any confidence.

Second, EPR signal kinetics measurements yield rates which are often highly dependent upon the microwave power used during the measurement. The decay times of signals measured near saturating microwave powers are underestimates of the true decay times [8]. The power dependence of the decay rates therefore need to be determined, so that the rates may be extrapolated to their values at zero microwave power. The power which we used in the kinetics experiments presented here was 0.1 mW, which is a saturating power level for the P700 triplet EPR signal. Thus we were in the difficult position of having to lower the light intensity so that we would not observe interference from the antenna triplet signal, but at the price of decreased signal/noise, which obviated a thorough study of the microwave power dependence of the decay kinetics.

The measurements of the P700 triplet signal kinetics which we have

presented here place an upper limit on the triplet lifetime observed at 0.1 mW. Because the decay rate observed optically is the average, K_{AV} , of the three zerofield sublevel decay rates, K_{AV} will be equal to K_Z if K_X and K_Y are much slower than K_Z . However, the value at 0.1 mW power also happens to be the lower limit with regard to the power dependence of the observed decay time.

Our results are nevertheless in agreement with recent EPR studies of the P700 triplet lifetime by Setif et al. [9] in photosystem I reaction centers which had been isolated by treatment with the detergent sodium dodecyl sulfate. The antenna triplet is not observed in this preparation. Thus, Setif et al. [9] were able to determine the complete magnetic field dependence of the P700 triplet state decay kinetics, as well as the microwave power dependence of the rates. The average triplet lifetime extrapolated to zero power was roughly 1 ms [9], in agreement with the optical measurements [2].

On the Light Intensity Dependence of the Antenna Triplet

There is an obvious difference between the light intensity dependence of the antenna triplet observed in samples in States A and B, as shown in Figure 5. At light intensities below about 10% of maximum, the antenna triplet amplitude in the sample in State B is dramatically suppressed relative to that of an identical aliquot of photosystem I fraction which has been prepared in State A. This difference constitutes evidence that the antenna triplet in redox State B may arise from a species which can donate excitation to P700, for the following reasons. At light intensities which are far from saturating for charge separation between P700 and A, photoexcited antenna chlorophyll which can transfer photoexcitation to P700

will do so, and will not undergo intersystem crossing to the triplet state. However, when the light intensity is high enough that the charge separation between P700 and A is approaching saturation, which corresponds to a saturation of the P700 triplet EPR signal, antenna molecules cannot pass their excitation to P700, and so will undergo intersystem crossing to the triplet state. Then, if the antenna triplet arises from a species which can transfer excitation to P700, one would expect to observe an attenuation of the antenna triplet EPR signal amplitude until the P700 EPR signal approaches light-saturation during measurements of the light intensity dependences of the two triplet signals. However, we can only know that the antenna signal amplitude is attenuated at low light intensities due to excitation transfer to P700 if we know the amplitude it would have in the case that donation of excitation to P700 is impossible. We can determine the amplitude the antenna triplet would have in the absence of excitation transfer to P700 by studying the light intensity dependence of the antenna triplet signal amplitude in a sample in which P700 is oxidized prior to measurement. The fact that the antenna triplet amplitude in the sample in State B is suppressed relative to the antenna triplet amplitude in the sample in state A at low light intensities suggests that the antenna triplet arises from a chlorophyll species which can transfer excitation to P700.

We note that in Figure 5c, the ratio of the absorptive to the emissive maxima (P/M) is a constant in the sample prepared in state A. However, the variation of P/M observed for the sample in state B gives a saturation profile for the P700 triplet signal. Thus, we can see from Figure 5c that the antenna signal begins to build in amplitude only as the P700 signal approaches saturation, which is again consistent with our hypothesis that

the antenna triplet signal arises from a species which is coupled to the reaction center.

However, it is necessary to be cautious about the results in Figure 5, because we know that the antenna triplet signal lowfield Z peak has an opposite polarity to that of the P700 lowfield Z peak, so that the two signals can cancel. Such a mutual cancellation would be an alternative explanation for the fact that the antenna triplet signal amplitude in Sample B is suppressed relative to that in sample A until the triplet signal from P700 has reached light saturation. We shall argue in the Appendix to this chapter that the differences between the light intensity dependence of the antenna triplet signals in redox States A and B cannot be due to a mutual cancellation of P700 and antenna triplet amplitudes, i.e. that the differences are real. Thus, we conclude that Figure 5 constitutes evidence that the antenna triplet arises from a species which can transfer excitation to the reaction center. We note, then, that Figure 5 is the first real justification we have for calling the antenna triplet by that very name.

On the Antenna and Radical Pair Triplet Photokinetics

We now wish to bring the work presented in this thesis to closure by showing how the theoretical analysis which we have developed is able to account for the experimental results presented in this chapter. We note before proceeding that it is easy to see in Figure 3 of Chapter Two that the lowfield signal for the Z canonical orientation corresponds to the signal S_+ and the highfield signal corresponds to S_- , where S_+ and S_- are given in Eq. 5 of this chapter. The lowfield signals for the X and Y

canonical orientations correspond to S_- , and the highfield signals correspond to S_+ .

We cannot say much about the P700 triplet signal kinetics observed at the Z peaks, except that the fact that the lowfield peak is absorptive and the highfield peak is emissive results from initial polarization by the radical pair mechanism, whereby $1/2(P_1 + P_{-1}) - P_0$ in Eq. 5 is $-\phi_T$. The fact that S_+ and S_- decay from absorption or emission to zero without crossing the baseline when the magnetic field is parallel to the Z molecular axis suggests either that the depopulation is not selective or that there is a decay polarization which corresponds to $K_Z < (K_X + K_Y)/2$.

Figures 9 a-c fortunately allow us to say quite a bit more about the antenna triplet kinetics. These kinetics are a combination of initial polarization and decay polarization. The signals are initially polarized with the pattern eee aaa, which corresponds to $\phi_X, \phi_Y > \phi_Z$, according to Table 1 in Chapter Two. This pattern in turn corresponds to $k_X, k_Y > k_Z$ for the sublevel population rates. All three lowfield emissive signals overshoot the baseline into absorption with a rate $3W_1$, whereas all three highfield absorptive signals overshoot the baseline into emission (data not shown). Thus, it is clear that the signals are also decay polarized. The decay polarization is rationalized as follows. The lowfield Z signal S_+ overshoots the baseline because $K_Z < (K_X + K_Y)/2$. The lowfield X and Y signals, S_- , overshoot because $K_X > (K_Y + K_Z)/2$ and $K_Y > (K_X + K_Z)/2$, respectively. Thus, it is clear that $K_Z < K_X, K_Y$, and therefore that $N_Z > N_X, N_Y$. This is consistent with the observed decay polarization pattern, which is aaa eee.

6. Conclusion

Recent results [10] have been reported which we wish to discuss before leaving the subject of triplet states in photosystem I reaction centers.

Schaafsma has suggested [10] that the antenna triplet may arise from chlorophyll a molecules whose bonding to the protein has been altered by treatment with Triton X-100. Evidence for Schaafsm's view is twofold. First, the antenna chlorophyll triplet is not observed via EPR in photosystem I reaction centers which have been isolated by treatment with sodium dodecyl sulfate [9,11]. Schaafsma [10] reports the observation by fluorescence detected magnetic resonance of a triplet whose $|D|$ value is 0.030 cm^{-1} , i.e. the same as the antenna triplet, in sodium dodecyl sulfate reaction centers. However, this triplet signal is not present when the samples are freshly prepared; it appears only after the sample has been exposed to the detergent for a prolonged period at room temperature [12]. Schaafsma has also reported measurements of chlorophyll a in vitro in which the central magnesium is ligated to one water molecule. This ligation to water increases the chlorophyll a $|D|$ value from 0.0278 to 0.030 cm^{-1} . However, he also reports that the triplet with the 0.030 cm^{-1} $|D|$ value in sodium dodecyl sulfate reaction centers is associated with a protein band on SDS-polyacrylamide gels, and therefore does not arise from free chlorophyll a. Based on these results, Schaafsm has suggested [10] that the antenna chlorophyll arises from a protein-bound chlorophyll a species whose ligation state is altered from the native state by detergent treatment.

Thornber has demonstrated [13] that protein-bound chlorophyll a is highly unstable in sodium dodecyl sulfate. Fully one-third of the chlorophyll a in SDS-extracts of chloroplasts is liberated from protein and

moves as free pigment during SDS-PAGE. It seems likely, then, that the reason the antenna triplet is not observed in SDS-extracts [9,11] is that it has been removed or destroyed by SDS-treatment. The results that we have presented in this chapter are suggestive that the antenna triplet is coupled to the reaction center. We have consistently observed that the antenna triplet recorded during light modulation experiments has a phase-shift corresponding to its decay time. However, the $|D|$ value of the signal at its maximum, i.e. of the phase-shifted signal, is also consistently 0.0278 cm^{-1} (data not shown). This suggests to us that the antenna triplet which we observe in Triton X-100 extracts is a convolution of two triplet signals arising from chlorophyll a antenna species. We mentioned in the Results section that the $|D|$ value of the triplet signal observed in the antenna fraction was 0.0278 cm^{-1} . We conclude therefore that there may indeed be some partial denaturation of the chlorophyll ligation state in Triton extracts, but that there is also native chlorophyll antenna protein present in these extracts which is removed by treatment with sodium dodecyl sulfate. Further experimental studies are required to settle this point.

Appendix

On the Mutual Cancellation of the Antenna and P700 Triplet Signal
Amplitudes

In this Appendix, we wish to show that the differences between the light intensity dependences of the amplitudes of the antenna triplet signals in redox States A and B cannot be due entirely to a mutual cancellation of P700 and triplet signal amplitudes in State B. The reason we need to show that the differences are real is that it is not at all obvious that they are real, for the following reasons. We noted in the Results and Discussion sections of this chapter that the lowfield Z peaks of the antenna and radical pair polarized P700 triplet EPR signals have opposite signs. The Z peak of the P700 triplet initially grows in absorptively with the instrument response time in 100 kHz experiments, whereas the Z peak of the antenna triplet initially grows in emissively with the instrument response time. Clearly, if the P700 triplet amplitude is large enough, it can cancel out the initial emissive amplitude of the antenna triplet. Without any question, cancellation of antenna triplet initial emission by P700 triplet initial absorption contributes to some of the reduction in amplitude of the antenna triplet emission in redox State B relative to that in redox State A. In the latter redox state, there is no P700 triplet signal. The question we are addressing here is whether mutual cancellation of antenna emission by P700 absorption can account for all of the reduction in emissive amplitude of the antenna triplet in redox State B relative to that in redox State A.

The argument we shall present is that if the P700 triplet signal is

large enough to account for the reduction in amplitude of the antenna emission in redox State B relative to that in redox state A, then there is a constraint imposed upon the amplitude of the maximum absorption in the kinetic trace. We will use the data of Figure 5 to calculate what the maximum of absorption is required to be if the P700 triplet absorption is large enough to account for the difference in the amplitude of emission between the antenna triplets observed in States A and B. We will compare the calculated amplitude of the maximum absorption to the observed amplitude, and show that the calculated value is too large.

We first present preliminary data which will be used in the subsequent calculation. We shall consider the kinetic traces in Figures 6a and b, which show the signal kinetics observed at 2985 Gauss and 2970 Gauss, respectively, at 6.5% light intensity in a sample in redox State B. Both signals start out in emission and then overshoot the baseline into absorption. Whereas the ratio between the emissive minimum and the absorptive maximum is characteristically 0.2 for the antenna triplet amplitude in the absence of any P700 triplet signal, the ratios in Figures 6a and 6b are 5.7 and 0.6, respectively. Thus it is clear that both Figures 6a and 6b are convolutions of antenna triplet and P700 triplet signal kinetics. The fact that the antenna triplet emission is observed at all in Figures 6 a and b implies that the amplitude of the initial antenna triplet emission is larger than the amplitude of the initial P700 triplet absorption, i.e., that the antenna triplet amplitude is larger than the P700 triplet amplitude in Figure 6. When the antenna triplet amplitude is larger than the P700 triplet amplitude, the observed signal cannot reach its absorptive maximum until after the antenna triplet signal crosses zero. The absorptive maximum in Figure 6a occurs less than 1.6 ms later than the

occurrence of the emissive minimum. During this time, the P700 triplet amplitude will decay from its initial value by a factor larger than $\exp(-1.6/1.8) = 0.41$.

Keeping these preliminary considerations in mind, we now note that when the antenna and radical pair polarized P700 triplet signals are convoluted, the observed emissive minima, $M_{OBS}(t_1)$, contain a negative contribution from the antenna triplet signal, $M_{ANT}(t_1)$, and a positive contribution from the P700 triplet signal, $P_{RP}(t_1)$, that is

$$(A1) \quad M_{OBS}(t_1) = M_{ANT}(t_1) + P_{RP}(t_1)$$

The observed absorptive maxima, $P_{OBS}(t_2)$, contain a positive contribution from the antenna triplet, $P_{ANT}(t_2)$, after the latter has crossed zero, and a positive contribution from the P700 triplet, $P_{RP}(t_2)$, that is

$$(A2) \quad P_{OBS}(t_2) = P_{ANT}(t_2) + P_{RP}(t_2)$$

According to the data plotted in Figure 5, the antenna triplet signal at 2985 Gauss in the sample in State B at 6.5% light intensity has an amplitude 0.04 times as large as its amplitude at 65% light intensity, whereas the antenna triplet signal at 2985 Gauss in the sample in State A at 6.5% light intensity has an amplitude 0.6 times as large as its amplitude at 65% light intensity. Let us suppose, then, that convolution of the antenna triplet with the radical pair triplet reduces the emissive amplitude of the antenna triplet to $0.04/0.6 = 0.07$ times its true value. Then from Eq. 6 we would have

$$(A3) \quad 0.07 M_{ANT}(t_1) = M_{ANT}(t_1) + P_{RP}(t_1)$$

or

$$(A4) \quad P_{RP}(t_1) = -0.93 M_{ANT}(t_1) = 0.93 \times 0.6 = 0.55$$

The observed maximum is therefore constrained to have a certain value. As we noted above, it occurs less than 1.6 ms later, when P_{RP} has dropped by a factor larger than 0.41 to $0.41 \times 0.55 = 0.23$. Therefore, $P_{RP}(t_2)$ in Eq. A2 must be at least 0.23 in the sample in State B at 6.5 % light intensity if mutual cancellation between the antenna and P700 triplet signals accounts for the reduction in amplitude of the antenna triplet in State B. As we said in the preceding paragraph, we know that the ratio of the absorptive maximum of the antenna triplet to its emissive maximum is 0.2. Thus $P_{ANT}(t_2)$ in Eq. A2 can be as large as $0.2 \times 0.6 = 0.13$ if the relative emissive amplitude of the antenna triplet is 0.6.

We therefore have the result that $0.23 < P_{OBS}(t_2) < 0.36$ if our hypothesis is correct that the true emissive amplitude of the antenna triplet signal at 6.5% light intensity is 0.6 times its amplitude at 65% light intensity. However, according to the data plotted in Figure 5, $P_{OBS}(t_2) = 0.09$, which is one half to one fourth as large as it needs to be in order to satisfy the latter hypothesis. An identical calculation using the data at 2970 Gauss in Figure 6b results in the requirement that $0.11 < P_{OBS}(t_2) < 0.24$; however, the observed value of $P_{OBS}(t_2)$ at 2970 Gauss is 0.06, which is again one half to one fourth as large as it needs to be in order to satisfy the hypothesis. The relative experimental error of the measurements in Figures 5 and 6 is less than 0.01. Thus we conclude that

the differences between the light intensity dependences of the antenna triplet amplitude in States A and B cannot be entirely due to mutual cancellation between the P700 and antenna triplet signals in the sample in State B. That is, we conclude that a significant fraction of antenna chlorophyll molecules are able to transfer excitation to P700 at light intensities which do not saturate the P700 A charge separation.

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CHAPTER SIX

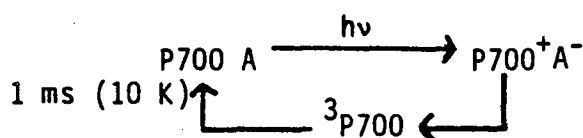
CONCLUSION

The work presented in this thesis divides naturally into two categories of investigation: first, experimental studies of chlorophyll triplet states in photosystem I; and, second, theoretical studies of triplet state photokinetics. In this final chapter, we shall discuss the overall conclusions which we have extracted from these studies.

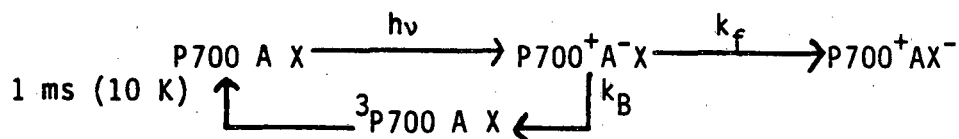
1. Major Conclusions of Experimental Studies of Triplet States in Photosystem I

The experimental studies presented in Chapters Three through Five allow us to make some general conclusions about the mechanism of primary photochemistry in photosystem I under conditions in which forward electron transfer from A^- to X is blocked.

The first conclusion we may make is that the triplet state of P700 serves as an intermediate state in the back reaction between the P700 cation and the anion of the earliest known acceptor, A. The results presented in Chapters Three through Five are suggestive that when forward photochemistry is blocked, the mechanism of primary events in photosystem I may be represented as



The results presented in Chapter Four suggest that when X is not reduced prior to measurement, photo-reduced A^- does not always transfer its electron to X: it often undergoes charge recombination with $P700^+$ and populates 3P700 in preference to forward electron transfer to X. A second conclusion we may make, therefore, is that there is either a heterogeneity in the population of photosystem I reaction centers, so that electron transfer from A^- to X is impossible in a portion of the reaction center population, or that there is a branch point in photosystem I primary photochemistry. In Chapter Four, we presented the latter branch point by the model



where $k_f = k_B$.

The results presented in Chapters Four and Five show that we observe the antenna triplet EPR signal under conditions which close the reaction center, i.e. under conditions of high light intensity, or when P700 is oxidized prior to illumination. A third conclusion we may make is thus that at low temperatures, antenna chlorophyll species which are connected to the photosystem I reaction center, but which cannot transfer their excitation to P700, may undergo intersystem crossing to the triplet state in order to dissipate excess energy.

2. Major Conclusions of the Theoretical Studies of Triplet State Photokinetics

The most important conclusion of the theoretical analysis presented in Chapters Two and Five is that the spin lattice relaxation rates between the

triplet spin sublevels are time dependent as a result of the metastability of the lowest lying triplet state, T^1 , that is, as a result of relaxation of triplet sublevel population to the ground state. When one includes the time dependence of the spin lattice relaxation rates in the analysis, one obtains solutions of the triplet sublevel kinetic equations which are simple and exact. The analysis of EPR triplet signal photokinetics which we presented in Chapter Five yielded a proposal of a new triplet EPR signal polarization mechanism, the decay polarization mechanism, which was described in Chapter Five.

Finally, we brought the two separate lines of investigation, the experimental and the theoretical lines, to full closure with respect to one another in Chapter Five by showing that our theoretical analysis is successful in describing the photokinetics of both the P700 and the antenna triplet EPR signals.

3. Future Directions

Research in higher plant photosynthesis will change qualitatively in the near future. Until very recently, workers have focussed on primary events in photosystems I and II separately, in order to determine the structure and function of each reaction center. This line of research will culminate when both reaction centers have been crystallized and when their structures can be determined by Xray analysis. Until then, the triplet EPR signal from P700 can be used as an observable of charge separation within photosystem I, to elucidate further the composition and structure of the reaction center.

However, the understanding of primary events in photosystems I and II

is already sufficiently well established that many workers have begun to tackle a higher order of chloroplast functioning: how photosystems I and II function together in chloroplasts. This is the general direction in which chlorophyll triplet state EPR studies can yield further progress in the understanding of photosynthesis, as will now be discussed.

In the Conclusion of Chapter Four, we speculated that heterogeneity within the photosystem I reaction center population could have implications regarding the participation of photosystem I in linear versus cyclic electron transport: the fraction connected to X and Centers A and B would participate with photosystem II in linear electron transport from water to NADP^+ , and the fraction not connected to X and Centers A and B would be coupled to the quinone-cytochrome-Rieske FeS oxidoreductase and would undergo cyclic electron transport. This is certainly an interesting idea and very relevant to the question of the coupling between photosystems I and II. However, some of the results presented in this thesis are inconsistent with the hypothesis that there is a heterogeneity within the photosystem I reaction center population with regard to the connection of P700 and A to the secondary acceptors, X and Centers A and B. If a fraction of the photosystem I population were not connected to X and Centers A and B, we would expect to observe the radical pair polarized P700 triplet EPR signal at low light intensities in the sample prepared in redox state $\text{P700}^+\text{Fd}_\text{A}^-$. The results we presented in Chapter Five show that (1) the antenna triplet P/M ratio observed during photokinetics measurements in the sample in redox state $\text{P700}^+\text{Fd}_\text{A}^-$ does not depend on the light intensity: the P/M ratio is an indicator of the contribution of the P700 triplet signal to the observed kinetics; and (2) the P/M ratio of the antenna triplet in reaction centers in the state $\text{P700}^+\text{Fd}_\text{A}^-$ is equal to that observed in a

P700-depleted chlorophyll protein fraction (data not shown in Chapter Five). Both of these results taken together are suggestive that we do not observe the radical pair polarized P700 triplet signal in samples prepared in the redox state $P700^+Fd_A^-$ prior to measurement, and thus that there is not a fraction of the photosystem I population in which A^- cannot transfer its electron to X.

The question of photosystem I heterogeneity cannot be resolved on the basis of existing data. Its resolution must be the subject of future investigation. In these investigations, the radical pair polarized P700 triplet EPR signal can be used as an indicator, not only of charge separation, but of charge recombination between $P700^+$ and A^- .

We have just been considering a question regarding the coupling of electron transport between photosystems I and II. There is also a related question regarding the transport of photoexcitation, not only within each photosystem, but between the two photosystems. It is important to understand excitation transfer within and among the antenna and light harvesting chlorophyll proteins in chloroplasts in order to arrive at a model of pigment organization and function. However, very little is known about the structures of the light harvesting and antenna chlorophyll complexes in higher plants, and this is where the antenna chlorophyll EPR triplet signal can be a useful observable. As we discussed in the conclusion to Chapter Five, the antenna triplet is not observed in the P700 chlorophyll protein which is extruded from the CP I band of SDS-polyacrylamide gels. Thus, the antenna triplet EPR signal may be characteristic specifically of a chlorophyll a antenna protein which is distinct from CP I. If so, then the antenna triplet EPR signal could be used in conjunction with chromatographic and optical studies to distinguish

between different chlorophyll a antenna species connected to photosystems I and II.

In summary, the EPR studies of chlorophyll triplet states presented in this thesis have contributed to the understanding of primary events and excitation transfer in photosystem I. In the future, EPR studies of chlorophyll triplet states can yield further information about primary photochemistry, about the coupling of electron transport between photosystems I and II, about the transport of photoexcitation to the reaction centers and the dissipation of excess excitation, and about the structure and organization of antenna and light harvesting chlorophyll protein complexes. The theoretical analysis of triplet state photokinetics presented in this thesis will be useful for understanding the photokinetics of triplet state EPR signals, not only in photosynthetic systems, but in any system of organic molecules in which triplet manifold population is possible following photoexcitation. The finding that the spin lattice relaxation rates are time dependent in metastable systems will also need to be incorporated into other theoretical analyses of EPR signal dynamics, such as the theory of chemically induced dynamic electron polarization (CIDEP).

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