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STRUCTURAL AND PHOTOCHEMICAL PROPERTIES OF THE PHOTOSYSTEM II CORE COMPLEX ISOLATED FROM SPINACH CHLOROPLASTS

The Ohio State University

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ABBREVIATIONS

1. Acryl/bis-acryl  acrylamide/N,N'-methylene-bis-acrylamide
2. Chl  chlorophyll
3. Chl a/b  ratio of chlorophyll a to chlorophyll b
4. CMC  critical micelle concentration
5. Cyt b-559  cytochrome b-559
6. DCIP  dichlorophenol-indophenol
7. DCMU  3-(3',4'-dichlorophenyl)-1,1-dimethylurea
8. DPC  diphenylcarbazide
9. I  light intensity
10. KAVE  distribution coefficient
11. kD  kilodalton
12. kD  rate constant for dark electron transport
13. kI  rate constant for light processes
14. LHC  light-harvesting complex
15. LiDS  lithium dodecylsulfate
16. PS I  photosystem I
17. PS II  photosystem II
18. P680  reaction center of photosystem II
19. P700  
20. SDS-PAGE  
21. TEMED  
22. Tris  
23. TSF-II  
24. TSF-IIa  
25. v  
26. WSC  

reaction center of photosystem I  
sodium dodecylsulfate-polyacrylamide gel electrophoresis  
N,N,N',N'-tetramethylethylene-diamine  
Tris (hydroxymethyl)aminomethane  
Triton X-100 subchloroplast fragment photosystem II  
Triton X-100 subchloroplast fragment photosystem II containing only chlorophyll a  
reaction rate  
water-soluble carbodiimide
INTRODUCTION

The process by which green plants utilize light energy to make carbohydrates from carbon dioxide and water, with oxygen evolved as a byproduct, was initially investigated two centuries ago. In 1780, Joseph Priestly demonstrated that plants evolved a substance which restored bad air (122). Thus the production of oxygen by plants was to be the first element of the mechanism of photosynthesis to be discovered. The discovery of the other basic components of the process followed in short order. The discovery of the need for light in photosynthesis by Jan Ingenhousz, and the consumption of CO₂ and water during the photosynthetic process, discovered by Jean Senebier and Theodore de Saussure, respectively, was accomplished by the end of the eighteenth century (122). Their investigations led to the establishment of the basic equation describing the photosynthetic process:

\[ \text{H}_2\text{O} + \text{CO}_2 + \text{light} \rightarrow (\text{CH}_2\text{O}) + \text{O}_2 \]

It was not until the twentieth century, however, that the true complexity of the photosynthetic process became clear. The overall process by which carbohydrate is
produced can be subdivided into two parts. One part consists of a series of chemical reactions whereby \( \text{CO}_2 \) is incorporated into stable organic compounds, as delineated by Calvin and his coworkers (13). The other part of photosynthesis consists of a series of light-initiated oxidation-reduction reactions which result in the consumption of water, the evolution of oxygen, and the production of NADPH and ATP. It is this latter part of photosynthesis, the light-dependent events, which will be the concern of the rest of this introduction.

**Chloroplast Structure**

The site of photosynthetic activity in higher plants resides within a subcellular organelle called a chloroplast. The chloroplast, as indicated by Figure 1, is comprised of a complicated system of inner membranous vesicles (thylakoid membranes), two separate aqueous phases, and a surrounding double-membrane envelope (6). The envelope membrane is generally regarded as being a passive mediator of chloroplast metabolites, however, it may function in the biosynthesis of the inner membrane system (6, 11). The aqueous phase, called the matrix or stroma, contains a vast array of soluble components, including the enzymes and organic intermediates of the Calvin cycle, RNA, DNA, and ribosomes, as well as insoluble inclusions of starch, lipid, and pigment precursors. The thylakoid membrane contains the entire apparatus for the light-driven events of
Figure 1

A schematic representation of a spinach chloroplast.
photosynthesis, including the components for oxygen evolution, ATP synthesis, NADPH production, and the pigment-protein complexes which are responsible for the collection of light. The activities of the inner aqueous phase, called the loculus, are unknown. This region may simply function as a repository for protons generated during the light dependent phase of photosynthesis (11).

The thylakoid membranes of higher plant chloroplasts are generally organized into ordered stacks of membranes, called grana, in which many individual thylakoid units are arranged on top of each other. These grana stacks are interconnected by membranous regions called stroma lamellae (6,100). The stroma lamellae are continuous with numerous thylakoids within the same grana stack and with thylakoids in different grana stacks (6,100). The particular structure of these membrane regions may in fact reflect functional aspects of the membrane components.

About half of the thylakoid membrane mass consists of lipids and the other half of protein (92). Of the lipid fraction, mono- and digalactosyldiglycerides represent about 40% of the total lipid content, with the remainder being comprised of chlorophylls (20%), phospholipids, and quinones (141). The protein portion of the membrane can be subdivided into two groups. Extrinsic membrane proteins, such as coupling factor (which catalyzes ATP synthesis), are easily removed from the surface of the membrane by high
salt concentrations or cation chelating agents. Intrinsic or integral membrane proteins, which include most of the electron transport components and the pigment-protein complexes, are securely bound within the membrane and released only after physical or chemical disruption of the membrane. Components of the Electron Transport System

A schematic representation of the components currently believed to be involved in photosynthetic linear electron transport is given in Figure 2. Two light reactions, mediated by photosystem I (PS I) and photosystem II (PS II), function in series to promote electron flow from water (the electron donor) to NADP⁺ (the electron acceptor) against an electrochemical gradient (37,38,104). The two photosystems, comprised of chlorophyll-protein complexes, absorb and transfer incident light energy to a specialized pair of chlorophyll a molecules (P680 for PS II and P700 for PS I) which excites them to transfer an electron to their respective acceptor molecules, Q and X, for PS II and PS I. In turn, Q, believed to be a plastoquinone molecule in a specialized environment (72), transfers its electron sequentially to a pool of plastoquinone molecules, cytochrome f, plastocyanin, and oxidized P700 (38). Likewise, X transfers its electron sequentially to ferredoxin, ferredoxin-NADP⁺ reductase, and finally NADP⁺. P680⁺, on the other hand, oxidizes water to give oxygen through a
Figure 2

A schematic representation of the linear electron transport system in the thylakoid membrane. The symbols are described in the text.
\[ \text{H}_2\text{O} \rightarrow \text{Z} \rightarrow \text{PSII} (\text{P680}) \rightarrow \text{PQ} \rightarrow \text{Cyt f} \rightarrow \text{PC} \rightarrow \text{PSI} (\text{P700}) \]
series of unknown steps (38). This series of electron transport steps not only results in the reduction of NADP⁺ but also generates a proton gradient across the thylakoid membrane which is believed to be the driving force behind ATP synthesis.

A great deal of evidence supports this concept of electron transport in higher plant photosynthesis (37, 38, 100, 104). The evidence is generally biophysical, biochemical, or genetic in nature. The biophysical evidence demonstrates the existence of two separate photosystems by measuring the wavelength dependency of the photosynthetic process. Illumination of chloroplasts at long wavelengths of light (\( \lambda > 685 \)) results in a decrease in the efficiency of the photosynthetic process. Additional illumination with shorter wavelengths of light results in a synergistic increase in the photosynthetic rate (33, 104). This enhancement effect is explained by postulating that the two photosystems are connected in series. Therefore, the efficiency of the process is limited by the rate of flow of electron through both PS I and PS II. Similarly, the reversible oxidation-reduction of cytochrome f, the extent of which depends on the wavelength of light (either preferentially PS II or PS I), supports the concept of two photosystems working in series. Genetics has been particularly useful in identifying components of the electron transport system. By selecting mutants lacking, or defective in, a single
component of the pathway, the identity or necessity of these components have been determined. The biochemical approach of disruption, purification and characterization of components have substantiated and extended the findings derived from the other two methods, and is the approach used to study PS II that is described in this dissertation.

**Pigment-Protein Complexes**

The existence of several distinct chlorophyll-protein complexes has been recognized for more than a decade (57, 128). The first evidence that chlorophyll was associated with specific proteins in the membrane came from studies employing electrophoresis of SDS-solublized chloroplasts (73,127,128). Chloroplasts solublized with SDS were separated into three distinct chlorophyll bands on polyacrylamide gels. The band of lowest electrophoretic mobility contained only chlorophyll a and a polypeptide with an apparent molecular weight of 70,000 (73,127). The second chlorophyll band contained equal amounts of chlorophyll a and b, and a polypeptide with a molecular weight of 25,000 (73, 127). The fastest moving band was found to be detergent-complexed, protein-free chlorophyll and carotenoids (73, 127). The supposition that the chlorophyll is actually associated with these proteins, not simply artifactually co-migrating with them, has been recently explicitly substantiated (4). Although neither chlorophyll-protein complex was photochemically active, it has been established by
various methods that the band of lowest mobility contained P700 and therefore was associated with PS I, while the second chlorophyll band represented a chlorophyll-protein complex which served in only a light-harvesting capacity for PS II (therefore, called the light-harvesting pigment-protein complex, or LHC). More recently this technique has been refined to such an extent by optimizing the solubilizing conditions, lowering the temperature, and shortening the electrophoresis time, that more than a half-dozen chlorophyll-protein bands have been resolved on SDS-gels (1,21,24,31,45,46,48,49,50,73,81,82,83). Most of these extra bands simply represent oligomeric forms of PS I and LHC, but at least one chlorophyll a band appears to represent the chlorophyll-protein containing P680. The apoprotein of this apparent PS II reaction center polypeptide has a molecular weight in the 40,000 to 50,000 range (48,49).

The photosystem I chlorophyll a-protein complex has been isolated in a photochemically active form from chloroplasts by several different techniques. These techniques employ either physical methods of fractionation of chloroplasts such as sonication or French press (57,100,137), or detergent solubilization with Triton X-100 (56,100,120) or digitonin (113,116,136). Following detergent solubilization, the extracts are further fractionated by sucrose density gradient centrifugation (113,136) or hydroxylapatite chromatography (120).
Similarly, homogenous preparations of the light-harvesting complex have been obtained by solublizing chloroplasts with SDS (73,128), Triton X-100 (128), or digitonin (116,136), followed by chromatography on hydroxylapatite (73) or DEAE-cellulose (136), or by sucrose gradient centrifugation (116,136).

The physical properties and chemical compositions of these two chlorophyll-protein complexes have been extensively studied and reviewed (128,129).

Isolation of the PS II chlorophyll-protein complex (or core complex) has proven to be more difficult. Generally, mild fractionation procedures result in the co-isolation of both the LHC and the PS II core complex. This finding has been used in fact to support other evidence that the LHC is physically associated with the PS II core complex in the membrane. More rigorous solublization techniques however destroy the PS II photochemical activity. In spite of these difficulties, photochemically active preparations of the PS II core complex have been obtained using either digitonin (113,117,136) or Triton X-100 (133,134). These PS II particles contain chlorophyll a and β-carotene as well as smaller amounts of other lipids and quinones. They catalyze the reduction of DCIP, an artificial electron acceptor, but require the presence of an artificial electron donor such as diphenylcarbazide (DPC) to provide electrons to the complex (a function normally provided by the
water-splitting apparatus in chloroplasts).

PS II particles prepared using Triton X-100 (133,134) are obtained by initially fractionating chloroplast membranes with high concentrations of the detergent. A membrane fragment highly enriched in PS II is pelleted by centrifugation at 144,000 x g for one hour. The sediment, designated TSF-II (Triton X-100 subchloroplast fragment containing PS II), contains both the LHC and PS II core complex. The PS II core complex, designated TSF-IIa, is then isolated from TSF-II by further centrifugation and subsequent chromatography on Bio-glas (porous glass) or sucrose gradient centrifugation. When fractionated on sucrose gradients, the particles are separated into three chlorophyll-containing bands (133). The band of lowest density, called TSF-IIa', contains only chlorophyll a but is photochemically inactive. The band of highest density, called TSF-IIb, is similar in structure and composition to the original TSF-II fraction. The middle chlorophyll band (almost entirely chlorophyll a) has a very high PS II activity and is devoid of PS I or LHC contamination. A similar TSF-IIa fraction (photochemically active) is obtained using Bio-glas (134).

Properties of TSF-IIa Particles

The physical composition and spectroscopic properties of TSF-IIa particles have been extensively investigated. The particles contain chlorophyll a and β-carotene, as well
as small amounts of other carotenoids (lutein, violoxanthin, neoxanthin) and plastoquinone (133). The content of manganese is very high (approx. 10 Chl's/Mn) (64). Under the electron microscope the TSF-II complex appears as discrete particles 11 to 12 nm in diameter, but displays a great tendency to form larger aggregates (133). SDS-gel electrophoresis of the particles indicates that they are composed of polypeptides with molecular weights of 63-, 51-, 44-, 34-, 29-, 24-, and 20 kD (68,69). The photochemical activity of the particles is inhibited by DCMU, a common PS II inhibitor, but requires higher concentrations than that needed to inhibit chloroplasts to a similar extent (91,133,134). The activity is also easily inactivated by heat (133).

The TSF-IIa particles also contain cytochrome b-559. Although this cytochrome is known to be associated with PS II activity in chloroplasts, it does not serve on the main electron transport chain. Its function in chloroplasts is still a mystery (25). In the particles, Cyt b-559 is present largely in the oxidized state, and it can be photo-reduced and oxidized by the particles (63,133,147). Potentiometric titrations indicate that the cytochrome present in the particles has a lower redox potential (approx. +60 mV) than in intact chloroplasts (+350 mV) (25,34). There appears to be one cytochrome b-559 molecule for each P680 in the particles based on the chlorophyll to Cyt b-559 ratio (about 40) (63,66).
TSF-IIa particles have a red absorption maximum at 673 nm and a fluorescence maximum at 685 nm (133). The particles also display delayed luminescence (91). Delayed luminescence is believed to result from the charge recombination of the primary donor and acceptor (P680⁺ Q⁻) of photosystem II in chloroplasts. In TSF-IIa particles, delayed light in the 0.5 to 10 ms time range has been found to be diminished by DCMU and 0-phenanthroline (an inhibitor of bacterial reaction centers) (91). Similarly, delayed luminescence from the particles is affected by temperature and the presence of electron donors and acceptors (91). In this regard, the TSF-IIa particles demonstrate many of the characteristics of photosystem II present in the chloroplast membrane.

Spectroscopic studies have indicated that the reaction center of TSF-IIa particles is designed very much like that of bacterial reaction centers. Light-minus-dark difference spectra demonstrated the presence of a chlorophyll cation radical (P680⁺) (64), implicating P680 as the primary electron donor. The primary acceptor has been tentatively identified as being a plastoquinone molecule (70,72). The plastoquinone molecule serves as a one electron carrier. As with bacterial reaction centers, a pheophytin intermediate and a functionally active carotenoid molecule have been identified in the TSF-IIa particles (70,71,85,121).
Clearly, a great deal is known about the properties of the TSF-IIa particles, especially that knowledge which can be gained by spectroscopic studies, yet little is known about their actual structural or physical (biochemical) properties. Although the polypeptide composition of these particles has been reported, no rigorous attempt has been made to substantiate that all of these polypeptides are actually part of the complex. Furthermore, neither the composition of each polypeptide (such as chlorophyll or plastoquinone) nor the arrangement of polypeptides in the complex have been investigated. It is to these questions that parts of this dissertation are addressed.

Cation Effects in Chloroplasts and Subchloroplast Particles

An overwhelming body of evidence has been accrued implicating a major role for mono- and divalent cations in the regulation of the entire photosynthetic process. Ion fluxes across the thylakoid membrane appear to influence Calvin cycle activities, ATP synthesis, and the primary photochemical processes. For an excellent review of all aspects of ionic regulation of photosynthesis the reader is referred to reference (10).

Although many of the details as to how cations affect individual components of the photosynthetic process are known, an overall understanding of how all of these individual effects come together to regulate photosynthesis is not available. Much of the problem arises from the fact that
most studies are performed on chloroplasts that have had their outer envelopes either damaged or completely removed during isolation. Studies performed with broken chloroplasts or thylakoid membranes therefore require the substitution of an artificial buffer medium for the native stroma. The artificial buffer conditions do not necessarily reflect native conditions and may induce spurious cation effects. Relatively recently, however, procedures have been devised by which completely intact chloroplasts can be obtained (135). The results obtained using thylakoid membranes need to be reconciled with the physiological status of cations, such as their distribution, concentrations, and movements, in the intact chloroplast.

Studies on the effects of cations on the primary processes of photosynthesis deal with the relationship between proton accumulation in the loculus and the concomitant movement of other ions (32,51) as well as cationic regulation of the distribution of absorbed light between the two photosystems (12,19,28,40,43,54,59,75,79,86,90,99,143,144,146). In the light, proton pumping, resulting from electron transport, can be seen as an alkalization of the suspending medium. In intact chloroplasts this amounts to a ΔpH across the thylakoid membrane of 2.5 units, with the loculus having a pH of 5.4 and the stroma a pH of 7.9 (11,51). The protons deposited in the loculus are bound to negative charges on
the membrane, probably proteins (11). Associated with the influx of protons, there appears to be an efflux of Mg\(^{2+}\) ions resulting in a significant increase in the stromal concentration of Mg\(^{2+}\) (51). The thylakoid membranes also undergo a considerable structural rearrangement in response to presence of cations (7, 41, 94, 97, 131, 142). This rearrangement includes the formation of grana stacks, shrinkage of the intrathylakoid compartment, and possibly even the reorganization of the photosystems within the membrane (11, 12, 92, 95).

Cationic regulation of electron transport in thylakoid membranes is directed at optimizing the rate of electron flow from water to NADP\(^+\). This is accomplished by controlling the distribution of absorbed quanta between PS I and PS II. Such a need arises from the fact that the absorption cross-section (the amount of chlorophyll) associated with each photosystem is unequal (20). As already indicated, the LHC, which accounts for approximately half of the total chlorophyll in the membrane, is primarily coupled to PS II. This tends to make the P680 turnover rate faster than the P700 turnover rate. Furthermore, the wavelengths of light used by each photosystem are slightly different (34, 78, 79, 87). Red light is absorbed by PS II more strongly than PS I, while the reverse is true for far red light. To achieve equal P680 and P700 turnover rates under different lighting conditions, the
amount of absorbed quanta which are directed to each reaction center must be controlled. This appears to be the function of metal cations.

Because of the vast array of studies, many results of which are conflicting, it is difficult to simply state the effect of cations in electron transport and energy distribution. However, the general consensus of these studies is that, in the presence of light absorbed preferentially by PS II, a portion of the light absorbed by the LHC is directed to PS I. This process is called spillover. In the presence of PS I preferential light, the energy is directed instead to the PS II core complex. Low concentrations of Mg$^{2+}$ ions (2-5 mM) or much higher concentrations of monovalent cations (100 mM) inhibit spillover. The effects of salts on the distribution of energy correlate with the observed effects of cations on the turnover rates of the individual photosystems (9,42,58,84,96,98).

As already indicated, accompanying the fluxes of ions and the redistribution of absorbed energy between photosystems, there are drastic cation-induced structural rearrangements of the thylakoid membrane. Under low salt conditions the thylakoids are not organized into grana. Addition of Mg$^{2+}$ or Na$^+$ causes the membranes to stack. This suggests that the cation-induced structural rearrangement of the thylakoid membranes is responsible for the observed cation effects on energy distribution. Supporting
this conclusion is the evidence that glutaraldehyde fixation of the membrane prevents both the cation-induced energy redistribution and structural rearrangement (58,90). However, this is not to suggest that all of the evidence supports the concept that conformational changes in the thylakoid membrane are related to energy distribution. Under some conditions, such as low pH, membrane stacking can be induced without the concomitant changes in energy distribution (12,36,89).

Gross and her colleagues, and others (3,18,77), have provided considerable evidence implicating the site of divalent cation action as being the LHC. Gross et al. found that thylakoid membranes contain at least two distinct binding sites for divalent cations (41). Covalent modification of these binding sites prevented the binding of cations to the membrane (102). The covalent modification also prevented membrane stacking and the fluorescence changes indicative of cation-induced inhibition of spillover (102). They further showed that the majority of the covalent modifier was incorporated into the LHC (103).

Cations also appear to affect the energy distribution in, and structural state of, the isolated pigment-protein complexes. In Photosystem I particles it has been found that mono- and divalent cations increase the rate of energy transfer from the antenna chlorophyll a molecules to P700 (39). Mg$^{2+}$ has also been shown to affect electron transport
in PS I particles (44). Davis and Gross have reported that the isolated LHC, like chloroplasts, has two distinct divalent cation binding sites (27,29). Cations also influence the aggregation state of the LHC (27,29). These studies support the concept that this protein plays an important role in grana stacking and the control of spillover in chloroplasts. Studies on isolated photosystem II particles (TSF-II), which retain the LHC, demonstrated two distinct cation effects; one was a specific divalent cation effect and the other was a ionic strength effect (27,30). Cations have been shown to regulate energy transfer and the quantum yield for photochemistry in these particles by acting on the LHC (30,55,148,149). All of these results taken together strongly indicate that the major site of cation effects resides in the LHC.

However, the other pigment-protein complexes may also be influenced by cations. As described above, isolated PSI particles show cation effects. Recent studies by Bose and Arntzen (16) and others (58,59,75,146) have suggested that the photosystem II core complex in chloroplasts is also responsive to cationic regulation. Furthermore, preliminary evidence by Davis et al. (30) has shown that cations also affect the activity of the isolated PS II core complex (TSF-IIa). These findings suggested that a closer examination of the effects of cations on the isolated PS II core complex was necessary. Such studies are described in this
Protease Treatment of Chloroplasts

Proteolytic digestion of chloroplast membranes has been used as a technique to study the organization of the photosynthetic apparatus. It has been used to determine both the orientation of membrane components as well as to assign functions to polypeptides by correlating the loss of specific activities with the digestion of certain polypeptides (105). Proteolytic treatment results in the inhibition of a number of photosynthetic functions. These include a loss of cation-induced inhibition of spillover (22,23,59), the loss of PS II sensitivity to DCMU (15,119,130), a change in redox potential of cytochrome b-559 (106), an inhibition of water oxidation activity (107,118), a loss of electron transport activity between the two photosystems (108,118), and the inhibition of ferredoxin-NADP⁺-reductase activity (106). However, the activities of the PS I and PS II reaction centers are not particularly affected (119). SDS-gel electrophoresis of membranes which have been treated with proteases indicates that most of the membrane proteins, except for the pigment-protein complexes, are susceptible to extensive proteolytic cleavage (88,124,125). This suggests that the pigment-protein complexes, unlike the other membrane proteins are largely buried within the membrane and therefore inaccessible to proteases.
In marked contrast to these studies, Jennings et al. (60) found that all of the polypeptides of the chloroplast membrane were digested by chymotrypsin, including the pigment-protein complexes. However, they found that both the LHC and PSI retained their normal electrophoretic mobilities and chlorophyll on SDS-gels under non-denaturing conditions. This indicated that although proteolytic treatment of the pigment-protein complexes resulted in their cleavage, the complexes retained their native structures. They concluded from this that chlorophyll stabilizes the structure of the protein-pigment complexes.

Obviously, proteolytic treatment of the isolated PS II core complex would provide valuable information as to the structural arrangement and proteolytic susceptibility of the polypeptides of the complex. Such experiments also may provide insight into the functions of the various polypeptides and possibly localize the photosynthetic activity to a particular polypeptide.

**Statement of Purpose**

As already indicated in various portions of the introduction, the system under study in this dissertation is the photosystem II core complex isolated from spinach chloroplasts. Because the photochemical processes in chloroplasts are modulated by cations, it is important to study how the photochemical activity of the isolated PS II core complex responds to the presence of ions. If the core
complex is responsive to cations, it is necessary to elucidate the biochemical basis of this effect. In the case of the isolated LHC, cations altered their aggregation state and absorption spectrum (27,29). In the case of isolated PS I particles, cations increased energy transfer within the complex apparently by changing the secondary structure of the complex (39). Obviously a similar analysis of the effects of cations on the structure of the PS II core complex is warranted. Specifically, the effects of protons, mono- and divalent metal cations on the photochemical activity of the complex as a function of illuminating light intensity will be determined. This technique will ascertain whether cations affect energy transfer or electron transport in these particles. Furthermore, the effects of cations on the apparent molecular weight of the complex will be determined by gel filtration.

In conjunction with these studies, the structures of the complex and the physical arrangement of its components will be analyzed. In particular, the polypeptide composition will be determined by SDS-gel electrophoresis of the complex after several different chromatographic steps. The structural arrangement of the polypeptides will be studied by proteolytic treatment and subsequent analysis of the activity and polypeptide composition of the complex.
These experiments should provide valuable insight into the structural and photochemical properties of the photosystem II core complex.
MATERIALS AND METHODS

Preparation of Chloroplast Lamellae

Chloroplast lamellae were prepared from approximately two pounds of washed and deveined spinach leaves. The leaves were ground in an isolation buffer consisting of 50 mM Tris HCl, pH 7.6, 350 mM sucrose for about 30 seconds in a Waring blender. The brei was pressed through several layers of cheesecloth and the lamellae were collected by centrifugation at 7,000 x g for 15 minutes in a Sorvall RC2-B centrifuge. The pelleted material was resuspended in a small volume (approx. 75 ml total) of the same buffer.

NaBr Wash of Chloroplast Lamellae

The chloroplast lamellae were washed twice with 2.0 M NaBr, following the procedure of Kamienietzky and Nelson (67), to remove coupling factor and other easily extractable proteins from the membranes. To the chloroplast lamellae suspension an equal volume of cold 4.0 M NaBr was added. The material was stirred for 30 minutes on ice then diluted with an equal volume of cold water. The lamellae were pelleted by centrifugation at 17,000 x g for 15 minutes. For complete removal of coupling factor, the membranes were resuspended in the isolation buffer and again subjected to the NaBr wash procedure. Finally, the washed membranes were freed of
residual NaBr by resuspension in isolation buffer and subsequent centrifugation at 17,000 x g for 10 minutes.

**Preparation of TSF-II**

The preparation of photosystem II particles may proceed from either chloroplast lamellae or NaBr washed chloroplast lamellae, with only minor differences. For experiments studying only the photochemical properties of the TSF-IIa particles, chloroplast lamellae were generally used. However, identical results were obtained with NaBr washed chloroplast lamellae. All studies involving structural aspects of PS II were performed with NaBr washed membranes.

TSF-II particles were prepared according to the method of Vernon et al. (134). Either chloroplast lamellae or NaBr washed lamellae were suspended in 50 mM Tris HCl, pH 8.0 and 0.5 M sucrose. The chlorophyll concentration was determined as described below and adjusted to 2.5 mg Chl/ml with buffer. Sufficient Triton X-100 was added to the suspension of lamellae to obtain a chlorophyll: Triton X-100 ratio of 40 mg Chl: 1 g. Triton X-100. In order to obtain a proper yield of TSF-II from NaBr washed membranes, the total amount of Triton X-100 added was decreased by 7.5%. The preparation was incubated with vigorous stirring for one hour. The solution was centrifuged for 5 minutes at 3,000 x g to remove unsolubilized material. The supernatant of the low speed centrifugation was subjected to a high speed centrifugation at 144,000 x g for 1 hour at 2°C in a Beckman L2-65B
preparative ultracentrifuge. The supernatant of this centri­
trifugation was discarded and the walls of the centrifuga­
tion tubes were rinsed with water. The pelleted material
(TSF-II) was stored in the tubes at -80° C until further use.

Preparation of TSF-IIa

The photosystem II core complex (TSF-IIa) was ob­
tained by suspending the TSF-II pellets in 5.0 mM Tris HCl, pH 8.0 containing 50 mM NaCl. The volume of buffer used was
approximately equal to that from which the material was
pelleted. The TSF-II fraction was gently resuspended with a
glass homogenizer (3-4 strokes) and then allowed to stand
for one hour at 2° C. After the incubation period, the
material was centrifuged at 144,000 x g for one hour. The
supernatant of this final centrifugation (TSF-IIa) was
carefully decanted. Occasionally, the supernatant was cen­
trifuged at high speed again for 30 minutes, if a portion
of the pellet decanted with the supernatant. If not used
immediately, the TSF-IIa particles were stored at -80° C.

Chromatography of TSF-IIa

The original procedure of Vernon et al. (133,134)
called for further purification of the TSF-IIa particles
on either sucrose gradients or absorption on Bio-glas -
1500. Generally, our preparations of TSF-IIa were suffi­
ciently pure after the differential centrifugation steps
so that these procedures could be omitted. However, when
particles of higher purity were required several different chromatographic procedures were employed. Ion-exchange chromatography of the TSF-IIa particles on aminoethyl cellulose was performed at 5°C in a column containing approximately 1.5 g. dry aminoethyl cellulose for each mg of chlorophyll applied. The column was initially washed with 20 mM Tris HCl, pH 8.2, containing 0.1% Triton X-100 and 0.5 M NaCl, then equilibrated with 20 mM Tris HCl, pH 8.2. The TSF-IIa particles were diluted 1:1 with water and applied to the column. The bound Chl fraction was washed successively with two column volumes each of 20 mM Tris HCl, pH 8.2 and 20 mM Tris HCl, pH 8.2, containing 0.5 M NaCl, then eluted with 20 mM Tris HCl, pH 8.2, containing 0.1% Triton X-100 and 0.5 M NaCl.

To remove Triton X-100 and salt from the TSF-IIa particles, the sample was passed through a Sephadex G-100 column (2.5 x 25 cm) at 4°C. The column was equilibrated with 5.0 mM Tris HCl, pH 8.2. All of the fractions containing chlorophyll were pooled and concentrated using an Amicon ultrafiltration cell with a XM-50 membrane. The Sephadex G-100 column was employed whenever the TSF-IIa particles were chromatographed on the aminoethyl cellulose column, but otherwise was used only as noted in the text of the Results and Discussion section.

Analytical gel filtration experiments were performed on either on a Sepharose CL-6B or CL-4B column (2.0 x 55 cm).
The column was eluted at a flow rate of 15 ml/h and 3.0 ml fractions were collected with a LKB Reditac Fraction Collector. Gel filtration was performed at 4°C unless otherwise noted in the text. The buffers used depended upon the nature of the experiment and will be detailed individually in the Results and Discussion section. The columns were calibrated with blue dextran (void volume), thyroglobulin (660,000), apoferritin (460,000), catalase (247,000), glucose oxidase (160,000), β-galactosidase (130,000), hexokinase (102,000), hemoglobin (64,000), and DNP-glycine (included volume). One and one-half milliliters of the TSF-IIa sample were loaded directly on the column without prior dialysis or concentration.

The elution volume was measured as the fraction number of the absorbance peak, times the fraction volume. The distribution coefficient ($K_{AVE}$) of each sample was calculated using the equation:

\[
\frac{\text{Sample Elution Vol.} - \text{Void Vol.}}{\text{Included Vol.} - \text{Void Vol.}} = K_{AVE}
\]

In experiments for which the elution conditions were such that the Chl was not localized about a single fraction but, rather, distributed between two distinct elution points (see figure 11 for example), the fraction of the total Chl associated with each elution peak was determined gravimetrically. The elution profile of Chl was plotted on graph paper, then the area under each peak was cut and weighed.
The distribution of Chl was expressed as the percentage of the weight of the individual peaks divided by the total weight of the elution profile.

Photochemical Assays

The DPC-dependent reduction of DCIP by TSF-IIa particles was assayed by measuring the absorption decrease at 600 nm. The standard assay medium consisted of 20 mM Tris-maleate, pH 6.0, containing 0.5 mM DPC and 25 μM DCIP. The sample chlorophyll concentration was kept between 1 and 2 μg/ml. Experiments performed to measure the activity of the TSF-IIa particles as a function of pH also used 20 mM Tris buffer, adjusted to the appropriate pH with concentrated maleic acid. The extinction coefficient at 600 nm of DCIP as a function of pH is listed in Table 1. Measurements of the effects of salts on PS II activity were performed in 5.0 mM Tris HCl, pH 8.0 plus the appropriate salt. Determinations of activity were made using an Aminco DW-2a spectrophotometer operated in the double-beam mode. Illumination of the sample compartment was provided by a projector lamp with a variable voltage supply. The output was passed through a water filter and a red cutoff filter (Corning CS-2-64). A 620 nm cutoff filter (Bausch and Lomb No. 90-1-620) protected the photomultiplier from the actinic light source. Light intensities were measured using a Kettering-Yellow Springs Instruments radiometer.
TABLE 1.

The Extinction Coefficient of DCIP vs. pH

<table>
<thead>
<tr>
<th>pH</th>
<th>E, mM⁻¹ cm⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.0</td>
<td>20.6</td>
</tr>
<tr>
<td>7.5</td>
<td>20.1</td>
</tr>
<tr>
<td>7.0</td>
<td>19.2</td>
</tr>
<tr>
<td>6.5</td>
<td>17.6</td>
</tr>
<tr>
<td>6.0</td>
<td>14.2</td>
</tr>
<tr>
<td>5.5</td>
<td>9.9</td>
</tr>
</tbody>
</table>

The reference extinction coefficient at pH 8.0 is from (17).
Data for activities as a function of light intensity were plotted in a double-reciprocal form as expressed by the equation:

\[
\frac{1}{V} = \frac{1}{k_D} + \frac{1}{k_L} \cdot I
\]

where \( V \) is the specific activity in \( \mu \text{mol DCIP reduced/mg Chl/h} \), \( I \) is the light intensity in \( \text{erg/cm}^2 \cdot \text{s} \), and \( k_D \) and \( k_L \) are reaction rate constants.

Photochemical assays of TSF-IIa particles immobilized on ethylenediamine Sepharose were performed using the Aminco DW-2a spectrophotometer operated in the dual-wavelength mode of operation. The measuring and reference wavelengths were 600 nm and 500 nm, respectively. The activity was calculated using a corrected extinction coefficient for DCIP of 10.0 at pH 6.0. Assays were conducted in a 1 x 1 cm stirred cuvette. The rates were corrected for the rate of DCIP reduction by DPC which occurred in the dark. Otherwise the filter arrangement and assay buffer conditions were as for the standard assay procedure.

The photoreduction and oxidation of cytochrome b-559 was also measured using the dual-wavelength mode of operation. The conditions used were adapted from those of others (63,74). The measuring and reference wavelengths were 559 nm and 570 nm, respectively. Absolute absorbance changes were calculated using an extinction coefficient of 21.0 \( \text{mM}^{-1} \cdot \text{cm}^{-1} \) (34). The assay buffer consisted of 10 mM Tris HCl, pH 6.0, 0.5 mM DPC, and chlorophyll at a concentration
of 25 to 35 μg/ml. The Triton X-100 was removed from the
TSF-IIa sample by gel filtration prior to assaying. The
light intensity was $1 \times 10^5$ erg/cm$^2 \cdot$ s provided through
the same filter combination as described above.

The P700 content of the TSF-IIa particles was mea-
sured using the method described by Gross (44). The spec-
trophotometer was operated in the double-beam mode of oper-
ation with 700 nm as the measuring wavelength. A Corning
4-96 filter and Bausch and Lomb 90-1-560 interference filter
were used to isolate the actinic light. A Schott 695 nm
cutoff filter protected the photomultiplier from stray
actinic light. An extinction coefficient of $64$ mM$^{-1}$ cm$^{-1}$
was used to calculate the P700 content (52). The assay
buffer contained 5.0 mM Tris HCl, pH 8.2, and 4.0 mM sodium
ascorbate with 20 to 40 μg/ml of chlorophyll.

**Absorption Spectra**

Visible absorption spectra were recorded on the
Aminco DW-2a spectrophotometer operated in the double-beam
mode (1 nm bandpass width). Photosystem II particles were
suspended in 5.0 mM Tris HCl buffer, pH 8.0 at a CHl con-
centration of 4 to 6 μg/ml. The buffer alone served as the
blank.

The reduced minus oxidized absorption spectrum of
cytochrome b-559 was obtained operating the spectrophotom-
eter typically on the 0.05 absorbance scale. The TSF-IIa
sample was suspended in buffer at a concentration of 10 to
20 μg Chl/ml in both the sample and reference cuvettes. A linear baseline was established in the 500 to 600 nm region. A few grains of sodium dithionite were added to the stirred sample cuvette and again the absorption spectrum was recorded. Prior oxidation of the cytochrome b-559 was not necessary. The millimolar extinction coefficient of the reduced molecule is 21 (A559 - A600 nm) (34).

**Polyacrylamide Gel Electrophoresis**

Sodium dodecylsulfate polyacrylamide gel electrophoresis was performed at room temperature in either slab or tube gels using the procedure of Kirchanski and Park (67). The gel conditions are given in Table 2. Tube gels were composed of a 9.0 cm long resolving gel and a 1.0 cm long stacking gel. Slab gels had a 15.0 cm long resolving gel with a 1.0 cm stacking gel. The sample buffer consisted of 2.0% SDS, 65.0 mM Tris HCl, pH 6.8, 2% mercaptoethanol, 10% glycerol, and 0.001% bromophenol blue. The sample volume was 50-200 μl. Electrophoresis was performed at 1.0 milliampere per tube or 25 milliampere for the slab gels. Samples containing TSF-IIa particles generally were not boiled before electrophoresis.

The gels were stained with Coomassie Brilliant Blue G-250 following a procedure adopted from that of Holbrook and Leaver (53). Initially the gels were fixed overnight in 50% methanol and 10% acetic acid, followed by several changes of 5.0% acetic acid. The gels were stained with
TABLE 2.

The Protocol for SDS-Polyacrylamide Gels^a

<table>
<thead>
<tr>
<th>Resolving Gel (10%)</th>
<th>Stock Solution</th>
<th>For Tube Gels (40 ml)</th>
<th>For Slab Gel (60 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide/Bis Acrylamide</td>
<td>30%/0.8% (w/v)</td>
<td>13.35 ml</td>
<td>20.0 ml</td>
</tr>
<tr>
<td>Tris-HCl, pH 9.8 (1.31 M)</td>
<td>2 M</td>
<td>26.4 &quot;</td>
<td>39.6 &quot;</td>
</tr>
<tr>
<td>SDS (0.1%)</td>
<td>20%</td>
<td>0.2 &quot;</td>
<td>0.3 &quot;</td>
</tr>
<tr>
<td>Ammonium Persulfate</td>
<td>10%</td>
<td>0.4 &quot;</td>
<td>0.5 &quot;</td>
</tr>
<tr>
<td>TEMED</td>
<td>-</td>
<td>0.16 &quot;</td>
<td>0.025&quot;</td>
</tr>
<tr>
<td>Stacking Gel (5%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acryl/Bis-acryl</td>
<td>30%/0.8% (w/v)</td>
<td>1.66 &quot;</td>
<td>3.32 &quot;</td>
</tr>
<tr>
<td>Tris-HCl, pH 6.1 (0.1M)</td>
<td>1 M</td>
<td>1.0 &quot;</td>
<td>2.0 &quot;</td>
</tr>
<tr>
<td>SDS (0.1%)</td>
<td>20%</td>
<td>0.05 &quot;</td>
<td>0.1 &quot;</td>
</tr>
<tr>
<td>H2O</td>
<td>-</td>
<td>7.3 &quot;</td>
<td>14.6 &quot;</td>
</tr>
<tr>
<td>TEMED</td>
<td>-</td>
<td>0.016 &quot;</td>
<td>0.025 &quot;</td>
</tr>
<tr>
<td>Ammonium Persulfate</td>
<td>10%</td>
<td>0.018 &quot;</td>
<td>0.05 &quot;</td>
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<tr>
<td>Running Buffer</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Glycine (0.192 M)</td>
<td>1 M</td>
<td>192 ml</td>
<td></td>
</tr>
<tr>
<td>Tris (0.025 M)</td>
<td>1 M</td>
<td>25 &quot;</td>
<td></td>
</tr>
<tr>
<td>SDS (0.1%)</td>
<td>20%</td>
<td>5 &quot;</td>
<td></td>
</tr>
<tr>
<td>H2O</td>
<td>-</td>
<td>778 &quot;</td>
<td></td>
</tr>
</tbody>
</table>

^aAdopted from reference (67).
0.04% Coomassie Brilliant Blue G-250 in 3.5% perchloric acid for 90 minutes at room temperature and then transferred again to a 5.0% acetic acid solution. Complete destaining was achieved with three charges of the 5.0% acetic acid solution over several days. Stained tube gels were scanned at 600 nm on a Guilford Gel Scanner. The scan rate was 2 cm/min. and the full scale optical density scale range was 0.25 to 1.5 O.D. units. Slab gels were either photographed after destaining or dried for preservation with a Bio-Rad Laboratories Gel Slab Dryer, Model 224. To prevent cracking, the gel was presoaked in 10% acetic acid, 1% glycerin for 30 minutes. The gel was placed on a sheet of filter paper then dried for 2.5 hours. After drying, the slab gel was pressed between two glass plates for several days to prevent curling.

The apparent molecular weights of polypeptides were calculated from a standard curve constructed using Bovine serum albumin (68,000), glutamate dehydrogenase (53,000), aldolase (40,000), chymotrypsinogen (25,700), RNase A (13,700), and cytochrome C (11,700). The relative mobility was measured as the distance traveled by each peptide, divided by the distance traveled by the bromophenol blue. The relative mobility of the standards were plotted as a function of the log of their molecular weights to obtain the standard curve.

In some experiments, the denaturing conditions of normal SDS-PAGE were not desired. To preserve the Chl-protein
interaction in the PS II particles during electrophoresis, several changes in the protocol were employed. Gel electrophoresis, under non-denaturing conditions, was performed at 3°C. Because sodium dodecylsulfate precipitates at this temperature, the soluble lithium salt (LiDS) was substituted for it in the reservoir buffer, stacking, and running gels, as suggested by Chua and Bennoum (24). Following the procedure of Camm et al. (21), neither SDS nor LiDS were added to the non-denatured samples before electrophoresis. The sample buffer consisted of 65.0 mM Tris HCl, pH 6.8, 0.1% mercaptoethanol, 10% glycerol, and 0.001% bromophenol blue. Of course, the samples were not boiled. Denatured TSF-IIa samples contained 2% LiDS and were gently heated (80°C, 1-2 min.). Under these conditions there is sufficient detergent present in the upper reservoir buffer to dissociate the individual polypeptides of the TSF-IIa particles yet the conditions are sufficiently mild so that at least a portion of the Chl is retained by the Chl a-binding polypeptide. The samples were subjected to electrophoresis for approximately 6 h., then the gels were removed from their tubes and scanned immediately at 670 nm. The gels were stained for protein as described above.

Proteolytic Treatment of the TSF-IIa Particles

The effects of limited proteolysis on the structural and photochemical properties of the TSF-IIa particles were studied using α-chymotrypsin (TLCK treated) or trypsin
(DPCC treated). The TSF-IIa particles were first purified on aminoethyl cellulose and Sephadex G-100 as described above, then treated with either protease. Proteolysis was carried out at 5\(^\circ\) C in a stirred container, generally for 20 hours. This temperature was chosen so as to preserve the activity of the TSF-IIa particles without significantly altering the activity of the protease. The reaction was performed in 5.0 mM Tris HCl, pH 8.0. The Chl concentration was approximately 50 \(\mu g/ml\).

Photochemical assays (DPC dependent DCIP reduction) were performed on the protease-treated samples by directly diluting an aliquot into the standard assay buffer (a 1:50 dilution).

To prepare TSF-IIa particles containing \(\alpha\)-chymotrypsin or trypsin for SDS gel electrophoresis, 100 \(\mu l\) samples were removed from the incubation mixture to which 10 \(\mu l\) of 50 mM phenylmethanesulfonyl fluoride (PMSF) in n-propanol was added. The sample was incubated 15 minutes on ice, then the SDS and electrophoresis buffer were added. The mixture was left at 5\(^\circ\) C until just prior to electrophoresis. The samples were not boiled.

For all experiments with the protease-treated TSF-IIa particles other than SDS electrophoresis and the standard photochemical assays, the sample at the end of the incubation period was again isolated on aminoethyl cellulose and Sephadex G-100 as described in the Chromatography section.
Immobilization of TSF-IIa on Either Aminoethyl Cellulose or Ethylenediamine Sepharose

Studies conducted with immobilized TSF-IIa particles were performed utilizing two slightly different support gels. The particles were immobilized on either ethylenediamine Sepharose or aminoethyl cellulose. The gels have essentially identical physical properties, except that the aminoethyl cellulose has a higher capacity. The aminoethyl cellulose is available commercially while the ethylenediamine Sepharose must be synthesized. In general, the properties of the immobilized particles were the same on either gel.

Ethylenediamine Sepharose was synthesized following the procedure of Liberatore et al. (76). Sepharose Cl-4B (10-40 ml packed gel) was washed with 0.1 M acetic acid, 1% w/v NaHCO₃, and water, then treated for 2 hours at room temperature with 0.01 M NaIO₄ (10 ml/ml gel). The oxidized gel was then washed with water.

Coupling of ethylenediamine to the support was achieved by mixing 0.1 M ethylenediamine in 0.1 M borate, pH 9.0 with the oxidized gel (2 ml solution/ml gel) for 1 hour at room temperature. After 20 and 40 minutes, NaBH₄ (0.25 mg/ml) was added. Finally, the gel was again washed with 0.1 M acetic acid, 1% NaHCO₃, and water.

Immobilization of TSF-IIa particles on ethylenediamine Sepharose was performed in the presence of 0.05% Triton X-100 (unbuffered) (3 ml solution/ml gel), approximately 10 ml
ethylenediamine Sepharose, and chlorophyll at a concentration of 30-50 μg/ml. Tris buffer was removed from the particles by ultrafiltration. Coupling of the particles to the gel was achieved by the addition of 0.05 g of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide to the reaction mixture. The stirred solution was adjusted continuously to pH 6.0 with dilute HCl and reacted on ice for 30 minutes. The gel was then pelleted by centrifugation at 3,000 x g for 1 minute (2°C) and washed free of non-immobilized TSF-IIa particles with 0.5 M NaCl containing 0.1% Triton X-100. The gel was washed with water several times and then resuspended in water.

Immobilization of the TSF-IIa particles on aminoethyl cellulose was similarly achieved, except for a few differences. The particles were absorbed onto the gel packed in a column, as described in the chromatography section, then washed with water. The gel was removed from the column and resuspended in water (1 ml/ml gel). Again, the particles were coupled with one addition of carbodiimide (0.05 g/ml gel) at pH 6.0 for 30 minutes on ice then washed as described for the TSF-IIa coupled ethylenediamine Sepharose gel.

The activity of the immobilized particles was measured as described in the Photochemical Assays section. The amount of TSF-IIa particles covalently bound to the gel was determined by extracting the Chl with 80% acetone (125). The Chl concentration was calculated using the equations of
Arnon (5) as described below.

180° Light Scattering Measurements

Light scattering experiments were conducted to determine the isoelectric point of the PS II core complex before and after proteolysis. They were performed in a stirred cuvette with a 1 cm pathlength. The sample contained 15 μg Chl/ml in 2.5 mM Tris HCl and 0.04% Triton X-100. The pH of the sample was adjusted in a stepwise fashion using 0.1 M HCl. The absorbance increase at 540 nm due to 180° light scatter was measured as a function of pH using a Model 139 Hitachi Perkin-Elmer spectrophotometer.

Protein Determinations

The protein content of the TSF-IIa particles was determined using the Bio-Rad Dye Binding Assay method (see Technical Bulletin 1051). To a 5.0 ml volume of dye reagent, a 0.1 ml sample of TSF-IIa particles (2.0-10.0 μg of chlorophyll) was added and vortexed quickly. The absorbance at 595 nm was measured against a blank containing dye reagent. The protein concentration was determined from a standard protein concentration curve constructed using catalase (10-100 μg). The protein content was generally expressed as a protein:chlorophyll ratio (μg/μg).

Chlorophyll Determinations

The chlorophyll content of either chloroplast lamellae or PS II particles was determined by the method of Arnon (5). To a 5.0 ml volume of 80% acetone, 0.05 ml of sample
was added. The solution was centrifuged to remove insoluble material and the absorbance was determined at 663 nm and 645 nm against an 80% acetone blank. The chlorophyll a and chlorophyll b concentration was calculated from these equations:

\[
\text{Chl a (mg/ml)} = 1.27 \times \text{A}_{663} - 0.269 \times \text{A}_{645}
\]

\[
\text{Chl b (mg/ml)} = 2.29 \times \text{A}_{645} - 0.468 \times \text{A}_{663}
\]

To determine dilute Chl sample concentrations, volumes up to 1 ml were added to 4 ml of 100% acetone. Sample volumes less than 1 ml were brought up to 1 ml with water.

Occasionally, Chl concentrations of TSF-IIa particles (in water) were estimated from their absorbance at 436 nm using the conversion ratio:

\[
\mu g \text{ Chl/ml} = 10.66 \frac{\text{OD}_{436}}{}
\]

Materials

The following chemicals were obtained from Sigma Chemical Company: Sepharose 4B and 6B; Aminoethyl Cellulose; Trypsin Type XI; α-Chymotrypsin Type VII; SDS; LiDS; Sephadex G-100; All protein standards; Digitonin; Ethylenediamine dihydrochloride. Triton X-100 was obtained from Research Products International Corp. All other chemicals were reagent grade or better.
RESULTS AND DISCUSSION

The following structural and photochemical properties of the isolated photosystem II core complex were studied:

1. To determine if cations regulate energy transfer or electron transport within the PS II core complex, the photochemical activity of the TSF-IIa particles was measured as a function of light intensity in the presence and absence of cations.

2. To determine if cations affect the structure of the PS II core complex, the aggregation state of the particles was measured by gel filtration.

3. The effect of Triton X-100 on the structure and photochemical activity of the TSF-IIa particles was determined by the same methods used to study cation effects.

4. The TSF-IIa particles were covalently immobilized in order to determine whether the monomeric state of the complex is active.

5. The polypeptide composition of the TSF-IIa particles was determined by SDS-gel electrophoresis.

6. The structural arrangement of the polypeptides in the TSF-IIa complex was studied by proteolytic treatment.
7. The polypeptides with which chlorophyll is associated were identified by LiDS-gel electrophoresis of the complex under non-denaturing conditions.

**Preparation of TSF-IIa**

The procedure developed by Vernon and co-workers (133) to produce TSF-IIa particles involved a series of differential centrifugation steps following the initial Triton X-100 treatment of the lamellae, as described in the Methods and Materials section. Originally, this procedure was followed by a sucrose density gradient step which separated the particles into three separate chlorophyll-containing complexes. Using their terminology, the top fraction, TSF-IIa', contained only Chl a but was photochemically inactive, the middle fraction (TSF-IIa) was the PS II core complex fraction, and the bottom fraction, TSF-IIb, was similar to the original TSF-II membrane fraction. The sucrose gradient, although apparently useful for further purification of the TSF-IIa complex, was soon discarded for an absorption step on a Bio-glas column (134). The sucrose gradient was discarded probably because it required long running periods, which decreased the activity of the particles, and allowed for only a relatively small preparation of TSF-IIa particle.

For these same reasons we did not utilize the sucrose gradient step either. However, in our hands, the Bio-glas step never worked. We found that the TSF-IIa particles
bound irreversibly to the column regardless of how the Bio-glas was pretreated, the nature of the equilibration buffer or the state of the TSF-IIa particles themselves. So, for most of the studies described in this work, the TSF-IIa particles were used as isolated after the final centrifugation step.

The total overall yield of TSF-IIa particles obtained by any particular preparation was surprisingly consistent, while the yield (total chlorophyll) for any individual step was quite variable. The yield of the TSF-IIa fraction varied from 20-50% of the initial chloroplast chlorophyll. On the other hand, the yield of TSF-IIa particles was consistently between 1.0-1.5% of the total chloroplast chlorophyll, regardless of the yield of TSF-II for that preparation. The reason for this is unknown but suggests that the TSF-II fraction is heterogenous with respect to its ability to yield TSF-IIa particles.

It was found that the composition of the buffer in which the TSF-II fragments were resuspended had a profound effect on the total yield of chlorophyll in any preparation of TSF-IIa particles as well as the amount of light-harvesting (LHC) contamination found to be associated with the TSF-IIa particles (Table 3). Low salt conditions led to a relatively high yield of TSF-IIa particles (on a per total chlorophyll basis) but they contained a significant amount of LHC, as indicated by the low Chl a/b ratio. The increased
Table 3

The Effect of Resuspension Buffer on the Yield and Chl a/b Ratio of TSF-IIa Particles\textsuperscript{a}

<table>
<thead>
<tr>
<th>Resuspension Buffer</th>
<th>Chl a/b Ratio</th>
<th>Chl Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 5.0 mM Tris HCl, pH 8.0</td>
<td>3.5</td>
<td>8.0%</td>
</tr>
<tr>
<td>2. 5.0 mM Tris HCl, pH 8.0, 50 mM NaCl</td>
<td>5.7</td>
<td>6.6%</td>
</tr>
<tr>
<td>3. 5.0 mM Tris HCl, pH 8.0, 25 mM MgCl\textsubscript{2}</td>
<td>6.0</td>
<td>2.6%</td>
</tr>
</tbody>
</table>

\textsuperscript{a}The yield is based on the amount of Chl remaining in the supernatant following centrifugation of the TSF-IIa fraction as described in the Methods and Materials section. The Chl a/b ratio of the supernatant was calculated using the equations of Arnon (5).
yield therefore was probably due to the presence of more LHC. Conversely, high concentrations of divalent cations produced particles with a very low LHC content, but the yield of particles was also greatly reduced compared to a sample containing high concentrations of monovalent cations. Therefore, resuspension of the TSF-II fragments in a high concentration of monovalent salts was considered to be optimal. Whether 50 mM NaCl or 50 mM Tris HCl was used appeared to make little significant difference with respect to the yield or LHC content of the particles.

The isolation procedure requires a one hour incubation period after resuspension of the TSF-II fragments. If the final centrifugation step is initiated immediately after resuspension, the LHC content of the TSF-IIa particles is increased appreciably. Incubation periods longer than one hour did not influence the LHC content of the preparation.

Both the effects of resuspension buffer and incubation period suggest that there is a salt- and time-dependent dynamic process which leads to the separation of the PS II core complex from the light-harvesting complex. The mechanism of this process, whether it is a rearrangement, aggregation, or dispersion of the pigment-protein complexes, is unknown. However, these effects may in fact be related to those seen by others on the distribution of pigment-protein complexes in detergent fractionated chloroplasts (2, 7). The effects are attributed to the influence of salts
on the structural arrangement of the membrane components (2,7).

The Effects of Ions on DCIP Reduction by TSF-IIa Particles

In order to analyze the effects of ions on photo-chemical activity of the TSF-IIa particles, the activity was measured as a function of light intensity. Lumry, Spikes, and Rieske (80,109) showed that the relationship between the rate of electron transport (DCIP reduction, in this case) and light intensity could be expressed by the equation

\[ \frac{1}{V} = \frac{1}{k_D} + \frac{1}{k_L I} \]

where \( V \) is the initial velocity of the reaction, \( I \) is the actinic light intensity, and \( k_L \) and \( k_D \) are rate constants.

Figure 3 illustrates the proposed series of light-induced oxidation-reduction reactions occurring in the presence of the TSF-IIa particles. The incident light (I) is absorbed by antenna chlorophyll molecules and transferred to the reaction center (P680). The efficiency of this process is reflected in the rate constant, \( k_L \). The energy absorbed by P680 initiates the electron transfer from P680 to the primary acceptor Q. In turn, the electron is transferred from Q to a secondary acceptor, R, then to DCIP. The oxidized P680 oxidizes DPC to complete the reaction. It is the rate of these secondary electron transfer steps which influence \( k_D \). Satoh et al. (114) have extended this analysis by demonstrating that agents which affect electron transport on
Figure 3

The proposed light-dependent electron transport pathway for TSF-IIa particles. The symbols are described in the text.
Reducing side of PS II

Oxidizing side of PS II

P680

Q \xrightarrow{k_D} R \xrightarrow{k_D} DCIP_{ox}

I \xrightarrow{k_I} \text{DPC}_{red}

\text{DCIP}_{red}

\text{DPC}_{ox}
the rate-limiting side of PS II affect $k_D$, whereas those which act on the opposite side of the light reaction affect $k_L$. So, the site of action of agents, such as ions, may be determined by measuring activity as a function of light intensity.

Before analyzing the effects of ions on PS II photochemistry, the optimum concentrations of DPC and DCIP were determined. As figure 4 indicates, maximal activity was achieved with 25 μM DCIP at both pH 6.0 and pH 8.0. This result is surprising since the assay conditions described by Vernon et al. (133) used 100 μM DCIP, although they made no claim that this was the optimal concentration for DCIP reduction activity. The reason for the apparent inhibition of activity by high concentrations of DCIP is unknown but may be due simply to shading by DCIP of the TSF-IIa particles from the actinic light. DPC at a concentration of 0.5 mM gave maximal activity as found by others (27,133). Higher concentrations of DPC did not increase the activity but did increase the PS II independent (dark) DCIP reduction rate. For these reasons, 25 μM DCIP and 0.5 mM DPC were chosen as the optimal assay conditions for TSF-IIa activity.

As can be seen from figure 4 the activity of the particles displayed a marked pH dependence. The rate of DCIP reduction by the TSF-IIa particles, as a function of light intensity and pH, is shown in figure 5. The rate of DCIP reduction increased approximately 20-fold as the pH was lowered
The rate of DCIP reduction by TSF-IIa particles as a function of DCIP concentration. The assay medium consisted of 20 mM Tris-maleate at either pH 6.0 or 8.0, and 0.5 mM DPC.
RATE OF DCIP REDUCTION (μ moles DCIP Reduced/mg CHI/hr)
from 8 to 6 (the pH optimum). The measurements were not extended below pH 5.5 because of the decrease in the extinction coefficient of DCIP. These results agree with those of Vernon et al. (235) who also found a low pH optimum (pH 6.5) for electron transport with the TSF-IIa particles. As Table 4 indicates, the major effect of increasing the H\textsuperscript{+} ion concentration was on the dark electron transport step (k\textsubscript{D}). The effect on the rate constant for the light processes (k\textsubscript{L}) was significant only at the higher pH values, and was still small compared to the changes in k\textsubscript{D}.

The effects of monovalent and divalent metal cations on the activity of the TSF-IIa complex varied, apparently depending on the Triton X-100 content of the particles. The TSF-IIa particles obtained after the final ultracentrifugation step (see Materials and Methods section) contained Triton X-100 at a concentration greater than 0.02%. The rate of electron transport by the particles in this state was stimulated by Mg\textsuperscript{2+} ions. The extent of this activation was typically in the range of 25-50% (at pH 8.0). Although there was considerable variation among various preparations of particles (a range of 0-300%), the activity always increased. Figure 6 shows the concentration dependence of MgCl\textsubscript{2} for the stimulation of DCIP reduction. The concentration for half-maximal stimulation was approximately 0.15 mM. The stimulation of activity by MgCl\textsubscript{2} was less at lower pH values, and could not be observed below pH 7.0. CaCl\textsubscript{2} was
Figure 5

A double-reciprocal plot of light intensity curves for DCIP reduction at different pH values. Data were plotted according to equation 1 (see text). Each point represents the average of three determinations. The range of values was less than or equal to the range covered by each symbol. The lines shown were determined by a least-squares analysis of all of the individual data points.
$V^{-1} (\mu \text{ moles DCIP Reduced/mg chl/hr})^{-1}$

$\text{pH}$

$8.0$

$7.5$

$7.0$

$6.5$

$6.0$

$(I \times 10^{-4})^{-1} (\text{erg/cm}^2/\text{sec})^{-1}$
Table 4

The Effects of pH on $k_L$ and $k_D^a$

<table>
<thead>
<tr>
<th>pH</th>
<th>$k_L$</th>
<th>$k_D$</th>
<th>Percent Increase $k_L$</th>
<th>Percent Increase $k_D$</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.0</td>
<td>15.8 ± 0.4</td>
<td>25 ± 1.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7.5</td>
<td>23.0 ± 0.9</td>
<td>61 ± 3.0</td>
<td>46%</td>
<td>144%</td>
</tr>
<tr>
<td>7.0</td>
<td>30.5 ± 0.7</td>
<td>120 ± 10</td>
<td>33%</td>
<td>121%</td>
</tr>
<tr>
<td>6.5</td>
<td>33.1 ± 0.5</td>
<td>320 ± 40</td>
<td>8.5%</td>
<td>164%</td>
</tr>
<tr>
<td>6.0</td>
<td>37.6 ± 1.0</td>
<td>600 ± 100</td>
<td>14%</td>
<td>86%</td>
</tr>
<tr>
<td>5.5</td>
<td>34.7 ± 0.7</td>
<td>575 ± 100</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

$^a$See text for definitions of $k_L$ and $k_D$. The values are calculated from data given in Figure 5.
Figure 6

MgCl₂ stimulation of TSF-IIa reduction of DCIP. The assay medium consisted of 5.0 mM Tris-maleate, pH 8.0, 0.5 mM DPC, 25 µM DCIP, and MgCl₂ at the appropriate concentration. The chlorophyll concentration was 1 µg/ml. The light intensity was $4.0 \times 10^5$ erg/cm²/s. Each point represents the average of 4-5 determinations.
Rate of DCIP Reduction (% Control)

[y-axis: MgCl2 (mM)]

[x-axis: ]
as effective as MgCl₂, but neither 1 mM Na₂SO₄ nor NaCl concentrations as high as 100 mM had any apparent effect on the rate of DCIP reduction (Table 5). These results clearly indicate that the stimulation of activity is specific for divalent cations.

Figure 7 shows the rate of DCIP reduction by the TSF-IIa particles in the presence and absence of 1 mM MgCl₂, as a function of light intensity. The values of k_D (which equal the inverse of the intercept on the ordinate) are 55.6 ± 1.5 and 34.7 ± 1.2¹ in the presence and absence of Mg²⁺ respectively, while the values of k_L (which equal the inverse of the slope) are 34.5 ± 0.8 and 27.5 ± 1.0 respectively. This represents a 60% increase in k_D and a 25% increase in k_L. Again, there was considerable variation among these parameters but the effect was always significantly greater on k_D than on k_L, more than 10-fold in some experiments. Thus, like H⁺ ions, Mg²⁺ ions affect primarily the dark electron transport steps. However, because there were slight changes in k_L in both cases, direct effects of H⁺ and Mg²⁺ ions on either energy transfer or the reaction center may be occurring as well.

The Triton X-100 can be removed from the TSF-IIa preparation by a simple gel filtration procedure on Sephadex G-100 (Figure 8).

¹The units for k_D are µmol DCIP reduced/mg chl/h. The units for k_L are µmol DCIP reduced/mg chl/h · erg/cm²-s⁻¹.
Table 5

The Effects of Various Ions on the Rate of DCIP Reduction by TSF-IIa Particles\(^a\)

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Percentage Increase in Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>control - no salt addition</td>
<td>-</td>
</tr>
<tr>
<td>1 mM MgCl(_2)</td>
<td>81</td>
</tr>
<tr>
<td>1 mM CaCl(_2)</td>
<td>86</td>
</tr>
<tr>
<td>1 mM Na(_2)SO(_4)</td>
<td>1</td>
</tr>
<tr>
<td>100 mM NaCl</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\)Assay conditions were as in Figure 6 except the light intensity was 2.6 \(\times\) 10\(^5\) erg/cm\(^2\)/s. Rates in the presence of ions were expressed as the percentage increase in rate from that obtained in the absence of ions.
A double-reciprocal plot of light intensity curves for DCIP reduction by TSF-IIa particles in the presence and absence of 1 mM MgCl₂. The lines shown were determined by a least-squares analysis of the data. The intercepts at the ordinate are 0.0180 ± 0.0005 and 0.0288 ± 0.0010 for the plus MgCl₂ and no salt lines, respectively. The slope was 0.0290 ± 0.0007 for the plus MgCl₂ line and 0.0363 ± 0.0013 for the no salt line.
Figure 8

The removal of Triton X-100 from TSF-IIa particles by gel filtration. A Sephadex G-100 column (2.5 x 25 cm) was equilibrated with 20 mM Tris HCl, pH 8.2 at room temperature. The flow rate was 15 ml/h and 2.3 ml fractions were collected. (A) A 2.0 ml sample of TSF-IIa (16.3 µg Chl/ml) was chromatographed. The fractions were monitored at 435 nm (•) for chlorophyll and at 275 nm (○) for Triton X-100 and protein. (B) A 2.0 ml sample of 0.1% Triton X-100 was chromatographed separately under the same conditions as in A.
Under these conditions the TSF-IIa particles eluted in the void volume while the bulk Triton X-100 (absorbance maximum at 275 nm) eluted at the same position as DNP-glycine. Since both protein and Triton X-100 have considerable absorbance at 275 nm it was not possible to conclude that the Triton X-100 was quantitatively removed by this procedure. However, the maximal Triton X-100 content of the particles can be calculated by assuming that all of the absorbance at 275 nm is due to Triton X-100. In this case the maximal Triton X-100 concentration would be 0.016%(W/V). Clearly, since the actual Triton X-100 content is obviously less than this value, the Triton X-100 content of the particles is greatly reduced by gel filtration.

The removal of Triton X-100 significantly altered the photochemical properties of the TSF-IIa particles. After the removal of Triton X-100, both 100 mM NaCl (figure 9) and 1 mM MgCl$_2$ inhibited the reduction of DCIP by the TSF-IIa particles. The addition of these ions affected both the intercept on the ordinate ($1/k_D$) and to a lesser extent the slope ($1/k_L$) of these lines. Again, therefore, the effect of these salts was primarily on the rate-limiting dark reaction step, accompanied, however, by a small effect on the rate constant for the light processes.

When Triton X-100 was added back to the sample of TSF-IIa particles from which the Triton X-100 had been removed, the inhibition of DCIP reduction was lost and the MgCl$_2$
A double-reciprocal plot of light intensity curves for DCIP reduction by TSF-IIa particles, after the removal of Triton X-100 from the sample, in the presence and absence of 100 mM NaCl. Conditions were as described in Materials and Methods. The lines were determined by a least squares analysis of the data. The intercepts at the ordinate are $0.0608 \pm 0.0020$ and $0.033 \pm 0.0015$ for the plus NaCl and no salts lines, respectively. The slopes and intercepts at the abscissa were $0.0786 \pm 0.0023$, $-0.774 \pm 0.034$ and $0.050 \pm 0.002$, $-0.668 \pm 0.037$ for the plus salt and no salt lines, respectively.
$V^{-1} \left( \mu \text{moles DCIP Reduced/mg chl/hr} \right)^{-1}$

![Graph showing relationship between $V^{-1}$ and other variables.](image-url)
stimulation effect was regained (Table 6).

Thus, it appears that the effects of salts on DCIP reduction may be either stimulatory or inhibitory, depending on the presence or absence of Triton X-100 in the preparation prior to dilution into the assay medium. In one case the effect is dependent strictly on divalent cations, while in the other it appears to be an ionic strength effect. However, in either instance salts predominantly affect only the rate-limiting dark steps of electron transport.

Not only were the effects of salts on TSF-IIa photochemistry changed by removal of Triton X-100, the pronounced dependence of the activity on pH was lost. As described previously, the activity of the particles showed a 20-fold stimulation going from pH 8.0 to 6.0. After gel filtration, the degree of stimulation was reduced to approximately 6-fold. It is not known whether this results from a decrease in the rate at pH 6.0 with a constant rate at pH 8.0, or an increase in the rate at pH 8.0 relative to a constant rate at pH 6.0.

The Effects of DCMU on DCIP Reduction by TSF-IIa Particles

The electron transport inhibitor DCMU is known to act on the reducing side of Photosystem II, between the primary acceptor and the plastoquinone pool in whole chloroplasts (8). Figure 10 illustrates the inhibition by 2 µM DCMU of DCIP reduction by TSF-IIa particles as a function of light intensity. DCMU at this concentration inhibits...
Table 6

The Effects of 1 mM MgCl₂ on the Rate of DCIP Reduction by TSF-IIa Particles, After Removal of Triton X-100, and Then Re-Addition of Triton X-100 to the Sample^a

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Activity (μmol DCIP Reduced/mg Chl·h)</th>
<th>Change in Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. No Triton X-100 Present</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Minus Mg²⁺</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>2. Plus Mg²⁺</td>
<td>14</td>
<td>44% decrease</td>
</tr>
<tr>
<td>B. Triton X-100 Present</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Minus Mg²⁺</td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td>2. Plus Mg²⁺</td>
<td>10.5</td>
<td>27% increase</td>
</tr>
</tbody>
</table>

^a Assays were performed as described in Figure 6.

(A) The Triton X-100 was removed from the sample as described in Figure 8.

(B) Triton X-100 was added back to the TSF-IIa sample to a final concentration of 0.04%. To assay, 0.2 ml of sample was diluted into the assay buffer (5.0 ml). The Triton X-100 concentration in the assay buffer therefore was 0.016% which is below the threshold concentration for inactivation of the particles (see Figure 12).
Figure 10

A double-reciprocal plot of light intensity curves for DCIP reduction by TSF-IIa particles in the absence or presence of 2.0 μM DCMU. The assay buffer consisted of 5.0 mM Tris-maleate, pH 6.0, containing 0.5 mM DPC, 25 μM DCIP, and 2.0 μM DCMU for the plus DCMU case. The values shown were the average of three determinations. The lines shown were determined by a least-squares analysis of all of the data points. The intercepts at the ordinate were 0.0040 ± 0.0004 and 0.0137 ± 0.0010 for the control and plus DCMU cases, respectively.
V\(^{-1}\) (µ moles DCIP Reduced/mg chl/hr\(^{-1}\))

(I\(\times\)10\(^{-4}\))\(^{-1}\) (erg/cm\(^2\)/sec\(^{-1}\))

PLUS DCMU

Control
the activity by approximately 50%, and obviously only influences $k_D$. Applying the analysis of Satoh, as described above, it is evident that electron transfer on the reducing side of Photosystem II is rate limiting in TSF-IIa particles. Furthermore, since cations also affect primarily $k_D$, they must also act on the reducing side of PS II.

**The Effect of Temperature, Salts and pH on the Apparent Molecular Weight of TSF-IIa Particles**

In addition to their effects on the photochemical activity of the TSF-IIa particles, both salts and pH had a profound effect on the apparent molecular weight of the particles as determined by gel filtration. The effects are summarized in Table 7. At room temperature the particles eluted in the void volume of a Sepharose CL-6B column, indicating a molecular weight greater than 800,000 for the particles. However, particles chromatographed at 4°C (pH 8.0) eluted as two separate peaks (figure 11). The first peak was, again, in the void volume of the column. The second peak eluted in the same elution volume as apoferritin, indicating that it had an apparent molecular weight of 460,000. Both peaks retained photochemical activity. Addition of either 1 mM MgCl$_2$, 1 mM NaSO$_4$ or 100 mM NaCl to the column buffer caused the particles to elute entirely in the void volume (figure 11). Additionally, simply lowering the pH of the buffer from pH 8.0 to 6.0 caused a similar increase in molecular weight.
Table 7

The Effects of Salts and pH on the Apparent Molecular Weight of TSF-IIa Particles

<table>
<thead>
<tr>
<th>pH</th>
<th>Salts</th>
<th>Percentage Void Volume</th>
<th>Percentage 460,000 Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.0</td>
<td>-</td>
<td>24</td>
<td>67</td>
</tr>
<tr>
<td>8.0</td>
<td>1 mM MgCl₂</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>8.0</td>
<td>100 mM NaCl</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>8.0</td>
<td>1 mM Na₂SO₄</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>7.0</td>
<td>-</td>
<td>43</td>
<td>57</td>
</tr>
<tr>
<td>6.0</td>
<td>-</td>
<td>78</td>
<td>22</td>
</tr>
<tr>
<td>6.0</td>
<td>1 mM MgCl₂</td>
<td>80</td>
<td>20</td>
</tr>
</tbody>
</table>

*a* Gel filtration on Sepharose CL-6B was performed at 4°C, as described under Materials and Methods. The elution pattern of the TSF-IIa particles was monitored by measuring the optical density of the fractions at 435 nm.
Figure 11

Gel filtration of TSF-IIa particles on a Sepharose CL-6B column at 4°C. Conditions were as described under Materials and Methods. The control elution buffer was 20 mM Tris HCl, pH 8.0. The elution buffer for the plus MgCl₂ case consisted of 20 mM Tris HCl, pH 8.0, and 1 mM MgCl₂.
Absorbance at 435 nm

<table>
<thead>
<tr>
<th>Fraction Number</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0.36</td>
</tr>
<tr>
<td>24</td>
<td>0.32</td>
</tr>
<tr>
<td>28</td>
<td>0.28</td>
</tr>
<tr>
<td>32</td>
<td>0.20</td>
</tr>
<tr>
<td>36</td>
<td>0.12</td>
</tr>
<tr>
<td>40</td>
<td>0.04</td>
</tr>
</tbody>
</table>

- **Control**
- **Plus 1mM MgCl₂**
These results indicate that temperature, salts, and pH influence the aggregation state of the TSF-IIa particles. The tendency of detergent extracted membrane proteins to aggregate after removal of the detergent is not uncommon however (47). The decrease in aggregation caused by lower temperature indicates that the aggregation is due to hydrophobic forces (126). The salt-induced aggregation is also consistent with this hypothesis.

The effect of pH on the aggregation state suggests that ionic forces are involved in controlling the structure of the complex. Although at pH 6.0 the complex is still well above its isoelectric point, a sufficient number of charged groups, most likely carboxyl groups, are neutralized, thus decreasing the charge repulsion between individual complexes. In this sense the pH effect is similar to the ionic strength effect on aggregation.

**Summary and Discussion of Ion Effects on the Photochemical Activity and Apparent Molecular Weight of TSF-IIa Particles**

The relationship between the aggregation state and the salt effects on electron transport is complex. Cations can either stimulate or inhibit the photochemical activity of the particles depending on the Triton X-100 content of the preparation. As will be discussed below, Triton X-100 has a profound effect on both the activity and aggregation state of the TSF-IIa complex. The salt-induced aggregation observed at 4° C corresponds most closely to the salt-induced inhibition
of electron transport observed in preparations from which the Triton X-100 had been removed prior to assaying. Obviously, the gel filtration procedure used to demonstrate aggregation, removes Triton X-100 from the particles in a manner essentially identical to that used to remove the Triton X-100 from the sample used in the assays. Furthermore, in either instance, both the activity decrease and aggregation increase appear to be a response to increased ionic strength. The stimulation of activity when Triton X-100 is present in the sample, on the other hand, is strictly dependent on divalent cations. Therefore, aggregation in the presence of salts coincides with inhibition of electron transport.

In contrast, aggregation of the particles accompanies an increase in the rate of electron transport as the pH is lowered. This suggests that changes in aggregation state are unrelated to pH effects on activity. However, as mentioned previously, the degree to which lower pH stimulated activity was also dependent on whether or not the Triton X-100 was removed from the preparation. Clearly, there is a relationship between the influence of pH on aggregation state and activity, but its exact nature remains obscure.

We can speculate on a number of possibilities as to how the photochemical activity of the TSF-IIa particles is influenced by its aggregation state. One possibility is that the aggregation state modulates the accessibility of
the electron donor (DPC) and acceptor (DCIP). However, experimental results, which will be detailed in the section describing the effects of proteases on the TSF-IIa complex, clearly demonstrate that the Km values for DPC and DCIP with highly aggregated particles are not significantly different from particles which are not aggregated. Since changes in accessibility should be reflected in altered Km values, it does not appear to be responsible for the changes in activity. An alternative possibility is that changes in the aggregation state of the particles impose a change in the conformational state of the individual complexes. In turn, the altered conformational state changes the rate limiting step for electron transport. As indicated earlier, the rate limiting step for electron transport is on the reducing side of PS II. However, it is not known how many electron transfer steps are actually involved in the transfer of electrons from the primary acceptor (Q) to DCIP. Therefore, although it was suggested that ions exert their influence directly on the rate-limiting electron transport step, the influence of ions may be such as to simply create new rate-limiting electron transfer steps.

Yamamoto and Ke (148,149) found a very similar effect of Mg$^{2+}$ on the activity of the TSF-IIa particles to those just described. They too concluded that cations affect principally the secondary electron transport steps and have no major influence on energy transfer in these particles.
They, however, placed instead the site of Mg$^{2+}$ regulation on the oxidizing side of PS II. The differences between their results and ours need to be reconciled by further experimentation.

Finally, the possible relationship between cation effects on activity observed in isolated photosystem II particles to those observed in chloroplasts should be mentioned briefly. As cited in the Literature Review section, cations have a myriad of effects on intact chloroplast membranes. Of these, under the proper conditions, cations cause an increase in the turnover rate of PS II. This effect is generally ascribed to alterations in the energy distribution between the pigment-protein complexes (PS I, PS II, and LHC). In uncoupled chloroplasts, no effect of cations on electron transport has been reported. In chloroplasts, the rate limiting step for electron transport is believed to be the re-oxidation of the plastoquinone pool (139). Our data indicate that cations can influence the rate-limiting electron transport step in isolated PS II particles. Obviously, the state of the plastoquinone in these particles is certainly different from that of the plastoquinone in chloroplasts, and extrapolation of results from one system to the other must be regarded cautiously, yet the findings suggest that cations may in fact regulate the rate of electron transport between PS II and PS I in chloroplasts.
The Effect of Triton X-100 on the Photochemistry of TSF-IIa Particles

The presence of Triton X-100 apparently has a significant influence on TSF-IIa particles, as demonstrated in the section describing the effects of cations on photochemistry. The effects of salts on DCIP reduction by TSF-IIa particles may be either stimulatory or inhibitory depending on the presence or absence of Triton X-100. However, the influence of Triton X-100 on TSF-IIa particles goes far beyond this simple effect. Triton X-100 was found to be an effective inhibitor of PS II photochemistry. The concentration dependence for this inhibition is illustrated in figure 12.

The half-maximal concentration for inhibition was found to be about 0.007% Triton X-100 (w/v). A double-reciprocal plot of DCIP reduction as a function of light intensity (figure 13), in the presence and absence of Triton X-100, reveals that Triton X-100 affects both $k_L$ and $k_D$. In fact, both lines (plus and minus Triton X-100) intersect the abscissa at the same point. The behavior can be understood by slightly modifying the equation of Lumry et al. (80,109) (Equation 1).

$$\frac{1}{v} = \frac{1}{k_D} + \frac{1}{k_L} I$$

When $1/v = 0$, Equation 1 reduces to

$$-I = \frac{k_D}{k_L}.$$  

If $-I$ is the same for both cases, there must be an equal change in $k_D$ and $k_L$. Both $k_D$ and $k_L$ depend on the fraction
Figure 12

Triton X-100 inhibition of DCIP reduction by TSF-IIa particles. Conditions were as described under Materials and Methods with Triton X-100 added to the desired concentration. The light intensity was $5.0 \times 10^5$ erg/cm$^2$-sec. Essentially the same results were obtained whether or not the Triton X-100 in the TSF-IIa sample was removed by gel filtration prior to dilution of the sample into the assay medium. Each value represents the average of three values.
Triton X-100 (%)

Rate of DCIP Reduction (% Control)

Triton X-100 (%)

0.005
0.010
0.015
0.020
0.025
0.030
0.035
0.040
0.045
0.050
0.055
0.060
0.065
0.070
0.075
0.080
0.085
0.090
0.095
0.100

0  20  40  60  80  100
Figure 13

A double-reciprocal plot of light intensity curves for DCIP reduction by TSF-IIa particles in the presence or absence of 0.007% Triton X-100. The assay buffer consisted of 5 mM Tris-maleate, pH 6.0, 0.5 mM DPC, 25 μM DCIP, and 0.007% Triton X-100 for the plus Triton X-100 case. The lines shown were determined by a least-squares analysis of the data. The intercepts at the ordinate and abscissa were 0.0040 ± 0.0004 and -0.081 ± 0.009, respectively, for the control and 0.0064 ± 0.0022 and -0.087 ± 0.029 for the plus Triton X-100 case.
$V^{-1} (\mu\text{moles DCIP Reduced/mg chl/hr})^{-1}$

$(I \times 10^{-4})^{-1} (\text{erg/cm}^2/\text{sec})^{-1}$

PLUS TX-100

Control
of active reaction centers (n):

\[ k_D = nK_D', \]  
\[ k_L = nK_L'. \]

\( K_D' \) and \( K_L' \) are new constants that do not depend on \( n \). Substituting these factors into Equation 1 we have:

\[ \frac{1}{v} = \frac{1}{n} \left( \frac{1}{K_D'} + \frac{1}{K_L'} \right) I. \]

However, the intercept on the abscissa will still be:

\[ -I = \frac{K_D'}{K_L'}. \]

Thus, a change in \( n \) will change both the slope and the \( y \)-intercept, but not the \( x \)-intercept. Our data indicate that Triton X-100 decreases the number of active reaction centers. A separate but equal effect of Triton X-100 on both electron transport (\( k_D \)) and energy transfer (\( k_I \)) can not be entirely ruled out however.

**The Effect of Triton X-100 on the Apparent Molecular Weight of TSF-IIa Particles**

The presence of Triton X-100 also had a profound effect on the apparent molecular weight of TSF-IIa particles. As described previously, at room temperature, in the absence of Triton X-100, the particles eluted in the void volume of the Sepharose 6B column. In the presence of 0.05% Triton X-100, however, the TSF-IIa particles eluted as a single Chl-containing peak with an apparent molecular weight of
250,000 (figures 14 and 15). The presence of 1 mM MgCl$_2$ in
the column buffer, in either case, did not alter the elution
pattern. Concentrations of Triton X-100 of less than 0.035%
in the elution buffer produced a pattern of two species
eluting from the column, with one eluting in the void vol-
ume and the other eluting with an apparent molecular weight
of 250,000. As indicated in Table 8, a concentration of
0.021% Triton X-100 produced an equal distribution between
the two species. (The CMC of Triton X-100 is about 0.015%
(93).) Although the concentration required to dissociate
the TSF-IIa particles 50% was three-fold higher than that
required to inhibit photochemistry by 50%, it suggests that
dissociation of the particles by Triton X-100 may be respon-
sible for the inhibition of photochemistry.

The Effect of Gel Filtration in the Presence of Triton X-100
on the Photochemistry and Structure of TSF-IIa Particles

After gel filtration of the TSF-IIa particles on a
Sepharose 6B column containing 0.05% Triton X-100, at 4° C,
the particles were completely photochemically inactive. This
contrasts sharply with control TSF-IIa particles incubated
in 0.05% Triton X-100 for the same period of time which
retained more than 50% of their activity. Even after removal
of the Triton X-100 from the particles by gel filtration on
Sephadex G-100, the TSF-IIa particles were still inactive.

Although the reason for this apparent irreversible
inactivation of the TSF-IIa particles by gel filtration in
Figure 14

The elution profile of TSF-IIa particles on Sepharose 6B in the presence of 0.05% Triton X-100. Chlorophyll was monitored at 436 nm (○–○) and protein was monitored at 280 nm (●●). The absorbance 280 nm nm due to Triton X-100 was subtracted from the values shown. Chromatography was performed as described under Materials and Methods, at 5°C. The fraction volume was 3.0 ml.
Figure 15

The molecular weight calibration curve for gel filtration of TSF-IIa particles on a Sepharose 6B column in the presence of 0.05% Triton X-100. Details of the calculation of the distribution coefficient for the protein standards and TSF-IIa particles, as well as the chromatographed conditions, are given under Materials and Methods.
Table 8

The Effect of Triton X-100 Concentration on the Apparent Molecular Weight of TSF-IIa Particles

<table>
<thead>
<tr>
<th>Percentage Triton X-100</th>
<th>Percentage Void Volume</th>
<th>Percentage 250,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>0.021</td>
<td>53</td>
<td>47</td>
</tr>
<tr>
<td>0.0225</td>
<td>35</td>
<td>65</td>
</tr>
<tr>
<td>0.025</td>
<td>22</td>
<td>78</td>
</tr>
<tr>
<td>≥0.035</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

aGel filtration of TSF-IIa particles on a Sepharose 6B column was performed, at room temperature, as described under Materials and Methods.

The column was equilibrated with 20 mM Tris HCl, pH 8.2 and the indicated concentration of Triton X-100.
the presence of Triton X-100 is unknown, several possibilities can be excluded. First, after gel filtration in the presence of Triton X-100, the particles were still able to self-aggregate, as indicated by gel filtration in the absence of Triton X-100. Therefore, the gel filtration procedure did not cause an irreversible disaggregation of the particles. Second, as will be detailed in a later section, neither the polypeptide composition nor the cytochrome b-559 content of the particles was not significantly altered. Third, occasionally an orange-colored material eluted in the void volume of the column. The visible absorption spectrum of this material is shown in figure 16. The spectrum suggests that this material consists of carotenoids. SDS-polyacrylamide gels of this material showed no polypeptide bands. Attempts to reconstitute the void volume with the inactivated TSF-IIa particles were unsuccessful in re-activating the photochemical activity of the particles. This, and the fact that the orange-colored material appeared only infrequently, indicates that separation of the void volume material from the TSF-IIa particles by gel filtration in the presence of Triton X-100 was not responsible for the inactivation of the particles.

The Stability of the Photochemical Activity of TSF-IIa Particles

Clearly, the influence of Triton X-100 upon the photochemical activity of TSF-IIa particles is both profound and
Figure 16

Absorption spectrum of the void volume material obtained by gel filtration of TSF-IIa particles on Sepharose 6B in the presence of 0.05% Triton X-100.
complex. Triton X-100 inhibits the photochemistry, apparently, in both an irreversible and reversible manner. The chloro-plasts, from which the TSF-IIa particles are isolated, are initially fractionated with 2.5% Triton X-100. Photosystem II is still bound within the membrane at this stage and therefore not necessarily as susceptible to the effects of Triton X-100. But during latter stages of the isolation, the isolated particles are exposed to concentrations of Triton X-100 up to 0.1% for several hours. In the presence of this much Triton X-100 the particles are presumably photochemically inactive, yet upon removal of the Triton X-100 by either dilution or gel filtration the particles are quite active. This contrasts sharply with the total, irreversible, loss of activity of the particles after gel filtration in the presence of Triton X-100.

The stability of the photochemistry, and particularly the effect of Triton X-100 on this stability, was studied more closely. As indicated by figure 17, the decay of photochemical activity with time approximated first-order kinetics. Therefore, the time required for the activity to decay 50%, or half-life, is a good measure of the stability of the photochemical activity. At 5°C, in the presence of Triton X-100, the TSF-IIa particles had a half-life of 5 to 10 hours. After removal of the Triton X-100 by gel filtration, the particles had a half-life of about 34 hours. The decay
Figure 17

The decay rate of the photochemical activity of TSF-IIa particles at 50°C. (A) The decay rate before removal of Triton X-100 from the sample and (B) after removal by gel filtration on Sephadex G-100. The activity was assayed in 20 mM Tris-maleate, pH 6.0 as described under Materials and Methods. The initial rates were 492 and 150 μmol DCIP reduced • mg Chl⁻¹ • h⁻¹ for lines A and B, respectively.
rate of the photochemical activity of the particles was further accelerated by dialysis in the presence of 0.05% Triton X-100 (Table 9). The increased decay rate upon dialysis was strictly dependent upon the presence of Triton X-100. Dialysis of TSF-IIa particles, from which the Triton X-100 had been removed, against buffer not containing Triton X-100 did not significantly increase the decay rate. So, like gel filtration but possibly not quite as efficiently, dialysis of TSF-IIa particles in the presence of Triton X-100 leads to an irreversible inactivation of their photochemical activity.

The stability of the photochemical activity of TSF-IIa particles was also sensitive to temperature. In the presence of Triton X-100, the particles had a half-life of only 20 minutes at room temperature. At 5° C the half-life was 5 to 10 hours, as already indicated. The half-life increased to approximately 22 hours at 0° C. The only temperature at which the particles were completely stable was -80° C. It was not determined whether removal of Triton X-100 from the sample would significantly increase the stability of the particles at room temperature.

Attempts to further stabilize the activity of the TSF-IIa particles at 5° C, after the removal of Triton X-100 from the sample, were unsuccessful. As indicated by Table 10, the addition of salts or lowering the pH of the sample did not increase the longevity of the particles.
Table 9

The Effect of Dialysis on the Stability of the Photochemical Activity of TSF-IIa Particles\textsuperscript{a}

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Activity (μmol DCIP·mg Chl\textsuperscript{-1}·h\textsuperscript{-1})</th>
<th>% Decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample contains Triton X-100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control\textsuperscript{b}</td>
<td>144</td>
<td>-</td>
</tr>
<tr>
<td>Sample dialyzed against Triton X-100\textsuperscript{c}</td>
<td>53</td>
<td>63</td>
</tr>
<tr>
<td>Triton X-100 removed from sample</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>94</td>
<td>-</td>
</tr>
<tr>
<td>Sample dialyzed against buffer\textsuperscript{d}</td>
<td>81</td>
<td>14</td>
</tr>
</tbody>
</table>

\textsuperscript{a}The photochemical activity was assayed at pH 6.0 as described under Materials and Methods. The samples either contained Triton X-100 at a concentration of approx. 0.05% or had the Triton X-100 removed by gel filtration on Sephadex G-100.

\textsuperscript{b}The controls were incubated in a test tube at 5\textdegree C for the same period of time as the dialyzed samples.

\textsuperscript{c}A 1 ml sample, containing Triton X-100, was dialyzed against 500 ml of 20 mM Tris HCl, pH 8.2, containing 0.05% Triton X-100 for 6 hours.

\textsuperscript{d}A 1 ml sample of TSF-IIa particles, not containing Triton X-100, was dialyzed against 500 ml of 20 mM Tris HCl, pH 8.2 for 20 hours.
Table 10

The Effect of Incubation Conditions on the Stability of the Photochemical Activity of TSF-IIa Particles

<table>
<thead>
<tr>
<th>Conditions</th>
<th>% of Initial Activity After 20 h.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>68</td>
</tr>
<tr>
<td>5 mM Tris-HCl, pH 8.2</td>
<td></td>
</tr>
<tr>
<td>2. 1% Digitonin</td>
<td>69</td>
</tr>
<tr>
<td>5 mM Tris HCl, pH 8.2</td>
<td></td>
</tr>
<tr>
<td>3. 10 mg/ml BSA</td>
<td>68</td>
</tr>
<tr>
<td>5 mM Tris HCl, pH 8.2</td>
<td></td>
</tr>
<tr>
<td>4. 100 mM NaCl, 10 mM MgCl₂</td>
<td>68</td>
</tr>
<tr>
<td>5 mM Tris HCl, pH 8.2</td>
<td></td>
</tr>
<tr>
<td>5. 50 mM Tris-maleate, pH 6.0</td>
<td>50</td>
</tr>
</tbody>
</table>

aTSF-IIa particles, from which the Triton X-100 had been removed by gel filtration on a Sephadex G-100 column, were incubated (at 5°C) in the medium indicated at a concentration of 18.6 µg Chl/ml. The activity was measured using the standard assay conditions described under Materials and Methods. The samples were pre-incubated for 4 hours in the indicated medium before their initial activity was determined. The pre-incubation was performed so as to eliminate the influence of the additions on the initial activity from the overall decay rate. The activity was again measured 20 hours after the end of the pre-incubation period.
Although neither BSA nor digitonin (a non-ionic detergent) stabilized the activity, they both increased the initial activity 20-35%. There are several possible reasons for the stimulation of activity by BSA and digitonin. They may remove or displace tightly bound Triton X-100 present in the TSF-IIa particles, or they may change the aggregation state of the particles which has been shown to affect the activity.

**Summary and Discussion of the Effects of Triton X-100 on TSF-IIa Particles**

The effects of Triton X-100 on the photochemical activity and structure of TSF-IIa particles can be summarized as follows:

1. Triton X-100 reversibly disaggregates the particles.
2. Triton X-100 reversibly inhibits the activity of the particles.
3. Gel filtration or dialysis in the presence of Triton X-100 rapidly and irreversibly inactivates the particles.
4. Incubation of the particles in the presence of Triton X-100 slowly inactivates the particles irreversibly.

Figure 18 illustrates two models of the possible relationship between the aggregation state and the activity of TSF-IIa particles. In both models, Triton X-100 reversibly disaggregates the particles to their 250,000 dalton, or monomer, state. The only real difference between the two
Figure 18

A schematic representation of the relationship between the activity of TSF-IIa particles and their aggregation state.

Key:
+ TX-100 in the presence of Triton X-100
- TX-100 the removal of Triton X-100
\[\text{an intact TSF-IIa complex}\]
\[\text{the Triton X-100 labile component}\]
\[\text{the TSF-IIa complex without the Triton X-100 labile component}\]
MODEL 1: ACTIVE INACTIVE INACTIVE INACTIVE

MODEL 2: ACTIVE ACTIVE INACTIVE INACTIVE
models is that model 1 suggests that dissociation of the particles is sufficient to cause immediate inactivation of the particles. This is supported by the similarity of the Triton X-100 concentrations required to dissociate the particles and inhibit the photochemical activity (reversibly). However, this concept is refuted by the fact that the particles can also re-aggregate and still be inactive, after gel filtration in the presence of Triton X-100. Alternately, model 2 suggests that the TSF-IIa monomer is active, but Triton X-100 progressively strips away from the complex a component necessary for activity. In either model, Triton X-100 eventually causes an irreversible inactivation of the particles. It is impossible to differentiate between these two models simply on the basis of the presented data. Certainly, more complex models can be devised, particularly if the effects of cations on activity and aggregation state were included, but further speculation would be futile with such limited information.

As already discussed, the identity of the Triton X-100 - labile component is not known. The Triton X-100 labile component is probably not a protein component of the complex. This is supported by the fact that both gel filtration and dialysis in the presence of Triton X-100 rapidly inactivates the complex without removing any polypeptides. One possibility is that the Triton X-100 - labile component is plastoquinone. Plastoquinone, which is believed to be the primary
acceptor of PS II, can be extracted from chloroplasts with non-aqueous solvents (72). Removal of plastoquinone inhibits PS II photochemistry. PS II activity can be reconstituted by the simple re-addition of plastoquinone to the extracted chloroplasts. In a similar manner, low concentrations of Triton X-100 may reversibly interrupt the interaction between the primary donor (P680) and the primary acceptor (Q - a plastoquinone molecule), but eventually Triton X-100 extracts and disperses the plastoquinone from the TSF-IIa particles. This would explain why there is both a reversible and irreversible inhibition of the photochemical activity of TSF-IIa particles by Triton X-100.

Immobilization of TSF-IIa Particles

As the proceeding section indicated, it is unclear as to whether the TSF-IIa particles in their dissociated state (the 250,000 complex will be called "monomer" from here on) are photochemically active. To determine if the monomer is active it is necessary to trap the particles in their monomeric state, in the absence of Triton X-100. One method by which this can be achieved is by covalently linking the TSF-IIa particles to an inert support gel (226).

Two very similar types of support gels were used to immobilize the TSF-IIa particles. Ethylenediamine Sepharose and aminoethyl cellulose both have primary amine functions available for reactions and only differ in the nature of the support material agarose in one case and cellulose in
the other. Except for the fact that aminoethyl cellulose had approximately a five-fold higher capacity for immobilized TSF-IIa particles, identical results were obtained with either gel.

The TSF-IIa particles were immobilized on either gel by addition of a water-soluble carbodiimide (WSC), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. The WSC initiates a condensation reaction between carboxyl groups on the particles and the amine functions of the support gel, to form an amide linkage. The TSF-IIa particles also bound to the gel in a non-covalent manner through ionic interactions, however, the particles could be removed by washing the gel with 0.5 M NaCl containing 0.1% Triton X-100. With ethylenediamine Sepharose, 10 to 15 μg Chl per ml of gel (packed volume) were covalently bound, while for aminoethyl cellulose up to 50 μg Chl/ml gel were immobilized. In the latter case, the efficiency for immobilizing the TSF-IIa particles was greater than 90%.

The TSF-IIa particles were still photochemically active after immobilization, although at a greatly reduced rate. As Table 11 indicates, simply absorbing the particles on aminoethyl cellulose decreased the activity of the particles. Covalent immobilization decreased the activity further. A decrease in activity is common for immobilized enzymes due to the inaccessibility of substrates. Although the activity of the immobilized TSF-IIa particles was low, it was both
Table 11

The Photochemical Activity of Absorbed or Covalently Immobilized TSF-IIa Particles

<table>
<thead>
<tr>
<th>Condition</th>
<th>Activity (μmol DCIP reduced/mg Chl·h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>244</td>
</tr>
<tr>
<td>absorbed</td>
<td>71</td>
</tr>
<tr>
<td>immobilized</td>
<td>19</td>
</tr>
</tbody>
</table>

aTSF-IIa particles were either absorbed or covalently linked to aminoethyl cellulose. Photochemical activity was assayed as described under Materials and Methods. The gels contained 54 and 50 μg Chl/ml gel for the absorbed and covalent cases, respectively. The cuvette contained 2 μg Chl/ml in either case.

bThe TSF-IIa particles were assayed in the presence of 0.1 ml Sepharose 4B.
DCMU and heat sensitive, indicating that the observed activity was still PS II specific.

It was found that salts did not affect the photochemical activity of the immobilized TSF-IIa particles. However, TSF-IIa particles which had first been absorbed onto, then removed from, aminoethyl cellulose generally did not display any salt effects either. The activity of immobilized particles were only slightly influenced by pH.

These studies suggest that the monomeric form of the TSF-IIa complex is in fact active. This conclusion must be qualified with the reservation that the observed activity may possibly be due to TSF-IIa particles which are not monomeric. Although all of the particles should be absorbed or covalently attached to the gel in the monomeric state, this condition can not be rigorously proven. Conceivably, simple absorption of the molecules allows for some interaction between monomers, thus the retention of a small amount of activity. Covalent immobilization may further decrease the interaction, with a concomitant decrease in activity. These cautionary comments notwithstanding, the fact that immobilized TSF-IIa particles retained some photochemical activity implies that the monomeric state of the particles is active.

The question of whether Triton X-100 would inhibit the photochemistry of immobilized TSF-IIa particles seemed to be particularly intriguing. Unfortunately, the experi-
mental conditions to assess this question were unobtainable. Triton X-100, like several other detergents tested, accelerated the DPC-dependent reduction of DCIP in the dark. Normally, using the spectrophotometer in the dual-beam mode automatically corrected for this dark reduction rate. Therefore, the presence of Triton X-100 did not significantly influence the observed PS-II-mediated reduction rate of DCIP. However, operating in the dual-wavelength mode, the rate of DCIP reduction in the dark was not automatically corrected and thus needed to be determined independently. Coupled with the already low rates of DCIP reduction by immobilized TSF-IIa particles, it was impossible to accurately correct for the Triton X-100 stimulation of DCIP reduction. Therefore it was not possible to determine if Triton X-100 inhibited the photochemistry of immobilized TSF-IIa particles.

As a curious aside to these studies on immobilized TSF-IIa particles, attempts to couple the TSF-IIa particles to support gels through the amino groups on the particles were completely unsuccessful. The methods or gels which failed are as follows: cyanogen bromide-activated Sepharose; ε-alanine-Sepharose and a water soluble carbodiimide; reductive alkylation with sodium cyanoborohydride in the presence of periodate oxidized Sepharose; and Affi· gel 10 (an activated ester). The fact that all of these amino-directed reagents were unable to react with the TSF-IIa particles indicates that the amino groups (the lysine side-chains and N-terminal
amino acids) present in the complex are buried within the complex.

The Polypeptide Composition of TSF-IIa Particles

As indicated by the earlier sections of this dissertation, gel filtration was used extensively to study the structure of the TSF-IIa particles. Therefore, it is important to know if the polypeptide composition of the TSF-IIa particles was altered by these procedures. Moreover, it is important to know the polypeptide composition of the TSF-IIa particles in order to understand the structure of the complex. To establish the polypeptide composition of the particles, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on the particles at various stages of isolation, including after gel filtration on Sepharose 4B and Sepharose 6B, and ion-exchange chromatography on aminoethyl cellulose.

Prior to the fractionation of the chloroplast membranes, coupling factor was removed from the membranes by a series of NaBr washes (61). This was done to insure that the coupling factor polypeptides did not contaminate the final preparation of TSF-IIa particles. The NaBr washes did not adversely affect the activity of the TSF-IIa particles, nor was the overall yield of particles influenced as long as the appropriate adjustment of the amount of Triton X-100 used to solubilize the chloroplast membranes was made as described in the Materials and Methods section.
As indicated by figure 19, the TSF-IIa preparation contained a large number of polypeptides. The major polypeptides present in these particles have molecular weights of 54-, 47-, 43-, and 30 kilodaltons. The particles contained a variable amount of one or more polypeptides with a molecular weight around 25,000 daltons. This latter set of polypeptides probably are light-harvesting pigment proteins. This is concluded from that fact that the amount of these polypeptides varied with the Chl b content of the particles and their molecular weight is consistent with that of authentic LHC (128).

The TSF-IIa preparation generally contained several other minor components. Typically, one polypeptide appeared between the 43- and 30 kilodalton polypeptides. There were also several minor polypeptides with molecular weights less than 25,000. In many preparations the amount of these polypeptides was negligible (see figure 21).

The TSF-IIa particles also contain cytochrome b-559. Cytochrome b-559 is composed of several very small polypeptides, 5-6 kilodaltons in size (35), and are not resolved into identifiable bands on 10% SDS-PAGE gels. Its presence is detected by an absorption peak at 559 nm in a dithionite reduced sample. The particles contain 50 ± 10 Chl molecules per cytochrome b-559. This Chl:cytochrome ratio is similar to that found by others for these types of particles (63, 74).
Figure 19

Gel scans of TSF-IIa particles on SDS-PAGE tube gels. Trace A is of TSF-IIa particles before gel filtration. Trace B is of TSF-IIa particles after gel filtration on Sepharose 4B, at 5°C. Trace C is of TSF-IIa particles after gel filtration on Sepharose 4B then on Sepharose 6B, in the presence of 0.05% Triton X-100. Approximately 5.0 μg Chl was applied to each gel.
The TSF-IIa preparation was also contaminated with a small amount of photosystem I. Values of 800 to 1800 Chls per P700 were found. Assuming a Chl:P700 ratio of 80 for the PS I contamination, this suggests that in some TSF-IIa preparations as much as 10% of the total Chl was actually associated with PS I. This PS I content is comparable to that found by Lach et al. (74) in their TSF-IIa preparations. Although the light-induced P700 signal is barely detectable, the presence of the PS I contamination is clearly evident on LiDS gels, as will be discussed in a subsequent section.

When chromatographed on a Sepharose 4B column equilibrated with 5.0 mM Tris HCl, pH 8.5, the particles eluted almost entirely as a single Chl peak with an apparent molecular weight of 460,000. This gel filtration procedure did not remove any of the major polypeptides, however, it did reduce or remove a few of the minor polypeptides (figure 19). Similarly, an additional gel filtration step on Sepharose 6B in the presence of 0.05% Triton X-100, in which the TSF-IIa particles eluted with an apparent molecular weight of 250,000, did not alter the polypeptide composition of the particles. Neither gel filtration procedure changed the cytochrome b-559 content of the particles. This suggests that the 54-, 47-, 43-, and 30 kD polypeptides, as well as cytochrome b-559, are all valid constituents of the PS II core complex. Furthermore, it indicates that the 460 kD species seen in the absence of detergent is simply a dimer of the 250 kD
species observed in the presence of Triton X-100.

Although the two gel filtration steps produced TSF-IIa particles relatively free of contaminants, the procedure required long periods of time to perform, had a relatively small capacity, and ultimately resulted in photochemically inactive particles. Therefore, an ion exchange step on aminoethyl cellulose was substituted for the gel filtration procedures. It allowed for the purification of large quantities of particles in a short period of time.

The aminoethyl cellulose column separated the TSF-IIa particles into two Chl containing fractions. The first Chl fraction, which eluted directly from the column, had no PS II photochemical activity. Its absorption spectrum showed a blue-shifted absorption maximum at 667.5 nm and a greater Chl b content, compared to the original TSF-IIa particles (figure 20). This fraction may be comparable to the inactive TSF-IIa' fraction obtained by Vernon et al. (133) on sucrose gradients. The second Chl fraction, which was eluted with 20 mM Tris HCl, pH 8.2 containing 0.5 M NaCl and 0.1% Triton X-100, had a slightly red-shifted absorption maximum and a specific activity 10-15% higher than the original TSF-IIa particles.

The polypeptide composition of the active TSF-IIa fraction was identical to that obtained by the gel filtration procedures (figure 21). The particles retained their cytochrome b-559 content as well as a small amount of LHC
Figure 20

Absorption spectra of TSF-IIa particles as isolated by differential centrifugation, and the photochemically inactive and active fractions from the aminoethyl cellulose column.
α-Chymotrypsin

Treated

Control

559

570

673

0.005 O.D.

0.1 O.D.

400 500 600 700
SDS-polyacrylamide gel electrophoretic patterns of TSF-IIa particles before (lane I) and after (lane II) chromatography on aminoethyl cellulose and Sephadex G-100. Lane III contains TSF-IIa particles which had been further chromatographed on a Sepharose 6B column in the presence of 0.05% Triton X-100. Each lane contained 5 μg of Chl. Lane IV contains protein molecular weight markers as described under Materials and Methods.
and PS I contamination. After chromatography on aminoethyl cellulose, the particles were in a highly aggregated state. They eluted in the void volume of a Sepharose 4B column in the absence of detergent, unlike the original TSF-IIa particles. However, in the presence of Triton X-100 these particles still eluted as a single Chl-containing peak with an apparent molecular weight of 250,000. Salts generally did not influence the activity of the particles at pH 8.0.

The polypeptide composition of TSF-IIa particles was reported previously by Klein and Vernon (68). The polypeptides present in that preparation had molecular weights of 63-, 57-, 51-, 44-, 20 kilodaltons. Their particles contained cytochrome b-559 as well. Obviously, there are some differences between their preparation of TSF-IIa particles and ours. The two high molecular weight polypeptides which they report are probably the α and β subunits of coupling factor (61). The other differences in polypeptide composition may be a result of the different procedures used to prepare the samples for SDS-PAGE.

Photosystem II particles similar to TSF-IIa particles have been purified from spinach chloroplasts fractionated with digitonin (112,113,136,138). The digitonin-prepared particles of Wessels and Borchert (136,138) contained polypeptides of 54-, 46-, 42-, 31-, and 27 kilodaltons as well as numerous other smaller polypeptides, including cytochrome b-559. The resemblance to the polypeptide composition of our TSF-IIa
particles is striking. Satoh (112,113) has indicated that his digitonin-prepared PS II particles are composed solely of two polypeptide, 43- and 27-kilodaltons, and cytochrome b-559. The 27 kilodalton polypeptide of Satoh is probably equivalent to our 30 kilodalton polypeptide.

Thus, there appears to be a great deal of structural similarity between PS II particles prepared using either digitonin or Triton X-100. Hopefully, this similarity results from the fact that the composition of the isolated particles reflects that of the PS II core complex present in the chloroplast membranes. The PS II particles of Satoh seem to be more highly resolved than our TSF-IIa particles. Perhaps future attempts at further purification of TSF-IIa particles will result in a simplified TSF-IIa particle as well.

The Effect of Protease Treatment on TSF-IIa Activity

The TSF-IIa particles were subjected to limited proteolysis in an attempt to further probe the structure of the complex and possibly identify those polypeptides which are crucial to the preservation of photochemical activity. The particles were first purified on aminoethyl cellulose, followed by gel filtration on Sephadex G-100 to remove salt and Triton X-100. The particles were then subjected to proteolytic digestion by either trypsin or α-chymotrypsin. The incubation was performed at 5°C so as to minimize the inactivation of the untreated control particles. As figure 22 indicates,
Figure 22

The effect of trypsin and α-chymotrypsin, at different concentrations, on the photochemical activity of TSF-IIa particles. The incubation period was for 20 hours, at 5°C. The Chl concentration was 50 μg/ml. The control rate was 69 μmol DCIP reduced • mg Chl⁻¹ • h⁻¹.
the activity of the TSF-IIa particles was remarkably stable to proteolytic inactivation. At a concentration of 5 μg/ml of either α-chymotrypsin or trypsin (Chl:protease ratio of 10), little or no inactivation of the particles occurred after a 20 hour incubation period. At higher concentrations of the protease, up to 100 μg/ml (Chl:protease ratio of 0.5), the activity was inhibited only 40% compared to an untreated sample. Treatment of the particles with either protease actually led to an initial 10-20% increase in the activity, followed by a decrease paralleling that of the control particles (figures 23 and 24).

If proteolysis was conducted on the complex without prior removal of Triton X-100 from the sample, activity was lost relatively quickly (within 6 hours). As indicated in an earlier section, Triton X-100 inhibits the photochemical activity of the TSF-IIa particles. This inhibition is reversible for short periods of time. However, 0.05% Triton X-100 also greatly accelerates an irreversible decay of activity. Apparently, proteases further accelerate this decay ratio, possibly by increasing the accessibility of the labile component to Triton X-100. Or conversely, Triton X-100 may increase the accessibility of the proteases to a component which is necessary for the photochemical activity of the complex.
Figure 23

The effect of α-chymotrypsin (5 μg/ml) on the photochemical activity of TSF-IIa particles as a function of the time of incubation. Conditions are given in the Materials and Methods section. The initial control rate was 150 μmol DCIP reduced · mg Chl⁻¹ · h⁻¹.
Rate of DCIP Reduction ( % of Initial Rate )

HOURS

20 40 60 80 100 120

HOURS

0 2 4 6 8 10 12 14 16 18

α-CHYMOTRYSIN (5µg/mL)

CONTROL
Figure 24

The effect of trypsin (5 μg/ml) on the photochemical activity of TSF-IIa particles as a function of the time of incubation. Conditions are given in the Materials and Methods section. The initial control rate was 136 μmol DCIP reduced · mg Chl⁻¹ · h⁻¹.
Rate of DCIP Reduction ( % of Initial Rate)

**Graph:**
- **X-axis:** Hours (20, 40, 60, 80, 100, 120)
- **Y-axis:** Rate of DCIP Reduction (% of Initial Rate)

- **Legend:**
  - **TRYPsin (5µg/mL)**
  - **CONTROL**

The graph shows the rate of DCIP reduction over time for two conditions: one with trypsin and one as a control, with the trypsin condition showing a higher rate of reduction compared to the control.
The Effect of Proteases on the Polypeptide Composition of the TSF-IIa Complex

Unlike the photochemical activity, the polypeptide composition of the TSF-IIa particles was considerably altered by protease treatment. SDS-gels of sample treated with α-chymotrypsin for different incubation periods (figure 25A) showed that all of the major polypeptides were extensively digested, with the exception of the 30 kD polypeptide. After 1 hour there was a complete loss of the 47 kD polypeptide and a considerable loss of the 54-, 43-, and 25 kD polypeptides. This corresponds to the same time period in which the activity of the particles was actually increasing. After 20 hours, essentially only the 30 kD polypeptide remained intact. Trypsin treatment of the TSF-IIa particles gave nearly identical results (figure 25B). It is not known how proteolysis affected the polypeptides of cytochrome b-559.

After proteolytic treatment, the TSF-IIa particles could still be isolated by the aminoethyl cellulose procedure. All of the chlorophyll in the sample bound to the column, suggesting that no free chlorophyll had been generated by proteolytic digestion. The protease was removed from the column with a 0.5 M NaCl wash and the TSF-IIa particles were removed with 0.5 M NaCl containing 0.05% Triton X-100. These particles were still photochemically active. After a further gel filtration step in Sephadex G-100, SDS-gels of the protease treated particles showed only the 30
Figure 25

The polypeptide composition of TSF-IIa particles on SDS-gels after different times during proteolysis. (A) The chymotrypsin concentration was 5 µg/ml, except for the bottom trace where 100 µg/ml was used. (B) The trypsin concentration was 5 µg/ml. Other conditions are given in Materials and Methods.
\( \alpha \)-CHYMOTRYPSIN

\( T = 0 \)

\( T = 1 \text{ h.} \)

\( T = 4 \text{ h.} \)

\( T = 20 \text{ h.} \)
$T = 8\ h.$
kD polypeptide (Figure 25A, bottom trace). However, there was a considerable amount of Coomassie blue staining material of higher mobility (molecular weights less than 15,000) which was not resolved into discrete bands. This material, which was not present in untreated samples, probably consists of peptide fragments, heterogeneous in size, which were not removed or resolved from the complex by the chromatographic procedures.

Considering the difference in the specificities of trypsin and α-chymotrypsin it is rather surprising to find such a similar pattern for proteolytic digestion of the particles. This suggests that the susceptibility of the individual polypeptides is based on the extent to which they are exposed to the solvent phase rather than their amino acid composition. With this assumption, it can be concluded that 47 kD polypeptide is more surface exposed than the other polypeptides because it is most readily cleaved, while the 30 kD polypeptide is confined entirely to the interior of the complex because it is most resistant to proteolytic cleavage.

The Effect of Proteolysis on Other Structural and Photochemical Properties of TSF-IIa Particles

Figure 26 demonstrates that the absorption spectrum of the TSF-IIa particles was unaffected by proteolysis. This is consistent with the fact that the photochemical activity was unaffected.
Figure 26

The absorption spectrum of untreated and α-chymotrypsin-treated (5 μg/ml, 20 h.) TSF-IIa particles. A few grains of sodium dithionite were added to the sample to obtain the reduced-oxidized absorption spectrum of cytochrome b-559.
The photosystem II reaction center can catalyze both a reversible photochemical reduction and oxidation of the cytochrome b-559 contained within the complex (63). The extent of the cytochrome b-559 photochemical reduction was 3.5 to 5.0% of the total cytochrome content of the particles, consistent with the findings of others (74). The signal was dependent upon the presence of the electron donor DPC, but was insensitive to DCMU. The photochemical reduction of cytochrome b-559 was found to be entirely inhibited by 0.05% Triton X-100. This supports the earlier conclusion that Triton X-100 inhibits PS II photochemistry at the primary processes level.

Proteolytic treatment of the TSF-IIa particles did not alter the chemically-induced cytochrome b-559 signal (figure 26), indicating that the cytochrome was not denatured or removed from the complex by the procedure. Proteolysis also did not change the photochemically-induced cytochrome b-559 signal (figure 27). This indicates that proteolysis did not disturb the close association between the PS II reaction center and cytochrome b-559.

Gel filtration of the protease-treated particles on Sepharose 4B in the absence of Triton X-100 showed that these particles were still highly aggregated, as they eluted in the void volume of the column. On the other hand, the particles eluted as a single chlorophyll peak with an apparent molecular weight of 100,000 ± 10,000 in the presence
Figure 27

Light-induced cytochrome b-559 reduction of control and α-chymotrypsin-treated samples of TSF-IIa particles. Conditions are as given in Materials and Methods.
CONTROL

ON OFF

0.001 A

a-CHYMOTRYPSIN TREATED

50 seconds
of 0.05% Triton X-100. This confirms that proteolysis did in fact reduce the molecular weight of the particles.

Analysis of the protein-to-chlorophyll ratio of the particles indicated that proteolysis with either trypsin or α-chymotrypsin decreased the ratio consistently by 40% (Table 12). This reduction in protein content is consistent with the 60% decrease in the size of the particles as determined by gel filtration. Thus, proteolytic treatment of the TSF-IIa particles removed some protein, but no chlorophyll, from the complex.

To determine whether proteolytic digestion altered the isoelectric point of the complex, the turbidity (180° light-scattering) was measured as a function of pH (figure 28). The light-scattering of the untreated particles was maximal at pH 4.55 ± .10 due to isoelectric precipitation. TSF-IIa particles which had been treated with α-chymotrypsin or trypsin had broad maximums at pH 4.4 ± .10. Within the sensitivity of this technique, this demonstrates that the isoelectric point of the complex was not significantly changed by proteolysis.

The effects of purification and proteolysis on the DCMU sensitivity of the PS II particles was very curious. The TSF-IIa particles before chromatography on aminoethyl cellulose were inhibited 50% by 1-2 μM DCMU (Figure 29). A similar sensitivity has been reported previously (133). After the aminoethyl cellulose and Sephadex G-100 chromato-
Table 12
The Effects of Proteolysis on the Protein to Chl Ratio of TSF-IIa Particles

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Protein:Chl (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Control</td>
<td>7.0</td>
</tr>
<tr>
<td>Chymotrypsin-treated</td>
<td>4.3</td>
</tr>
<tr>
<td>B. Control</td>
<td>9.3</td>
</tr>
<tr>
<td>Trypsin-treated</td>
<td>5.7</td>
</tr>
</tbody>
</table>

*TSF-IIa particles were treated with either 100 μg/ml of α-chymotrypsin or trypsin for 20 hours then chromatographed on aminoethyl cellulose and Sephadex G-100. Procedures are described in Materials and Methods.*
Figure 28

The 180° light scatter of TSF-IIa particles as a function of pH. The control (●—●) and α-chymotrypsin-treated samples (5 μg/ml ○○ and 100 μg/ml ▲▲) were all titrated at a concentration of 15 μg Chl/ml.
Figure 29

The DCMU concentration dependence for inhibition of DCIP reduction by TSF-IIa particles (A) before and (B) after chromatography on aminoethyl cellulose, and (C) TSF-IIa particles treated with 5 μg/ml α-chymotrypsin for 20 hours.
graphic steps however, the particles were about 10-fold less sensitive to DCMU ($K_i = 20 \mu M$). The reason for this change is not clear, but one possible explanation is that prior to purification approximately 50% of the PS II particles have a low $K_i$ (less than 0.5 $\mu M$) for DCMU while the rest have a higher $K_i$ (approx. 20 $\mu M$). Purification of the TSF-IIa particles then converts the low $K_i$ particles to the high $K_i$ state. The difference in the two states may simply reflect two different modes of inhibition by DCMU.

Treatment of the particles with either chymotrypsin or trypsin only slightly decreases the sensitivity of the particles to DCMU (figure 29). This finding is not entirely inconsistent with results obtained by others with trypsin treatment of chloroplasts. Such treatment of chloroplasts changes the $K_i$ of DCMU from approximately 50 nm to 3 $\mu M$ and allows direct donation of PS II electrons to ferricyanide (130). In our purified PS II particles the $K_i$ for DCMU is already higher than it is for trypsin-treated chloroplasts, and they can already use ferricyanide as an electron acceptor. Therefore, a major change in DCMU sensitivity would not necessarily be expected. Nonetheless, the fact that we still do observe some DCMU-inhibition after protease treatments stands in direct contrast with the results of Croze et al. (26) who found that trypsin treatment of PS II membrane fragments (TSF-II) completely abolished its DCMU sensitivity. Further
experiments are necessary to reconcile these two sets of results.

As may be expected, neither the purification nor protease treatment altered the sensitivity of the activity to Triton X-100. Similarly, the $K_m$'s for DPC and DCIP were not altered (Table 13). This indicates that the accessibility of the donors and acceptors to their reaction sites was not changed.

The sum of these results indicates that the TSF-IIa complex, as a whole, can undergo extensive proteolytic cleavage without significantly altering its photochemical properties. This leads to either one of two conclusions. Either the reaction center polypeptide, and therefore the Chl-containing species, is the 30 Kd polypeptide, which does not undergo cleavage, or the reaction center polypeptide does undergo cleavage without denaturing or loss of activity.

To differentiate between these two possibilities, identification of the Chl-containing polypeptides was necessary. To achieve this, the particles were subjected to electrophoresis on LiDS-gels under non-denaturing conditions (4). Figure 30A shows the Chl pattern prior to the staining of the gels. The untreated TSF-IIa particles showed five Chl bands. The Chl band of lowest mobility (apparent molecular weight of 90 kD) consists of PS I contamination. The next two Chl bands (apparent molecular weights of 47- and 43 kD)
Table 13

The Effects of Aminoethyl Cellulose Chromatography and Proteolytic Treatment of TSF-IIa Particles on the Km Values of DCIP and DPC

<table>
<thead>
<tr>
<th>Sample</th>
<th>Km Values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DCIP (μM)</td>
</tr>
<tr>
<td>TSF-IIa</td>
<td>8.1</td>
</tr>
<tr>
<td>TSF-IIa AE</td>
<td>4.9</td>
</tr>
<tr>
<td>TSF-IIa • Protease</td>
<td>7.1</td>
</tr>
</tbody>
</table>

aParticles were assayed either before (TSF-IIa) and after (TSF-IIa AE) purification on aminoethyl cellulose, or following proteolytic treatment with 5 μg/ml α-chymotrypsin for 20 h. (TSF-IIa • protease). The Km values for DPC and DCIP were determined at pH 6.0.
were barely resolved from each other. These are the PS II Chl-containing polypeptides. The third Chl band consists of LHC contamination and the fourth Chl band consists of free pigments, the latter representing the vast majority of the total Chl molecules present. The apparent amount of both PS I and LHC Chl contamination of the TSF-IIa particles is greatly exaggerated on these gels because the Chl associated with PS II is much more readily dissociated under these conditions, compared to the Chl associated with either PS I or LHC. Stained LiDS-gels (figure 30B) show that the Chl is associated with polypeptides in the 40-50 kD range. No Chl was found to be associated with the 30 kD polypeptide.

TSF-IIa samples treated with chymotrypsin showed a similar Chl pattern on LiDS-gels except that there was a single Chl band between the PS I and LHC bands. This Chl band had a slightly higher mobility than the original PS II Chl-containing polypeptides. After staining with Coomassie blue, a diffuse protein band was apparent under the Chl. This protein band was clearly different from the 30 kD polypeptide which was not digested. As with the SDS-gels, none of the higher molecular weight polypeptides (43-, 47-, and 54 kD) were observed on the LiDS-gels of the samples treated with proteases. The diffuse protein band and its associated Chl were present only under very mild non-dissociating conditions.
Figure 30

Electrophoresis of control and α-chymotrypsin-treated (100 μg/ml, 20 h.) TSF-IIa particles on LiDS-gels under non-denaturing conditions. Approx. 10 μg Chl were applied to each gel. (A) The gels were scanned at 670 nm for Chl before staining and (B) scanned at 600 nm after staining with Coomassie blue.
LHC
PS I  P S n  FREE Chi
CONTROL

a-CHYMOTRYPSIN TREATED

A

PS I  PS II  FREE Chl
LHC

B

CONTROL

a-CHYMOTRYPSIN TREATED

47 30 kD
54 43

0.02 A
0.20 A
The sum of these results indicate that the reaction center polypeptide of PS II is cleaved during proteolysis but the vital peptide fragments do not dissociate, leaving the photochemical apparatus intact.

**Summary and Discussion of the Effects of Proteases on TSF-IIa Particles**

Several investigators have reported finding PS II associated Chl a-containing polypeptides isolated from detergent-fractionated chloroplasts on either SDS- or LiDS-gels (21,24,50,115). These Chl a complexes have molecular weights in the 40 to 50 kD region. For example, Camm and Green reported two Chl a complexes in addition to others, with molecular weights of 47 and 43 Kd (21). Additionally, Satoh has reported that the Chl a is associated with the 43 Kd polypeptide in his digitonin prepared particles (115). Our results generally concur with these findings. However, it can not be unhesitatingly stated that there are two separate Chl a-containing polypeptides in the PS II complex. The two Chl a bands observed on the LiDS-gels may actually be due to a single Chl a-containing polypeptide in two slightly different states of denaturation.

The isolated photosystem II core complex, in general, is very stable in the presence of proteases. Although most of the major polypeptides were proteolyzed, the basic photochemical activities and structural state of the complex was retained. During proteolysis some peptide fragments (approx.
40%) were removed from the particles while others, presumably those associated with the reaction center, remained intact or at least retained their native conformation. This interaction between the peptide fragments was sustained even in the presence of mild denaturants. Such a strong interaction may in fact depend on the presence of the chlorophyll which is associated with these fragments.

The ability of chlorophyll-protein complexes to remain intact after proteolysis is not without precedence. Jennings et al. (60) report that chymotrypsin treatment of spinach chloroplast membranes does not alter the electrophoretic mobility of either PS I or the LHC, yet the polypeptides components of both are extensively digested. Our findings extend their results to include photosystem II, and demonstrates for the first time, such an ability in an isolated chlorophyll-protein complex.
We have found that the photochemical activity of the isolated photosystem II core complex (TSF-IIa) is responsive to the presence of protons, mono- and divalent cations. The activity of the TSF-IIa particles is maximal at pH 6.0. The activity is approximately 20-fold less at pH 8.0. The response of the photochemical activity to the presence of mono- and divalent metal cations is dependent upon the presence or absence of Triton X-100 in the sample. When Triton X-100 is present in the sample, divalent cations stimulate the activity of the particles while monovalent metal cations have no effect. After removal of the Triton X-100 from the TSF-IIa particles by gel filtration, the activity is inhibited by both mono- and divalent cations. By analyzing the effect of cations on the activity as a function of light intensity, it has been determined that protons, mono- and divalent cations influence the activity of the particles by acting on the rate-limiting electron transport step. The rate-limiting electron transport step is on the reducing side of PS II (the electron transfer steps leading from \( Q^- \) to DCIP) in these particles. We conclude that cations do not significantly affect energy transfer.
within the PS II core complex. These studies confirm the findings of others (see Introduction) that the primary site of cationic control of energy transfer resides in the light-harvesting complex. However, these experiments do suggest that cations may influence electron transfer rates between the two photosystems in the thylakoid membrane.

Cations also influence the aggregation state of the TSF-IIa particles. The particles have an apparent molecular weight of 460,000, as determined by gel filtration under low ionic strength, high pH conditions. Increasing the ionic strength or lowering the pH of the buffer medium results in the aggregation of the particles (molecular weight greater than 800,000). Increasing the temperature also causes the particles to aggregate. This suggests that the aggregation is due to hydrophobic forces. The salt-induced aggregation is consistent with this hypothesis.

If the cation effects on photochemical activity are related to the cation effects on the aggregation state of the particles, the relationship must be complex. The salt-induced aggregation corresponds most closely with the salt-induced inhibition of electron transport observed in samples from which the Triton X-100 has been removed. In contrast, aggregation accompanies an increase in the activity as the pH is lowered. The apparent contradiction of these results can be resolved by suggesting that the structure of the two aggregates may be different. Future experiments may clarify
We found that Triton X-100 inhibits the photochemistry of the TSF-IIa particles by acting on the reaction center. The concentration of Triton X-100 required for half-maximal inhibition of photochemistry is 0.007%. The inhibition of photochemistry by Triton X-100 is reversible, at least for short periods of time. However, incubation of the particles in the presence of 0.05% Triton X-100 leads to an irreversible inactivation of the activity. Subjecting the particles to either gel filtration or dialysis in the presence of 0.05% Triton X-100 also rapidly inactivates the particles in an irreversible manner. Because the polypeptide composition of the complex is not altered by these procedures, we suggest that the irreversible inactivation occurs due to the removal of a non-protein component such as plastoquinone.

Triton X-100 also has an effect on the molecular weight of the TSF-IIa complex. Specifically, Triton X-100 dissociates the complex into 250,000 dalton subunits. The concentration of Triton X-100 required to dissociate 50% of the particles is 0.021%. The particles are able to reassociate after the removal of the Triton X-100. The 460,000 dalton TSF-IIa particle, observed in the absence of Triton X-100, and the 250,000 dalton species have the same polypeptide composition.

Therefore, the 460,000 species is simply a dimer of the 250,000 dalton TSF-IIa particle. The disaggregation caused by Triton X-100 may be responsible for the observed reversible
inactivation of the reaction center activity. However, the concentration of Triton X-100 required to inhibit the activity and disaggregate the particles both by 50% is different by 3-fold. Furthermore, TSF-IIa particles immobilized on a support gel, apparently in their monomeric state, are photochemically active. These results indicate that the 250,000 dalton species is photochemically active.

The TSF-IIa particles can be purified by a series of gel filtration procedures performed in the presence and absence of Triton X-100. The particles can also be purified by chromatography on aminoethyl cellulose. An aminoethyl cellulose column separates the TSF-IIa particles into two chlorophyll-containing fractions. One fraction does not have any PS II photochemical activity while the other fraction has a specific activity higher than the original particles. The TSF-IIa particles purified by either method are composed of the same polypeptides. The molecular weights of the polypeptides are 54,000, 47,000, 43,000, and 30,000 daltons as determined by SDS-gel electrophoresis. The complex also contains cytochrome b-559. There are approximately 50 chlorophyll molecules for every cytochrome b-559 molecule. We suspect that the polypeptides found in the TSF-IIa particles represent at least the minimal number of polypeptides associated with the PS II core complex in the thylakoid membrane.

The structure of the TSF-IIa complex has been probed using proteolytic enzymes. Prolonged treatment of the particles
with either trypsin or α-chymotrypsin results in extensive digestion of all of the polypeptides except the 30,000 dalton polypeptide. The 47,000 dalton polypeptide is the most rapidly digested polypeptide, using either protease, suggesting that it is located on the surface of the complex. By the same line of reasoning, we conclude that the 30,000 dalton polypeptide is buried within the complex.

Proteolytic treatment reduces the size of the complex from 250,000 daltons to 100,000 daltons as determined by gel filtration in the presence of 0.05% Triton X-100, and reduces the protein:Chl ratio of the complex by 40%. It does not alter the isoelectric point of the particles.

In spite of the drastic structural changes brought about by proteolysis, the TSF-IIa particles retain their photochemical activity. Proteolysis does not change the absorption spectrum of the particles, nor does it significantly change the sensitivity of the particles to DCMU and Triton X-100. The TSF-IIa particles have the same Km values for DPC and DCIP, and are still able to photochemically reduce cytochrome b-559, after proteolytic treatment. These results indicate that although proteolytic treatment digests the polypeptides and reduces the size of the complex, it does not alter the photochemical properties of the complex.

Finally, by subjecting the TSF-IIa particles to LiDS-gel electrophoresis under non-denaturing conditions, we have demonstrated that chlorophyll is associated with both the
47,000 and 43,000 dalton polypeptides. Obviously, one of these polypeptides must be the reaction center polypeptide of photosystem II. Both polypeptides are extensively cleaved upon treatment with proteolytic enzymes. We conclude that the reaction center polypeptide of PS II is cleaved during proteolysis but the peptide fragments retain their native conformational state, therefore, the photochemical activity of the complex remains unimpaired.

Clearly, such studies only touch the surface of what there is to be known about the structure of the PS II core complex. The first tenuous steps towards the elucidation of any complex system may appear trivial and unsatisfyingly vague about the information that they bring. Yet, the process must begin somewhere. In many instances, the important result of such experiments does not answer what is - but rather, suggests only what is possible. It is hoped that the studies described in this dissertation will help lay the foundation for future, more elucidating, studies of the structural and photochemical properties of the photosystem II core complex of spinach chloroplasts.
LIST OF REFERENCES


