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Minireview

The identification of the Photosystem II reaction center: a personal story *

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Abstract

This minireview is about the path that led me to the identification of the Photosystem II reaction center in oxygenic photosynthesis. It is based mostly on my own experiences and viewpoints. Thus, the article is essentially a personal account, and does not include all contributions that led to the identification of this functional unit of Photosystem II.

Abbreviations: Chl – chlorophyll; CP-43 – 43 kDa chlorophyll *a* binding protein of Photosystem II core antenna; CP-47 – 47 kDa chlorophyll *a* binding protein of Photosystem II core antenna; EDTA – ethylene diamine tetra acetic acid; PS – photosystem; P-680 – the primary electron donor Chls of Photosystem II; P-700 – the primary electron donor Chls of Photosystem I; Q_B – the secondary plastoquinone acceptor of Photosystem II

Early history

From the early days of the chemical identification of chlorophyll (Chl), many biologists and biochemists believed that this pigment was associated with proteins in its functional state *in vivo* (e.g., Emil Smith 1938; see historical note by Govindjee 1988). Thus, numerous studies were devoted to the direct isolation of the pigment in its natural environment in leaves, but success initially was limited partly due to the lack of knowledge of the photosynthetic mechanisms and of the purification techniques for membrane proteins. (see Albertsson, this issue, for a history of some of the methods). The fragmentation of chloroplasts was also attempted in order to identify the minimum unit capable of photosynthesis (Jan B. Thomas et al. 1953).

A remarkable achievement in chloroplast fragmentation was the success by Keith Boardman and Jan Anderson (1964), in Australia, in separating digitonin-solubilized spinach chloroplasts into two fractions, each enriched in one of the two photosystems (PS), supporting the prevailing concept of two photochemical reactions in photosynthesis, i.e., Photosystem I (PS I) and Photosystem II (PS II) (see Anderson 2002). In this fractionation, PS II activity was associated with the membrane fraction enriched in Chl *b*, which is now known as the grana fraction of chloroplasts. A few years later, *p*oly*a*crylamide *g*el *e*lectrophoresis (PAGE) under partially denaturing conditions was introduced in this field by Teruo Ogawa et al. (1966) using the ionic-detergent SDS (sodium dodecyl sulfate), leading to the successful separation of two chlorophyll-protein complexes (see Ogawa, this issue). This led to the identification of the P-700 apoprotein of PS I, but did not allow the isolation of a PS II pigment-protein complex with photochemical activity. Instead, these studies established that the majority of Chl *b* molecules in chloroplasts are associated with a specific thylakoid protein which according to a proposal by Phil Thornber and Highkin (1974) is now known as *l*ight-*h*arvesting *c*hlorophyll a/b *p*rotein (LHCP) complex.

Identification of the Photosystem II core complex

Isolation

The search for a Chl-protein complex with PS II activity was continued by using less denaturing non-ionic detergents. Huzisige et al. (1969), in Japan, and Wessels et al. (1973), in the Netherlands, used digi-

* This paper is dedicated to the late Professor Hirosi Huzisige (May 9, 1916–March 21, 2003) (see Figure 7).

tonin, whereas Leo Vernon et al. (1971), in the USA, used Triton X-100. The latter two groups demonstrated that PS II activity is associated with fractions that have a high Chl *a*/*b* ratio rather than with those enriched in Chl *b*.

I came into the field at this stage of the investigation, when I was visiting Warren L. Butler at the University of California at San Diego (see Govindjee et al., 1987, for a special issue dedicated to Butler). The initial purpose of my study was to isolate the three Chl-protein complexes that are responsible for one of the three fluorescence emission bands of chloroplasts observed at low temperature (77 K), i.e., F-685, F-695, and F-735 emission bands (for a historical account of these emission bands, see Govindjee 1995). At that time, Warren Butler and his coworkers had proposed a kinetic model called the 'Tripartite model' to explain excitation energy transfer interactions among three different pigment systems in photosynthesis, i.e., PS I, PS II, and LHCP (Butler and Strasser 1977). The model was based on the assumption that the F-685, F-695, and F-735 emissions originate from LHCP, PS II, and PS I, respectively, and I was to substantiate the model biochemically, i.e., to establish these three pigment complexes as definite chemical entities to correlate with the three fluorescence emission bands observed at low temperatures. However, since Chls were thought to be bound to proteins mostly by hydrophobic interactions and, at the same time, Chl-binding proteins by themselves are intrinsic membrane components, the project seemed rather difficult as the wavelength and the yield of Chl fluorescence are extremely sensitive to the molecular environment. Thus, we had to look for very specific conditions that permitted the breakage of some interactions, but not others of similar kinds. We finally came to use a mild non-ionic detergent, digitonin, which was used by Boardman and Anderson (1964) and Wessels et al. (1973), but in a well defined condition by preparing 'lyophilized digitonin' that is freely soluble or dispersible in buffer solution (see description on the solubility of digitonin, for example, in the *Merck Index*), in order to avoid drastic changes in the molecular environment of pigments within protein-pigment complexes and to obtain reproducible results. We employed exhaustive methods such as linear sucrose gradient centrifugation (Figure 1) and isoelectric focusing in order to purify all major pigment complexes in the detergent-extract all at once.

By introducing these procedures, we established a systematic method to isolate Chl-protein complexes with minimum modifications in the environment of pigments as demonstrated by the retention of spectroscopic forms of Chl, i.e., *in vivo* absorption peaks of Chls (Satoh and Butler 1978; Satoh 1982). As a byproduct of this work, we isolated a PS II pigment-protein complex with photochemical activity (now called the 'PS II core complex'), which contains only Chl *a* and β-carotene, but neither Chl *b* nor xanthophylls. This established the PS II complex as a chemical entity with definite pigment and cofactor composition (e.g., Satoh 1983), which, in essence, is the pigment protein complex of PS II originally described by Wessels et al. (1973).

Polypeptide composition

The path toward a final conclusion regarding the polypeptide composition of the isolated PS II core complex, however, was lined by several technical difficulties that I did not recognize for several years. The first problem was that the components of ATP synthase (CF_0/CF_1) in the extracts behaved just like the PS II core complex in the purification procedure that we had developed. Therefore, we had to improve the extraction procedure to include NaBr- or EDTA-treatment prior to the digitonin-solubilization of thylakoid membranes (Satoh 1979). The second problem was the tendency of the PS II polypeptides to form aggregates upon heat-treatment in the sample buffer for SDS-PAGE conducted according to the standard procedure described by Laemmli (1970). After considerable time, we realized that we should not heat-treat the sample in the resolving buffer (Satoh 1979).

The most serious problem, which finally was overcome, was related to the abnormal behavior of PS II protein bands around 30 kilodalton (kDa) in the SDS-PAGE. In those days, thylakoid membrane proteins of the green alga *Chlamydomonas reinhardtii* had been given numbers (Chua and Bennoun 1975) in order to exhaustively identify the gene and function of each polypeptide components (for example, number five was given to the apo-CP47, number six was for the apo-CP-43, etc.). However, two proteins now known as the D1 and D2 proteins were not included in this count, because of the fact that they do not form distinct sharp bands in Coomassie Blue-stained gels in the SDS-PAGE. They form *diffusive* bands, and that is why they have the prefix 'D.' When analyzing the polypeptide composition of the purified PS II core complex from spinach, we also encountered this problem in this molecular mass region. However, this

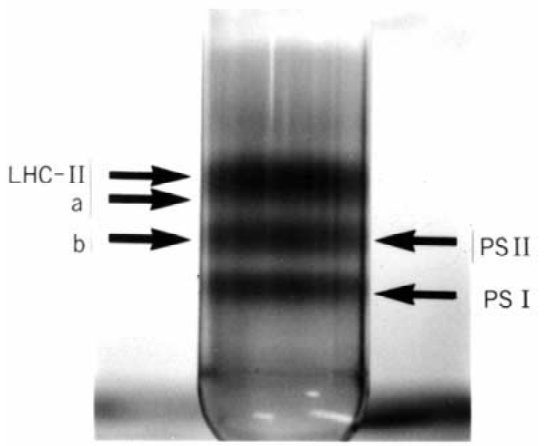

Figure 1. Separation of chlorophyll-protein complexes in thylakoid membranes by linear sucrose density gradient centrifugation (data from Satoh and Butler 1978). For a color version of this figure, see color section in the front of the issue.

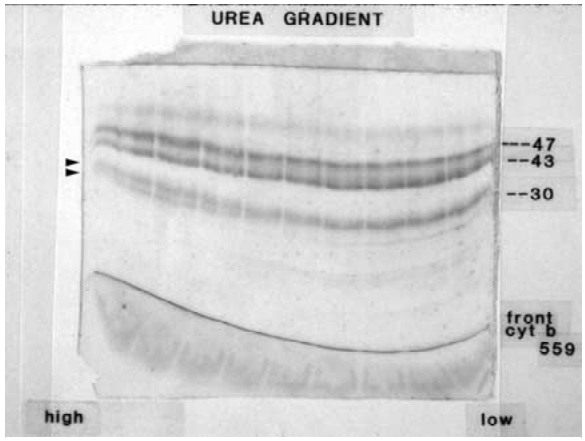

Figure 2. Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) of the Photosystem (PS) II core complex in the presence of a continuous urea gradient (modified from Satoh et al. 1983). For a color version of this figure, see color section in the front of the issue.

was finally resolved by including urea in the running gel upon SDS-PAGE. This procedure was developed for thylakoid proteins when I was visiting Charles Arntzen at Michigan State University, and we used it for the purified PS II core complex (Satoh et al. 1983). It happened as follows. In a gel prepared on the day before the Midwest Photosynthesis Conference of the state in 1981, Jan Watson and the late Kit Steinback applied a urea-gradient to the analyzing gel for electrophoresis of the PS II core complex, and we found that a band around 30 kDa in the purified complex was split into two sharp bands at higher concentrations of urea (Figure 2). (Figure 2 of Satoh et al. 1983; Photograph of Arntzen, Watson and Steinback appear in a paper by Allen 2002.) This was a demonstration of the separation of D1 and D2 proteins by SDS-PAGE. The gene coding for the D2 protein actually was identified several years later, when *psbD* gene was discovered in chloroplast genome as an open reading frame that seemed to code for a '32 kDa (D1)-like' protein in higher plants and *Chlamydomonas* (Alt et al. 1984; Holschuh et al. 1984; Jean David Rochaix et al. 1984; also see Rochaix, 2002). The 32 kDa band in the purified PS II core complex was eventually identified to correspond with the *psb*D gene product (Nixon et al. 1986).

By using urea-gradients in SDS-PAGE, we came to the conclusion that the second polypeptide in the 30 kDa range in the purified PS II core complex is the component known as the 'Herbicide or Q_B binding protein' (Pfister et al. 1981) and, at the same time, the protein reported by Ellis (1977) to be rapidly metabolized in illuminated chloroplasts (Satoh et al. 1983). This protein is now known as D1 protein and is encoded by *psb*A gene (see e.g., Zurawski et al. 1982). (For a molecular biology history, see Bogorad, this issue.)

At the end of this wandering path of discovery, the PS II core complex was firmly established to be a definite chemical entity in the thylakoid membrane. It consists of six polypeptide subunits, i.e., CP-47, CP-43, D1, D2, and the cytochrome b559 heterodimer (Satoh 1983). However, according to subsequent studies, several additional polypeptides were shown to be associated with the core in some preparations (Ikeuchi 1992).

Oxygen evolution

When identifying the PS II core complex as a functional unit of PS II, a basic problem we faced was the fact that the isolated complex does not exhibit any water oxidation activity that is characteristic of PS II. However, this was resolved through the work by Xiao-Song Tang and myself in a paper dedicated to the late Warren Butler (Tang and Satoh 1985). In this work, the isolation of a preparation similar to the core complex was described, but in this preparation one additional polypeptide corresponding to the 33 kDa protein, involved in the oxygen-evolution of photosynthesis (*psb*O gene product), was retained by just adjusting the pH of the extraction and purification medium form 7.2 to 6.0. This was the first evidence to show that the oxygen-evolving activity of PS II is as-

sociated with a pigment-protein complex, rather than with membranes.

Search for 'P-680 apo-protein'

The chemical establishment of the PS II complex made it possible to analyze the structure and function from various aspects, e.g., the selective iodo-labeling of D1 and D2 polypeptides identifying the site of oxidative equivalents in the PS II (Takahashi et al. 1986). However, a central aspect was the identification of the site of primary charge separation since the isolated core complex contained 50-60 Chl *a* molecules per reaction center suggesting that the complex contains both antenna and the reaction center. At the beginning of study, the CP-47 was regarded to be the site of charge separation in PS II, since most experimental evidence present at the time seemed to support that idea. For example, upon partially disintegrating and fractionating PS II preparations, the activity of PS II always paralleled the CP-47 band, rather than CP-43 (Camm and Green 1983; de Vitry et al. 1984; Yamagishi and Katoh 1985). In addition, the isolated holo-complex of CP-47 associated with Chls was shown to exhibit F-695 emission that was thought to originate from Chl *a* around the reaction center of PS II, and this complex also exhibited activities attributed to primary reactions in PS II (Nakatani et al. 1984). The notion seemed to be realistic since the predicted secondary structure of this polypeptide has Histidine residues that could be Chl *a* ligands in transmembrane helices in a manner suitable to accommodate the reaction center Chl, P-680 (Morris and Herrmann 1984). Thus this protein subunit of the PS II core complex was referred to as the 'P-680 apo-protein' in the literature and in textbooks.

Isolation of D1/D2/cyt b559 complex

A further subfractionation of the PS II core complex was attempted also in my laboratory focusing on the isolation of the CP-47 holo-complex with reasonably high PS II activity. However, success was very limited. On the other hand, non-denaturing gel-electrophoresis of digitonin extracts gave me an impression that the D1 and D2 proteins co-migrate. Thus, we attempted to isolate this complex directly from detergent extracts using column chromatography with a newly developed material from Toyo Soda (DEAE-Toyopearl) – with this material almost all green pigmented substances adsorbed on the top of column could be eluted (Yamada et al. 1985). For the extraction of Chl proteins, we used a high concentration of Triton X-100 (4%), instead of digitonin, in order to attain exhaustive extraction of Chl-protein complexes from thylakoid membranes and in the hope to separate the reaction center from the antenna.

By looking at the results of column chromatography conducted by Osamu Nanba (Figure 3), one of my undergraduate students, I noticed that the D1 and D2 proteins were enriched in fractions that eluted later, after extensive washing the column with a high concentration of NaCl. Thus I made up my mind to simply follow the fraction even though there was no evidence for photochemical activity or pigment association, as the co-existence of these two polypeptides in an apparent complex by itself was interesting as discussed in a review article (Satoh 1985). The isolation of a D1/D2 complex actually was very simple compared to my previous experiences in the purification of proteins, i.e., only one-step purification after extraction was sufficient. We simply had to be patient and wait until almost all, let us say more than 98%, of Chls present in the extracts had passed through the column; the subsequent fractions were enriched in the D1/D2 proteins.

By looking at the purification process and the purified material, I felt convinced that the fraction was something entirely new. This came especially from the unique color that I could perceive even with my color-blind eyes, in addition to the unexpected polypeptide composition. The polypeptide composition of the purified material was quite simple – in addition to two polypeptide bands in the 30 kDa region, which

Figure 3. A snapshot of Osamu Nanba at lab bench in 1986 (photograph taken by N. Inagaki).

Figure 4. The author (Kimiyuki Satoh, left) and Achim Trebst at a meeting in 1988 (Table Ronde Roussel Uclaf No. 63 – Structure, Function and Molecular Mechanisms in Photosystem II, in Paris).

Figure 5. A photograph of the author (KS, extreme right) with Mike Seibert (extreme left) and Govindjee (center) at a Gordon Conference. There had been many exchanges between us regarding details of methods of preparation and stabilization of PS II reaction centers.

presumably correspond to the D1 and D2 proteins (see later), we only found a band at the pigment front under the experimental condition used. By looking at the absorption spectrum, I was surprised that the position of the Soret peak was not that of Chl *a*. However, I soon realized that the absorption peak might be due to pheophytin *a* contributions, puzzling me that this could be due to harsh treatment by Triton X-100 in the extraction procedure resulting in the pheophytinization of original Chl *a* present in the complex. We hurried to measure the photochemical activity and to quantify the pigment composition, and, on the other hand, proved immunologically that there is no contamination by the fragment of CP-47 in question, as well as by CP-43. The *h*igh *p*erformance *l*iquid *c*hromatographic (HPLC) analysis clearly demonstrated the presence of pheophytin *a*, with Chl *a* to pheophytin *a* ratio of about 2.6 (Nanba and Satoh 1987). For the PS II activity, we could only measure, in our laboratory, the steady-state photoaccumulation of reduced pheophytin *a* in the presence of dithionite and methyl viologen under continuous illumination as developed by Klimov et al. (1977) (For a personal account of the discovery of pheophtin as the primary electron acceptor of PS II, see Klimov, this issue.) The light minus dark difference spectrum, that we obtained, clearly demonstrated the formation of pheophytin anion (Nanba and Satoh 1987). This was an exciting moment, convincing us that the isolated pigment-protein complex of unusual pigment and polypeptide composition exhibits the activity of PS II reaction center. It was spring of 1986 in Okayama in Japan.

Based on the similarity of D1 and D2 proteins to the subunits of purple bacterial reaction center whose structure was emerging those days by crystallographic analysis (Deisenhofer et al. 1985), it had been suggested at that time, with no direct experimental support, that these two proteins, i.e., D1 and D2 proteins, could form the reaction center of PS II (Michel and Deisenhofer 1985; Trebst 1986). Trebst (1986) wrote: 'therefore to suggest that the 32 and 34 kDa peptides form also the reaction center of Photosystem II. However, so far the 47 kDa peptide is thought to be the reaction center peptide.' (See Figure 4 for a photograph of the author with Achim Trebst.)

I was asked to present our results at the 're-arranged' symposium on the 'Structure of Molecular Complexes' at the VIIth International Congress on Photosynthesis held in the summer of 1986 at Brown University (Providence, Rhode Island) in the USA. (Satoh and Nanba 1987). On my way to Providence, I stopped at the laboratory of George Feher and Melvin Okamura at UC San Diego. Here, while using preparations consisting of D1, D2, and cytochrome b559, we detected the EPR triplet signal ascribed to originate from charge recombination between the primary radical pair in the PS II reaction center (Okamura et al. 1987). Needless to say, there was a hot discussion on D1/D2 *versus* CP-47 as the PS II reaction center at the Congress.

After the Congress in Providence, I visited Hans van Gorkom and the late Arnold Hoff in Leiden (The Netherlands) to conduct fast spectroscopy measurements in order to demonstrate that the primary charge separation between P-680 and pheophytin takes place in our preparation (Danielius et al. 1987). Then, I sent one of my colleagues (Takahashi) to Paul Mathis at Saclay to analyze the early events of PS II taking place in our preparation (Takahashi et al. 1987). During my stay in Leiden, Achim Trebst asked me to visit Bochum and gave site-specific antibodies for D1 and D2

Figure 6. Warren Butler (5th from left in the first row, in white suit) at a 1975 conference on the 'photosynthetic unit' in Marburg (Germany), organized by Horst Saenger. The quality of the photograph is not good enough to recognize several persons, mentioned in the text here. Among others, 8th from the left in the first row is Teruo Ogawa; and 8th from the left in the second row is Keith Boardman (wearing a tie); Hans Gaffron (wearing glasses and looking towards the camera) is just above Boardman in the 3rd row; see Homann, this issue, for some of the discoveries by Gaffron); to his right is Govindjee. Photo provided by Govindjee.

Figure 7. Hirosi Huzisige (left), my supervisor and one of pioneers in this research field and Yoshihiko Fujita, in 1998.

proteins to finally convince ourselves and others that D1 and D2 proteins are present in the PS II preparation (Satoh et al. 1987).

This long journey of collaborations thus resulted in the identification of the D1 and D2 proteins as the principal components of the PS II reaction center (Evans 1987; Satoh 1988), a decade after the successful isolation of the PS II core complex in Warren Butler's laboratory at UC San Diego. This set the stage to focus on the structure of the complex to understand the function and dynamics of the PS II reaction center. (See Seibert and Wasielewski, this issue, for the historical account of the first picosecond measurements by Mike Wasielewski, Mike Seibert, Doug Johnson and Govindjee on the PS II RC particles; see Figure 5.)

I end this perspective by paying tribute to Warren Butler. I show him in his environment of friends and colleagues gathered at Marburg, Germany, in 1975, the year I joined him (see Figure 6). He is fifth from the left in the front row.

Acknowledgments

I am grateful to my teacher, the late Hirosi Huzisige (Figure 7), who instilled in me an interest in photosynthesis research, for the enthusiastic guidance during the early part of my research career. I am indebted to the late Warren L. Butler for his interest and support in the initiation of this study, to my students in Okayama University, especially to Xiao-Song Tang, Yoshiaki Yamada and Osamu Nanba, for their significant contribution to the progress in this line of study, and to collaborators, especially to Charles J. Arntzen, Melvin Y. Okamura, George Feher, Paul Mathis, Hans J. van Gorkom and the late Arnold J. Hoff, for their valuable help in reaching the goal described here. It is also a pleasure to acknowledge the assistance of Yumiko Yamamoto. This article was written in Tempe, Arizona, when I was visiting Wim Vermaas's laboratory, and I wish to thank him for his warm hospitality and help in reading this manuscript. This paper was edited by Govindjee.

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