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# Resolution and Reconstitution of the Photosystem I Reaction Center: Structure and Function of the Terminal Electron Acceptor FA/FB Polypeptide

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**RESOLUTION AND RECONSTITUTION OF THE PHOTOSYSTEM I  
REACTION CENTER:STRUCTURE AND FUNCTION OF THE  
TERMINAL ELECTRON ACCEPTOR F<sub>A</sub>/F<sub>B</sub> POLYPEPTIDE**

**by**

**TETEMKE MEHARI**

**A dissertation submitted in partial fulfillment of the  
requirements for the degree of**

**DOCTOR OF PHILOSOPHY  
in  
ENVIRONMENTAL SCIENCES AND RESOURCES:  
CHEMISTRY**

**Portland State University  
1990**

TO THE OFFICE OF GRADUATE STUDIES:

The members of the Committee approve the dissertation of Tetemke Mehari presented  
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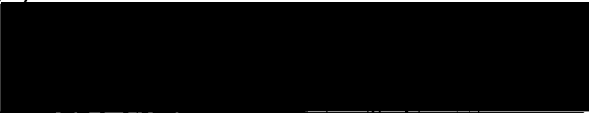
  
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AN ABSTRACT OF THE DISSERTATION OF Tetemke Mehari for the Doctor of  
Philosophy in Environmental Sciences and Resources: Chemistry presented July 6, 1990.

Title: Resolution and Reconstitution of the Photosystem I Reaction Center: Structure and  
Function of the Terminal Electron Acceptor F<sub>A</sub>/F<sub>B</sub> Polypeptide.

APPROVED BY THE MEMBERS OF THE DISSERTATION COMMITTEE:

  
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The Photosystem I core protein containing P700, A<sub>0</sub>, A<sub>1</sub>, and F<sub>X</sub> has been isolated  
from *Synechococcus* sp. PCC 6301 and spinach Photosystem I complexes with 6.8 M urea

followed by sucrose density ultracentrifugation. The Photosystem I core protein has retained > 90% of  $F_X$  and 100% of P700 (determined by optical spectroscopy) but is totally devoid of iron-sulfur centers  $F_A$  and  $F_B$  (determined by optical and ESR spectroscopy). SDS-PAGE indicates the retention of the 82- and 83-kDa reaction center polypeptides. The loss of  $F_A$  and  $F_B$  is further reflected in the decline of acid labile sulfide from  $11.8 \pm 0.4$   $S^{2-}/P700$  in the Photosystem I complex to  $4.6 \pm 0.3$   $S^{2-}/P700$  in the Photosystem I core protein. This preparation represents the first isolation of an intact reaction center core incorporating the redox centers P700 through  $F_X$  but totally lacking  $F_A$  and  $F_B$ . Complete restoration of electron flow from P700 to  $F_A/F_B$  was achieved by incubating a P700 and  $F_X$ -containing Photosystem I core protein with a freshly isolated 8.9-kDa,  $F_A/F_B$  polypeptide. When illuminated during freezing, both  $F_A$  and  $F_B$  become quantitatively reduced, and the ESR spectrum is nearly indistinguishable from  $F_A$  and  $F_B$  in the control Photosystem I complex. In the reconstituted Photosystem I complex  $F_X$  is photochemically reduced only in the presence of  $F_A^-$  and  $F_B^-$ , and the broad high field resonance of  $F_X$  in the core protein appears upon reconstitution to be indistinguishable from  $F_X$  in the control Photosystem I complex. Optical flash photolysis after extensive washing confirms the complete restoration of the  $P700^+ [F_A/F_B]^-$  backreaction, indicating quantitative rebinding of the 8.9-kDa polypeptide. This procedure represents the first reconstitution of the Photosystem I complex from a purified Photosystem I core protein and a freshly isolated 8.9 kDa  $F_A/F_B$  holoprotein, and makes possible independent manipulation of the two subunits that carry the entire electron acceptor system of Photosystem I. The  $F_A/F_B$  iron-sulfur clusters in the 8.9 kDa polypeptide are easily degraded to the level of zero-valence sulfur during isolation from the Photosystem I complex. The level of degradation is minimized by working under anaerobic conditions and low temperature. It has been found that incubation of the purified Photosystem I core protein and the low molecular mass polypeptides (which includes the 8.9-kDa  $F_A/F_B$

apoprotein) with a solution of  $\text{FeCl}_3$ ,  $\text{Na}_2\text{S}$  and  $\beta$ -mercaptoethanol restores the light induced charge separation between P700 and  $\text{F}_\text{A}/\text{F}_\text{B}$ . The optical and spectroscopic properties are indistinguishable from the control Photosystem I complex. When a rebuilt spinach or *Synechococcus* sp. PCC 6301  $\text{F}_\text{A}/\text{F}_\text{B}$  polypeptide is rebound to a Photosystem I core protein from the same species, the reconstituted complexes show light-induced ESR spectra of  $\text{F}_\text{A}/\text{F}_\text{B}$  with  $g$ -values identical to their respective controls. However, when the rebuilt spinach  $\text{F}_\text{A}/\text{F}_\text{B}$  polypeptide is rebound to a *Synechococcus* sp. PCC 6301 Photosystem I core protein, the hybrid spinach-*Synechococcus* sp. PCC 6301 Photosystem complex shows a light-induced ESR spectrum of  $\text{F}_\text{A}/\text{F}_\text{B}$  with  $g$ -values that differ slightly, but characteristically, from those of both spinach and *Synechococcus* sp. PCC 6301 control complexes. The hybrid spinach-*Synechococcus* sp. PCC 6301 Photosystem I complex was completely functional in light-induced charge separation between P700 and  $\text{F}_\text{A}/\text{F}_\text{B}$ , and showed a normal 30-ms room temperature charge recombination between  $\text{P700}^+$  and  $[\text{F}_\text{A}/\text{F}_\text{B}]^-$ . Accordingly, rebuilding of the  $\text{F}_\text{A}$  and  $\text{F}_\text{B}$  iron-sulfur clusters is now possible and reconstitution of the  $\text{F}_\text{A}/\text{F}_\text{B}$  holoprotein after chemical modification of the  $\text{F}_\text{A}/\text{F}_\text{B}$  apoprotein or genetic modification of the *psaC* gene should reveal new findings about Photosystem I structure and function.

## **DEDICATION**

**This dissertation is dedicated to my wife Hanna Belete who literally made it possible for me to pursue my graduate education.**

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## ABBREVIATIONS AND DEFINITIONS

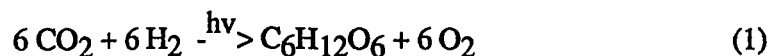
PS I, Photosystem I; Chl, chlorophyll; ESR, electron spin resonance; Tris, Tris(hydroxymethyl)aminomethane; DCPIP, 2,6-dichlorophenolindophenol; LDS, lithium dodecyl sulfate; Tiron, 4,5-dihydroxy-1,3-benzene-disulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; NHI, non-heme iron; NADPH, nicotinamide adenine dinucleotide phosphate; APS, ammonium persulfate; TEMED, N,N,N',N'-tetramethylethylenediamine; LHCI, light harvesting chlorophyll protein complex, part of PS I.

Photosystem I complex: multiprotein reaction center isolated with Triton X-100, containing P700 and electron acceptors  $A_0$ ,  $A_1$ ,  $F_X$ ,  $F_B$  and  $F_A$ ; Photosystem I Core Protein: reaction center heterodimer of the *psaA* and *psaB* polypeptides isolated from the Photosystem I complex with chaotropes, containing P700 and acceptors  $A_0$ ,  $A_1$  and  $F_X$ ;  $F_A/F_B$  polypeptide: 8.9-kDa polypeptide of *psaC* containing iron-sulfur clusters  $F_A$  and  $F_B$ .

## CHAPTER I

### INTRODUCTION

Photosynthesis is the process by which light energy from the sun is converted to chemical energy. This energy is conserved in the form of ATP and NADPH, which can then be used to reduce inorganic CO<sub>2</sub> to glucose. The net reaction of photosynthesis is described by the following deceptively simple equation (1).



It is imperative that all photosynthetic organisms possess machinery for capturing sunlight with the correct bandgap. This machinery consists of specialized pigment-protein complexes associated with the photosynthetic membrane, with variation in the type of pigment and protein composition among organisms that operate under different physiological and environmental conditions. The most important type of pigment found in photosynthetic systems is chlorophyll; however, phycobillins also function as pigments in cyanobacteria. Carotenoids, in addition to absorbing sunlight like chlorophyll and phycobillins, also perform a very important function in protecting the reaction center by quenching singlet oxygen, which otherwise would destroy it. Each reaction center is a multiprotein complex where a photon absorbed by antenna pigments is transferred to, and converted to, charge separation between a primary electron donor and primary electron acceptor. The membrane-bound pigment-protein complex which includes the reaction center is called a photosynthetic unit.



Thylakoid membranes of green plants and cyanobacteria (formerly known as blue green algae) have been found to contain two types of photosynthetic units: Photosystem I and Photosystem II. The physical organization of the Photosystems as well as the thylakoid membranes within the chloroplasts of green plants has been extensively reviewed [1 and references. therein] and is shown in Figure 1. The use of Photosystem I and Photosystem II in series by the higher plants and cyanobacteria has made highly-abundant water the appropriate reductant with concomitant production and release of oxygen to the atmosphere, thereby changing the once-reducing atmosphere to an oxidizing one. Plants and cyanobacteria are thought to have evolved from the earliest and most simple photosynthetic anaerobic bacteria that contain a single photosynthetic unit. Since this dissertation deals with oxygenic photosynthesis, the bacterial reaction center will not be discussed further. Except where relevant, Photosystem II also will not be discussed in its own right. Photosystem I in higher plants and cyanobacteria will be the primary topic of this work (for reviews on Photosystem I see References. 2 and 3).

The photosynthetic reaction that converts atmospheric CO<sub>2</sub> to glucose is a complex one. This process can be simplified by breaking it down into two phases: a light phase and a dark phase. The light phase involves the production of ATP and NADPH using light energy. It is at this phase that solar energy is transformed into chemical energy. NADPH, the reducing power in photosynthesis, is produced by oxidizing water to molecular oxygen. Since the reduction of NADPH by water is non-spontaneous (endergonic), light energy is used to drive the reaction. It is here that the two Photosystems I and II are needed to trap two photons of red light (~1.8 eV each) in series. The reaction retains ~1.22 eV of energy in the form of reduced NADPH at an efficiency of ~30% [3]. This is summarized in equation (2).



A good portion of the remaining energy is stored in the form of ATP with the rest being degraded to heat. The photophosphorylation of ADP to give ATP is coupled to the protonmotive force generated due to the vectorial translocation of protons across the thylakoid membrane. This process accompanies cyclic or non-cyclic electron flow in the electron transport chain. Since the quantum yield of photosynthesis is  $\sim 1$ , every photon captured by the antenna pigments results in stable charge separation. It is, therefore, interesting to consider how energy is transferred over large spatial distances in the antenna in a manner that competes successfully with other radiative and non-radiative decay channels. Like any other biological membranes, the thylakoid membrane is thought to be asymmetric and dynamic in terms of the orientation of the pigment-protein components with respect to the reaction center. Components of the spinach Photosystem I holocomplex are shown in Table 1 [4 and references therein] and a proposed structure of Photosystem I, based on limited biochemical and spectroscopic evidence, is shown in Figure 2.

**TABLE I**  
**COMPONENTS OF THE SPINACH PHOTOSYSTEM I HOLOCOMPLEX**

<u>Gene<sup>a</sup></u>	<u>Coded<sup>b</sup></u>	<u>Residues<sup>c</sup></u>	<u>Mass<sup>d</sup></u>	<u>#<sup>f</sup></u>	<u>Cofactors</u>	<u>Function/Properties</u>
<u>Hydrophobic Photosystem I Core Proteins</u>						
<i>psaA</i>	C	750	83.0	~ 100	Chl <i>a</i>	{ Antenna Chlorophyll & Charge Separation }
<i>psaB</i>	C	734	82.4	12-16	b-Carotene	Antenna & Photoprotection
				2	Quinone <sup>e</sup>	Charge Stabilization
				1	[4Fe-4S]	Charge Stabilization
<i>psaI</i>	C	36 (Barley)	4.0		?	Transmembrane Helix
<i>psaJ</i>	C	44 (Tobacco)	5.1		?	Transmembrane Helix
<i>psaK</i>	N	87	8.4		None	Intrinsic Membrane Protein
<u>Hydrophilic Photosystem I Peripheral Proteins</u>						
<i>psaC</i>	C	80	8.9	2	[4Fe-4S]	Terminal Electron Acceptor
<i>psaD</i>	N	162	17.9		None	Ferredoxin-Docking Protein
<i>psaE</i>	N	91	9.7		None	?
<i>psaF</i>	N	154	17.3		None	Plastocyanin-Docking Protein
<i>psaG</i>	N	98	10.8		None	?
<i>psaH</i>	N	95	10.2		None	LHCI Linker Protein ?
<u>Hydrophobic Chlorophyll a/b Binding Proteins (Eukaryotes only)</u>						
<i>Cab-6A/B</i>	N	201/2	22		Chl <i>a</i> & <i>b</i>	Antenna Chlorophyll (LHCIIa)
<i>Cab-7</i>	N	?	24		Chl <i>a</i> & <i>b</i>	Antenna Chlorophyll
<i>Cab-8</i>	N	?	24		Chl <i>a</i> & <i>b</i>	Antenna Chlorophyll

<sup>a</sup> See Plant Molecular Biology Reporter [R. Hallick]

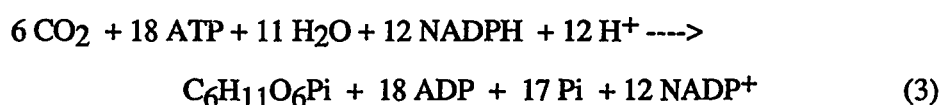
<sup>b</sup> C: Chloroplast; N: Nucleus

<sup>c,d</sup> Deduced from gene sequence, in kDa

<sup>e</sup> per reaction center heterodimer

<sup>f</sup> See text

Even though the requirement of light energy as a regulator for the fixation of carbon seems to be firmly established [5,6], the production of glucose from CO<sub>2</sub> does not require the use of light energy (provided NADPH and ATP are available). Hence, during the so called "dark" phase of photosynthesis, the ATP and NADPH, produced during the light phase, are used to convert CO<sub>2</sub> to glucose. This process, known as the Calvin cycle, is shown in equation (3).

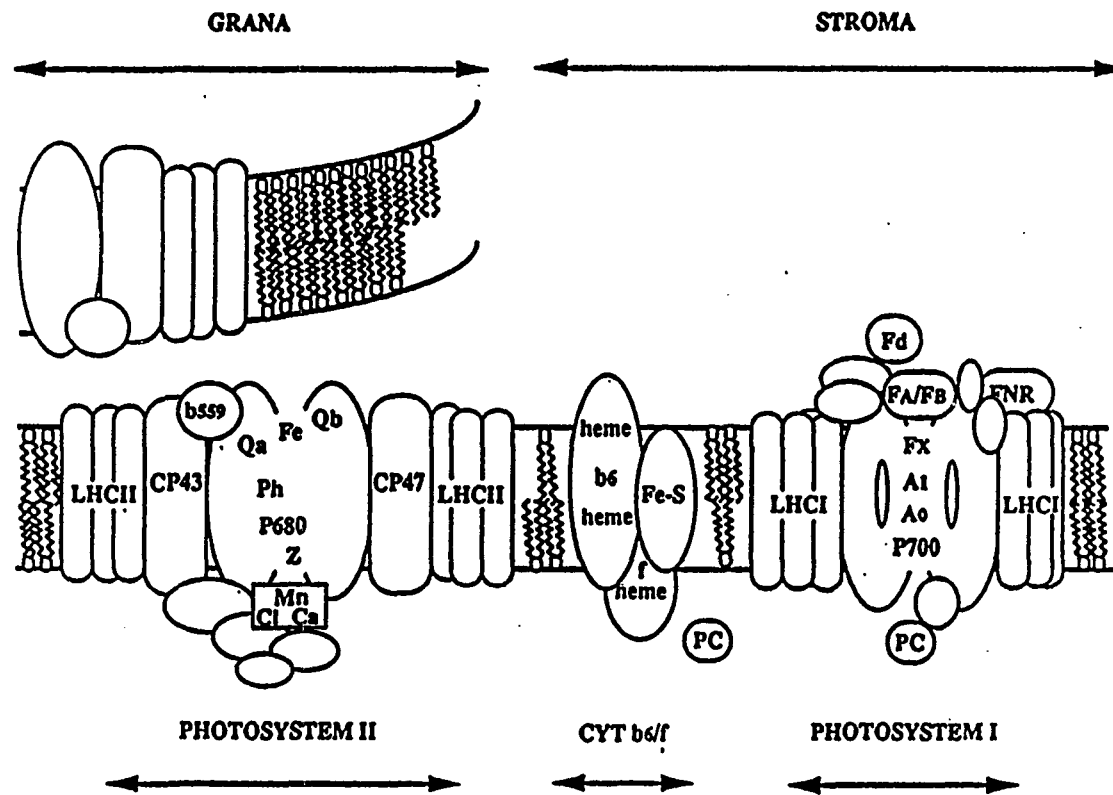


The Photosystem I reaction center can be considered to be a light-driven plastocyanin: ferredoxin-oxidoreductase. The Photosystem I holocomplex has a complex structure containing 10 or 11 polypeptides [6], 100-200 antenna chlorophyll molecules, a chlorophyll primary electron donor P700, a chlorophyll primary electron acceptor A<sub>0</sub>, a quinone intermediate electron acceptor A<sub>1</sub>, and three iron-sulfur centers F<sub>X</sub>, F<sub>B</sub> and F<sub>A</sub> (see reference 7-9, for review). The redox partners of Photosystem I, plastocyanin and ferredoxin, are soluble proteins that interact with specialized binding sites of the complex during forward electron transport.

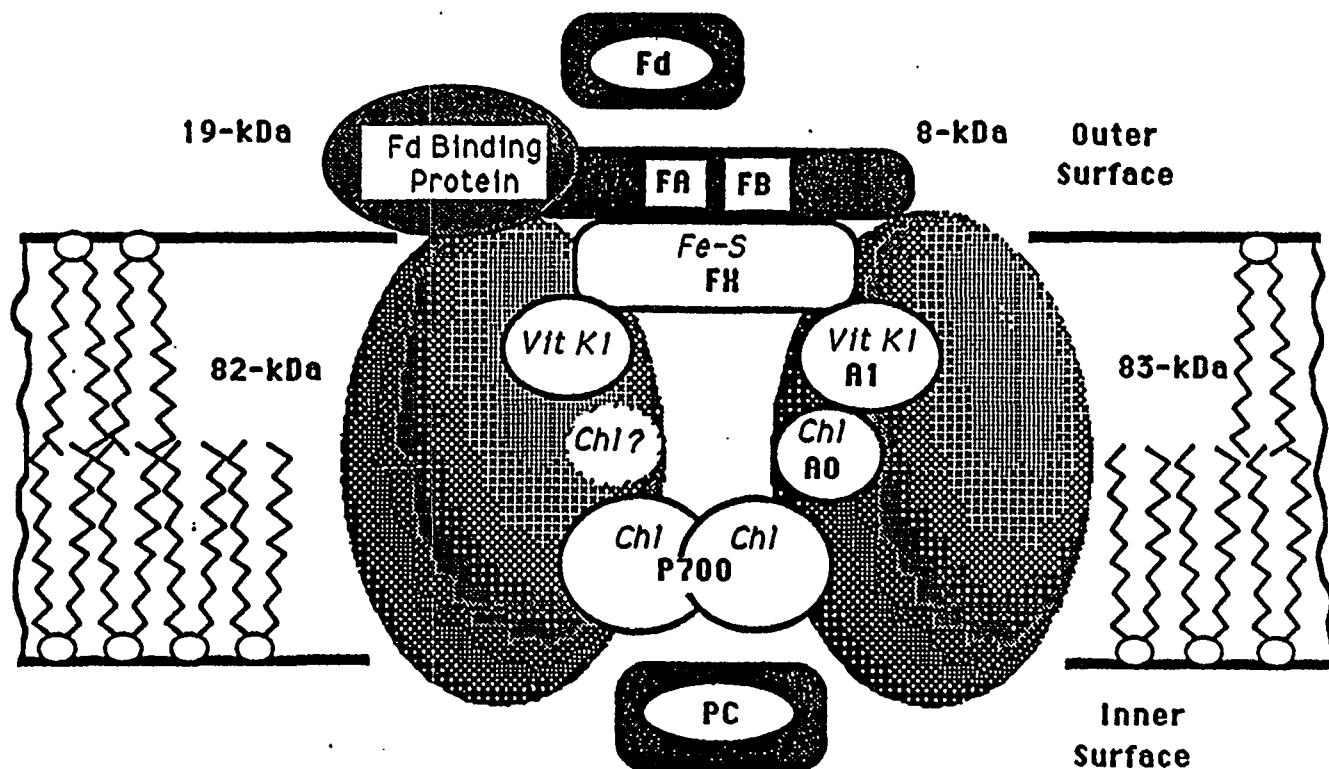
The primary events in Photosystem I include the absorption of a photon of light by an antenna chlorophyll molecule, the migration of the exciton to the photochemical trapping center, and the resulting charge separation between the primary donor, P700 and primary acceptor, A<sub>0</sub>. The quantum efficiency of this photochemical event is nearly 1. This is achieved by passing the electron from the primary acceptor to the iron-sulfur terminal electron acceptors, thereby preventing charge recombination by delocalizing the electron away from P700<sup>+</sup>. Upon absorption of the exciton from the antenna chlorophyll molecules, the primary donor, P700, becomes an excited singlet state. This state has a

midpoint potential of  $\sim -1300$  mV, which is low enough to reduce the primary acceptor,  $A_0$ , (another chlorophyll molecule) in a picosecond time scale. The forward electron transfer between adjacent redox partners is much faster than their corresponding backreaction with  $P700^+$ . Eventually the electron reaches the iron-sulfur  $F_A/F_B$  terminal electron acceptors by passing through the intermediate electron acceptors,  $A_1$ , and  $F_X$ . All the electron transport components, except  $F_A$  and  $F_B$ , have a sufficient gap in midpoint redox potential to dictate the direction and sequence of electron flow. However,  $F_A$  is only  $\sim 40$ -50 mV higher in midpoint potential than  $F_B$ , and in some species is even reversed [see reference 10]. This may be expected since  $F_A$  and  $F_B$ , both  $[4Fe-4S]$  clusters, are located on a single 8.9 kDa polypeptide. Selective removal of one cluster without influencing the other is a very difficult task to perform. However, Golbeck and Warden [11] selectively inactivated  $F_B$  and could still observe forward electron flow to  $F_A$ ; this led to the conclusion that  $F_A$  and  $F_B$  function in parallel. Malkin [12], on the other hand, proposed that forward electron flow proceeds from  $F_X$  to  $F_A$  only through  $F_B$ . This longstanding controversy is still unresolved and the terminal electron acceptors shall be represented as  $F_A/F_B$  throughout this dissertation to acknowledge this uncertainty.

This dissertation deals with Photosystem I and its electron transport protein components: the 8.9 kDa polypeptide and its cofactor, the  $F_A/F_B$  iron-sulfur clusters, are at the heart of the discussion. Chapter II deals with a technique of preparing a Photosystem I reaction center core containing P700 and iron-sulfur center  $F_X$  by removing the 8.9 kDa polypeptide. Chapter III shows how a fully functional Photosystem I complex can be reconstituted from the Photosystem I core protein and the isolated  $F_A/F_B$  holoprotein. In Chapter IV, a protocol for reconstituting the  $F_A/F_B$  holoprotein from the apoprotein using inorganic iron and sulfur will be discussed. ESR spectral differences in the  $F_A/F_B$  resonances and similarities between same species and cross-species reconstituted Photosystem I complexes are also investigated.



**Figure 1.** Physical organization of the photosynthetic electron transfer components in the thylakoid membrane of higher plants or cyanobacteria (reprinted from reference 2)



**Figure 2.** A proposed structural model for the Photosystem I reaction center complex and its oxidation-reduction partners based on spectroscopic and biochemical evidence (reprinted from reference 2).

## **CHAPTER II**

### **ISOLATION OF THE INTACT PHOTOSYSTEM REACTION CENTER CORE CONTAINING P700 AND IRON-SULFUR CENTER F<sub>X</sub>**

The Photosystem I reaction center complex of both plants and cyanobacteria consists of a chlorophyll primary electron donor, P700, a chlorophyll primary electron acceptor, A<sub>0</sub>, a quinone intermediate electron acceptor, A<sub>1</sub>, and three iron-sulfur centers, F<sub>A</sub>, F<sub>B</sub>, and F<sub>X</sub> (See references. 7-9 for review]. It has been suggested that A<sub>1</sub> and F<sub>X</sub> are located on the P700- and A<sub>0</sub>-containing reaction center polypeptides [13-17], and that F<sub>A</sub> and F<sub>B</sub> are located on a peripheral 8.9 kDa polypeptide [18,19].

In an earlier series of papers from this laboratory [13,16,17] it was reported that iron-sulfur centers F<sub>A</sub> and F<sub>B</sub> are removed from a spinach Photosystem I particle after addition of 0.5 to 1% LDS at pH 8.3. In the resulting reaction center core, light-induced charge separation takes place between P700 and F<sub>X</sub> without the need for prereduction of F<sub>A</sub> and F<sub>B</sub>. However, even under the best of conditions, the differential effect of LDS on the three iron-sulfur clusters led to a mixture of products: 20% were in the native state [P700...A<sub>0</sub>A<sub>1</sub>F<sub>X</sub>F<sub>A</sub>F<sub>B</sub>], 60% were in the intact 'core' state [P700...A<sub>0</sub>A<sub>1</sub>F<sub>X</sub>], and 20% were in the 'CP1' state [P700...A<sub>0</sub>]. The conclusions reached on the polypeptide location of F<sub>X</sub>, as well as the number of iron-sulfur pairs present in F<sub>X</sub>, were complicated by the heterogeneous nature of this preparation.

Earlier, it was shown that F<sub>B</sub> was the most susceptible component, and F<sub>X</sub> was the least susceptible component, to oxidative denaturation following treatment of a spinach



Photosystem I reaction center with 2 or 4 M urea [11]. Since the denaturants LDS and urea interfere with the patterns of ion-ion associations in proteins, I decided to investigate the ability of high concentrations of urea to remove the low molecular weight polypeptides carrying  $F_A$  and  $F_B$  without affecting the integrity of  $F_X$ . In this chapter, 6.8 M urea is used to isolate an intact Photosystem I reaction center core containing the entire complement of electron components from P700 to  $F_X$ . A cyanobacterial reaction center was chosen for study because it responded to urea with minimal destruction of  $F_X$  and because it contains fewer low molecular weight polypeptides than spinach [20].

## MATERIALS AND METHODS

### Particle Preparation

Photosystem I reaction center particles containing the full complement of electron acceptors ( $A_0$   $A_1$   $F_X$   $F_A$   $F_B$ ) were isolated from *Synechococcus* sp. PCC 6301 (*Anacystis nidulans* TX-20). The cells were grown in Krantz and Myers Medium C and incubated at 35-39° under 1%  $CO_2$  in air and harvested 24 hrs after inoculation. The cells were centrifuged, resuspended to 0.7 mg/ml Chl in 0.05 M Tris, pH 7.5 and 2 mM EDTA, and broken in a French Pressure Cell (2500 on PSI scale). The eluate was spun at 3000 RPM for 3 min and the supernatant was re-centrifuged at 19,000 RPM for 60 min (SS-34 rotor). The resulting pellet was resuspended to 100 µg/ml Chl in 50 mM Tris, pH 8.3, containing 0.2 M KCl and 1% Triton X-100, and after 24 hr of incubation, the suspension was centrifuged at 14,000 RPM for 30 min. The supernatant was concentrated to 30 ml over a YM-100 ultrafiltration membrane (Amicon) and centrifuged for 48 hrs at 24,000 RPM (SW-27 rotor) in a 0.1 to 1.0 M sucrose gradient containing 50 mM Tris, pH 8.3, and 0.1% Triton X-100. The lower green band was isolated, dialyzed against 50 mM Tris, pH 8.3, containing 1% Triton X-100 and loaded onto a DEAE Bio-Gel A column that had been pre-equilibrated with the same buffer. The column was washed until the eluant was

colorless, and the Photosystem I particle was removed with a pulse of 1 M NaCl in 50 mM Tris, pH 8.3, containing 0.1% Triton X-100. The Photosystem I particle was dialyzed, concentrated over a YM-100 membrane and recentrifuged on a 0.1 to 1 M sucrose gradient in the absence of Triton X-100. The chlorophyll to P700 ratio at this stage was 92 when determined by chemical difference spectroscopy, and 91 when determined photochemically. The sample was dialyzed against 50 mM Tris, pH 8.3, and placed in 0.1 M glycine buffer, pH 10.0 containing 6.8 M urea at 250  $\mu$ g/ml Chl for 10 min. The material was dialyzed against 50 mM Tris, pH 8.3 for 24 hours, concentrated over a YM-100 membrane, and centrifuged for 48 hrs at 24,000 RPM (SW-27 rotor) in a 0.1 to 1 M sucrose gradient containing 50 mM Tris, pH 8.3 and 0.1% Triton X-100. The lower green band, which contains the reaction center in the state [P700...A<sub>0</sub> A<sub>1</sub> FX], was removed, dialyzed against 50 mM Tris, pH 8.3, and stored at -80° C in 20% glycerol.

#### Analytical Assays

Chlorophyll was determined in 80% acetone [21]. Acid labile sulfide was determined as described in ref [22]. Spinach ferredoxin at a purity ratio of 0.48 (A<sub>420</sub>/A<sub>276</sub>) served as absolute standard for acid labile sulfide [23].

#### Optical Absorption Spectroscopy

Flash-induced absorption transients were determined at 698 nm as described in reference 4. The recovery time of the DC-coupled amplifier (EG&G Model 113) following a saturating laser flash was ~10  $\mu$ s; thus photochemical P700 is defined for the purpose of this study as any 698 nm absorption transient with a lifetime of  $\geq 10 \mu$ s.

#### Electron Spin Resonance Spectroscopy

ESR studies were performed on a Varian E-109 spectrometer equipped with an Air Products LTD liquid helium transfer cryostat. The spectrometer was interfaced to a Nicolet 4094A Digital Oscilloscope and a Macintosh Plus computer for signal averaging and baseline subtraction. Sample temperatures were monitored with a gold chromel thermistor

situated directly below the sample tube. Light-minus-dark difference spectra were obtained by illuminating the sample with a 150-Watt Xenon lamp (Oriel).

### Electrophoresis

Electrophoresis (PAGE) was performed in a 8 cm x 1.5 mm slab gel containing a linear 10-15% polyacrylamide (bis:acrylamide of 1:20) gradient (Hoefer Model SE-200). The control and urea-treated Photosystem I reaction centers (1  $\mu\text{g}/\mu\text{l}$  protein) were incubated in 0.0625 M Tris (pH 6.8), 2% SDS, 10% glycerol and 5%  $\beta$ -mercaptoethanol for 24 h at 30 C [24]. The samples were applied to the stacking gel at a protein concentration of 20  $\mu\text{g}/\text{well}$ . Electrophoresis was carried out at 20 C at a constant current of 12 ma for 2.5 hours. Gels were stained with Coomassie Brilliant Blue and scanned with an LKB laser densitometer.

## **RESULTS**

### Effect of 6.8 M Urea on Photosystem I: Flash-induced Absorption Changes at 698 nm

Figure 3A shows the flash-induced absorption transient at 698 nm in a Photosystem I complex isolated from *Synechococcus* sp. PCC 6301. The kinetics were measured in the presence of an electron donor (ascorbate/DPIP; 30 ms half-time for reduction of  $\text{P700}^+$ ) but in the absence of an electron acceptor; under these conditions, the 30-ms decay of the absorption transient indicates charge recombination between  $\text{P700}^+$  and the terminal electron acceptors  $[\text{F}_\text{A}/\text{F}_\text{B}]^-$ . Figure 3B shows the flash-induced absorption change in the same sample after 10-min of incubation with 6.8 M urea at pH 10.0. The 1.2-ms decay of the absorption transient is typical of a reaction center in which  $\text{F}_\text{A}$  and  $\text{F}_\text{B}$  have been removed prior to flash photolysis [13]; under these conditions electron flow terminates at  $\text{F}_\text{X}$ , resulting in a characteristic backreaction with  $\text{P700}^+$ . The kinetics and magnitude of the absorption change indicate that the urea protocol removes  $\text{F}_\text{A}$  and  $\text{F}_\text{B}$  entirely from the Photosystem I reaction center core without affecting  $\text{F}_\text{X}$ .

### Isolation and Purification of the Photosystem I Reaction Center Core Protein

Unlike treatment with 1% LDS [13], prolonged urea treatment of the Photosystem I reaction center does not result in the simultaneous loss of F<sub>X</sub> relative to F<sub>A</sub> and F<sub>B</sub>; typically, >90% of F<sub>X</sub> is retained after 120-min of exposure to 6.8 M urea. This relative stability makes it feasible to remove the urea by overnight dialysis without degradation of the iron-sulfur cluster. Also unlike LDS, the dialyzed, urea-treated reaction center is stable in 0.1% Triton X-100. The ability to withstand detergents allows purification strategies to be devised that depend on solubilization of the Photosystem I reaction center. We found that the dissociated reaction center can be purified by ultracentrifugation in a 0.1% Triton-containing, 0.1 to 1.0 M sucrose gradient; after 48 hr at 48,000 x g the treated reaction center separates into an upper light-green band, and a lower dark green band. The lower band is photochemically active in electron flow from P700 to F<sub>X</sub> and shows flash-induced absorption changes identical to that of Figure 3B.

### Characterization of the Photosystem I Core Protein by Optical and ESR Spectroscopy

Figure 4A shows the ESR spectrum of F<sub>A</sub> ( $g = 2.05, 1.94, 1.86$ ) and F<sub>B</sub> ( $g = 2.05, 1.92, 1.89$ ) in the native reaction center after reduction with dithionite/methyl viologen at pH 10.0 and illumination at 12 K. The interaction between the two iron-sulfur clusters is seen by the shift of the high field resonance of F<sub>A</sub> from  $g = 1.86$  to 1.89. If the sample is illuminated at 12 K with ascorbate/DPIP as reductant, only F<sub>A</sub> becomes photoreduced (not shown). Figure 4B shows that F<sub>A</sub> and F<sub>B</sub> are absent in the reaction center core isolated by ultracentrifugation after 6.8 M urea treatment. Similar to Figure 4A, the core particle was reduced with dithionite/methyl viologen at pH 10.0 prior to illumination at 12 K. To ensure complete reduction of F<sub>A</sub> and F<sub>B</sub> the core sample was illuminated while cooling to 12 K; no indication of F<sub>A</sub> or F<sub>B</sub> was found (not shown).

Figure 5 shows the ESR spectrum of F<sub>X</sub> in the urea-treated, ultracentrifuged reaction center after incubation with ascorbate/DPIP at pH 10.0 and illumination at 6 K. The spectrum was resolved by subtracting four light-off scans from four light-on scans and

amplifying the difference by a factor of four. The characteristic  $g$ -values (2.07, 1.88 and 1.79) and the altered (~30% broader) lineshape is typical of  $F_X$  in a reaction center deficient in  $F_A$  and  $F_B$  [16,17]. The same spectrum was seen after reduction of the sample with dithionite/methyl viologen at pH 10.0; however, unlike  $F_A$  and  $F_B$ ,  $F_X$  was not chemically reduced at -600 mV (vs  $H_2$ ).

#### Labile Sulfide Content of the Photosystem I Core Protein

The loss of  $F_A$  and  $F_B$  is also reflected in the decline of acid-labile sulfide from  $11.8 \pm 0.4 S^{2-}/P700$  ( $n=3$ ) in the control reaction center to  $4.6 \pm 0.3 S^{2-}/P700$  ( $n=3$ ) in the reaction center core preparation. This ratio is calculated on the basis of photochemical P700; the chemical difference measurement of P700 indicates that nearly all of the available P700 is photoactive on this timescale. The negligible contamination with  $F_A$  and  $F_B$ , along with the precision of the P700 and labile sulfide measurements, permits the existence of four  $S^{2-}/P700$  to be established in the core preparation with a high degree of confidence.

#### Polypeptide Composition of the Photosystem I Core Protein

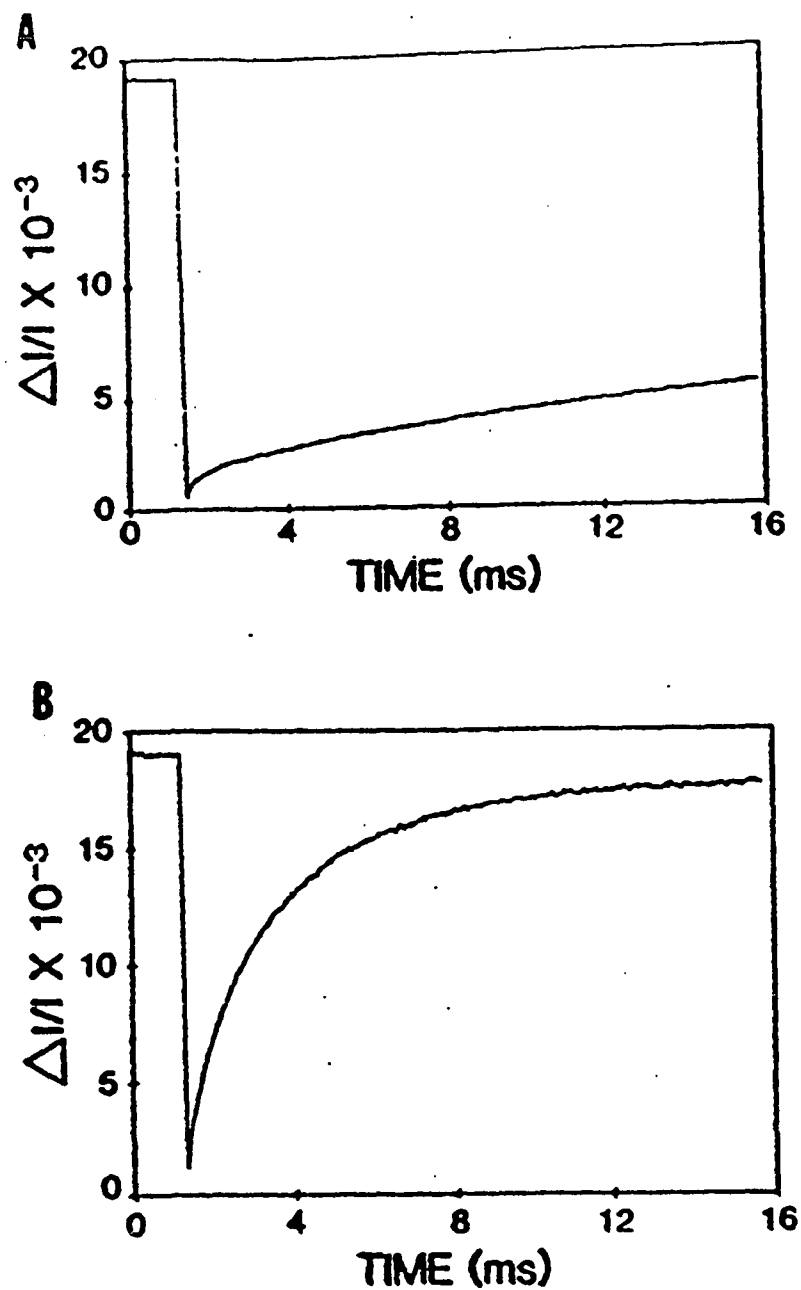
The polypeptide composition of the control and urea-treated preparations are shown in Figure 6. The control *Synechococcus* sp. PCC 6301 reaction center contains low molecular weight polypeptides at 16.4-kDa and 8.1-kDa in addition to the reaction center polypeptides at 90-kDa and 57-kDa (the 90-kDa band contains chlorophyll, and under more rigorous treatment conditions, degrades to the more diffuse 57-kDa band). There are also minor bands at 13.4-kDa and 17.2-kDa. In the urea-treated and ultracentrifuged reaction center the 90-kDa and 57-kDa bands are present in the same amount as the control, but the 16.4-kDa and 8.1-kDa bands are totally missing. The minor polypeptides are still present to some degree; the 13.4-kDa band is depleted to 35-50% of the control and the 17.2-kDa band is 75-85% retained. If the 8.1-kDa polypeptide corresponds to the 8.9 kDa polypeptide that has been identified as an [8Fe-8S] protein in spinach, the loss of ~8 moles

of labile sulfide per mole of P700 may be understood as the complete removal of the polypeptide carrying F<sub>A</sub>/F<sub>B</sub>.

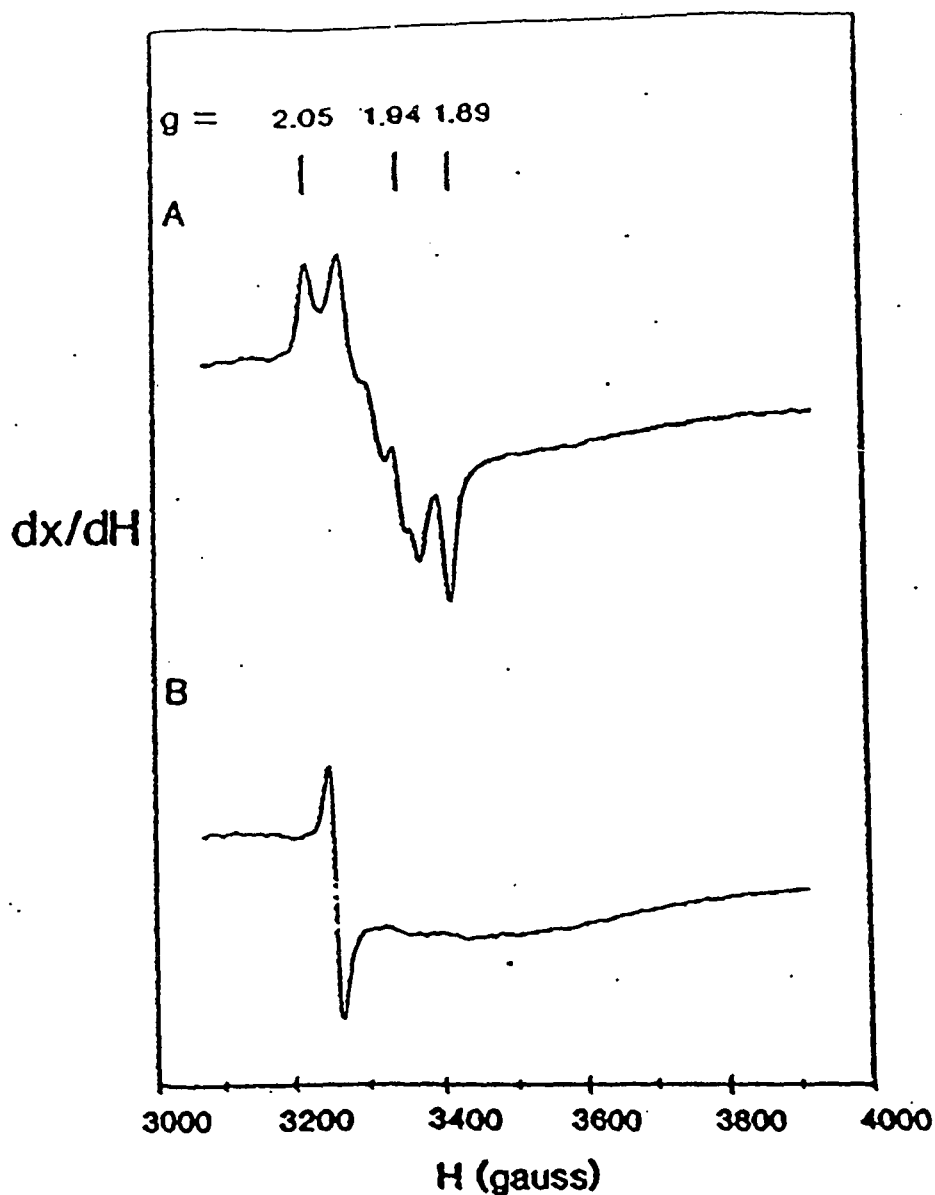
## DISCUSSION

I suggest the cyanobacterial reaction center reported in this chapter represents the intact Photosystem I core incorporating the components P700 and F<sub>X</sub>. The data indicate that little deterioration of the reaction center has taken place throughout the course of purification: even after extensive sample handling we find nearly the entire complement of F<sub>X</sub> present in the core preparation as was present in the control particle. The retention of four S<sup>2-</sup>/P700 in the core preparation agrees with a previous estimate of the labile sulfide content of F<sub>X</sub> [17] and indicates the existence of four Fe-S pairs in Photosystem I distinct from that present in F<sub>A</sub> and F<sub>B</sub>. This is enough labile sulfide for one [4Fe-4S] cluster or two [2Fe-2S] clusters. Since F<sub>X</sub> has been proposed to consist of a [4Fe-4S] cluster [25,26], the data are consistent with the existence of one molecule of F<sub>X</sub> per molecule of P700.

The polypeptide composition is more problematical since there are reported differences in the number as well as molecular weights of the polypeptides in various cyanobacterial Photosystem I preparations [20,27-29]. Most reaction centers contain at least two low molecular weight polypeptides and a diffuse protein band at about 55-70-kDa. Our data indicate that the 6.8 M urea protocol removes the 16.4 and 8.1-kDa polypeptides entirely from a *Synechococcus* sp. PCC 6301 Photosystem I reaction center. As such, we assert that F<sub>X</sub> resides on the high molecular weight polypeptides in Photosystem I.

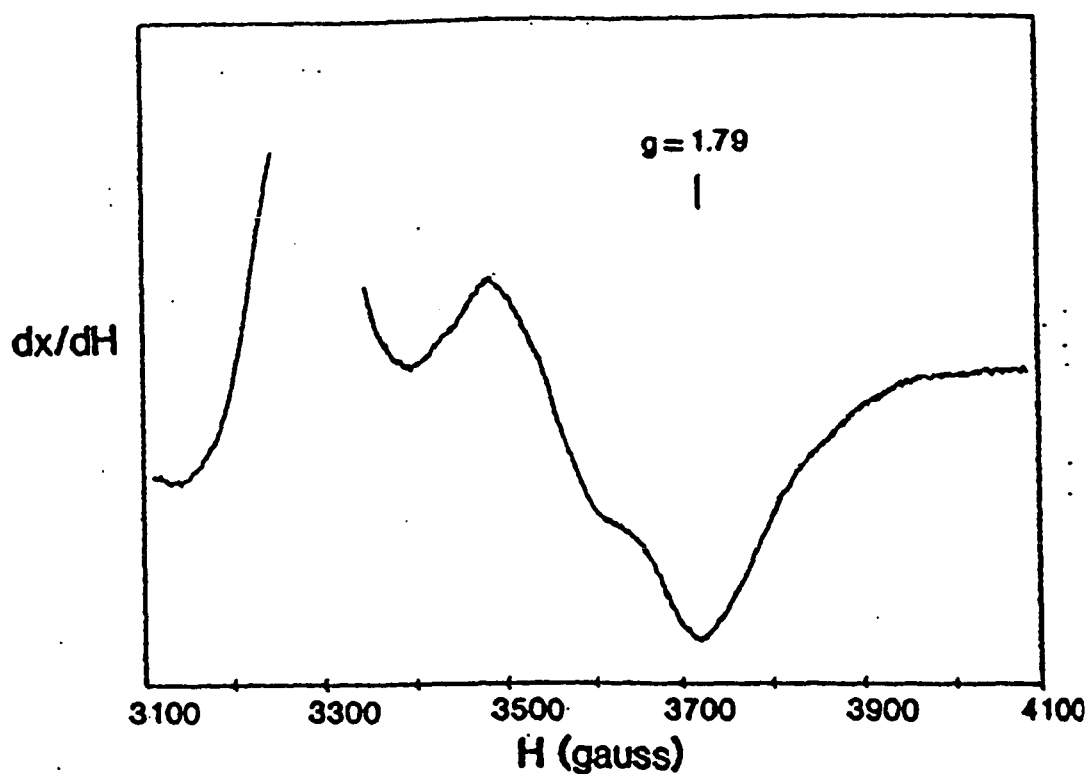


**Figure 3** Flash-induced absorption change at 698 nm before (A) and after (B) addition of 6.8 M urea to a *Synechococcus* sp. PCC 6301 Photosystem I reaction center. All measurements were made at 10  $\mu\text{g/ml}$  Chl in 0.1 M glycine, pH 10.0, containing 1.7 mM ascorbate and 0.033 mM DCPIP. Both samples were allowed to incubate for 10 min in darkness prior to flash photolysis.

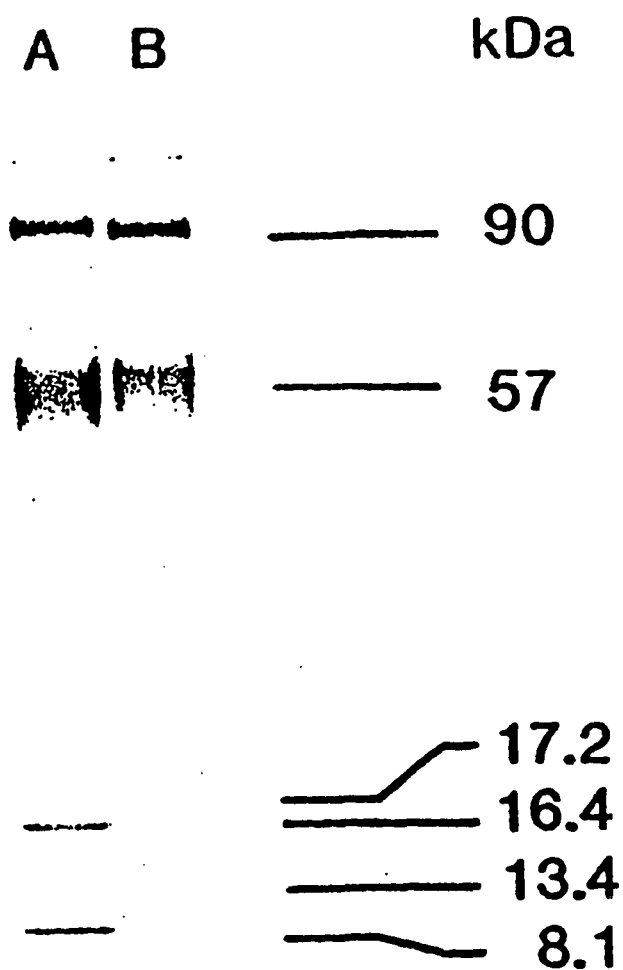


**Figure 4.** X-band ESR spectrum of  $F_A$  and  $F_B$  in control (A) and 6.8 M urea-treated, ultracentrifuged (B) Photosystem I reaction centers. Both samples were prepared by addition of 1 mM sodium dithionite and 10  $\mu$ M methyl viologen to the core preparation at 300  $\mu$ g/ml Chl in 0.1 M glycine, pH 10.0. There was no significant increase in the amount of  $F_A$  or  $F_B$  upon illumination of the sample at 12 K. Spectrometer conditions: temperature, 12 K; microwave power, 10 mW; microwave frequency, 9.18 GHz; receiver gain,  $5.0 \times 10^3$ ; modulation amplitude, 10 G at 100 kHz.





**Figure 5.** Light-minus-dark (before light) X-band ESR spectrum of Fx in the 6.8 M urea-treated, ultracentrifuged Photosystem I reaction center. The sample contained 1 mM ascorbic acid and 0.8 mM DCPIP in 0.1 M glycine at pH 10.0. Spectrometer conditions: temperature, 6 K; microwave power, 40 mW; microwave frequency, 9.25 GHz; receiver gain,  $5 \times 10^3$ ; modulation amplitude, 40 G at 100 kHz. The spectrum represents the average of four 4-minute sweeps each for the light and dark samples, and a 5-fold enlargement in software.



**Figure 6.** Polypeptide composition of the control (A) and the 6.8 M urea-treated, ultracentrifuged (B) Photosystem I reaction centers. Molecular weights are calculated on the basis of the  $R_f$  values of soluble proteins from 6.21 kDa to 66.0 kDa treated identically to the Photosystem I particles but run in alternate wells. Details of the electrophoresis are given in Materials and Methods.

## **ACKNOWLEDGEMENTS**

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## CHAPTER III

### RECONSTITUTION OF THE PHOTOSYSTEM I COMPLEX FROM THE P700 AND F<sub>X</sub>-CONTAINING REACTION CENTER CORE PROTEIN AND THE F<sub>A</sub>/F<sub>B</sub> POLYPEPTIDE

As indicated in Chapter I, the Photosystem I complex of plants and cyanobacteria is a chlorophyll-containing, light-driven, plastocyanin:ferredoxin oxidoreductase. The bound electron transport components consist of a chlorophyll primary electron donor, P700, a chlorophyll primary electron acceptor, A<sub>0</sub>, a quinone intermediate electron acceptor, A<sub>1</sub>, and three iron-sulfur centers, F<sub>X</sub>, F<sub>B</sub>, and F<sub>A</sub> [see references 2,7,9 for review]. According to current understanding, a singlet exciton migrates from an antenna chlorophyll to the reaction center trap, bringing about charge separation between P700 and A<sub>0</sub>. The electron is then passed through the intermediate acceptors A<sub>1</sub> and F<sub>X</sub> to the terminal electron acceptors, F<sub>B</sub>/F<sub>A</sub>.

The polypeptide location of these electron transport components is known; A<sub>1</sub> [14,30,31] and F<sub>X</sub> [13,17, see also 32-34] are associated with the P700 and A<sub>0</sub>-containing [15] reaction center proteins, and F<sub>A</sub> and F<sub>B</sub> are located on a peripheral 8.9-kDa polypeptide [35,36]. Wynn and Malkin [36] recently confirmed an earlier procedure [35] for the purification from spinach of the 8.9-kDa polypeptide that carries the electron acceptors F<sub>A</sub> and F<sub>B</sub>. The isolated polypeptide shows an ESR spectrum characteristic of an iron-sulfur cluster, but with significant differences from the spectrum found in an intact Photosystem I complex. As shown in Chapter II, chaotropic agents such as urea are extremely effective in removing the low molecular weight proteins, including the F<sub>A</sub>/F<sub>B</sub> polypeptide, from a cyanobacterial Photosystem I complex [37,38]. In the

resulting Photosystem I core protein, transient charge separation and recombination occurs between P700 and iron-sulfur center  $F_X$ .

In this chapter, I report that the Photosystem I complex can be reconstituted from the isolated Photosystem I core protein from *Synechococcus* sp. PCC 6301 and the isolated  $F_A/F_B$  polypeptide from spinach. Electron flow from P700 to  $F_A/F_B$  at both 16 K and at room temperature is completely restored, and  $F_X$  photoreduction occurs only in the presence of prereduced  $F_A^-$  and  $F_B^-$ . Most significantly, the ESR spectra of  $F_A$ ,  $F_B$  and  $F_X$  revert to their original characteristics and are nearly indistinguishable from a control Photosystem I complex.

## MATERIALS AND METHODS

The Photosystem I complex containing the full complement of electron acceptors ( $A_0 A_1 F_X F_A F_B$ ) was isolated from *Synechococcus* sp. PCC 6301 (*Anacystis nidulans* TX-20) membranes [37]. Treatment with 6.8 M urea results in loss of the low molecular weight polypeptides and in the purification of the Photosystem I core protein containing only the reaction center heterodimer *psaA* and *psaB*, and the electron acceptors  $A_0 A_1$  and  $F_X$  [37,38]. The  $F_A/F_B$  polypeptide was isolated according to published procedures [35,36] except that excess dithionite was present throughout the isolation procedure. The reconstitution protocol consisted of mixing the Photosystem I core with the  $F_A/F_B$  polypeptide in an approximate 1:1 molar ratio and incubating in the presence of 0.1 %  $\beta$ -mercaptoethanol for 3 min. Chlorophyll concentration was determined in 80% acetone [21]. Flash-induced absorption transients were determined at 698 nm [37] and ESR studies were performed on a Varian E-109 spectrometer as described in reference [32].

## RESULTS

### Characterization of the Reconstituted Photosystem I Complex by ESR Spectroscopy

The ESR spectrum of the Photosystem I core protein from *Synechococcus* sp. PCC 6301 is shown in Figure 7A. The absence of ESR resonances characteristic of  $F_A$  and  $F_B$  confirms the loss of the low molecular weight polypeptides, including the 8.9-kDa,  $F_A/F_B$  polypeptide [37,38], from the cyanobacterial reaction center (the broad, shallow resonance of  $F_X$  is barely visible under the conditions optimal for observation of iron-sulfur centers  $F_A$  and  $F_B$ ; see Figure 10A). The ESR spectrum of the chemically-reduced 8.9-kDa,  $F_A/F_B$  polypeptide from spinach is shown in Figure 7B. When compared with the control Photosystem I complex, the resonances of  $F_A$  and  $F_B$  are significantly broader, and all but the  $g_X$  resonances are shifted to a slightly lower field (compare, Figure 9B).

When the Photosystem I core protein is incubated for 3 min at an approximate 1:1 molar ratio with the unreduced  $F_A/F_B$  polypeptide and frozen in darkness, the low temperature photoreduction of  $F_A$  ( $g = 2.056, 1.956$  and  $1.872$ ) and  $F_B$  ( $g = 2.072, 1.934$  and  $1.892$ ) is observed with nearly equal spin concentrations (Figure 8A). In contrast, when the control Photosystem I complex is illuminated under identical conditions, iron-sulfur center  $F_A$  ( $g = 2.056, 1.949, 1.865$ ) is predominantly photoreduced (Figure 8B). There are additional small differences in the peak positions, but the most significant feature is that the spectrum of the reconstituted  $F_A/F_B$  polypeptide has narrowed and appears quite similar to the control. The integrated signal size induced on illumination at 16 K is the same in the reconstituted Photosystem I complex as in the control Photosystem I complex. This, and the absence of diffusion-controlled reactions at 16 K, suggests that the  $F_A/F_B$  polypeptide is rebound to the reaction center core.

When the reconstituted Photosystem I complex is illuminated during freezing, the low field and high field resonances of  $F_A$  ( $g = 2.056, 1.949$  and  $1.899$ ) and  $F_B$  ( $g =$

2.056, 1.934 and 1.899) have merged and show the characteristic interaction between the two clusters (Figure 9A). When the control Photosystem I complex is illuminated during freezing under identical conditions (Figure 9B), the spectrum also shows the full extent of interacting iron-sulfur centers  $F_A$  ( $g = 2.048, 1.949$  and  $1.892$ ) and  $F_B$  ( $g = 2.048, 1.927$  and  $1.892$ ), but the peak positions are slightly shifted. The narrow  $F_A$  and  $F_B$  resonances (compare, Figures 9A, 7B) indicate that the majority of the 8.9-kDa polypeptide is not photoreduced 'in solution' but is rebound to the Photosystem I core protein. However, the slight difference between the spectrum of the reconstituted Photosystem I complex and the control Photosystem I complex (compare the mid field regions of Figures. 9A,B) may be due to a small contribution of 'free' photoreduced  $F_A^-/F_B^-$  that may be present in excess to the Photosystem I core protein (see also Figure 9 in reference 35).

A further indication of reconstitution can be found by observing the low temperature behavior of iron-sulfur center  $F_X$ . The ESR spectrum of the Photosystem I core protein after freezing in darkness with sodium ascorbate and DCPIP at pH 8.3 and illumination at 6 K shows the characteristic  $g$ -values (2.05, 1.87 and 1.78) and broader resonances typical of iron-sulfur center  $F_X$  in a reaction center deficient in  $F_A$  and  $F_B$  [32]. When the spectrum of the reconstituted Photosystem I complex is measured under identical conditions, the low temperature photoreduction of  $F_X$  does not occur (spectra not shown). When the Photosystem I core protein is illuminated during freezing in the presence of sodium ascorbate and DCPIP at pH 8.3, the full reduction of  $F_X^-$  occurs (Figure 10A). However, the presence of sodium dithionite and methyl viologen at pH 10 are required, as in the control Photosystem I complex, for photoreduction of  $F_X$  in the reconstituted Photosystem I complex (Figure 10B) [note that the  $F_A/F_B$  resonances are distorted at the conditions optimal for  $F_X$ ]. The ESR signal of  $F_X$ , especially in the high field region, is indistinguishable from that of  $F_X$  in the untreated Photosystem I complex (see Reference

2). This behavior is also consistent with efficient binding of the  $F_A/F_B$  polypeptide to the Photosystem I core protein.

#### Characterization of the Reconstituted Photosystem I Complex by Optical Spectroscopy

The flash-induced absorption transient at 698 nm in a Photosystem I core protein from *Synechococcus* sp. PCC 6301 is shown in Figure 11A. The 1.2-ms optical absorption transient is typical of a reaction center in which  $F_A$  and  $F_B$  have been removed prior to flash photolysis [13]; under these conditions electron flow terminates at  $F_X$ . The P700 absorption transient after addition of the spinach  $F_A/F_B$  polypeptide is shown in Figure 11B. The restoration of the ~30-ms optical transient occurs within the mixing time of 1 min and indicates that efficient electron flow from P700 to  $F_A/F_B$  has been reestablished. The extent and kinetics of the ~30-ms transient remain intact despite extensive washing over an Amicon YM-100 ultrafiltration membrane, implying tight rebinding of the 8.9-kDa polypeptide. Addition of 6 M urea reverses the effect, causing restoration of the 1.2-ms,  $P700^+ F_X^-$  backreaction at the expense of the 30-ms,  $P700^+ [F_A/F_B]^-$  backreaction (Figure 11C).

## DISCUSSION

The protocol described in this chapter represents the first reconstitution of the Photosystem I complex from the P700 and  $F_X$ -containing Photosystem I core protein and the  $F_A/F_B$  polypeptide. The restoration of cryogenic and room temperature electron flow from P700 to the terminal iron-sulfur clusters  $F_A/F_B$ , and the near-normal ESR spectrum of  $F_A$  and  $F_B$  in the reconstituted Photosystem I complex indicates that the 8.9-kDa polypeptide and the Photosystem I core protein reconstitute to their native configuration. It is important to note that the isolated  $F_A/F_B$  polypeptide shows a severely distorted ESR signal (Figure 7B). This suggests that after isolation, the iron-sulfur clusters might be exposed to a hydrophilic environment, which may cause a structural alteration of  $F_A$



and/or  $F_B$ . The change is reversible, since we obtained completely normal ESR signals after reconstitution of  $F_A/F_B$  onto the Photosystem I core protein (Figures.8A, 9A). This proves that the isolated  $F_A/F_B$  polypeptide is not denatured even though it has an abnormal ESR spectrum. The ESR signal of  $F_X$ , which was rather broad in the Photosystem I core protein (cf. Figure 10A), narrowed to resemble the control spectrum after reconstitution with the  $F_A/F_B$  polypeptide (Figure 10B), suggesting that the microenvironment around the iron-sulfur cluster of  $F_X$  also recovers. There might be discrete binding sites for  $F_A/F_B$  on the Photosystem I core protein, and the binding of  $F_A/F_B$  at this site allows recovery of the normal configuration of both  $F_A/F_B$  and  $F_X$ . The observation that  $F_B$  as well as  $F_A$  was reduced on illumination at 16 K in the reconstituted Photosystem I complex (compare Figures 8A,8B) suggests that the  $F_A/F_B$  polypeptide rebinds with an orientation resulting in a closer location of  $F_B$  to  $F_X$  than in the control. The ease of reconstitution shows that the binding affinity of the  $F_A/F_B$  polypeptide to the Photosystem I core protein must be high and that the binding sites have not been irreversibly altered by the isolation procedures.

The only features that distinguish the reconstituted Photosystem I complex from the control Photosystem I complex are the different ratios of photoreduced  $F_A$  and  $F_B$  at 16 K, and the slightly different  $g$ -values of  $F_A$  and  $F_B$ . These differences are minor and may be due to the use of a spinach rather than a *Synechococcus* sp. PCC 6301  $F_A/F_B$  protein. However, there are no significant differences in the ESR or EXAFS spectra of  $F_A$ ,  $F_B$  and  $F_X$  between spinach and *Synechococcus* sp. PCC 6301 despite the nearly two billion year evolutionary gap separating these organisms [39]. Recent sequence analysis has also shown that the *Synechococcus* sp. PCC 7002 and tobacco *psaC* polypeptides differ in only 8 of 80 amino acid residues - 6 of which are extremely conservative (Table II) [4]. The *psaA* and *psaB* genes are remarkably similar between *Synechococcus* sp. PCC 7002 and spinach (Tables III,IV) [4] especially in the region of (putative) helix VIII, which contains the cysteine residues that are postulated to coordinate iron-sulfur center  $F_X$ . We would

expect that this region would be important for the interaction with the  $F_A/F_B$  protein, especially since the  $F_A/F_B$  and  $F_X$  iron-sulfur clusters are probably in very close contact. It may not be surprising, therefore, that reconstitution of the spinach  $F_A/F_B$  polypeptide with the *Synechococcus* sp. PCC 6301 Photosystem I core protein occurs with demonstrated high efficiency.

TABLE II

COMPARISON OF THE AMINO ACID SEQUENCES OF THE FA/FB PROTEIN  
(psaC GENE PRODUCT) IN SEVERAL SPECIES OF PROKARYOTIC  
AND EUKARYOTIC ORGANISMS.

	10	20	30	40	50	
7002	MSHSVKIYDTCIGCTQCVRACPLDVLEMVPWDGCKAGQIASSPRTEDCVGCKRCET					
Nostoc	T		T	A		
6803			T	I	K	A
6301	/		/			
S. vu	/A T	? ? ?	? /			
C. pa	A T		T	R N	A	S
toba			T	I	K	A
live	A A		T	I	K	A
spin			T	I	K	A
pea			T	I	G	K
maiz		H	T	I	K	A
whea			T	I	K	A
barl			T	I	K	A

	60	70	80	Ref.
7002	ACPTDFLSIRVYLGAETTRSMGLAY*			[40]
Nost			*	[40]
6803		V	WH	*
6301				
S. vu				[42]
C. pa			G	*
toba		V	WH	*
live	R	V	N	S
spin		V	WH	G
pea		V	WH	*
maiz		V	P	A S
whea			P	A S
barl		V	P	A S

Only amino acids which differ from those of *Synechococcus* sp. PCC 7002 are shown. Slashes indicate the beginning or ends of determined sequences; stars indicate the C-terminus; question marks indicate undetermined amino acids. The *Synechococcus* sp. PCC 6301 sequence is unpublished [Bryant, D.].

Nost = Nostoc; S. vu = *S. vulcanus*; C. pa = *C. paradoxa*; toba = tobacco; live = liverwort; spin = spinach; maiz = maize; whea = wheat; barl = barley

TABLE III

COMPARISON OF THE AMINO ACID SEQUENCES OF *psaA*  
GENE PRODUCTS OF SEVERAL PROKARYOTIC AND  
EUKARYOTIC ORGANISMS

	10	20	30	40	50	60
Sy	MAVKVDKDPVPTSFEEKWGKPGHFDRTLARG-PKTTTWIWNLHADAHDFD					
Ma	MIIRSSEP-----	EVKIA R IK	E AR	S I K N D		
Sp	MIIRSSEP-----	EVKIL R K	A A	S I K E		
Pe	MIIRSPEPKVQILADPEVKIL	R IK	Q A	S I K D	W	
To	MIIRSSEP-----	EVKIL R K	E AR	S I K D		
Li	MTIRSSEP-----	EVKIV E K	A	S K S		
Eu	MTITPPEQQVKR----	VR AFVSN E	SR	S L SK N		

	70	80	90	100	110	120	130
Sy	SQTSDLEDISRKIFSAHFGLAVVFVWLSGMFYHGAKEFSNYEAWLNNPTVIKPSAQVWVPVVGQGI						
Ma	H G E V	Q SII L		R	SD H G	I E	
Sp	H E	Q SII L		R	SD H G	I E	
Pe	H E V	Q SII L		R W	WD H R	I E	
To	H E V	Q SII L		R	SD H G	I E	
Li	H N E V	P II I		R	SD H	I E	
Eu	NH T V	Q IIEI	F R		LD IHV I	I E	

	140	150	160	170	180	190
Sy	LNGDVGGGFSGIQITSGLFYLWRAAGFTNSYQLYVTAIGGLVMAALMVFAGWFFHYHKAAPKLEWF					
Ma	R	F QI	S I SEL	C A IF S L		A
Sp	R	F QI	S I SEL	C A F L		A
Pe	R	F QI	S I SEL	C A F L		V
To	R	F Q	S SEL	C A F L		A
Li	Q	F Q	S I SEL	S F L		A
Eu	N Q	Q	SC I SEF	I LT IFS VLF		

	200	210	220	230	240	250	260
Sy	FQNAEAMMNHLSVLLGCGSLGWTGHLIHVSLPVNKLDDSGVAAKDIPLPHEF-FDASVMAELYP						
Ma	DV S L	AG L	S A Q	I QF A	DP E	ILNRDLL Q	
Sp	DV S L	AG L	S A Q	I QF NA	DP E	LILNRDLL Q	
To	DV S L	AG L	S A QV	I QF NA	D-----	Q	
Li	DV S L	AG L	S A QV	I QF NA	DP E	ILNRDLL Q	
Eu	V S L	G	L C S A Q	I	NPAEL	D IL K LISQ	

	270	280	290	300	310	320
Sy	FAQGLKPFPTLDWAAYSDFLTFKGGNLPTTGSWLSDVAHHHLAIAVLFIAGHMYRTGFGIGH					
Ma	E AT	N SK AE	S R D I G	I	I L	NW
Sp	E AT	N SK A	R D V G	T T	I L	NW
Pe	EQAT	N SK A	R D L G	T I	I L	NW
To	E AT	N SK A	R D V G	T I	I L	NW
Li	K T	N SE	R V G	T T	LV	NW
Eu	SK A	FH SE	R NV G	T	L L	K NWK

**TABLE III**  
**COMPARISON OF THE AMINO ACID SEQUENCES OF *psaA***  
**GENE PRODUCTS OF SEVERAL PROKARYOTIC AND**  
**EUKARYOTIC ORGANISMS**  
 (continued)

	330	340	350	360	370	380	390
Sy	SMKEILEAHKGPFTGEGHKGLYEILTTSWHAQLAVNLALLGSLTIVIAHHMYSMPYPYMATDYGT						
Ma	GL D	Q		SL M T V			L
Sp	GL D	Q		L M	V	A	L
Pe	GI D	Q		SIW M	IQQ	IA I	LQ
To	GL D	Q		SL M	V		L
Li	F			L M	IV	A	L
Eu	DI GL	T Q	F N	SL MM S	IV Q		I I

	400	410	420	430	440	450
Sy	QLSLFTHHTWIGGFLIVGAGAHGAIFMVRDYPKAKVNNLLDRVIRHRDAIISHLNWVCIFLGF					
Ma	M	A A		TTRY D	L	
Sp	M	A A		TTRY D	L	A
Pe	M	A A		TTRY D	L	
To	M	D A A		TTRY D	L	A
Li	M	A A		TTQYK	L	
Eu	E Y	C A A		L F	LL	L

	460	470	480	490	500	510	520
Sy	HSFGLYIHNDTMRALGRPQDMFSDSAIQLOPIFAQWIQNIHTLAPGGTAPNAIASASQVF-GGDVV						
Ma		S	A		AG V	G TT T	LTWG EL
Sp		S	T	V	T A SA	G T T	LTWG S L
Pe		S	T	V	WT A T	G TT T	LIWG L
To		S	T	V	T A AA	G T T	LTWG L
Li		S	T	V	T A NF	L T	LTWG I
Eu		LS		V	T Y TL	F LVSPTTP	W-

	530	540	550	560	570	580
Sy	AIGNKVALMPITLGTADFVHHIHAFTIHVTVLILLKGLLYSRSSRLVPDKGQLGFRFPDGP					
Ma	G L P	L		V FA	I	AN
Sp	V G L P	L		V FA	I	AN
Pe	SV G L P	L		V FA	I	AW
To	V G L P	L	A	V FA	T	AK
Li	V S L P	L		V FA	I	AN
Eu	S SG M	K L		V F	I	A

	590	600	610	620	630	640	650
Sy	RGGTCQVSGWDHVFGLGLFWMYNSLSIVIFHFSWKMQSDVWGTILPDGVSHTGNGFATSAITING						
Ma	A		I V		SDQ I T I		Q S
Sp	A		I V		S SDQ V T I		Q S
Pe	A V		AI V		G NDQ V T I	R	Q S
To	A		AI V		SVSDQ V T I		Q S
Li	A		I V		SEQ V T I		Q
Eu	A		I VA		V AN-K I		SQGS

TABLE III

COMPARISON OF THE AMINO ACID SEQUENCES OF *psaA*  
GENE PRODUCTS OF SEVERAL PROKARYOTIC AND  
EUKARYOTIC ORGANISMS  
(continued)

	660	670	680	690	700	710
Sy	WLRDFLWAQSSQVINSYGSALSAYGIMFLAGHFVFAFSLMFLFSGRGYWQELIESIVWAHNKLIK					
Ma	A	Q	S	LF GA	W	
Sp	A	Q	S	LF GA	W	
Pe	A	Q	S	LF GA	W	
To	A	Q	S	LF GA	W	
Li	A	Q	S	LL GA	W	
Eu		Q	P	L GA	W T	

	720	730	740	750
Sy	LAPAIQPRALSIVQGRAVGVAHYLLGGIVTTWAFFLARSLSIG*			
Ma	V T	I	T	A IIAV *
Sp	V T		T	A IIAV *
Pe	V T		T	A IIAV *
To	V T	I	T	A IIAV *
Li	V	T		A IIAV *
Eu	V N	T		A S II V *

Only amino acids which differ from those of *Synechococcus* sp. PCC 7002 are shown. Slashes indicate the beginning or ends of determined sequences; stars indicate the C-terminus; question marks indicate undetermined amino acids; hyphens indicate insertion/deletions created to optimize the sequence homology. Names of organisms and references: *Synechococcus* sp. PCC 7002, Bryant et al., 1987; maize, Fish et al., 1985, 1986; spinach, Kirsch et al., 1986; Pea, Lehmbeck et al., 1986; tobacco, Shinozaki et al., 1986; liverwort, Ohyama et al., 1986; *Euglena gracilis*, Cushman et al., 1987.

Sy = *Synechococcus*; Ma = maize; Sp = Spinach; Pe = Pea; To = Tobacco; Li = liverwort; Eu = *Euglena*.

TABLE IV

COMPARISON OF THE AMINO ACID SEQUENCES OF *psaB*  
GENE PRODUCTS OF SEVERAL PROKARYOTIC  
AND EUKARYOTIC ORGANISMS

	10	20	30	40	50	60
Sy	MATKFPKFSQDLAQDPTTRRIWYGIATAHDFETHDGMTEENLYQKIFASHFGHLAIIIFLWTSGLTF					
Ma	ELR R G	F	S D R N	Q	N	
Sp	LR R G	F	S DI R N	Q	N	
Pe	LRI R GI	F	S DI GR N	Q	N	
To	LR R G	F	S DI R N	Q	N	
Li	SFR R G S	F	S D R	Q	N	
Eu	G	F S S	N	Q	N	

	70	80	90	100	110	120	130
Sy	HVAWQGNFEQWIKDPLNVRPIAHAIWDPHFGQGAIDAFQAGASNPVNIAYSGVYHWFYTIGMTN						
Ma	S Q H		P VE RG AG	Q W LR			
Sp	S VQ H		P VE RG LG	Q W LR			
Pe	A VQ FH		P VE RG LG	Q W LR			
To	S VQ H		P VE RG AG	Q W LR			
Li	S A GQ H G		P VE RG G	Q W LR			
Eu		HI S	P E SQFPG	Q W LR			

	140	150	160	170	180	190
Sy	ADLYQGAVFLLILSSLFLFAGWLHLQPKFRPSLAWFKDAESRLNHHLAGLFGVSSSLAWTGHVH					
Ma	E T L F T S IG	WK S N	S			
Sp	E T L F VIS LG	WK VS N	S			
Pe	E T I F FIS L	WK VS N	S		A	
To	E T L F AIS I	WK VS N	S			
Li	Q N L V IS I	H WK KVS N	S			
Eu	V N SM FIAT P	E YS KVS	SA L S I			

	200	210	220	230	240	250	260
Sy	VAIP-ESRGVHVGVNDNFLSVKPHPAGLMPFFFTGNWGVYAQNPDTAGHVFGTSQAGTAILTFLGGF						
Ma	GS EY R N D L Y Q G LL Q NL	SSN L T	L				
Sp	G EY R N D L Q G L Q NL	SSS L Q	L				
Pe	G EY R N D L Y Q G LL Q NL	SSSN L T	L				
To	A EY R N D L Q G L Q NL	SSS L A	L				
Li	E R TKL E G A Q NI	V SSN A	I				
Eu	I K QL S E K SL SE	SLT AS V					

	270	280	290	300	310	320
Sy	HPQTESLWLTDIAHHHLAIAVLFIAGHMYRTNVRIGHSEIKELNAHQPPSGKLGAGHKGLYDT					
Ma	Q	FI L	FG	DL E T G R R		
Sp	Q M	FV LV	FG	M DL E I G R R		
Pe	Q V	F L G L	FG	Y E I G R R		
To	Q Q	FI LV	FG	M DL D I G R R		
Li	Q	V	FG	ET T G R R		
Eu	E K	V	FA R DD	KA L F E		

**TABLE IV**  
**COMPARISON OF THE AMINO ACID SEQUENCES OF *psaB***  
**GENE PRODUCTS OF SEVERAL PROKARYOTIC**  
**AND EUKARYOTIC ORGANISMS**  
 (continued)

	330	340	350	360	370	380	390
Sy	VNNSLHFQ	LGLALASGLVIT	SLVAQHMYSLPSYAF	IAKDYYT	TQAALYTHH	QYIAGFL	MVGAF
Ma	I	I		A	Q F		I T
Sp	I			A	Q F		I T
Pe	I	I		A	Q F	QS	I T P
To	I			A	Q F		I T
Li	I			P	L Q F		I T
Eu	I	LG		SP	LIQ R M		I T

	400	410	420	430	440	450
Sy	IFFVRDYDPEANK	DNVLYRML	EHKEALISHLSW	SLFLG	FHTLGLYVHND	VVAFGTPEKQILI
Ma	I	N Q E	A D	I	A	P ML
Sp	I	N Q E	A DL	I	A	ML
Pe	I	N Q A	A	I	A	ML
To	I	N Q E	A	I	A	ML
Li	I	N Q	A	I	A	ML P
Eu	I	F E K G	SRI D	I	T	MQ

	460	470	480	490	500	510	520
Sy	EPVFAQFVQAASG	KALYGFDVLL	SNADSIA-SN	ASAAYLP	GWLD	AINSGSNS	FLNIGPGDFIVHH
Ma	I	WI S H TT	I	STNGPTFNAGR	NTW	N V EN	T
Sp	I	WI S H TS		STSGP FNAGR	SIW	N V EN	T
Pe	I	WI S H TT	IP	STNGP LNAGR	WIW	N EN	T
To	I	WI S H TS		STSGP FNAGR	SIW	N V EN	T
Li	I	WI S H		STNNP FNAGR	SIW	NN	T
Eu		WI S H NI	ELNI	SDSSN F ASQ	IW	NG DK T	Q

	530	540	550	560	570	580
Sy	AIALGLHTTT	LILVKGALD	ARGSKLMPDK	KDFGYSFPCD	GPRGGTCD	ISAWDAFYLAMFWMLN
Ma						V
Sp						V
Pe						D V M
To						V
Li				E		V
Eu			R			V

	590	600	610	620	630	640	650
Sy	TLGWLTFYWHK	HLGIWQGN	VAQFNEKSTY	LMGWFRDY	LWANS	AQLINGYN	PNPYNLSVWAWMFL
Ma	I	V	ITL	S S	L	L S	F M S
Sp	I	V	AITL	S S	L	L S	F M S
Pe	I	V	ITL R	S S	L	L S	IT LVC S
To	I	V	ITL	S S	L	L S	F M S
Li	I	V	ITL	A	S	L L S	F M S
Eu	I	T	ITL	G	S	L L S	F M S A G



**TABLE IV**  
**COMPARISON OF THE AMINO ACID SEQUENCES OF *psaB***  
**GENE PRODUCTS OF SEVERAL PROKARYOTIC**  
**AND EUKARYOTIC ORGANISMS**  
 (continued)

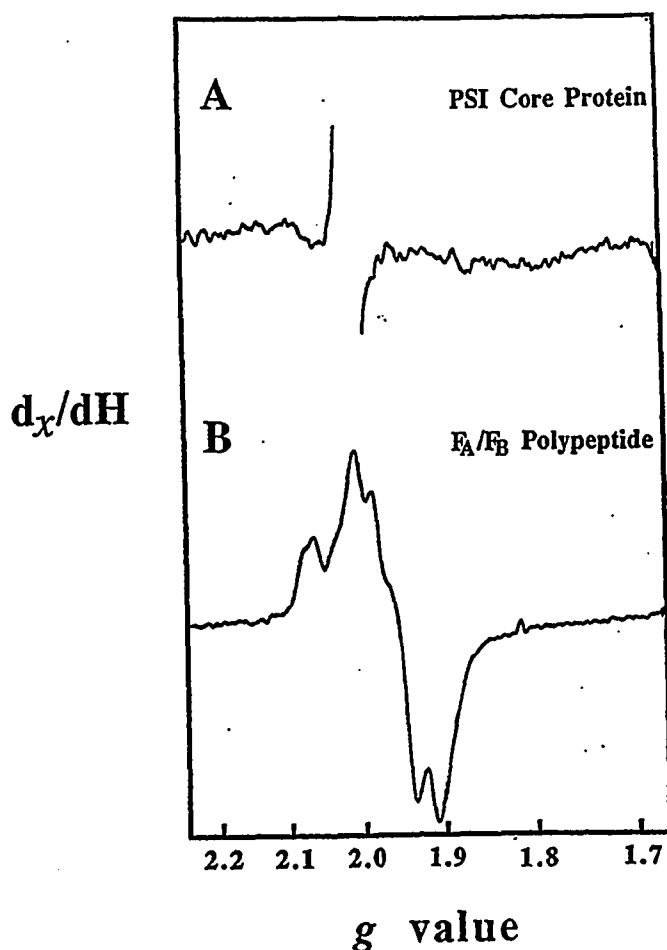
	660	670	680	690	700	710	
Sy	FGHLVWATGFMFLISWRGYWQELIETIVWAHERTP	ANIVRWKDKPVALSIVQARLVGLAHFTV					
Ma			LA	LI R	V		S
Sp			LA	LI R			S
Pe			LA	LI		V	S
To			LA	LI R			S
Li			LA	LS			S
Eu			L	IT VFK T			S

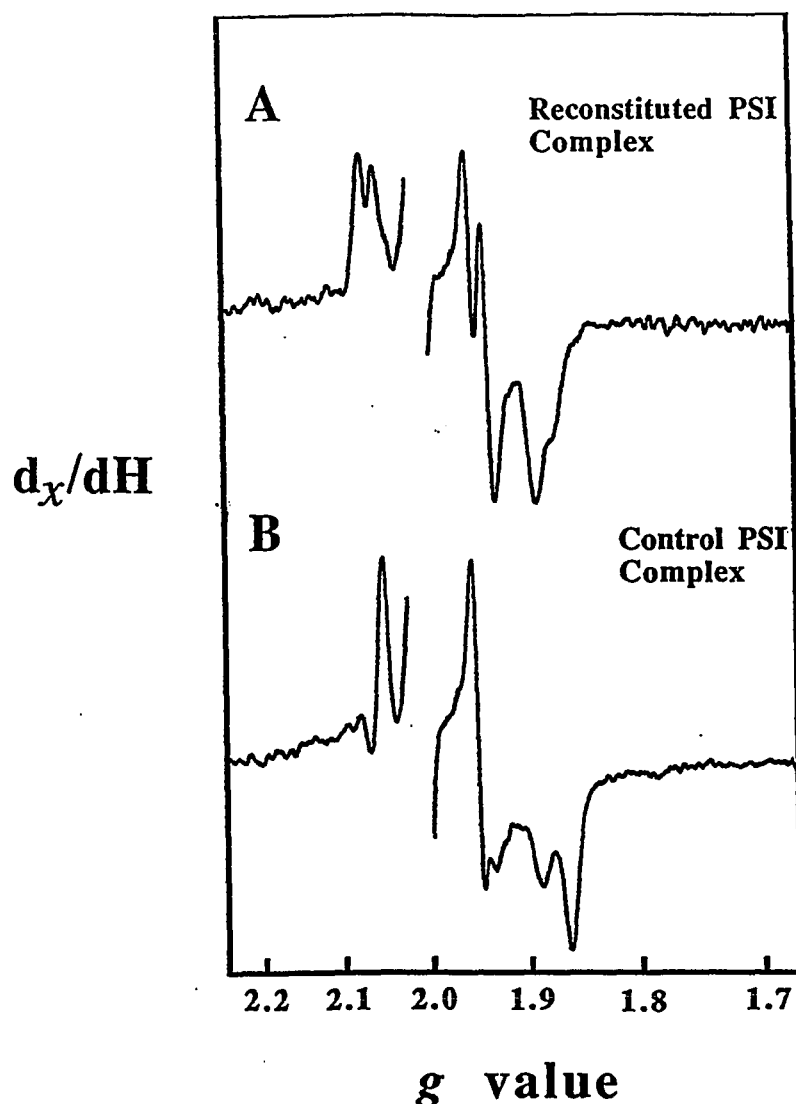
	720	730	
Sy	GYVLTAAFLIASTSGKFG*		
Ma	IF	*	
Sp	IF	*	
Pe	IF	*	
To	IF	*	
Li	IF	*	
Eu	F	A E*	

Only amino acids which differ from those of *Synechococcus* sp. PCC 7002 are shown. Slashes indicate the beginning or ends of determined sequences; stars indicate the C-terminus; question marks indicate undetermined amino acids; hyphens indicate insertion/deletions created to optimize the sequence homology. Names of organisms and references: *Synechococcus* sp. PCC 7002, Bryant et al., 1987; maize, Fish et al., 1985, 1986; spinach, Kirsch et al., 1986; Pea, Lehmbeck et al., 1986; tobacco, Shinozaki et al., 1986; liverwort, Ohyama et al., 1986; *Euglena gracilis*, Cushman et al., 1988.

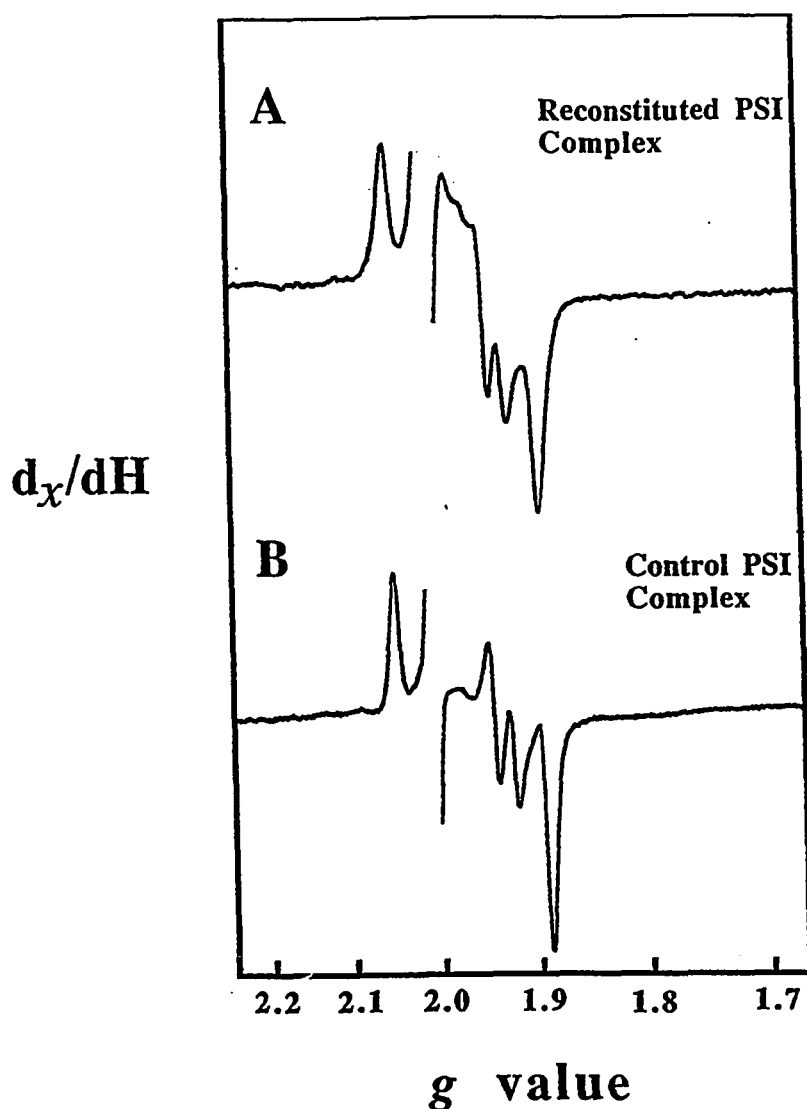
Sy = *Synechococcus*; Ma = maize; Sp = Spinach; Pe = Pea; To = Tobacco; Li = liverwort; Eu = *Euglena*.



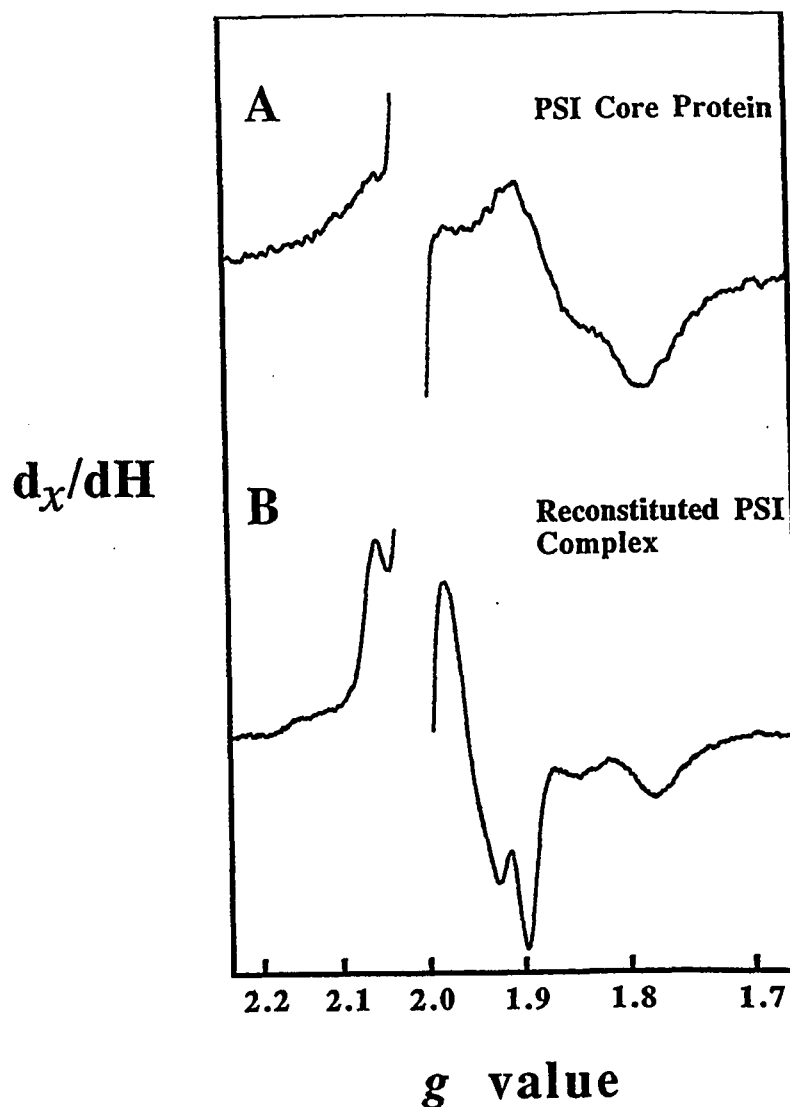
**Figure 7.** ESR spectrum of the *Synechococcus* sp. PCC 6301 Photosystem I core protein and the spinach  $F_A/F_B$  polypeptide. (A) Spectrum of the Photosystem I core protein after illumination at 16 K. The sample was suspended in 50 mM Tris, pH 8.3 containing 1 mM ascorbate and 0.3 mM DCPIP at 500  $\mu\text{g/ml}$  Chl. The spectrum was resolved by subtracting the light-off from the light-on spectrum and amplifying 50-fold in software. The broad dip in the high field region represents the weak signature of  $F_X$  under conditions optimal for observation of  $F_A$  and  $F_B$ . (B) Spectrum of the  $F_A/F_B$  polypeptide after 4 min incubation with sodium dithionite and 0.033 mM methyl viologen in 0.1 M glycine, pH 10.0. The spectrum was resolved by subtracting the oxidized from the (chemically) reduced spectrum and amplifying 12-fold in software. Spectrometer conditions: temperature, 16 K; microwave power, 20 mW; microwave frequency, 9.140 GHz; receiver gain,  $5.0 \times 10^3$ ; modulation amplitude, 10 G at 100 kHz.



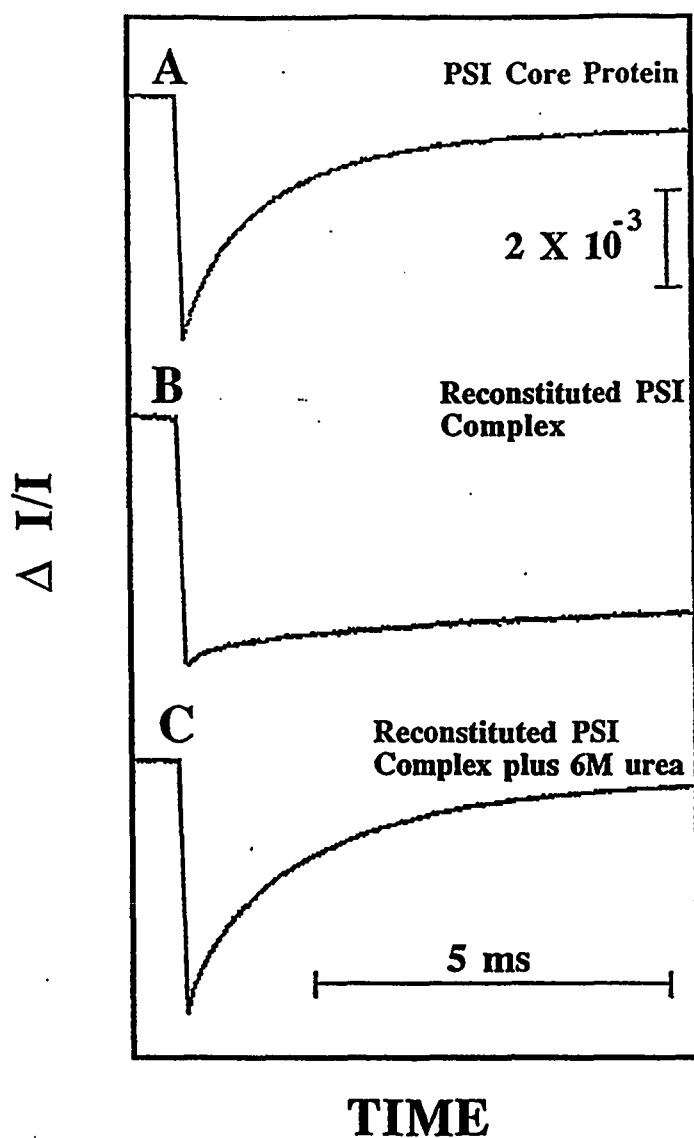
**Figure 8.** ESR spectrum of the reconstituted and the control Photosystem I complexes after freezing in darkness and illumination at 16 K. (A) Light-minus-dark ESR spectrum after addition of the spinach FA/F<sub>B</sub> polypeptide to the *Synechococcus* sp. 6301 Photosystem I core protein. The spectrum was resolved by subtracting the light-off (before light on) from the light-on spectrum and amplifying 50-fold in software. (B) Control Photosystem I complex isolated from *Synechococcus* sp. PCC 6301. The spectrum was resolved by subtracting the light-off (before light on) from the light-on spectrum and amplifying 30-fold in software. Both samples were suspended in 50 mM Tris buffer, pH 8.3 containing 1 mM sodium ascorbate and 0.3 mM DCPIP at 500 µg/ml Chl. Spectrometer conditions were the same as in Figure 3.1.



**Figure 9.** ESR spectrum of the reconstituted and the control Photosystem I complexes after illumination during freezing. (A) Light-minus-dark ESR spectrum after addition of the the spinach  $F_A/F_B$  polypeptide to the *Synechococcus* sp. 6301 Photosystem I core protein. The spectrum was resolved by subtracting the light-off (before light on) from the light-on spectrum and amplifying 15-fold in software. (B) Control Photosystem I complex isolated from *Synechococcus* sp. PCC 6301. The spectrum was resolved by subtracting the light-off (before light on) from the light-on spectrum and amplifying 6-fold in software. Both samples were suspended in 50 mM Tris buffer, pH 8.3 containing 1 mM sodium ascorbate and 0.3 mM DCPIP at 500  $\mu\text{g/ml}$  Chl. Spectrometer conditions were the same as in Figure 3.1.



**Figure 10.** ESR spectrum of Fx in the Photosystem I core protein and the reconstituted Photosystem I complex after illumination during freezing. (A) Light-minus-dark ESR spectrum of the *Synechococcus* sp. 6301 Photosystem I core protein. The sample was suspended in 50 mM Tris, pH 8.3 containing 1 mM ascorbate and 0.3 mM DCPIP at 500  $\mu$ g/ml Chl. The spectrum was resolved by subtracting the light-off (before light on) from the light-on spectrum and amplifying 5-fold in software. (B) Light-minus-dark ESR spectrum of the reconstituted Photosystem I complex. The sample was incubated for 4 min with sodium dithionite and 0.033 mM methyl viologen in 0.1 M glycine, pH 10.0. The spectrum was resolved by subtracting the light-off (before light on) from the light-on spectrum and amplifying 6-fold in software. Spectrometer conditions: temperature, 6 K; microwave power, 40 mW; microwave frequency, 9.100 GHz; receiver gain,  $5 \times 10^3$ ; modulation amplitude, 40 G at 100 kHz.



**Figure 11.** Flash-induced absorption changes in the Photosystem I core protein and in the reconstituted Photosystem I complex. (A) Absorption transient at 698 nm in the *Synechococcus* sp. PCC 6301 Photosystem I core protein. (B) Absorption transient 1 min after addition of the spinach  $F_A/F_B$  polypeptide. (C) Absorption transient 10-min after addition of 6 M urea to the reconstituted Photosystem I complex. All measurements were performed at 5  $\mu\text{g/ml}$  Chl in 50 mM Tris buffer, pH 8.3, containing 1.7 mM ascorbate and 0.033 mM DCPIP.

## ACKNOWLEDGEMENTS

The work presented in this chapter has been performed in collaboration with Kevin Parrett and lead to the publication : Golbeck, J. H., Mehari, T., Parrett, K.G. and Ikegami, I. (1988) "Reconstitution of the Photosystem I Complex from the P700 and F<sub>X</sub>-containing Reaction Center Core Protein and the F<sub>A</sub>/F<sub>B</sub> Polypeptide", FEBS Lett. 240, 9-14.

## CHAPTER IV

### RECONSTITUTION OF THE IRON-SULFUR CLUSTERS IN THE ISOLATED $F_A/F_B$ POLYPEPTIDE: ASSEMBLY OF SAME-SPECIES AND CROSS-SPECIES PHOTOSYSTEM I COMPLEXES

In Chapter III I showed that the P700 and  $F_X$ -containing Photosystem I core protein could be isolated intact from a *Synechococcus* sp. PCC 6301 Photosystem I complex by chaotropic treatment followed by sucrose gradient ultracentrifugation [37,38]. I also indicated that the spinach  $F_A/F_B$  polypeptide, isolated from thylakoid membrane fragments by acetone and methanol precipitation [35,36], could be rebound to the *Synechococcus* sp. PCC 6301 Photosystem I core protein, leading to complete restoration of electron flow from P700 to  $F_A$  and  $F_B$  [40]. However, the isolated spinach  $F_A/F_B$  protein was extremely sensitive to heat and chemical oxidants, and degraded within minutes after removal from the thylakoid membrane. I questioned, therefore, whether the  $F_A$  and  $F_B$  iron-sulfur clusters in the denatured apoprotein could be reconstituted synthetically from  $Fe^{3+}$  and  $S^{2-}$  as well as from  $Fe^{3+}$  and  $Se^{2-}$ . Although reconstitution of water-soluble [2Fe-2S] and [4Fe-4S] proteins such as spinach and *Clostridial* ferredoxins has been accomplished [41,42], previous attempts to reconstitute the ferredoxin-like iron-sulfur clusters of  $F_A$  and  $F_B$  in an oxidatively-denatured Photosystem I complex have been unsuccessful [43]. The similarity between sulfur and selenium in chemical and physical properties made selenium a good candidate for substituting sulfur in the cluster.



## MATERIALS AND METHODS

### Isolation of the Photosystem I core protein and the F<sub>A</sub>/F<sub>B</sub> polypeptide

The Photosystem I complex was isolated from spinach, *Synechococcus* sp. PCC 6301, *Synechococcus* sp. PCC 6803, *Synechocystis* sp. PCC 7002, and *Synechococcus elongatus* membrane fragments with Triton X-100 followed by sucrose density ultracentrifugation [40]. The Photosystem I core protein was isolated from spinach and *Synechococcus* sp. PCC 6301 Photosystem I complexes with 6.8 M urea followed by ultrafiltration over a YM-100 membrane and sucrose-density ultracentrifugation in 0.01% Triton X-100 [44]. The spinach and *Synechococcus* sp. PCC 6301 F<sub>A</sub>/F<sub>B</sub> holoproteins were isolated using methanol/acetone extraction treatment of lyophilized thylakoid fragments [35,36] and butanol extraction of lyophilized thylakoid fragments [45,46] except that dithionite was present throughout the isolation procedure [40]. The extracted material was centrifuged to remove small thylakoid fragments and the clear supernatant was concentrated over a YM-5 ultrafiltration membrane with ultrapure nitrogen. The iron-sulfur clusters were found to degrade rapidly if any additional purification steps were included. To prevent iron-sulfur denaturation, the crude protein fraction from spinach and *Synechococcus* sp. PCC 6301 was used directly after solvent extraction of the thylakoid membranes. The elapsed time from addition of the solvent to freezing of the preparation was 1 hr. One consequence of this abbreviated isolation procedure is that in addition to the F<sub>A</sub>/F<sub>B</sub> protein, the supernatant contains many of the peripheral low molecular mass polypeptides which are associated with the Photosystem I complex. At least two of these polypeptides, the *psaE* and *psaD* gene products, also rebind to the Photosystem I core protein upon reconstitution with the F<sub>A</sub>/F<sub>B</sub> holoprotein (see reference 44).

### Oxidative denaturation of the $F_B$ and $F_A$ iron-sulfur clusters

The  $F_A/F_B$  apoprotein was prepared by allowing air-oxidation of the iron-sulfur clusters in the freshly-isolated protein. Preliminary studies, using ESR spectroscopy of the chemically-reduced protein as the assay, indicated that the clusters degrade with a half-time of about 30 min at 37°C. The apoprotein could also be prepared through controlled oxidation of the iron-sulfur clusters by treating the freshly-isolated  $F_A/F_B$  protein for 2 hr with 3 M urea and 5 mM  $K_3Fe(CN)_6$  in 50 mM Tris, pH 8.3 (see 43,47). The urea and  $K_3Fe(CN)_6$  were removed by dialysis for 24 hr in 50 mM Tris, pH 8.3 and 5 mM Tiron, followed by dialysis for an additional 24 hr in 50 mM Tris, pH 8.3 to remove the iron-Tiron chelate and the excess Tiron. The fraction containing the  $F_A/F_B$  apoprotein was concentrated over a YM-5 membrane.

### Reconstitution of the $F_B$ and $F_A$ iron-sulfur clusters

Reconstitution of the  $F_A/F_B$  iron-sulfur clusters was performed according to the following protocol: (i) a solution of 50 mM Tris, pH 8.3, was purged with oxygen-free nitrogen in a closed reaction vessel; (ii) after 2 hours,  $\beta$ -mercaptoethanol was added through a septum to a final concentration of 1%; (iii) the  $F_A/F_B$  apoprotein was added to a concentration of 5  $\mu$ g/ml (~1 nmol/ml iron-sulfur cluster) and the solution was purged with oxygen-free nitrogen; (iv) after 10 minutes, an aliquot of 30 mM  $FeCl_3$  was slowly added to a final concentration of 0.3 mM; (v) after 5 minutes, an aliquot of 30 mM  $Na_2S$  was slowly added to a final concentration of 0.3 mM. This solution was allowed to incubate in the dark and at room temperature for 12 hr. The reaction vessel was uncapped and the solution was transferred under a flow of oxygen-free nitrogen to an ultrafiltration cell equipped with a YM-5 membrane and concentrated with ultrapure nitrogen at 4°C.  $\beta$ -mercaptoethanol (0.01%) was present throughout the entire procedure.

#### Rebinding of the $F_A/F_B$ holoprotein to the Photosystem I core protein

The native- $F_A/F_B$  and reconstituted- $F_A/F_B$  proteins were rebound at a ~100-fold excess molar ratio to the Photosystem I core protein (10  $\mu\text{g Chl/ml}$ ) in 50 mM Tris, pH 8.3, and 0.1%  $\beta$ -mercaptoethanol. After 1 hour incubation at room temperature, the solution was washed twice over a YM-100 membrane with 50 mM Tris, pH 8.3, and 0.01% Triton X-100 to remove unbound  $F_A/F_B$  protein. The reconstituted Photosystem I complex was subject to sucrose-density ultracentrifugation in 0.04% Triton X-100. In some cases, the preparation was used directly for spectroscopy after the ultrafiltration step.

#### Reconstitution of the $F_A/F_B$ apoprotein in the presence of the Photosystem I core protein

The reconstituted- $F_A/F_B$  polypeptide was rebound to the Photosystem I core protein by incubating the  $F_A/F_B$  apoprotein with  $\text{FeCl}_3$ ,  $\text{Na}_2\text{S}$  and  $\beta$ -mercaptoethanol as described above, but in the presence of Photosystem I core protein at 10  $\mu\text{g Chl/ml}$ . After 12 hr, the reaction vessel was uncapped and the solution transferred under a flow of oxygen-free nitrogen to an ultrafiltration cell equipped with a YM-100 membrane and washed twice with 50 mM Tris, pH 8.3, containing 0.01% Triton X-100. The washed solution was diluted to 500  $\mu\text{g Chl/ml}$  followed by dialysis for 12 hr in 50 mM Tris, pH 8.3, containing 5 mM Tiron. The residual Tiron and Tiron-iron chelate were removed by dialysis for 12 hr in 50 mM Tris, pH 8.3, containing 0.01% Triton X-100. The reconstituted Photosystem I complex was concentrated to 1000  $\mu\text{g Chl/ml}$ .

#### Se-analogue $F_A/F_B$ Reconstitution

Prior to attempting to incorporate Fe-Se clusters into the  $F_A/F_B$  apoprotein, the iron and sulfur in the native protein were removed by acidification ( $\text{S}^{2-}$  is eliminated as  $\text{H}_2\text{S}$ ) [48,49]. Dialysis of the reaction mixture in 50 mM Tris, pH 8.1, containing 0.05% Triton X-100, made it possible to remove the iron. The apoprotein (1-2 mg/ml) in 0.1 M Tris, pH 7.7 was incubated with 50-fold molar excess of DTT for 30 min at room temperature. To

the mixture was then added, 30-fold molar excess of  $\text{FeCl}_3$  solution that is purged with oxygen-free nitrogen; this was immediately followed by addition of 30-fold molar excess of  $\text{Se}^{2-}$ . The  $\text{Se}^{2-}$  was generated *in situ* by reducing  $\text{SeO}_3^{2-}$  with 10-fold molar excess of DTT in a separate vessel and then adding it to the reaction mixture after 5 minutes incubation. Both  $\text{SeO}_3^{2-}$  and DTT could as well be added to the reaction mixture to form  $\text{Se}^{2-}$  in the presence of iron and the apoprotein, even though the yield is somewhat lower. This protocol has been repeated as outlined above to reconstitute a Photosystem I complex whose  $\text{F}_\text{A}/\text{F}_\text{B}$  clusters contain selenium instead of sulfur. The particles were studied by optical and ESR spectroscopy before and after washing with 50 mM Tris, pH 8.1 containing 0.05% Triton X-100.

#### Spectroscopic methods

Flash-induced absorption transients were determined at 698 nm using a single beam spectrophotometer consisting of a 250-watt quartz-tungsten source, a monochromator before and after the sample cuvette, and a silicon diode (United Detector Technology PIN-10D). A shutter opened the measuring beam 1 ms prior to the flash, and a sample-and-hold circuit nulled the measuring beam after the shutter opening but just prior to the flash. The photocurrent was changed in to a voltage with a 1 K resistor, amplified with a 300-kHz bandwidth voltage amplifier (EG&G Model 113), and digitized with a Nicolet 4094A Digital Oscilloscope. The data were transferred to a Macintosh plus computer over an RS-232 line using software written in-house. The sample was repetitively flashed with a Phase-R DL 1200 flashlamp-pumped dye laser (50 mJ pulse energy for 400 ns FWHM at 660 nm with sulforhodamine B as the organic dye). The optical measurements were performed at 20°C in a 1 cm pathlength cuvette containing 5 µg Chl/ml, 0.033 mM DCPIP or PMS, and 1.7 mM ascorbate in 50 mM Tris, pH 8.3. ESR studies were performed on a Varian E-109 spectrometer equipped with an Air Products LTD liquid helium transfer cryostat. The spectrometer was interfaced to a Macintosh Plus computer via a Keithley

digital voltmeter (Model 195A) and an IEEE-488 bus controller (I/O Tech Mac 488A) for signal averaging and baseline subtraction. The frequency was measured with a Hewlett-Packard Model 5340A microwave frequency counter, the magnetic field strength was measured with a Varian Gaussmeter. The precision of the measurement, using diphenylpicrylhydrazyl as a field marker and a calibrated time base, allows  $g$ -values to be determined with an relative error of no more than  $\pm 0.0015$  at  $g = 2.000$ . Sample temperatures were monitored with a thermistor situated directly below the sample tube. Light-minus-dark difference spectra were obtained by illuminating the sample with a 150-Watt Xenon lamp.

#### Analytical methods

Acid-labile sulfide and P700 were determined as described in reference [22]. Chlorophyll was determined in 80% acetone [21].

## RESULTS

#### ESR spectra of isolated spinach and cyanobacterial Photosystem I complexes

The ESR spectra of the  $F_A/F_B$  clusters in isolated spinach and *Synechococcus* sp. PCC 6301 Photosystem I complexes are shown in Figure 12. When the sample is illuminated during freezing, both iron-sulfur clusters become photochemically reduced, leading to resonances at  $g = 2.051$ , 1.941, 1.924 and 1.887 in spinach and  $g = 2.047$ , 1.937, 1.917 and 1.879 in *Synechococcus* sp. PCC 6301 (Figure 12A). Within the tolerances of the measurement (see Materials and Methods), identical  $g$ -values were determined for spinach Photosystem I complexes measured on five separate occasions and for *Synechococcus* sp. PCC 6301 complexes measured on four separate occasions. In the fully reduced sample, the differences in  $g$ -values between spinach and *Synechococcus* sp. PCC 6301 are most apparent in the high- and mid-field resonances. When the complexes are frozen in darkness and illuminated, 85% of  $F_A$  and 15% of  $F_B$  is photoreduced.

Consistent with the above analysis, the  $g = 1.86$  resonance of  $F_A^-$  in spinach and *Synechococcus* sp. PCC 6301 shows reproducible differences (the former is located downfield of the latter). The above-tabulated differences are not related to the nature of the Photosystem I preparation, nor to the method of reduction of the iron-sulfur clusters. For example, the same  $g$ -values are found for the  $F_A/F_B$  clusters in digitonin Photosystem I complexes and in Triton Photosystem I complexes (Figure 12B), as well as in intact spinach thylakoid membranes (not shown). Likewise, identical  $g$ -values are found in Photosystem I complexes during reduction in darkness with dithionite at pH 10.5 and during photochemical reduction by freezing during illumination at pH 8.3 (not shown).

The ESR spectra of the  $F_A/F_B$  iron-sulfur clusters in isolated *Synechococcus* sp. PCC 6803, *Synechococcus* sp. PCC 7002, and *Synechococcus elongatus* Photosystem I complexes are shown in Fig 4.2. The  $g$ -values of the mesophilic and thermophilic species of *Synechococcus* (Figure 13A) are nearly identical: the latter occur at  $g = 2.051, 1.937, 1.920$  and  $1.879$ . *Synechocystis* sp. 6803 and *Synechococcus* sp. PCC 7002 (Figure 13B) also show similar  $g$ -values of  $2.047, 1.937, 1.917$  and  $1.880$  and  $2.049, 1.937, 1.917$  and  $1.877$ , respectively. In general,  $g$ -values of the  $F_A/F_B$  clusters are similar in the four different cyanobacterial Photosystem I complexes and are shifted consistently up-field compared with those in the spinach Photosystem I complex. The precise relationship of these  $g$ -values to those found in the cross-species spinach-*Synechococcus* sp. PCC 6301 Photosystem I complex will be discussed later.

#### Oxidative Denaturation and Reconstitution of the $F_A/F_B$ Iron-Sulfur Clusters

The ESR spectrum of the isolated spinach  $F_A/F_B$  polypeptide is shown in Figure 14. To minimize the effect of prep-to-prep variations, three traces are shown which represent  $F_A/F_B$  holoproteins isolated on three different occasions. Depending on whether acetone/methanol or butanol is used in the isolation procedure, the appearance of the

spinach  $F_A/F_B$  holoprotein is slightly different. In the case of the acetone-isolated protein (Figure 14A), the iron-sulfur clusters in the isolated  $F_A/F_B$  protein are most probably intact. Previous studies have shown that the Photosystem I complex reconstituted with this protein is capable of quantitative chemical reduction of  $F_A$  and  $F_B$  at pH 10.5 and photochemical reduction of  $F_A$  and  $F_B$  by freezing during illumination at pH 8.3 [40]. In the case of the butanol-isolated protein (Figure 14B), the ratio of the  $F_B$  resonance at  $g = 1.93$  to the  $F_A$  resonance at  $g = 1.96$  is clearly smaller when compared with the acetone-isolated protein. In spite of the fact that both holoproteins were liberated from the membrane in the presence of excess dithionite, the lower yield of  $F_B^-$  reduction in the reconstituted Photosystem I complex (not shown) indicates a certain amount of  $F_B$  has degraded during the butanol isolation procedure. These results are consistent with the original reports that the acetone/methanol procedure yields an  $F_A/F_B$  holoprotein that is nearly intact, containing 6.4 moles of acid labile sulfide [36], whereas the butanol procedure yields a holoprotein that is significantly degraded, containing only 3.2 moles of non-heme iron and labile sulfide [46].

The iron-sulfur clusters in the acetone-liberated spinach  $F_A/F_B$  protein are, nevertheless, quite sensitive to oxidative denaturation after the protein is liberated from the Photosystem I complex. As shown in Figure 15A, the ESR resonances of  $F_A$  and  $F_B$  degrade with an approximate half-time of about 30 min after exposure to 37° C, even under highly reducing conditions. There does not appear to be any significant differential sensitivity of either the  $F_A$  or  $F_B$  cluster to this mode of denaturation. The iron-sulfur clusters can also be forced into oxidative denaturation at 0° C by exposure to 3 M urea and 5 mM  $K_3Fe(CN)_6$  for 10 min; this method allows for controlled denaturation under known conditions [42]. In both instances, the acid-labile sulfide is oxidized to the level of zero-valence sulfur and the non-heme iron is lost from the protein (data not shown).

We found that the oxidatively-denatured iron-sulfur clusters can be reconstituted by incubating the spinach  $F_A/F_B$  apoprotein with  $FeCl_3$  and  $Na_2S$  in the presence of  $\beta$ -mercaptoethanol (see reference 41). The ESR spectra of the native  $F_A/F_B$  protein, the  $F_A/F_B$  apoprotein and the reconstituted  $F_A/F_B$  protein are shown in Figure 15. The chemically-reduced spectrum of the native  $F_A/F_B$  protein shows principal resonances at  $g = 2.052, 1.963, 1.931$  and  $1.890$  (Figure 15A). After oxidative denaturation with urea and  $K_3Fe(CN)_6$ , little or no chemically-reduced  $F_A$  or  $F_B$  is observed (Figure 15B). The lack of iron-sulfur clusters is also reflected in the loss of the chemical oxidized-minus-reduced difference spectrum of the denatured protein (data not shown). However, after reconstitution with  $FeCl_3$  and  $Na_2S$  in the presence of  $\beta$ -mercaptoethanol, the characteristic ESR spectrum with interacting  $F_A$  and  $F_B$  is regained (Figure 15B). The efficiency of reconstitution of the  $F_A/F_B$  clusters is  $>90\%$  after 12 hr of incubation with  $FeCl_3$  and  $Na_2S$  and  $\beta$ -mercaptoethanol. In the absence of added sodium sulfide, yield decreases to 40%; the sulfide for reconstitution is derived from the protein-bound zero-valence sulfur released by nucleophilic displacement with  $\beta$ -mercaptoethanol. No reconstitution is observed in the absence of either  $\beta$ -mercaptoethanol or added iron. However, if the apoprotein is not dialyzed against Tiron, some residual iron is present, and a 10% yield of  $F_A$  and  $F_B$  reconstitution is observed in the presence of  $\beta$ -mercaptoethanol alone. The only difference with the freshly-isolated  $F_A/F_B$  holoprotein is that there appears to be a broadening or merging of the high- and mid-field resonances after *in vitro* reinsertion of the iron-sulfur clusters. Although the reconstituted  $F_A/F_B$  spectrum appears more axial than the control  $F_A/F_B$  spectrum, we believe that the shape of the spectrum is due to a freezing-in of a large variety of conformational states rather than to a change in the symmetry of the spin system..



### Rebinding of the reconstituted- $F_A/F_B$ polypeptide to the Photosystem I core protein

In confirmation of the work presented in Chapter III, we found that when the freshly-isolated spinach  $F_A/F_B$  holoprotein is rebound to the *Synechococcus* sp. PCC 6301 Photosystem I core protein, there is a transition in the flash-induced absorption change from a 1.2-ms optical transient due to the  $P700^+ F_X^-$  backreaction to a 30-ms optical transient due to the  $P700^+ [F_A/F_B]^-$  backreaction. When an attempt is made to rebind the spinach  $F_A/F_B$  apoprotein to the *Synechococcus* sp. PCC 6301 Photosystem I core protein, there is no change in the 1.2-ms backreaction between  $P700^+$  and  $F_X^-$ . However, when the rebuilt spinach  $F_A/F_B$  protein is rebound to the *Synechococcus* sp. PCC 6301 Photosystem I core protein, the 1.2-ms optical transient due to the  $P700^+ F_X^-$  backreaction is replaced (within a mixing time of <1 min) by the 30-ms transient characteristic of the  $P700^+ [F_A/F_B]^-$  backreaction (not shown). The iron-sulfur clusters in the spinach  $F_A/F_B$  apoprotein can also be reconstituted in the presence of the *Synechococcus* sp. PCC 6301 Photosystem I core protein (see reference 44). Under these conditions, electron flow from P700 to  $F_A/F_B$  is also reestablished, except that 12 hrs of incubation is required due to the relatively slow kinetics of reinsertion of the iron-sulfur clusters into the  $F_A/F_B$  apoprotein.

The ESR spectrum of the reconstituted spinach-*Synechococcus* sp. PCC 6301 Photosystem I complex is compared to the native spinach Photosystem I complex and to the native *Synechococcus* sp. PCC 6301 Photosystem I complex in Figure 16. This cross-species complex was prepared with  $F_A/F_B$  apoprotein isolated by treating a spinach Photosystem I complex with chaotropes followed by rebuilding the iron-sulfur clusters in the isolated  $F_A/F_B$  apoprotein and rebinding to the *Synechococcus* sp. PCC 6301 Photosystem I core protein. When the sample is illuminated during freezing, the  $F_A/F_B$  resonances in the reconstituted spinach-*Synechococcus* sp. PCC 6301 Photosystem I complex appear as sharp as those in the control complex. The spectrum is characterized by

interaction between  $F_A$  and  $F_B$  which gives rise to  $g$ -values of 2.052, 1.940, 1.922 and 1.886. This spectrum is similar to that of the reduced  $F_A/F_B$  clusters in the spinach Photosystem I complex ( $g$ -values of 2.051, 1.941, 1.924 and 1.887; Figure 16A) but is quite unlike the native *Synechococcus* sp. PCC 6301 Photosystem I complex ( $g$ -values of 2.047, 1.937, 1.917 and 1.880; Figure 16B). The  $g$ -values, moreover, do not depend on whether the reconstituted spinach-*Synechococcus* sp. PCC 6301 Photosystem I complex is photochemically reduced at pH 8.3 or chemically reduced in the dark at pH 10.5 (compare Figure 16 A, B). The spectrum is identical to the reconstituted Photosystem I complex prepared using a solvent-extracted  $F_A/F_B$  holoprotein (note that the  $g$ -values of the reconstituted cross-species Photosystem I complex shown here are slightly different from those shown in ref [40] due to more precise calibration of the ESR spectrometer in this study). Indeed, when the solvent-isolated spinach  $F_A/F_B$  holoprotein is rebound to the *Synechococcus* sp. PCC 6301 Photosystem I core protein, frozen in darkness, and illuminated at 16 K,  $F_A$  ( $g$ -values of 2.05, 1.94 and 1.86) is 85% photoreduced, and  $F_B$  ( $g$ -values of 2.07, 1.92 and 1.89) is about 15% photoreduced (not shown). This ratio is similar to the native spinach and *Synechococcus* sp. PCC 6301 Photosystem I complexes reduced under identical conditions, and differs significantly from the earlier reported rebinding of the spinach  $F_A/F_B$  protein to the *Synechococcus* sp. PCC 6301 Photosystem I core protein where 50% of  $F_A$  and 50% of  $F_B$  were photoreduced [40].

To determine whether the  $g$ -values of the cross-species ESR spectrum are due to inherent features of the spinach-*Synechococcus* sp. PCC 6301 reconstitution or to an altered  $F_A/F_B$  protein, I performed same-species reconstitution studies. The results of reconstituting a *Synechococcus* sp. PCC 6301  $F_A/F_B$  protein with a *Synechococcus* sp. PCC 6301 Photosystem I core protein are shown in Figure 17A. The yield of the *Synechococcus* sp. PCC 6301  $F_A/F_B$  holoprotein was consistently low; nevertheless, we were able to isolate enough protein to show that the ESR spectrum of the reconstituted

Photosystem I complex was identical to that of the native *Synechococcus* sp. PCC 6301 Photosystem I complex. The results of reconstituting a spinach  $F_A/F_B$  protein with a spinach Photosystem I core protein are shown in Figure 17B. The difficulty encountered in this experiment was that the yield of the spinach Photosystem I core protein was low: the spinach complex appears to be more stable than the *Synechococcus* sp. PCC 6301 complex, and there is considerable denaturation of  $F_X$  in the process of ensuring that most of the  $F_A/F_B$  polypeptide is removed.. A similar destruction of  $F_X$  was noted by Hoshina et al. [50] when ethylene glycol was used to remove the  $F_A/F_B$  protein from a spinach Photosystem I complex. Nevertheless, as shown in Figure 17B, the  $g$ -values after rebinding the spinach  $F_A/F_B$  protein to the spinach Photosystem I core (Figure 17B) are identical to those in the native spinach Photosystem I complex (the low yield of both *Synechococcus* sp. PCC 6301  $F_A/F_B$  protein and spinach Photosystem I core protein precluded the reciprocal cross-species reconstitution study). We conclude that the ESR spectrum of the cross-species, spinach-*Synechococcus* sp. PCC 6301 Photosystem I complex is due to inherent protein-protein interactions between the  $F_A/F_B$  polypeptide and the Photosystem I core protein and is not due to damage incurred to the  $F_A/F_B$  protein during isolation.

## DISCUSSION

I have shown that the  $F_B$  and  $F_A$  iron-sulfur clusters in the isolated 8.9-kDa protein from Photosystem I can be reconstituted from the oxidatively-denatured apoprotein with  $FeCl_3$  and  $Na_2S$  in the presence of  $\beta$ -mercaptoethanol. Evidence for the *in vitro* reconstitution of the  $F_A$  and  $F_B$  iron-sulfur clusters includes: (i) regeneration of the chemically-reduced ESR spectrum of  $F_A$  and  $F_B$  in the isolated protein, (ii) restoration of the 30-ms optical transient between  $P700^+$  and  $[F_A/F_B]^-$  after rebinding to the Photosystem I core protein, and (iii) regeneration of the characteristic light-induced ESR

spectrum of interacting  $F_A$  and  $F_B$  after rebinding to the Photosystem I core protein and illumination before and during freezing. The ESR spectrum after freezing in the dark and illumination at 16 K appears to be similar to a control spinach or *Synechococcus* sp. PCC 6301 Photosystem I complex, with 85% photoreduction of  $F_A$  and 15% photoreduction of  $F_B$ . This differs from the 50% ratio of photoreduction of  $F_A$  and  $F_B$  reported earlier [40]; the difference is that the latter sample was reconstituted in the ESR tube and frozen *in situ* after 10 min of incubation, whereas the sample in this study was ultrafiltered and dialyzed before freezing. Differences in the ratios of photoreduced  $F_A$  and  $F_B$  have been found between species [10,51], and even in the same species, the presence of 60% glycerol leads to a reversal of the roles of the low temperature photoreduction of  $F_A$  and  $F_B$  [52]. Perhaps there is a subtle, time-dependent reorientation of the  $F_A/F_B$  protein on the reconstituted Photosystem I core protein that leads to the preferential photoreduction of one iron-sulfur cluster over another. In any event, the results of this study show that after incubating for a sufficient period of time, the cross-species reconstitution of a spinach  $F_A/F_B$  protein with a *Synechococcus* sp. PCC 6301 Photosystem I core protein leads to photochemical properties similar to either the control spinach or *Synechococcus* sp. PCC 6301 complex.

One difficulty with the reconstitution protocol shown here is that an accurate estimation of the yield of reconstitution of the  $F_A/F_B$  protein is difficult to assess: the native or reconstituted holoprotein is extremely labile in the absence of the Photosystem I core protein. Since the  $F_A/F_B$  apoprotein apparently does not bind to the Photosystem I core protein, any given preparation can suffer a significant degradation of the  $F_A/F_B$  clusters before the remaining  $F_A/F_B$  protein is incapable of rebinding to the Photosystem I core protein. We have ensured quantitative rebuilding of the  $F_A/F_B$  iron-sulfur clusters and rebinding to the Photosystem I core protein because we have added the  $F_A/F_B$  protein back to the Photosystem I core in large excess. Indeed, as I showed in Chapter III, the

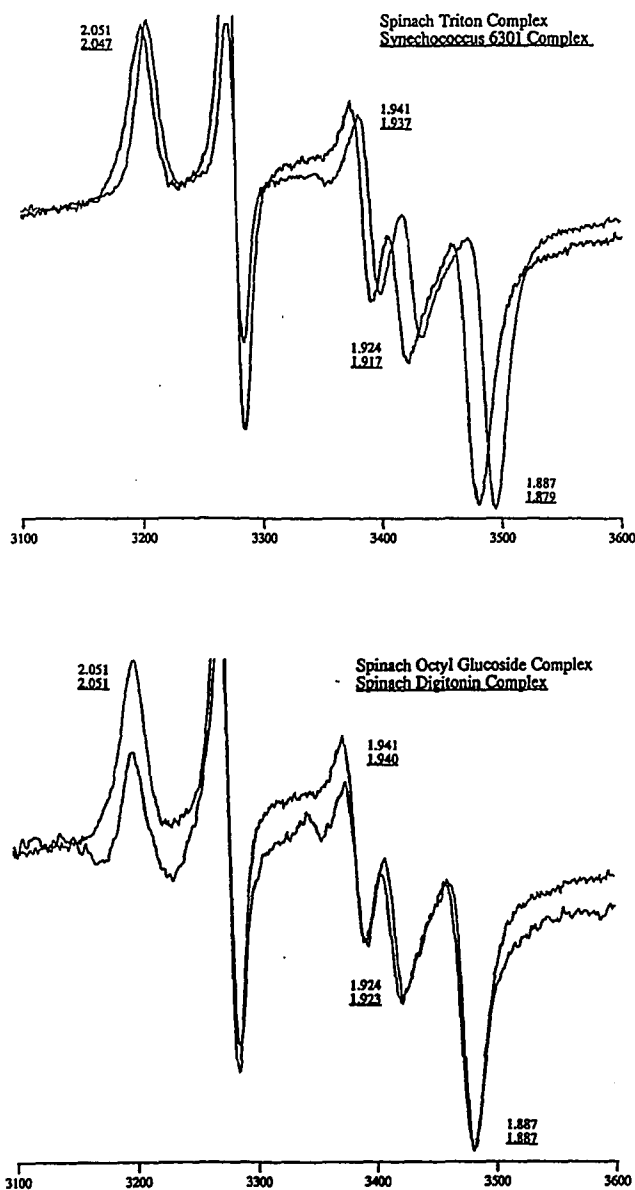
affinity of the  $F_A/F_B$  protein to the Photosystem I core protein is so high that only a slight excess is necessary to guarantee complete reconstitution of the Photosystem I complex. The reconstitution of the  $F_A/F_B$  clusters in the presence of the Photosystem I core protein avoids these problems, since the affinity of the  $F_A/F_B$  holoprotein to the Photosystem I core protein is extremely high, and since the insertion of the  $F_A/F_B$  clusters into the apoprotein is probably the rate-limiting step in the reaction. One additional consequence is that since the isolated  $F_A/F_B$  protein is extremely labile to heat and mild oxidants, the reconstitution protocol outlined here allows isolation of the  $F_A/F_B$  apoprotein followed by rebuilding the iron-sulfur clusters to produce a competent  $F_A/F_B$  protein. This, in turn, can be followed by rebinding to the Photosystem I core protein to produce a functional Photosystem I complex. Part or all of the acid labile sulfide in iron-sulfur proteins can be replaced by selenium without loss of biological activity [53,54]. This, however, has not been achieved in the case of the  $F_A/F_B$  protein in Photosystem I complex. Even though selenium and sulfur have similar properties, the fact that selenium has more mass and is larger than sulfur might render the  $[4Fe-4Se]$  cluster less stable than the corresponding sulfur-containing clusters [55]. The exchange of this highly unstable, artificial cluster into the  $F_A/F_B$  apoprotein would require the correct conformation (among many other factors) on the part of the apoprotein. This may be unlikely in the case of the  $F_A/F_B$  apoprotein where the two clusters interact delicately. A slight modification of one cluster could denature the other, thereby facilitating its own degradation. Chances are better if reconstitution of the apoprotein is carried out in the presence of the Photosystem I core protein. This has been shown with ~50% reconstitution when the Photosystem I core protein was present, as opposed to none in its absence. Washing the 'reconstituted' particle with 50 mM Tris, pH 8.1, and 0.05% Triton X-100 over YM-100 membrane lowered the reconstitution drastically. This could be due to many complex reasons, among which: (i) the bound and unbound clusters could be in dynamic equilibrium; which simply

means removing the unbound clusters would initiate the dissociation of the bound clusters. (ii) the terminal electron acceptors from P700 could be soluble species other than  $F_A/F_B$  holoproteins.

Turning our attention finally to the consequences of the cross-species reconstitution, it is of interest to know whether these differences are inherent to the native Photosystem I complexes or whether these differences result entirely from specific environmental parameters created within the  $F_A/F_B$  protein. This question can be addressed, in part, by reconstitution studies of the Photosystem I complex. The studies shown in this chapter indicate that the  $g$ -values of the spinach  $F_A/F_B$  clusters differ slightly but characteristically from those in *Synechococcus* sp. PCC 6301. Moreover, when the spinach  $F_A/F_B$  protein is reconstituted with the *Synechococcus* sp. PCC 6301 Photosystem I core protein, the resulting ESR spectrum is more like the native spinach Photosystem I complex than the *Synechococcus* sp. PCC 6301 complex. Although rebinding to the Photosystem I core protein is necessary for the  $F_A/F_B$  resonances to narrow and sharpen, this limited study might suggest that the ESR spectral differences originate within the  $F_A/F_B$  protein itself and not from the specific interactions between this protein and the Photosystem I core.

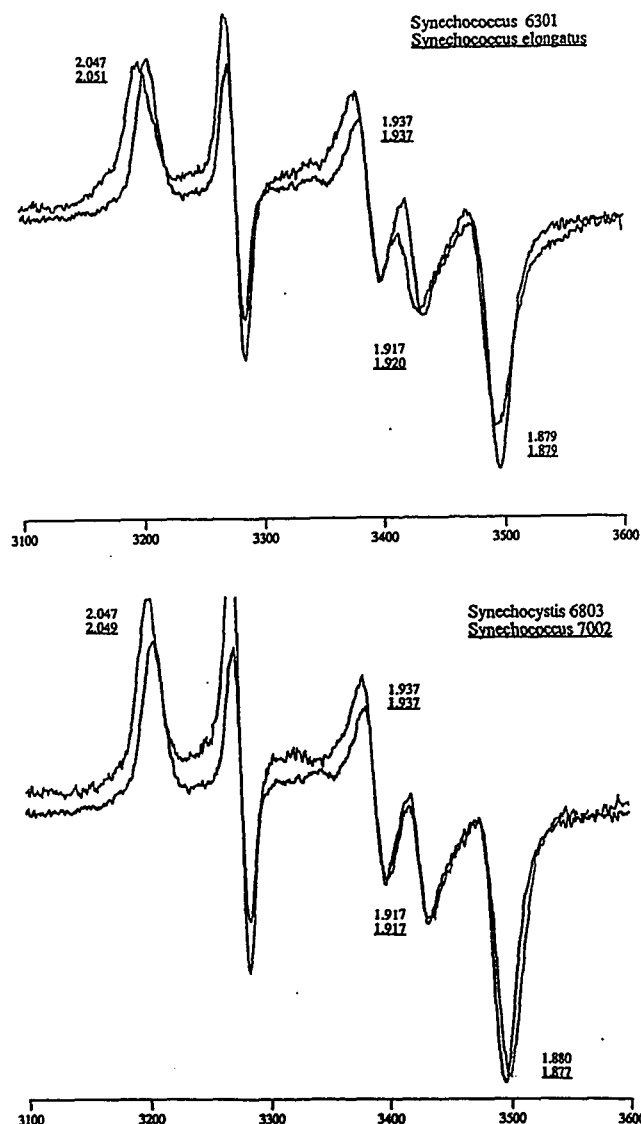
This conclusion, however, is weakened considerably by consideration of the amino acid sequences of the  $F_A/F_B$  proteins. As shown in Table II, the  $F_A/F_B$  proteins are very highly conserved whether encoded in the plastid genomes of eucaryotes or the procaryotic cyanobacteria (the amino acid sequence for the *Synechococcus* sp. PCC 6301 is, unfortunately, not available). The higher plant sequences are approximately 95% identical, while the cyanobacterial sequences (with the sole exception of *Synechocystis* sp. PCC 6803) exhibit >90% identity. Virtually all amino acid substitutions within a class are conservative replacements. Each gene predicts a protein of 81 amino acids (8.8 kDa), although it appears that the N-terminal methionine is generally missing from the mature

protein. The protein from all sources is predicted to contain nine cysteine residues, eight of which are expected to participate in ligating the two [4Fe-4S] centers  $F_A$  and  $F_B$ . The sequences can be divided into three groupings which arise from specific amino acid replacements at two positions in the proteins. These are positions 37 and 70-71. At position 37, all higher plants have a lysine residue while *Cyanophora paradoxa*, *Synechococcus* sp. PCC 7002, and *Nostoc* sp. PCC 8009 have non-charged amino acids. At positions 70-71, some species have tryptophan-histidine, while other species have non-aromatic amino acids (either glycine-alanine, glycine-asparagine, or glycine-proline). This apparent division between prokaryotes and eukaryotes, however, is broken by *Synechocystis* sp. PCC 6803, which is plant-like in that it contains lysine at position 37 and tryptophan-histidine at positions 70 and 71. In fact, spinach and *Synechococcus* sp. PCC 6803 only differ by one amino acid at the C-terminus of the protein: at position 80, spinach contains glycine, whereas *Synechococcus* sp. PCC 7002 contains alanine. Yet, in spite of this extremely minor difference, there are large differences in the  $g$ -values of the  $F_A/F_B$  clusters between these two species. It is difficult to believe that this sole substitution, at the C-terminus of the protein, leads to the changes seen in the  $g$ -values of both the  $F_A$  and  $F_B$  clusters. This one instance is therefore sufficient to indicate that in addition to narrowing the resonances, the Photosystem I core protein has an important influence on the ESR spectrum of the  $F_A/F_B$  clusters.

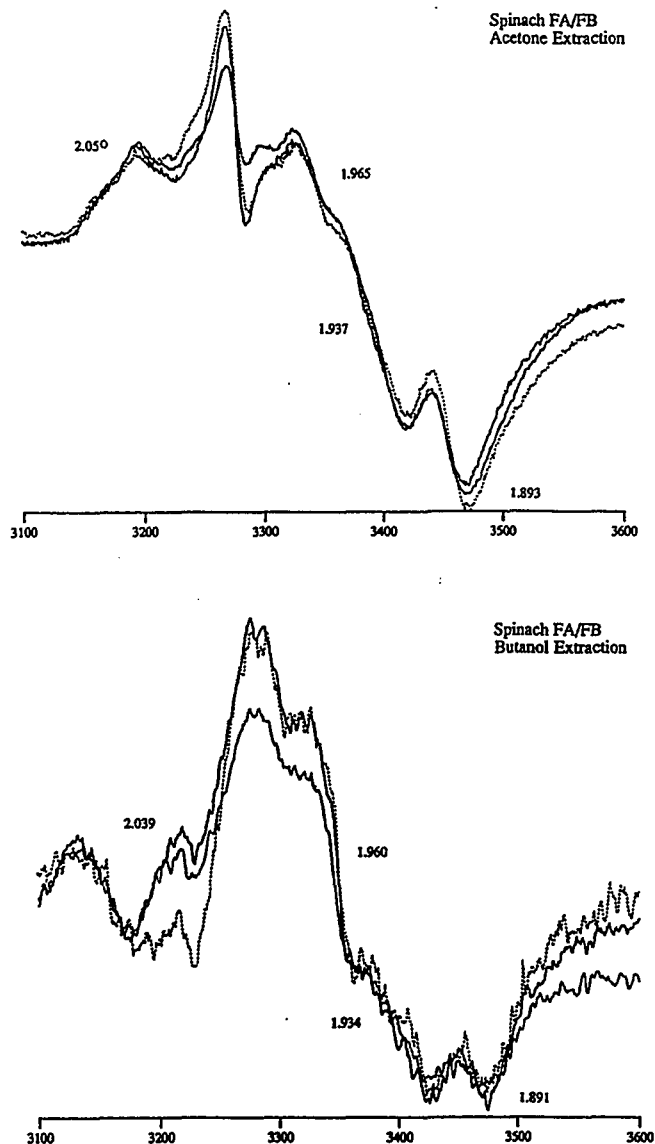


**Figure 12.** ESR spectra of spinach and *Synechococcus* sp. PCC 6301 Photosystem I complexes isolated with Triton X-100 (A) and spinach Photosystem I particles isolated with octyl glucoside and digitonin (B). The samples were illuminated during freezing and the resonances were resolved by subtracting the light-off (before light on) from the light-on spectrum. All samples were suspended in 50 mM Tris buffer, pH 8.3 containing 1 mM sodium ascorbate and 0.03 mM DCPIP at 300  $\mu$ g Chl/ml.

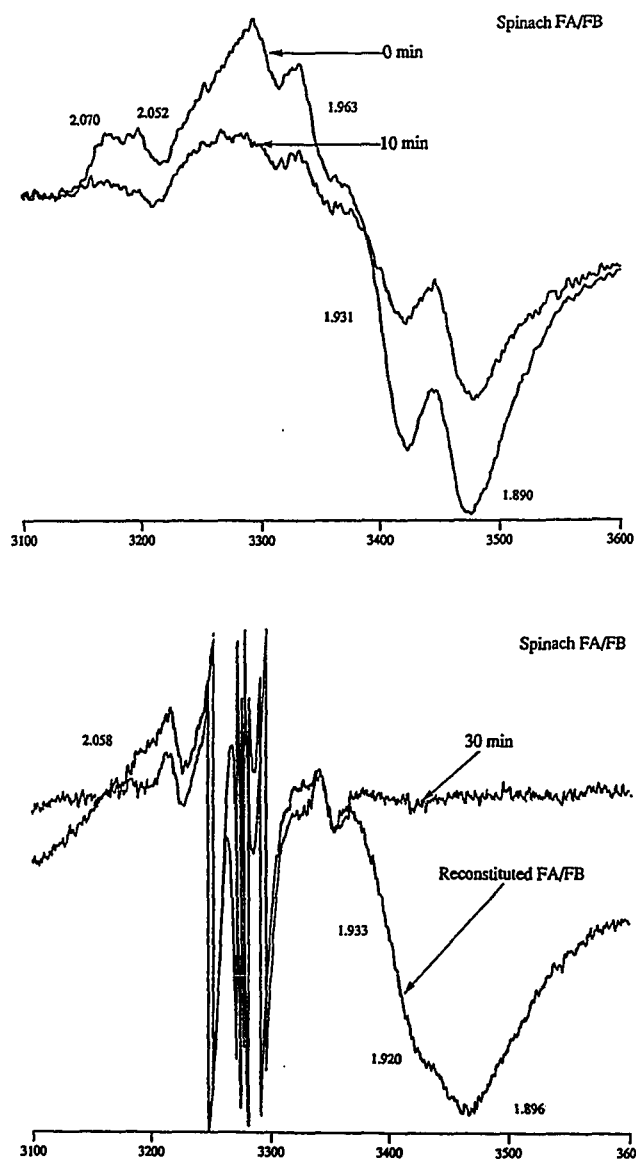




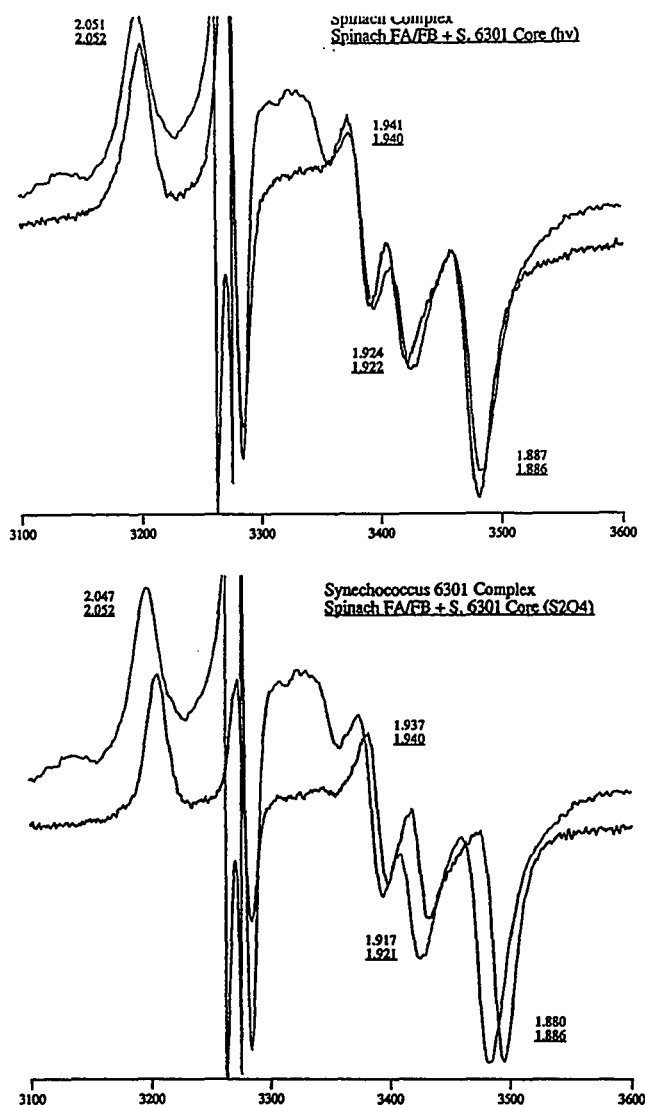
**Figure 13.** ESR spectra of the chemically-reduced FA/FB protein from mesophilic and thermophilic species of *Synechococcus* (A) and *Synechococcus* sp. PCC 6803 and *Synechococcus* sp. PCC 7002 (B). The samples were reduced directly in the ESR sample tube in the presence of excess sodium dithionite and 0.033 mM methyl viologen in 0.1 M glycine, pH 10. The spectra were resolved by subtracting the oxidized spectrum from the chemically reduced spectrum and amplifying the difference 5-fold in software. Spectrometer conditions: temperature, 19 K; microwave power, 20 mW; microwave frequency, 9.128 GHz; receiver gain,  $5.0 \times 10^3$ ; modulation amplitude, 10 G at 100 kHz.



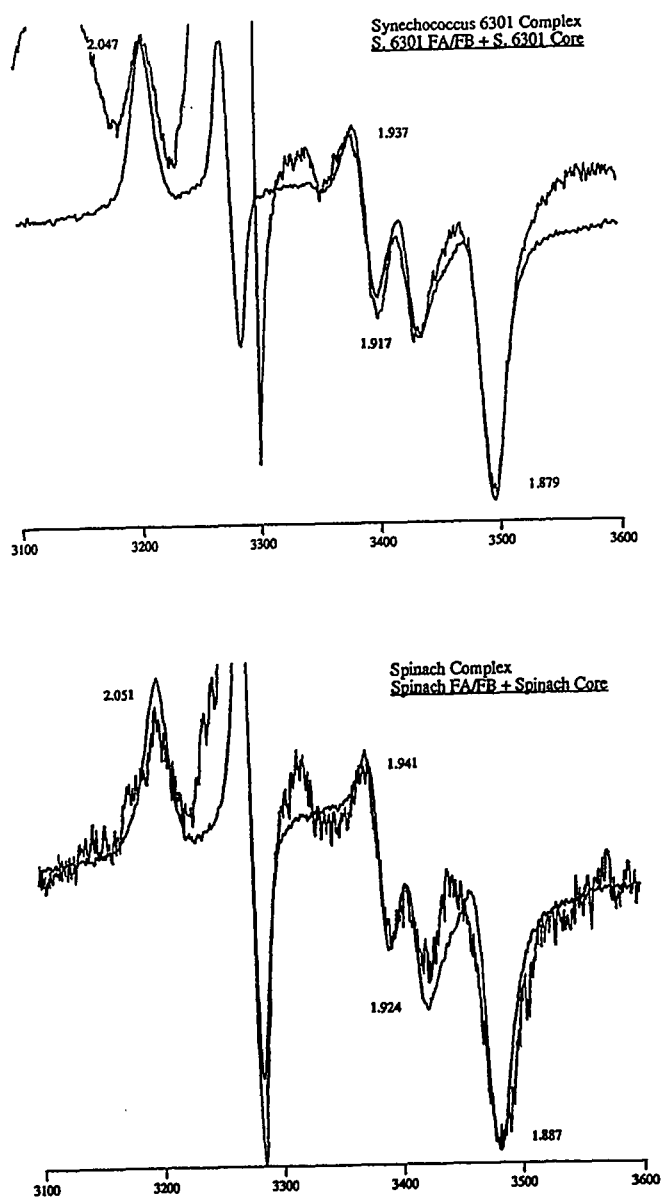
**Figure 14 .** ESR spectra of the chemically-reduced FA/F<sub>B</sub> protein freshly isolated from lyophilized spinach thylakoid membranes by acetone/methanol (A) and butanol (B) extraction. The samples were reduced directly in the ESR sample tube in the presence of excess sodium dithionite and 0.033 mM methyl viologen in 0.1 M glycine, pH 10. The spectra were resolved by subtracting the oxidized spectrum from the chemically reduced spectrum and amplifying the difference 5-fold in software. Spectrometer conditions: temperature, 19 K; microwave power, 20 mW; microwave frequency, 9.128 GHz; receiver gain,  $5.0 \times 10^3$ ; modulation amplitude, 10 G at 100 kHz. Three independent determinations are shown to minimize possible prep-to-prep variations in the appearance of the spectra. The spectra were normalized to appear the same intensity.



**Figure 15.** ESR spectrum of the chemically-reduced FA/FB protein after 0, 10 and 30 min of heating to 37 C (A) and after reconstitution with FeCl<sub>3</sub>, Na<sub>2</sub>S and β-mercaptoethanol (B). The samples were warmed directly in the ESR sample tube in the presence of excess sodium dithionite and 0.033 mM methyl viologen in 0.1 M glycine, pH 10. The spectra were resolved by subtracting the oxidized spectrum from the chemically reduced spectrum and amplifying the difference 5-fold in software. Spectrometer conditions: temperature, 19 K; microwave power, 20 mW; microwave frequency, 9.128 GHz; receiver gain,  $5.0 \times 10^3$ ; modulation amplitude, 10 G at 100 kHz.



**Figure 16.** ESR spectra of the photochemically-reduced spinach Photosystem I complex and the reconstituted spinach-Synechococcus sp. PCC 6301 complex (A), and the Synechococcus sp. PCC 6301 complex and the chemically-reduced spinach-Synechococcus sp. PCC 6301 Photosystem I complex (B). The light-reduced samples were suspended in 50 mM Tris buffer, pH 8.3 containing 1 mM sodium ascorbate and 0.03 mM DCPIP at 300  $\mu$ g Chl/ml. The spectra were resolved by subtracting the light-off (before light on) from the light-on spectrum. The chemically-reduced sample was reduced directly in the ESR sample tube in the presence of excess sodium dithionite and 0.033 mM methyl viologen in 0.1 M glycine, pH 10. The spectrum was resolved by subtracting the oxidized spectrum from the chemically reduced spectrum. Spectrometer conditions: temperature, 19 K; microwave power, 20 mW; microwave frequency, 9.128 GHz; receiver gain,  $5.0 \times 10^3$ ; modulation amplitude, 10 G at 100 kHz.



**Figure 17.** ESR spectra of the photochemically-reduced reconstituted *Synechococcus-Synechococcus* Photosystem I complex compared with the control *Synechococcus* PCC 6301 complex (A) and reconstituted spinach-spinach Photosystem I complex compared with the control spinach complex.(B). The spectra were resolved by subtracting the light-off (before light on) from the light-on spectrum. The samples were suspended in 50 mM Tris buffer, pH 8.3 containing 1 mM sodium ascorbate and 0.03 mM DCPIP at 300  $\mu$ g Chl/ml.

## **ACKNOWLEDGMENTS**

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## CHAPTER V

### GENERAL CONCLUSIONS

This dissertation has attempted to shed light on some important problems concerning electron transport components of Photosystem I. Urea and other selected chaotropes have been found to dissociate the low molecular mass polypeptides from the Photosystem I complex with little or no effect on the Photosystem I core protein and its cofactors. This has been proved by reconstituting a functional same-species Photosystem I complex from the Photosystem I core protein and the  $F_A/F_B$  holoprotein, with identical  $g$ -values for the light-induced ESR spectra of  $FX$  and  $F_A/F_B$  as the control. However, chaotropic treatment degrades the  $F_A/F_B$  iron-sulfur clusters which might lead to a conformational change of the polypeptide. This is no longer a major obstacle in isolating the  $F_A/F_B$  holoprotein since the apoprotein is renatured upon incubation with  $FeCl_3$  and  $Na_2S$ . The percentage of rebuilt holo- $F_A/F_B$  protein from the apoprotein varies from preparation to preparation; the best always being when the apoprotein is freshly isolated at  $-20^\circ C$  and anaerobic conditions. There is no procedure to measure the chaotropic effect on the other low molecular mass proteins since they seem not to be involved directly in binding the redox centers that are involved directly in forward electron transport. Lack of this direct involvement with the redox centers makes the rest of the low molecular mass proteins difficult and to assay. Cross-linking experiments have indicated that the 17- and 19-kDa polypeptides serve as binding sites for the soluble plastocyanin and ferredoxin proteins, respectively. Similar cross-linking experiments could be, and have been attempted to study the spatial configuration of the other polypeptides. Since the 17- and 19-

kDa proteins have been implicated to serve as binding sites for the soluble ferredoxin and plastocyanin, it maybe that one or more of the other low molecular mass proteins might serve to bind the 8.9-kDa protein (perhaps in the presence of the other low molecular mass proteins). The experiment needs to be carried out by different orders-of-mixing experiments and by incubating the fractions in the absence of one or more of low molecular mass proteins to see if one or another is necessary for the binding of the 8.9 kDa protein to the Photosystem I core.protein. This experiment was not successful here because of the difficulty in obtaining "pure" and undamaged  $F_A/F_B$  holoprotein. However, since all reconstitution attempts using 'purified'  $F_A/F_B$  protein gave a poor yield, it is possible that at least one other low molecular mass protein is required for the rebinding of the 8.9-kDa polypeptide. It is also likely that a degraded 8.9-kDa protein does not rebind to the Photosystem I core protein. Future work should also engage in identifying a more gentle purification technique along with the already-outlined reconstitution procedures.

The iron and labile sulfur can be removed from the  $F_A/F_B$  protein by various physical and chemical treatments. It is possible to reconstitute the clusters with different isotopes of iron and sulfur in the presence of a thiol-containing ligand Genetic or chemical modifications of the polypeptide can now be performed before the reconstitution of the holoprotein. The rebuilt holo- $F_A/F_B$  protein could be added back to the Photosystem I core protein to regenerate a functional Photosystem I complex. Careful analysis of the backreaction kinetic parameters along with other spectroscopic evaluations of the reconstituted complex could reveal the type of amino acid residues present at the active site.

Another interesting experiment (that was not a success) was an attempt to reconstitute a Photosystem I complex where the acid-labile sulfur in the  $F_A/F_B$  clusters is replaced by selenium. Such substitution is known to take place at least in some related iron-sulfur proteins [53-55,66,67]; some plants and microorganisms require selenium as a constituent of protein [68]. The iron-selenium containing  $F_A/F_B$  holoprotein is either very



unstable, which may have led to difficulties in preparing enough sample for ESR and resonance Raman analysis or the artificial clusters are soluble and non-bound to the protein. This experiment could have helped delineate whether the terminal electron acceptors  $F_A$  and  $F_B$  act in parallel or in series. The fact that  $F_A$  and  $F_B$  are situated close to each other on the same polypeptide has made selective manipulation of one of the clusters without affecting the other very difficult. ESR spectra, optical kinetic parameters and redox potentials depend critically on distances between redox centers and are greatly affected by rather small modifications of the reaction center. Since the  $F_B$  center has been found to be more susceptible to change than the  $F_A$  center [11,12], it is possible that  $F_B$  resides on a more hydrophilic part of the protein.

In conclusion, despite the fact that much remains to be learned about the individual components before a good picture of Photosystem I can be drawn, the resolution and reconstitution experiments pioneered here will undoubtedly play a major role in future studies. It is encouraging that cyanobacterial Photosystem I complexes have been crystalized; hopefully the 3-dimensional structure will be solved by X-ray crystallography in the near future [69,70].

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## APPENDIX

FORTH program used for Acquisition, Transfer, Manipulation and Storage of ESR spectra  
from Varian E-109 Spectrometer to Macintosh Plus Computer

: IT ;

DECIMAL

CREATE MY.TYPES "TEXT , "DATA ,

: STD.FILE.OPEN

REPLY 74 ERASE

75 75 XY>POINT

" HELLO WORLD"

MY.TYPES 2 REPLY (GET.FILE)

REPLY C@ BOOLEAN DUP

IF REPLY 6+ <W@ VOLREF# !

IODIR OFF REPLY 10+ OPEN\$

THEN ;

: STD.FILE.PUT

REPLY 74 ERASE

75 75 XY>POINT

" SELECT NEW FILE NAME"

" EPR[]"

REPLY (PUT.FILE)

REPLY C@ BOOLEAN DUP

IF REPLY 6+ <W@ VOLREF# !

IODIR OFF REPLY 10+ 4000 SWAP NEW.FILE

THEN ;

CREATE DISKFILE , " DATA:" 31 ALLOT

: FILENAME DISKFILE 6+ ;

: HOLD.FILENAME DUP C@ 5+ DISKFILE C!

DUP COUNT FILENAME SWAP CMOVE ;

: NEW.FILE.OPEN

REPLY 74 ERASE

75 75 XY>POINT

" HELLO WORLD"

MY.TYPES 2 REPLY (GET.FILE)

REPLY C@ BOOLEAN DUP

IF REPLY 6+ <W@ VOLREF# !

IODIR OFF REPLY 10+ HOLD.FILENAME OPEN\$

```

        THEN ;
:NEW.FILE.PUT
    REPLY 74 ERASE
    75 75 XY>POINT
    " SELECT NEW FILE NAME"
    " EPR[]"
    REPLY (PUT.FILE)
        REPLY C@ BOOLEAN DUP
    IF     REPLY 6+ <W@ VOLREF# I
        IODIR OFF REPLY 10+ HOLD.FILENAME 4000 SWAP NEW.FILE
    THEN ;

```

VARIABLE SPEC.FILE

```
:STD.FILE.PUTTER NEW.FILE.PUT SPEC.FILE I DROP ;
```

```
:STD.FILE.OPENER NEW.FILE.OPEN SPEC.FILE I DROP ;
```

```
token.for sysbeep ' ((ERROR)) 16+ w!
```

```

: >ERR.MSG ( addr\count -- )
    63 MIN DUP ERR.MSG C!
    ERR.MSG 1+ SWAP CMOVE ;

```

```

:RECOVER.SYS.WINDOW
    parent status =
    front.window ?wptr not and
    if outfile on
        sys.window ?active.wptr not
        if sys.window add.window then
            sys.window dup show.window select.window
        then ;

```

```
TOKEN.FOR ERROR.ALERT (ERROR) I
```

```
TOKEN.FOR ABORT.ALERT (ABORT) I
```

```

axe recover.sys.window
axe >err.msg

```

```

:ALERT.FILE
    " CREATE A NEW FILE?" ' 0 ' 0 ' 0
    4 0 2
    ISSUEALERT
    DISCARD.ACTIVATES
    CASE
        1 OF          STD.FILE.PUTTER          ENDOF
        2 OF          STD.FILE.OPENER          ENDOF
        3 OF
    ENDCASE ;

```

```
:ALERT.FILE1
  " CREATE A NEW FILE?" '0' '0' '0'
  4 0 2
  ISSUEALERT
  DISCARD.ACTIVATES
  CASE
    1 OF      STD.FILE.PUTTER      ENDOF
    2 OF      STD.FILE.OPENER      ENDOF
    3 OF      ABORT                ENDOF
  ENDCASE;
```

ALERT.FILE1

FORTH DEFINITIONS

50000 MINIMUM.VOCAB

```
1024 1 1ARRAY TYPE.BUFFER
2000 2 1ARRAY SPEC.BUFFER
8 2000 2 2ARRAY TEMP.BUFFER
```

VARIABLE SLOPE

CREATE NO.FLASHES 0 C,

CREATE LINE.VAR 260 ,

CREATE SERIAL.FILE# 100 ,

decimal

anew SERIAL  
75000 minimum.object

\ some default settings... ( modify them as you require )  
create (BITS) 8 , \ 5,6,7 or 8 are valid  
create (STOP) 3 , \ 1,2,3 = 1,1.5,2 stop bits  
create (PARITY) 0 , \ 0,1,2,3 = none,odd,none,even  
create (BAUD) 9600 ,

create SERIAL.OPTIONS hex  
FF c, \ Xon/Xoff output flow control flag  
00 c, \ CTS hardware handshake flag  
11 c, \ Xon character  
13 c, \ Xoff character  
00 c, \ errors that abort  
00 c, \ status changes that cause events  
00 c, \ Xon/Xoff input flow control flag  
00 c, \ not used

decimal

1024 constant INPUT.SIZE ( Default input buffer size - easily changed ! )



```

create INPUT.BUFFER here input.size dup allot erase

0 wconstant SERIAL.IN
0 wconstant SERIAL.OUT \ RefNums for serial devices

: (OPEN.SERIAL) ( -- | opens serial driver if it is not already )
  serial.in not
  IF " .AIN" open$ ' serial.in w! \ use .BIN for printer port
    " .AOUT" open$ ' serial.out w! \ use .BOUT for printer port
  THEN ;

hex
: SETUP.SERIAL ( #stop\parity\#data bits\baud rate\RefNum -- )
  ( configures serial driver for above list of parameters )
  5 needed
  locals| REFNUM BAUD #BITS PARITY #STOP |
  1C200 baud / 2- \ assign baud bits
  0270 #bits 5- 2* scale 0C00 and \ assign #bits bits
  or
  parity 3 and 0C scale \ assign parity bits
  or
  #stop 3 and 0E scale \ assign #stop bits
  or \ now we have complete word
  10 scale \ put in high order word
  refnum 8 sp@ 8+ fscontrol \ equivalent to SerReset
  drop ;
decima!

: OPEN.SERIAL ( ADDR\CNT -- | Opens pair of file#'s)
  ( For serial port A. Addr\cnt specifies buffer )
  swap
  (open.serial) \ make sure serial drivers are open

  serial.in 9 sp@ 8+ fscontrol 2drop \ equivalent to SerSetBuf
  serial.options 2@ serial.in 10 sp@ 8+
  fscontrol 2drop ; \ equivalent to SerHShake ( set handshake options )

: S.TYPE ( ADDR\CNT -- | Serial Equivalent to TYPE )
  SERIALOUT WRITE.TEXT ;

: S.EXPECT ( ADDR\CNT -- | Non-editing Equivalent to EXPECT )
  SERIAL.IN READ.TEXT ;

: S.?TERMINAL ( -- N | returns # of CHARS available in buffer)
  0 0 0 0 0 serial.in 2 sp@ 8+ fsstatus \ equivalent to SerGetBuf
  >R 2drop 2drop drop R> ;

: S.STATUS ( -- STAT2\STAT1 | Error and handshake status)
  0 0 0 0 0 0 serial.in 8 sp@ 8+ fsstatus \ equivalent to SerStatus
  >R >R 2drop 2drop R> R> ;

: S.?READY ( -- flag | true when ready for output)

```

```

S.STATUS SWAP DROP NOT ;

: S.KEY ( -- CHAR | Get CHAR from Serial port )
  0 SP@ 3+ 1 S.EXPECT ;

: S.EMIT ( CHAR -- | Send CHAR to Serial port)
  SP@ 3+ 1 S.TYPE DROP ;

: S.BREAK ( -- | send break char for 6 ticks )
  0 0 serial.out 12 sp@ 8+ fscontrol \ equivalent to SerSetBrk
  tickcount 6+ BEGIN dup tickcount < UNTIL drop \ delay 6 ticks
  0 0 serial.out 11 sp@ 8+ fscontrol ; \ equivalent to SerClrBrk

: BAUD ( baud.rate -- | does all the work in setting up )
  ( Before using serial ports, should call 300 BAUD, 1200 BAUD, or whatever)
  (baud) !
  input.buffer input.size open.serial \ initialize the serial drivers
  (stop) @ (parity) @ (bits) @ (baud) @ serial.in
  setup.serial ; \ make the settings

: BINARY 2 BASE ! ;

: S.DROP S.KEY DROP ;

: S.CRLF CRLF 2 S.TYPE ;

: SET.MAC
  0 SERIAL.OPTIONS CI
  0 SERIAL.OPTIONS 1+ CI
  11 SERIAL.OPTIONS 2+ CI
  13 SERIAL.OPTIONS 3+ CI ;

SET.MAC 9600 BAUD

: EMPTY.SERIAL 0 TYPE.BUFFER S.?TERMINAL DUP CR . S.EXPECT ;

: TYPE.IT CR S.?TERMINAL DUP 0 TYPE.BUFFER SWAP S.EXPECT
  0 TYPE.BUFFER SWAP TYPE CR ;

: HELLO " HE" COUNT S.TYPE CRLF 2 S.TYPE TYPE.IT ;

: FREE.488A " @" COUNT S.TYPE CRLF 2 S.TYPE ;

: ?ERROR " STA" COUNT S.TYPE S.CRLF 30 DELAY TYPE.IT ;

: CLEAR.488A " @@" COUNT S.TYPE
  60 DELAY ?ERROR ;

: USE.REMOTE " REMO16" COUNT S.TYPE S.CRLF ;

: BAUD=96 " BA;9600" COUNT S.TYPE S.CRLF 9600 BAUD ;
: BAUD=192 " BA;19200" COUNT S.TYPE S.CRLF 19200 BAUD ;

```

```

: S.?T S.?TERMINAL ;

CLEAR.488A 30 DELAY USE.REMOTE 30 DELAY

: XQT COUNT S.TYPE " X" COUNT S.TYPE ;

: DCV      " OU16#03;F0" XQT ; DCV 30 DELAY
: 2VOLTS   " OU16#03;R3" XQT ; 2VOLTS 30 DELAY
: RATE     " OU16#03;S1" XQT ; RATE 30 DELAY
: NO.FILTER " OU16#03;P0" XQT ; NO.FILTER 30 DELAY
: NO.DELAY  " OU16#03;W0" XQT ; NO.DELAY 30 DELAY
: READ.INPUT " OU16#04;Q00" XQT ; READ.INPUT 30 DELAY
: FORMAT.DATA " OU16#03;G1" XQT ; FORMAT.DATA 30 DELAY
: NO.TERMINATOR " OU16#02;YX" XQT ; NO.TERMINATOR 30 DELAY
: SRQ.ON    " OU16#04;M32" XQT ; SRQ.ON

: DISP" " OU16;D " COUNT S.TYPE [COMPILE] " XQT S.CRLF ;
: DISP.OFF " OU16#02;DX" COUNT S.TYPE S.CRLF ;

: K.STATUS " EN16#03;'U0'18"
      ." TFRKQQSMZWWAJGBPYY" 60 DELAY
      TYPE.IT ;

?ERROR ?ERROR ?ERROR ?ERROR

: CLEAN  LOWER.LEFT 20 100 XYSIZE CARTESIAN ON ;
: LEGENDS 250 0 2250 255 FRAME RECTANGLE ;
: XHATCHES 2250 250 DO I 1 MOVE.TO I 5 DRAW.TO 200 +LOOP ;
: YHATCHES 255 1 DO 250 I MOVE.TO 270 I DRAW.TO 51 +LOOP ;

CREATE AMPLIFY 1 ,
: VOLTS* DUP AMPLIFY I
      @PEN DROP 50 - 5 * 250 + 260 MOVE.TO
      . BS EMIT 42 EMIT ;

CREATE SETOFF 0 ,
: +SIGNAL 20 SETOFF +I ;
: -SIGNAL -20 SETOFF +I ;

CREATE TIME 0 ,
: TIMED 0 DO LOOP ;

CREATE MINS 0 ,

: MINUTE MINS I
      MINS @ CASE
          4 OF 5058 TIME I ENDOF
          8 OF 21400 TIME I ENDOF
          16 OF 0 TIME I ENDOF
      ENDCASE ;

: ?PARAMETERS CR

```

AMPLIFY @ . BS EMIT 42 EMIT CR  
MINS @ . ." Minute Run" CR  
TIME @ ." Time Constant=" . CR CR ;

: 2.MIN.SET " OU16#03;T4" XQT ;  
: SET.TRIGGER " OU16#03;T5" XQT ;  
: 2.MIN.TRIG " EN16'X';22000" COUNT S.TYPE S.CRLF ;  
: TRIGGER " EN16'X';11" COUNT S.TYPE S.CRLF ;

CLEAR.488A USE.REMOTE SET.TRIGGER

: 2.MINUTE.RUN  
2 MINS I  
2.MIN.SET  
CLEAN PAGE LEGENDS  
?PARAMETERS  
." To Begin Run Hit [Return]" 1 SYSBEEP  
BEGIN KEY 13 = UNTIL  
@CLOCK  
2.MIN.TRIG  
2000 0 DO  
DO.EVENTS DROP  
PAD 11 S.EXPECT  
PAD 7 \$>NUMBER  
IF  
I SPEC.BUFFER WI  
I 250 +  
I SPEC.BUFFER W@ AMPLIFY @ \* 78 / SETOFF @ + DOT  
ELSE  
." Invalid Number"  
1 SYSBEEP  
LEAVE  
THEN  
LOOP  
@CLOCK SWAP - 60 /MOD  
0 127 MOVE.TO  
." Minutes " ." Seconds" CR  
CLEAR.488A USE.REMOTE SET.TRIGGER  
1 SYSBEEP ;

: TRANSFER  
SET.TRIGGER  
CLEAN PAGE LEGENDS  
?PARAMETERS  
." To Begin Run Hit [Return]" 1 SYSBEEP  
BEGIN KEY 13 = UNTIL  
@CLOCK  
2000 0 DO  
DO.EVENTS DROP  
TRIGGER  
PAD 13 S.EXPECT  
PAD 7 \$>NUMBER  
IF

```

I SPEC.BUFFER WI
  I 250 +
I SPEC.BUFFER W@ AMPLIFY @ * 78 / SETOFF @ + DOT
ELSE
  ." Invalid Number"
1 SYSBEEP
LEAVE
THEN
TIME @ TIMED
LOOP
@CLOCK SWAP - 60 /MOD
0 127 MOVE.TO
.." Minutes " .." Seconds" CR
1 SYSBEEP ;

```

```

CREATE NORMALIZED 2 ALLOT
CREATE NORMALIZER 32768 W,

```

```

:NORMALIZE.DATA 10 SPEC.BUFFER W@ NORMALIZER W@ -
  NORMALIZED WI
  2000 0 DO I SPEC.BUFFER W@
    NORMALIZED W@ -
    I SPEC.BUFFER WI
  LOOP ;

```

```

:PLOT.DATA NORMALIZE.DATA
  CLEAN 250 0 MOVE.TO
  1950 0 DO
    I SPEC.BUFFER W@ 78 / 255 AND
    I 250 + SWAP DOT
  LOOP 0 257 MOVE.TO ;

```

```

:DISPLAY.DATA PAGE PLOT.DATA LEGENDS XHATCHES YHATCHES ;

```

```

:CHANGE.NORMALIZE 78 * NORMALIZER WI DROP DISPLAY.DATA ;

```

```

:NEW.LINE 0 -10 LINE.VAR +I LINE.VAR @ ;

```

```

VARIABLE FILE.POSITION

```

```

:INPUT.FILE.NUMBER BEGIN NEW.LINE MOVE.TO ." RECORD #" 1 SYSBEEP
  3 ASK.NUMBER UNTIL 4000 * FILE.POSITION I ;

```

```

:WRITE.TO.DISK
  INPUT.FILE.NUMBER
  0 SPEC.BUFFER 4000 FILE.POSITION @ SPEC.FILE @ WRITE.VIRTUAL
  DISPLAY.DATA
  CR ." Data Written To Disk"
  SPEC.FILE @ CURRENT.POSITION 4000 - 4000 / CR
  ." # " .
  SPEC.FILE @ CLOSE DISKFILE OPEN$ SPEC.FILE I ;

```

```
: READ.FROM.DISK
  INPUT.FILE.NUMBER
  0 SPEC.BUFFER 4000 FILE.POSITION @ SPEC.FILE @ READ.VIRTUAL
  DISPLAY.DATA
  SPEC.FILE @ CURRENT.POSITION 4000 - 4000 / CR
  ." # " . ;

: STORE.DATA WRITE.TO.DISK ;

: CLEAN.ZOOM LOWER.LEFT 80 100 XYSCALE CARTESIAN ON ;

: PLOT.ZOOM
  CLEAN.ZOOM
  0 0 MOVE.TO
  992 0 DO
    I SPEC.BUFFER W@ 20 /
    I 125 + SWAP DOT
  LOOP CR ;

: X.SCALE PAGE CLEAN.ZOOM PLOT.ZOOM
  CLEAN LEGENDS XHATCHES YHATCHES ;

: LINEAR.SPEC 260 LINE.VAR I READ.FROM.DISK ;

: OVERLAY.FILE
  INPUT.FILE.NUMBER
  0 SPEC.BUFFER 4000 FILE.POSITION @ SPEC.FILE @ READ.VIRTUAL
  PLOT.DATA ;

: OVERLAY.TEMP
  BEGIN
    ." Enter Buffer Number [0-7]"
    1 ASK.NUMBER
  UNTIL
    2000 0 DO DUP I TEMP.BUFFER W@
      I SPEC.BUFFER WI
    LOOP DROP
  PLOT.DATA ;

: HOLD.TEMP LOCALS| X|
  2000 0 DO
    I SPEC.BUFFER W@
    X I TEMP.BUFFER WI
  LOOP ;

: FETCH.TEMP LOCALS| X|
  2000 0 DO
    X I TEMP.BUFFER W@
    I SPEC.BUFFER WI
  LOOP
  DISPLAY.DATA ;
```

VARIABLE SCALER

```
:INPUT.SCALE BEGIN CR 0 260 MOVE.TO ." % SCALE"  
4 ASK.NUMBER UNTIL SCALER !;
```

```
:Y.SCALE INPUT.SCALE CR DUP .  
2000 0 DO  
I SPEC.BUFFER W@  
SCALER @ * 100 /  
I SPEC.BUFFER W!  
LOOP  
DISPLAY.DATA ;
```

```
:OVERLAY.ZOOM  
BEGIN  
." Enter Buffer Number [0-7]"  
1 ASK.NUMBER  
UNTIL  
2000 0 DO DUP I TEMP.BUFFER W@  
I SPEC.BUFFER W!  
LOOP DROP  
PLOT.ZOOM 257 0 MOVE.TO ;
```

```
:AVERAGE.SPEC.0-1  
2000 0 DO 0 I TEMP.BUFFER W@  
1 I TEMP.BUFFER W@ +  
2 I SPEC.BUFFER W!  
LOOP DISPLAY.DATA ;
```

```
:AVERAGE.SPEC.0-3  
2000 0 DO 0 I TEMP.BUFFER W@  
1 I TEMP.BUFFER W@ +  
2 I TEMP.BUFFER W@ +  
3 I TEMP.BUFFER W@ +  
4 I SPEC.BUFFER W!  
LOOP DISPLAY.DATA ;
```

```
:AVERAGE.SPEC.0-7  
2000 0 DO 0 I TEMP.BUFFER W@  
1 I TEMP.BUFFER W@ +  
2 I TEMP.BUFFER W@ +  
3 I TEMP.BUFFER W@ +  
4 I TEMP.BUFFER W@ +  
5 I TEMP.BUFFER W@ +  
6 I TEMP.BUFFER W@ +  
7 I TEMP.BUFFER W@ +  
8 I SPEC.BUFFER W!  
LOOP DISPLAY.DATA ;
```

```
:DIFFERENCE.SPEC  
2000 0 DO 0 I TEMP.BUFFER W@
```

```

        1 I TEMP.BUFFER W@
          - 128 +
I SPEC.BUFFER WI
  LOOP DISPLAY.DATA ;

:SCALEX
  SWAP 10 - 25 * SWAP ;

:?SLOPE
  ROT SWAP - 10000 * ROT ROT - / SLOPE ! ;

:ADJUST.BASELINE
  SCALE.X 2SWAP SCALE.X 2OVER ?SLOPE SWAP
    2000 0 DO
      2DUP I SWAP - SLOPE @ * 78 * 10000 / +
      I SPEC.BUFFER W@ SWAP -
      I SPEC.BUFFER WI
        LOOP DROP DROP
      DISPLAY.DATA ;

: LINE
  SCALE.X 2SWAP SCALE.X 2OVER ?SLOPE SWAP
    2000 0 DO
      2DUP I SWAP - SLOPE @ * 10000 / +
      I 250 + SWAP DOT
      LOOP DROP DROP
    0 260 MOVE.TO ;

: STR$ DUP ABS <# #S 2 ROLL SIGN #> ;

VARIABLE DATA.FILE

:NEW.CRICKET.FILE
  REPLY 74 ERASE
  75 75 XY>POINT
  " Select File Name:"
  " #"
  REPLY (PUT.FILE)
    REPLY C@ BOOLEAN DUP
  IF    REPLY 6+ <W@ VOLREF# I
    IODIR OFF REPLY 10+ 0 SWAP NEW.FILE
  THEN ;

: STORE.TEXT.1000
  2000 0 DO
    I SPEC.BUFFER W@ STR$ DATA.FILE @ WRITE.TEXT
    CRLF 1 DATA.FILE @ WRITE.TEXT
  2 +LOOP ;

: STORE.TEXT.2000
  2000 0 DO
    I SPEC.BUFFER W@ STR$ DATA.FILE @ WRITE.TEXT
    CRLF 1 DATA.FILE @ WRITE.TEXT
```



```

LOOP ;

: FILE.DATA.1000
  NEW.CRICKET.FILE DATA.FILE I
  DATA.FILE @
  IF
    STORE.TEXT.1000
    CR ." Data Stored on Disk"
    DATA.FILE @ CLOSE
  ELSE
    CR ." Cricket Data Not Created."
    THEN 1 SYSBEEP ;

: FILE.DATA.2000
  NEW.CRICKET.FILE DATA.FILE I
  DATA.FILE @
  IF
    STORE.TEXT.2000
    CR ." Data Stored on Disk"
    DATA.FILE @ CLOSE
  ELSE
    CR ." Cricket Data Not Created."
    THEN 1 SYSBEEP ;

VARIABLE SETTING.STUFF 99 ALLOT

STRUCTURE SETTING.STRUCTURE
  9 STRING: +date
  25 STRING: +samp
  25 STRING: +conds
  LONG:      +conc
  LONG:      +temp
  LONG:      +field
  LONG:      +scan
  LONG:      +freq
  LONG:      +power
  LONG:      +gain
  LONG:      +mod
STRUCTURE.END

: BOILERPLATE.INFO
  CR ." Maximum # of Characters in [ ]" CR CR ;

: DATE.INFO
  CR ." Enter Date: "
  SETTING.STUFF +date 9 INPUT.STRING ;

: SAMP.INFO
  CR ." Enter Sample Description: "
  SETTING.STUFF +samp 25 INPUT.STRING ;

: CONDS.INFO
  CR ." Enter Sample Conditions: "
  SETTING.STUFF +conds 25 INPUT.STRING ;

```

```

:CONC.INFO          CR ." Enter Concentration in ug/ml: " 5 ASK.NUMBER DROP
                    SETTING.STUFF +conc ! ;

:TEMP.INFO          CR ." Enter Temperature in K: " 4 ASK.NUMBER DROP
                    SETTING.STUFF +temp ! ;

:FIELD.INFO         CR ." Enter Field Setting in Gauss: " 5 ASK.NUMBER DROP
                    SETTING.STUFF +field ! ;

:SCAN.INFO          CR ." Enter Scan Range in Gauss: " 5 ASK.NUMBER DROP
                    SETTING.STUFF +scan ! ;

:FREQ.INFO          CR ." Enter Microwave Frequency in MHz: " 5 ASK.NUMBER DROP
                    SETTING.STUFF +freq ! ;

:POWER.INFO         CR ." Enter Microwave Power in mW: " 4 ASK.NUMBER DROP
                    SETTING.STUFF +power ! ;

:GAIN.INFO          CR ." Enter Gain Setting: " 6 ASK.NUMBER DROP
                    SETTING.STUFF +gain ! ;

:MOD.INFO           CR ." Enter Modulation Amplitude in Gauss: " 3 ASK.NUMBER
DROP
                    SETTING.STUFF +mod ! ;

:READ.INFO
PAGE BOILERPLATE.INFO
    ." Date [9]: "
    SETTING.STUFF
+date COUNT TYPE CR
    ." Sample [25]: "
    SETTING.STUFF
    +samp COUNT TYPE CR
    ." Conditions [25]: "
    SETTING.STUFF +conds
COUNT TYPE CR
    ." Concentration (ug/ml) [5]: "
    SETTING.STUFF
+conc ? CR
    ." Temperature (K) [4]: "
    SETTING.STUFF
    +temp ? CR
    ." Field Setting (G) [5]: "
    SETTING.STUFF
    +field ? CR
    ." Scan Range (G) [5]: "
    SETTING.STUFF
    +scan ? CR
    ." Frequency (MHz) [5]: "
    SETTING.STUFF
    +freq ? CR
    ." Microwave Power (mW) [4]: "
    SETTING.STUFF
    +power ? CR
    ." Instrument Gain [6]: "
    SETTING.STUFF
    +gain ? CR
    ." Amplitude Modulation (G) [3]: "
    SETTING.STUFF
+mod ? CR
;

:INITIALIZE.INFO
DATE.INFO

```

SAMP.INFO  
 CONDS.INFO  
 CONC.INFO  
 TEMP.INFO  
 FIELD.INFO  
 SCAN.INFO  
 FREQ.INFO  
 POWER.INFO  
 GAIN.INFO  
 MOD.INFO  
 CR ." Settings Entered Into Memory" CR

;

:DEFAULT.INFO

0	SETTING.STUFF	+date	CI
0	SETTING.STUFF	+samp	CI
0	SETTING.STUFF	+conds	CI
0	SETTING.STUFF	+conc	!
0	SETTING.STUFF	+temp	!
0	SETTING.STUFF	+field	!
0	SETTING.STUFF	+scan	!
0	SETTING.STUFF	+freq	!
0	SETTING.STUFF	+power	!
0	SETTING.STUFF	+gain	!
0	SETTING.STUFF	+mod	!

;

DEFAULT.INFO

VARIABLE STAMP.POSITION

:CALC.STAMP.POSITION FILE.POSITION @ 3900 + STAMP.POSITION ! ;

:STAMP.TO.DISK

CALC.STAMP.POSITION  
 SETTING.STUFF 99 STAMP.POSITION @ SPEC.FILE @ WRITE.VIRTUAL  
 SPEC.FILE @ CLOSE DISKFILE OPEN\$ SPEC.FILE !  
 READ.INFO  
 CR ." Sample and Instrument Parameters Stored on Disk" ;

:STAMP.FROM.DISK

CALC.STAMP.POSITION  
 SETTING.STUFF 99 STAMP.POSITION @ SPEC.FILE @ READ.VIRTUAL  
 READ.INFO ;

9 CONSTANT SAMPLE.INFO

:SAMPLE.MENU SAMPLE.INFO DELETE.MENU

0 " Specs " SAMPLE.INFO NEW.MENU

" DISPLAY SPECS;INITIALIZE SPECS;-("

" RETRIEVE SPECS;STORE SPECS;-("DATE;"

" SAMPLE;CONDITIONS;"

APPEND.ITEMS

SAMPLE.INFO APPEND.ITEMS

SAMPLE.INFO APPEND.ITEMS

SAMPLE.INFO

" CONCENTRATION;TEMPERATURE;"  
 " MAGNETIC FIELD;SCAN RANGE;FREQUENCY;POWER;"  
 " ESR GAIN;AMPLITUDE MOD"

SAMPLE.INFO APPEND.ITEMS  
 SAMPLE.INFO APPEND.ITEMS  
 SAMPLE.INFO APPEND.ITEMS

DRAW.MENU.BAR

SAMPLE.INFO MENU.SELECTION: 0 HILITE.MENU

CASE	1	OF	READ.INFO	ENDOF
	2	OF	INITIALIZE.INFO	ENDOF
	3	OF		ENDOF
	4	OF	STAMP.FROM.DISK	ENDOF
	5	OF	STAMP.TO.DISK	ENDOF
	6	OF		ENDOF
	7	OF	DATE.INFO	ENDOF
	8	OF	SAMP.INFO	ENDOF
	9	OF	CONDS.INFO	ENDOF
	10	OF	CONC.INFO	ENDOF
	11	OF	TEMP.INFO	ENDOF
	12	OF	FIELD.INFO	ENDOF
	13	OF	SCAN.INFO	ENDOF
	14	OF	FREQ.INFO	ENDOF
	15	OF	POWER.INFO	ENDOF
	16	OF	GAIN.INFO	ENDOF
	17	OF	MOD.INFO	ENDOF

ENDCASE ;  
 SAMPLE.MENU

VARIABLE FIELD.MID  
 VARIABLE SCAN.RANGE  
 VARIABLE KLYSTRON.FREQ

: H.INFO  
 SETTING.STUFF +FIELD @ FIELD.MID !  
 SETTING.STUFF +SCAN @ SCAN.RANGE !  
 SETTING.STUFF +FREQ @ KLYSTRON.FREQ !  
 ;

: STORE.SPECTRA WRITE.TO.DISK STAMP.FROM.DISK ;

: G.VALUE DROP 10 - 1000 \* 80 / H.INFO SCAN.RANGE @  
 \* 1000 / DUP .  
 SCAN.RANGE @ 2 / FIELD.MID @ SWAP -  
 + DUP CR . ." Gauss" CR  
 KLYSTRON.FREQ @ 1000 \* SWAP / 7145 \*  
 10000 / . ." g-Value\*1000" ;

: 1.QUAD.G.VALUE  
 DROP DROP 10 - 1000 \* 80 / H.INFO SCAN.RANGE @ 4 / \* 1000 /  
 SCAN.RANGE @ 2 / FIELD.MID @ SWAP - + DUP CR . ." Gauss" CR  
 KLYSTRON.FREQ @ 1000 \* SWAP / 7145 \*  
 10000 / . ." g-value \*1000" ;

: 2.QUAD.G.VALUE

```
DROP DROP 10 - 1000 * 80 / H.INFO SCAN.RANGE @ 4 / * 1000 /
SCAN.RANGE @ 2 / FIELD.MID @ SWAP -
SCAN.RANGE @ 4 / + + DUP CR .." Gauss" CR
KLYSTRON.FREQ @ 1000 * SWAP / 7145 *
10000 / . ." g-value *1000" ;
```

:3.QUAD.G.VALUE

```
DROP DROP 10 - 1000 * 80 / H.INFO SCAN.RANGE @ 4 / * 1000 /
SCAN.RANGE @ 2 / FIELD.MID @ SWAP -
SCAN.RANGE @ 4 / 2 * + + DUP CR .." Gauss" CR
KLYSTRON.FREQ @ 1000 * SWAP / 7145 *
10000 / . ." g-value *1000" ;
```

:4.QUAD.G.VALUE

```
DROP DROP 10 - 1000 * 80 / H.INFO SCAN.RANGE @ 4 / * 1000 /
SCAN.RANGE @ 2 / FIELD.MID @ SWAP -
SCAN.RANGE @ 4 / 3 * + + DUP CR .." Gauss" CR
KLYSTRON.FREQ @ 1000 * SWAP / 7145 *
10000 / . ." g-value *1000" ;
```

10 CONSTANT DATA.NUMBER

:ACQUIRE.MENU DATA.NUMBER DELETE.MENU

0 "Acquire" DATA.NUMBER NEW.MENU

" DISPLAY SPECTRUM;STORE SPECTRUM;SELECT FILE;-(" DATA.NUMBER

APPEND.ITEMS

" 1xVOLTS/1;2xVOLTS/2;" DATA.NUMBER

APPEND.ITEMS

" 4xVOLTS/4;8xVOLTS/8;-(:2 MINUTES;4 MINUTES;" DATA.NUMBER

APPEND.ITEMS

" 8 MINUTES;16 MINUTES;-(:OFFSET+/-;OFFSET-/-;RUN" DATA.NUMBER

APPEND.ITEMS

DRAW.MENU.BAR

DATA.NUMBER MENU.SELECTION: 0 HILITE.MENU

CASE	1	OF	DISPLAY.DATA	ENDOF	
	2	OF	STORE.SPECTRA	ENDOF	
	3	OF	ALERT.FILE	ENDOF	
	4	OF		ENDOF	
	5	OF	1 VOLTS*	ENDOF	
	6	OF	2 VOLTS*	ENDOF	
	7	OF	4 VOLTS*	ENDOF	
	8	OF	8 VOLTS*	ENDOF	
	9	OF		ENDOF	
	10	OF	2.MINUTE.RUN	ENDOF	
	11	OF	4 MINUTE TRANSFER	ENDOF	
	12	OF	8 MINUTE TRANSFER	ENDOF	
	13	OF	16 MINUTE TRANSFER	ENDOF	
	14	OF		ENDOF	
	15	OF	+SIGNAL	ENDOF	
	16	OF	-SIGNAL	ENDOF	
	17	OF	TRANSFER	ENDOF	

ENDCASE ;

ACQUIRE.MENU

```

11 CONSTANT TEMP.NUMBER

:INTO.TEMP DUP HOLD.TEMP
  1+ -1 TEMP.NUMBER ITEM.CHECK ;

:EMPTY.TEMP 9 1 DO
  I 0 TEMP.NUMBER ITEM.CHECK
  LOOP ;

:TEMP.MENU TEMP.NUMBER DELETE.MENU
  0 " Temps" TEMP.NUMBER NEW.MENU
  " HOLD 0;HOLD 1;HOLD 2;HOLD 3;HOLD 4;HOLD 5;" TEMP.NUMBER
  APPEND.ITEMS
  " HOLD 6;HOLD 7;-(:FETCH 0;FETCH 1;FETCH 2;FETCH 3;" TEMP.NUMBER
  APPEND.ITEMS
  " FETCH 4;FETCH 5;FETCH 6;FETCH 7;-(:EMPTY;" TEMP.NUMBER
  APPEND.ITEMS
DRAW.MENU.BAR
TEMP.NUMBER MENU.SELECTION: 0 HILITE.MENU
CASE
  1 OF 0 INTO.TEMP ENDOF
  2 OF 1 INTO.TEMP ENDOF
  3 OF 2 INTO.TEMP ENDOF
  4 OF 3 INTO.TEMP ENDOF
  5 OF 4 INTO.TEMP ENDOF
  6 OF 5 INTO.TEMP ENDOF
  7 OF 6 INTO.TEMP ENDOF
  8 OF 7 INTO.TEMP ENDOF
  9 OF ENDOF
  10 OF 0 FETCH.TEMP ENDOF
  11 OF 1 FETCH.TEMP ENDOF
  12 OF 2 FETCH.TEMP ENDOF
  13 OF 3 FETCH.TEMP ENDOF
  14 OF 4 FETCH.TEMP ENDOF
  15 OF 5 FETCH.TEMP ENDOF
  16 OF 6 FETCH.TEMP ENDOF
  17 OF 7 FETCH.TEMP ENDOF
  18 OF ENDOF
  19 OF EMPTY.TEMP ENDOF
ENDCASE ;
TEMP.MENU
2000 2 1ARRAY NEWSPEC.BUFFER

:3.MID.PT.AVG
  1999 1 DO I SPEC.BUFFER W@
    I 1- SPEC.BUFFER W@
    I 1+ SPEC.BUFFER W@
    + + 3 /
    I NEWSPEC.BUFFER W!
  LOOP ;

:3.SMOOTH.SPEC 3.MID.PT.AVG
  2000 0 DO I NEWSPEC.BUFFER W@

```

I SPEC.BUFFER WI LOOP  
DISPLAY.DATA ;

: 7.MID.PT.AVG

```
1996 4 DO I SPEC.BUFFER W@
    I 1- SPEC.BUFFER W@
    I 2- SPEC.BUFFER W@
    I 3- SPEC.BUFFER W@
    I 1+ SPEC.BUFFER W@
    I 2+ SPEC.BUFFER W@
    I 3+ SPEC.BUFFER W@
    + + + + + 7 /
    I NEWSPEC.BUFFER WI
    LOOP ;
```

: 7.MID.PT.SMOOTH

```
7.MID.PT.AVG
2000 0 DO
    I NEWSPEC.BUFFER W@
    I SPEC.BUFFER WI
    LOOP
    DISPLAY.DATA ;
```

: 7.SMOOTH.SPEC 7.MID.PT.SMOOTH ;

: 15.MID.PT.SMOOTH

```
1992 9
DO I SPEC.BUFFER W@
    I 1+ SPEC.BUFFER W@
    I 2+ SPEC.BUFFER W@
    I 3+ SPEC.BUFFER W@
    I 4+ SPEC.BUFFER W@
    I 5+ SPEC.BUFFER W@
    I 6+ SPEC.BUFFER W@
    I 7+ SPEC.BUFFER W@
    I 1- SPEC.BUFFER W@
    I 2- SPEC.BUFFER W@
    I 3- SPEC.BUFFER W@
    I 4 - SPEC.BUFFER W@
    I 5 - SPEC.BUFFER W@
    I 6 - SPEC.BUFFER W@
    I 7 - SPEC.BUFFER W@
    + + + + + 15 /
    I NEWSPEC.BUFFER WI
    LOOP ;
```

: 15.SMOOTH.SPEC 15.MID.PT.SMOOTH 2000 0 DO

I NEWSPEC.BUFFER W@ I SPEC.BUFFER WI LOOP DISPLAY.DATA ;

: 25.MID.PT.SMOOTH

1986 13

```

DO I SPEC.BUFFER W@
  I 1+ SPEC.BUFFER W@
  I 2+ SPEC.BUFFER W@
  I 3+ SPEC.BUFFER W@
  I 4+ SPEC.BUFFER W@
  I 5+ SPEC.BUFFER W@
  I 6+ SPEC.BUFFER W@
  I 7+ SPEC.BUFFER W@
  I 8+ SPEC.BUFFER W@
  I 9 + SPEC.BUFFER W@
  I 10 + SPEC.BUFFER W@
  I 11 + SPEC.BUFFER W@
  I 12 + SPEC.BUFFER W@
      + + + + + + + + + + 13 /
  I 1- SPEC.BUFFER W@
  I 2- SPEC.BUFFER W@
  I 3- SPEC.BUFFER W@
  I 4 - SPEC.BUFFER W@
  I 5 - SPEC.BUFFER W@
  I 6 - SPEC.BUFFER W@
  I 7 - SPEC.BUFFER W@
  I 8 - SPEC.BUFFER W@
  I 9 - SPEC.BUFFER W@
  I 10 - SPEC.BUFFER W@
  I 11 - SPEC.BUFFER W@
  I 12 - SPEC.BUFFER W@
      + + + + + + + + + + 12 /
      + 2 /

I NEWSPEC.BUFFER W!
LOOP ;

:25.SMOOTH.SPEC 25.MID.PT.SMOOTH 2000 0 DO
  I NEWSPEC.BUFFER W@ I SPEC.BUFFER W! LOOP DISPLAY.DATA ;

:SHOW.1.QUAD
  PAGE CLEAN.ZOOM
  499 0 DO
    I SPEC.BUFFER W@ 78 / 255 AND
    I 62 + SWAP DOT
  LOOP
    CLEAN LEGENDS XHATCHES YHATCHES ;

:SHOW.2.QUAD
  PAGE CLEAN.ZOOM
  499 0 DO
    I 499 + SPEC.BUFFER W@ 78 / 255 AND
    I 62 + SWAP DOT
  LOOP
    CLEAN LEGENDS XHATCHES YHATCHES ;

:SHOW.3.QUAD
  PAGE CLEAN.ZOOM
  499 0 DO

```



```

      I 999 + SPEC.BUFFER W@ 78 / 255 AND
      I 62 + SWAP DOT
    LOOP
      CLEAN LEGENDS XHATCHES YHATCHES ;

:SHOW.4.QUAD
  PAGE CLEAN.ZOOM
  499 0 DC
    I 1499 + SPEC.BUFFER W@ 78 / 255 AND
    I 62 + SWAP DOT
  LOOP
    CLEAN LEGENDS XHATCHES YHATCHES ;

( .....INTEGRATING ROUTINES.....)

2000 4 1ARRAY INTEGRATE.BUFFER1

2000 4 1ARRAY INTEGRATE.BUFFER2

0 VARIABLE BASELINE.VALA

0 VARIABLE BASELINE.VALZ

0 VARIABLE BASELINE.SLOPE

0 VARIABLE INTEGRAL

: ?BASELINE 0 INTEGRAL !
      0 BASELINE.VALA !
      40 20 DO I SPEC.BUFFER W@ BASELINE.VALA +I LOOP
      BASELINE.VALA @ 20 / BASELINE.VALA !
      0 BASELINE.VALZ !
      1940 1920 DO I SPEC.BUFFER W@ BASELINE.VALZ +I LOOP
      BASELINE.VALZ @ 20 / BASELINE.VALZ !
      BASELINE.VALZ @ BASELINE.VALA @ - 1000 * 1950 / BASELINE.SLOPE !
;

: INTEGRATE.PEAKS1 0 INTEGRAL !
      1949 0 DO I SPEC.BUFFER W@
      BASELINE.VALA @ I BASELINE.SLOPE @ * 1000 / +
      - INTEGRAL +I
      INTEGRAL @ I INTEGRATE.BUFFER1 I LOOP ;

: INTEGRATE.PEAKS2 0 INTEGRAL !
      1949 0 DO I INTEGRATE.BUFFER1 @ 1000 /
      INTEGRAL +I
      INTEGRAL @ I INTEGRATE.BUFFER2 I LOOP ;

: PLOT.INTEGRATION1
  CLEAN 0 0 MOVE.TO
  1950 0 DO I 250 + I INTEGRATE.BUFFER1 @ 10000 /
  DOT LOOP ;

```

```

:PLOT.INTEGRATION2
  CLEAN 00 MOVE.TO
  1950 0 DO I 250 + I INTEGRATE.BUFFER2 @ 10000 /
  DOT LOOP ;

:TOTALINTEGRAL 0 255 MOVE.TO ." Total Integral is " INTEGRAL @ . CR ;

:1INTEGRATOR ?BASELINE INTEGRATE.PEAKS1 PLOT.INTEGRATION1 ;

:2INTEGRATOR ?BASELINE INTEGRATE.PEAKS2 PLOT.INTEGRATION2 TOTALINTEGRAL ;

: RESCALE 10 - 2000 * 80 / ;

:WHICH.PEAK DROP SWAP DROP RESCALE SWAP RESCALE ;

: DISPLAY.VALUE CR ." Peak Value: " . ;

:PERCENT.VALUE ." % of Total: " . ;

:PEAK.VALUE WHICH.PEAK
  INTEGRATE.BUFFER2 @ SWAP INTEGRATE.BUFFER2 @ - ABS
  DUP DISPLAY.VALUE
    100 * INTEGRAL @ /
    PERCENT.VALUE
  ;

:FLAT.LINE WHICH.PEAK DUP SPEC.BUFFER W@ ROT ROT DO DUP I SPEC.BUFFER WI
  LOOP DROP DISPLAY.DATA ;

12 CONSTANT SPEC.NUMBER
:SPECTROMETRY.MENU SPEC.NUMBER DELETE.MENU
  0 " Analyze" SPEC.NUMBER NEW.MENU
" RETRIEVE SPECTRUM;CHANGE NORMALIZER;ADJUST BASELINE;-;" SPEC.NUMBER
APPEND.ITEMS
" AVERAGE [0->1];AVERAGE [0->3];AVERAGE [0->7];" SPEC.NUMBER
APPEND.ITEMS
" DIFFERENCE [0-1];-;(OVERLAY FILE;OVERLAY TEMP;-;" SPEC.NUMBER
APPEND.ITEMS
" 3 POINT SMOOTH;7 POINT SMOOTH;" SPEC.NUMBER
APPEND.ITEMS
" 15 POINT SMOOTH;25 POINT SMOOTH;-;(Y ZOOM %;" SPEC.NUMBER
APPEND.ITEMS
" SHOW.1.QUAD;SHOW.2.QUAD;SHOW.3.QUAD;SHOW.4.QUAD;" SPEC.NUMBER
APPEND.ITEMS
" OVERLAY ZOOM 4X" SPEC.NUMBER APPEND.ITEMS
DRAW.MENU.BAR
  SPEC.NUMBER MENU.SELECTION: 0 HILITE.MENU
    CASE 1 OF CR LINEAR.SPEC ENDOF
      2 OF CR DROP CHANGE.NORMALIZE -1 ENDOF
      3 OF CR DROP ADJUST.BASELINE -1 ENDOF
      4 OF CR ENDOF
      5 OF CR AVERAGE.SPEC.0-1 ENDOF

```

```

6 OF CR AVERAGE.SPEC.0-3      ENDOF
7 OF CR AVERAGE.SPEC.0-7      ENDOF
8 OF CR DIFFERENCE.SPEC        ENDOF
9 OF CR                        ENDOF
10 OF CR OVERLAY.FILE          ENDOF
11 OF CR OVERLAY.TEMP          ENDOF
12 OF CR                        ENDOF
13 OF CR 3.SMOOTH.SPEC          ENDOF
14 OF CR 7.SMOOTH.SPEC          ENDOF
15 OF CR 15.SMOOTH.SPEC         ENDOF
16 OF CR 25.SMOOTH.SPEC         ENDOF
17 OF CR                        ENDOF
18 OF CR Y.SCALE                ENDOF
19 OF CR SHOW.1.QUAD            ENDOF
20 OF CR SHOW.2.QUAD            ENDOF
21 OF CR SHOW.3.QUAD            ENDOF
22 OF CR SHOW.4.QUAD            ENDOF
23 OF CR OVERLAY.ZOOM           ENDOF

```

ENDCASE ;  
SPECTROMETRY.MENU

13 CONSTANT COMPUTE.NUMBER  
: COMPUTE.MENU COMPUTE.NUMBER DELETE.MENU  
0 " Comp" COMPUTE.NUMBER NEW.MENU

" g-Value;-;"

COMPUTE.NUMBER APPEND.ITEMS

" 1 Quad g-value;2 Quad g-value"

COMPUTE.NUMBER

APPEND.ITEMS

" 3 Quad g-value;4 Quad g-value;-;"

COMPUTE.NUMBER

APPEND.ITEMS

" LINE;FLATLINE;-;"

COMPUTE.NUMBER APPEND.ITEMS

" 1 INTEGRAL;2 INTEGRAL;"

COMPUTE.NUMBER APPEND.ITEMS

" PEAK INTEGRAL VALUE;-;"

COMPUTE.NUMBER APPEND.ITEMS

" 1000 pt FILE;2000 pt FILE"

COMPUTE.NUMBER APPEND.ITEMS

DRAW.MENU.BAR

COMPUTE.NUMBER MENU.SELECTION: 0 HILITE.MENU

```

CASE 1 OF DROP G.VALUE -1 ENDOF
      2 OF                ENDOF
      3 OF 1.QUAD.G.VALUE -1 ENDOF
      4 OF 2.QUAD.G.VALUE -1 ENDOF
      5 OF 3.QUAD.G.VALUE -1 ENDOF
      6 OF 4.QUAD.G.VALUE -1 ENDOF
      7 OF                ENDOF
      8 OF DROP LINE      -1 ENDOF
      9 OF DROP FLAT.LINE -1 ENDOF
      10 OF                ENDOF
      11 OF 1INTEGRATOR    ENDOF
      12 OF 2INTEGRATOR    ENDOF
      13 OF DROP PEAK.VALUE -1 ENDOF
      14 OF                ENDOF

```

```

15      OF FILE.DATA.1000      ENDOF
16      OF FILE.DATA.2000      ENDOF
ENDCASE ;
COMPUTE.MENU

NEW.WINDOW COMPUTE.WINDOW
  " Computation Window" COMPUTE.WINDOW W.TITLE
  40 10 335 525      COMPUTE.WINDOW W.BOUNDS
  CLOSE.BOX SIZE.BOX + COMPUTE.WINDOW W.ATTRIBUTES
  SYS.WINDOW      COMPUTE.WINDOW W.BEHIND

COMPUTE.WINDOW ADD.WINDOW

:COMPUTATION.PROGRAM
  CR @MOUSEXY 1 PICK . 2 PICK . ;

:KEYSTROKE.PROGRAM
  CR ." OK IT JUST HAPPENED" ;

:ACTIVATE.COMPUTE
  IF
    BEGIN
      DO.EVENTS
      CASE MOUSE.DOWN OF COMPUTATION.PROGRAM ENDOF
        ?KEYSTROKE IF
          KEYSTROKE.PROGRAM THEN
            ENDCASE
      AGAIN
    ELSE ." COMPUTATION PROGRAM CLOSED"
      THEN ;

COMPUTE.WINDOW ON.ACTIVATE ACTIVATE.COMPUTE

CREATE (STOP1) 1 ,

: (OPEN.SERIAL1) ( -- | opens serial driver if it is not already )
  serial.in not
  IF ".BIN" open$ ' serial.in w! \ use .AIN for modem port
    ".BOUT" open$ ' serial.out w! \ use .AOUT for modem port
  THEN ;

: OPEN.SERIAL1 ( ADDR\CNT -- | Opens pair of file#'s
  ( For serial port A. Addr\cnt specifies buffer )
  swap
  (open.serial1) \ make sure serial drivers are open

  serial.in 9 sp@ 8+ fscontrol 2drop \ equivalent to SerSetBuf
  serial.options 2@ serial.in 10 sp@ 8+
  fscontrol 2drop ; \ equivalent to SerHShake ( set handshake options

: BAUD1 ( baud.rate -- | does all the work in setting up )
  ( Before using serial ports, should call 300 BAUD, 1200 BAUD, or whatever)

```

```

(baud) !
input.buffer input.size open.serial1      \ initialize the serial drivers
(stop1) @ (parity) @ (bits) @ (baud) @ serial.in
setup.serial ;                          \ make the settings

: RESET.PLOTTER 0 ' serial.in w! 0 ' serial.out w! ;

VARIABLE XSTART
VARIABLE X RANGE

CREATE TITLE$ ," 123456789012345678901234567890"
CREATE ON$ ," 1." 27 ON$ 1+ CI
CREATE OFF$ ," 1.)" 27 OFF$ 1+ CI
CREATE X.ON$ ," 1.180;;17:" 27 X.ON$ 1+ CI
CREATE X.OFF$ ," 1.N;19:" 27 X.OFF$ 1+ CI

: FETCH 8 ASK.NUMBER DROP ;

: PLOT.INFO
  SETTING.STUFF +FIELD @ SETTING.STUFF +SCAN @ 2 / - XSTART !
  SETTING.STUFF +SCAN @ X RANGE !
  CR ." Record#?" TITLE$ 30 INPUT.STRING ;

: P.TYPE COUNT S.TYPE ;

: P.SCALE.DATA " IN;SC -250,2250,-2480,22480,PA;" P.TYPE ;

: P.SCALE.GRID " IN;SC -250,2250,-32,288,PA;" P.TYPE ;

: GRID P.SCALE.GRID
  " PU 0,0,PD,2000,0,2000,255,0,255,0,0;" P.TYPE ;

: CHECK BEGIN S.?READY TRUE = UNTIL ;

: ON.PLOTTER
  1 SERIAL.OPTIONS CI
  17 SERIAL.OPTIONS 2+ CI
  19 SERIAL.OPTIONS 3+ CI
  1 SERIAL.OPTIONS 6 + CI
  RESET.PLOTTER 9600 BAUD1
  ON$ P.TYPE X.ON$ P.TYPE X.OFF$ P.TYPE 17 S.EMIT ;

: OFF.PLOTTER OFF$ P.TYPE
  0 SERIAL.OPTIONS CI
  100 SERIAL.FILE# !
  0 ' SERIAL.IN W!
  0 ' SERIAL.OUT W!
  9600 BAUD ;

: LEFTPEN " SP1;" P.TYPE ;

: RIGHTPEN " SP2;" P.TYPE ;

```

```

: RETURNPEN " PU,SP0;" P.TYPE ;

: ENDLABEL 3 S.EMIT ;

: XDIR " DR 1,0;" P.TYPE ;
: YDIR " DR 0,1;" P.TYPE ;

: XINC X RANGE @ 10 / ;

: XTICKS
  10 1+ 0 DO CHECK
    2000 XINC * I * X RANGE @ /
    " PU" P.TYPE STR$ S.TYPE " ,0,XT;" P.TYPE

    XSTART @ XINC I * +
    " CP -1,-1;LB" P.TYPE STR$ S.TYPE ENDLABEL
  LOOP ;

: XLABEL
  " PU 1000,0" P.TYPE
  " CP -9.5,-2.7;LB H (gauss)" P.TYPE ENDLABEL ;
: YLABEL
  " PU 0,127" P.TYPE
  " CP -6.5,3.6;LB dx/dH" P.TYPE ENDLABEL ;

: HEADING
  YDIR TITLE$ COUNT
  " PU -240,190;" P.TYPE " LBRECORD # " P.TYPE S.TYPE ENDLABEL XDIR ;

: LABEL YDIR YLABEL CHECK XDIR XLABEL CHECK HEADING ;

: PLOTDATA
  P.SCALE.DATA
  " PA PU,0,0,PD;" P.TYPE
  1950 0 DO CHECK
    I SPEC.BUFFER W@
    I
    " PD" P.TYPE STR$ S.TYPE " ," P.TYPE STR$ S.TYPE " ," P.TYPE
  LOOP ;

: FAST.PLOTDATA
  P.SCALE.DATA
  " PA PU,0,0,PD;" P.TYPE
  1950 0 DO CHECK
    I SPEC.BUFFER W@
    I
    " PD" P.TYPE STR$ S.TYPE " ," P.TYPE STR$ S.TYPE " ," P.TYPE
  8 +LOOP ;

: MANUAL.PLOTDATA
  " DF;SC 0,2000,0,20000,PA;" P.TYPE
  " PA PU,0,0,PD;" P.TYPE
  1950 0 DO CHECK

```

```

I SPEC.BUFFER W@
I
" PD" P.TYPE STR$ S.TYPE " ," P.TYPE STR$ S.TYPE " ;" P.TYPE
LOOP ;

:PLOT.GRID
PLOT.INFO ON.PLOTTER LEFTPEN GRID XTICKS
LABEL RETURNPEN ;

:PLOT.FROM.BUFFER
PLOT.GRID ON.PLOTTER LEFTPEN PLOTDATA RETURNPEN ;

:PLOT.POINTS-L ON.PLOTTER LEFTPEN PLOTDATA RETURNPEN ;

:PLOT.POINTS-R ON.PLOTTER RIGHTPEN PLOTDATA RETURNPEN ;

:PLOT.FROM.BUFFER.FAST
PLOT.GRID ON.PLOTTER LEFTPEN FAST.PLOTDATA RETURNPEN ;

:PLOT.POINTS-L.FAST ON.PLOTTER LEFTPEN FAST.PLOTDATA RETURNPEN ;

:PLOT.POINTS-R.FAST ON.PLOTTER RIGHTPEN FAST.PLOTDATA RETURNPEN ;

:MANUAL ON.PLOTTER LEFTPEN MANUAL.PLOTDATA RETURNPEN ;

15 CONSTANT PLOT.NUMBER
:PLOT.MENU PLOT.NUMBER DELETE.MENU
0 " Plot " PLOT.NUMBER NEW.MENU
" PLOT FROM BUFFER;PLOT POINTS-L" PLOT.NUMBER
APPEND.ITEMS
" PLOT POINTS-R;-(;PLOT FROM BUFFER FAST" PLOT.NUMBER
APPEND.ITEMS
" PLOT-L FAST;PLOT-R FAST;-(;MANUAL PLOT" PLOT.NUMBER
APPEND.ITEMS
DRAW.MENU.BAR
PLOT.NUMBER MENU.SELECTION: 0 HILITE.MENU
CASE 1 OF PLOT.FROM.BUFFER ENDOF
2 OF PLOT.POINTS-L ENDOF
3 OF PLOT.POINTS-R ENDOF
4 OF ENDOF
5 OF PLOT.FROM.BUFFER.FAST ENDOF
6 OF PLOT.POINTS-L.FAST ENDOF
7 OF PLOT.POINTS-R.FAST ENDOF
8 OF ENDOF
9 OF MANUAL ENDOF
ENDCASE OFF.PLOTTER ;
PLOT.MENU

```