Isolation and Characterization of Spinach Photosystem II Membrane-Associated Catalase and Polyphenol Oxidase†

Yelena G. Sheptovitsky and Gary W. Brudvig*

Department of Chemistry, Yale University, New Haven, Connecticut 06520-8107

Received June 11, 1996; Revised Manuscript Received October 22, 1996⊗

ABSTRACT: Photosystem II (PSII) membranes exhibit catalase and polyphenol oxidase (PPO) activities. Mild heat treatment of PSII membranes for 90 min at 30 °C releases most of these enzyme activities into the supernatant, accompanied by a 7-fold activation of PPO. In contrast, mild heat treatment of thylakoid membranes does not release significant amounts of either activity, indicating that both enzymes are bound to the luminal surface of the thylakoid membrane. The heat-released PSII membrane-associated catalase and PPO have been purified and characterized. Catalase activity was correlated with a 63 kDa polypeptide which was purified by batch adsorption to anion-exchange beads followed by gel filtration. The PSII membrane-associated catalase is unstable in solution, probably due to irreversible aggregation. The enzyme was characterized in terms of molecular and subunit size, amino-acid composition, UV−visible absorption, heme content, pH optimum, inhibitor sensitivity, and K_m value for H_2O_2. Its properties indicate that the PSII membrane-associated catalase is a luminal thylakoid membrane-bound heme enzyme that has not been identified previously. The residual catalase activity of PSII membranes after mild heat treatment is irreversibly inhibited with 3-amino-1,2,4-triazole, a specific inhibitor of heme catalases, without inhibition of O_2-evolution activity. This result indicates that little, if any, of the catalase activity from PSII membranes in the dark is catalyzed by the O_2-evolving center of PSII. PPO activity was correlated with a 48 kDa polypeptide. However, the 48 kDa polypeptide and another heat-released polypeptide of 72 kDa have the same N-terminal sequence, which is also identical to that of a known 64 kDa protein [Hind, G., Marshak, D. R., & Coughlan, S. J. (1995) *Biochemistry 34,* 8157−8164]. During heat treatment of PSII membranes and further manipulations it was found that the 72 kDa polypeptide was largely converted into the 48 kDa polypeptide. Thus, the 72 kDa polypeptide appears to be a latent precursor of the active 48 kDa PPO. The PSII membrane-associated PPO was purified by anion-exchange chromatography and was characterized in terms of substrate specificity, pH optimum, inhibitor sensitivity and native molecular weight. The heat-released PPO appears to be identical to the enzyme previously isolated from spinach thylakoid membranes [Golbeck, J. H., & Cammarata, K. V. (1981) *Plant Physiol. 67,* 977−984].

Due to its unique properties which include light harvesting, charge separation, and water oxidation, photosystem II (PSII)[1] has become the most studied thylakoid membrane-protein complex. Because 80% of the PSII complexes are located in the chloroplast grana, it is possible to isolate PSII in pure and active form by isolating the grana portion of thylakoid membranes. During the last two decades, a number of PSII preparations with different degrees of subfractionation have been developed. The procedure known as the BBY preparation (Berthold et al., 1981) has become a standard method for isolating PSII membranes because it allows the appressed region of the grana, where PSII is located (Yu et al., 1992), to be isolated without loss of light-harvesting, charge-separation, or water-oxidation activity.

At present 27 polypeptides are identified as either PSII subunits or proteins associated with the PSII complex (Andersson & Styring, 1991; Irrgang et al., 1993; Kuwabara & Hashimoto, 1990; Kuwabara, 1995; Zhang et al., 1995). Only two of the 27 proteins, the heterodimer of the D1 and D2 subunits which forms the reaction center, bind all of the redox components that are necessary for primary charge separation and water oxidation. At least nine polypeptides bind chlorophyll a/b and function in the light-harvesting process. The function of the other proteins associated with PSII still remains obscure.

In addition to the primary water-plastoquinone oxidoreductase activity, it has been found that the PSII complex of higher plants can carry out other enzymatic activities. For example, it is known that PSII samples of higher plants can catalyze the decomposition of hydrogen peroxide in the dark. This catalase activity has been attributed, in part, to contamination of a thylakoid membrane-bound heme catalase in PSII samples (Frasch & Mei, 1987; Mano et al., 1987, 1993). Although there are a few reports of the isolation of heme catalases from spinach leaves (Galston et al., 1951; Gregory, 1968; Hirasawa et al., 1987, 1989), the putative catalase from PSII samples has never been identified or

† This work was supported by the National Institutes of Health (GM 32715).

⊗ Abstract published in *Advance ACS Abstracts,* December 1, 1996.

[1] Abbreviations: AU, arbitrary units; bis-Tris propane, 1,3-bis[tris-(hydroxymethyl)methylamino]propane; Chl, chlorophyll; dopa, 3,4-dihydroxyphenylalanine; dopamine, 3-hydroxytyramine; FPLC, fast protein liquid chromatography; HPLC, high-pressure liquid chromatography; kDa, kilodalton; LHCII, light-harvesting chlorophyll protein associated with PSII; MES, 2-(*N*-morpholino)ethanesulfonic acid; OD, optical density; PPO, polyphenol oxidase; PSI, photosystem I; PSII, photosystem II; PSII-30 °C, pellet fraction following incubation of PSII membranes at 30 °C for 90 min; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; snt-30 °C, supernatant fraction following incubation of PSII membranes at 30 °C for 90 min; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

S0006-2960(96)01384-0 CCC: $12.00

purified. Also, several groups have concluded that PSII can catalyze the disproportionation of hydrogen peroxide in the dark by a reaction cycle involving the S_{-1} and S_1 states of the O_2-evolving center (Frasch & Mei, 1987, 1987; Mano et al., 1993; Taoka et al., 1993; Velthuys & Kok, 1978). Thus, the question of whether the catalase activity of PSII samples is due to the presence of a heme catalase, is endogenous to the O_2-evolving center of PSII, or both remains to be elucidated.

PSII preparations also exhibit polyphenol oxidase activity. Polyphenol oxidase (PPO) is a copper enzyme found in plants. It catalyzes the oxidation of hydroxyphenols to their quinone derivatives which then spontaneously polymerize. The molecular weight of active plant PPOs is in the 40−50 kDa range. The active form of PPO is known to originate from a latent precursor protein of higher molecular weight. Although the physiological route of PPO activation is not known, *in vitro* activation of PPOs has been achieved by treatment with detergents, denaturing agents, proteolytic enzymes, fatty acids, high or low pH, or by aging (Golbeck & Cammarata, 1981). The existence of a latent PPO in plant thylakoid membranes was first reported by Arnon (1949). Since then the knowledge about thylakoid PPO has significantly advanced due to efforts of a number of research groups [for review, see Trebst and Depka (1995)]. Spinach thylakoid membrane-bound PPO was isolated, purified and characterized by Golbeck and Cammarata (1981). It was found to be specific only to o-dihydroxyphenols when it was activated with linolenic acid and to exist in two interconvertible forms: a tetramer and a monomer with a subunit molecular weight of 42.5 kDa.

Recently the complete sequence of a 64 kDa protein purified from spinach thylakoid membranes was reported (Hind et al., 1995). For many years this polypeptide was thought to be a thylakoid LHCII kinase (Coughlan & Hind, 1986), but later investigations revealed that the 64 kDa polypeptide is just a substrate for a not-yet-identified protein kinase (Race et al., 1995). Because the mature protein sequence showed 50% similarity to several PPOs, it was concluded that the 64 kDa polypeptide is a latent thylakoid PPO and the precursor of the active 42.5 kDa PPO purified by Golbeck and Cammarata (1981).

Spinach thylakoid PPO has also been correlated with dithiothreitol-sensitive proteases (39 and 60 kDa) which were extracted with 1 M NaCl from spinach PSII membranes (Kuwabara & Hashimoto, 1990; Kuwabara, 1995). These proteins had the same N-terminal sequence, APILPDVEK-, as the above-mentioned 64 kDa protein. The 39 and 60 kDa proteins showed protease activity at low-salt conditions with a pH optimum between 7 and 9. Since 1,10-phenanthroline inhibited the protease activity, a metal was suggested to be at the active site of these enzymes.

The localization of the 64 kDa PPO protein on thylakoid membranes has been studied (Lax & Vaughn, 1991; Yu et al., 1992). Using different experimental techniques, both groups found the 64 kDa protein at the edges of the grana.

In this study, we report that a mild heat treatment of BBY PSII membrane preparations results in release of catalase and PPO activities into the supernatant. The proteins responsible for these enzymatic activities have been identified and purified to electrophoretic homogeneity. Both enzymes have been characterized and their properties are compared to those of previously reported proteins.

MATERIALS AND METHODS

Sample Preparation. PSII membranes were isolated from market spinach leaves as described in Beck et al. (1985) by a modified version of the BBY procedure (Berthold et al., 1981). All preparations were done on ice under dim green light. Prior to use, PSII membranes were stored at 3−5 mg of Chl/mL in liquid nitrogen. Unless otherwise stated, the buffer for PSII samples contained 15 mM NaCl, 20 mM MES, pH 6.5, and 30% ethylene glycol. The Chl concentration was determined by the method of Arnon (1949). O_2-evolution rates of PSII samples, measured with a Clark-type electrode, were in the range of 300−450 μmol of O_2/(mg of Chl·h). Extrinsic polypeptides were depleted from PSII membranes by 1 M $CaCl_2$ treatment (Ono & Inoue, 1983).

PSII membranes (1.5−2.0 mg of Chl/mL) were heat-treated by incubation in a thermostated cell for 90 min with stirring at 30 °C. After incubation, the PSII membranes were centrifuged for 30 min at 37000*g*. The supernatant (snt-30 °C) was saved, and the pellet of PSII membranes (PSII-30 °C), following two washes with buffer, was resuspended in buffer to a Chl concentration of 3−5 mg of Chl/mL.

All electrophoretic and blotting procedures were carried out by using standard procedures (Ausubel et al., 1995). Polyacrylamide gel electrophoresis was performed with/without sodium dodecyl sulfate using a Bio-Rad Modular Mini Electrophoresis System. Dilute protein samples were precipitated with 10% TCA prior to solubilization in sample buffer. Gels were fixed and stained with Coomassie blue or silver. Polypeptide molecular weights were estimated by using the low molecular weight standards from Bio-Rad which included lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), bovine serum albumin (66.2 kDa), and phosphorylase *b* (97.4 kDa). Proteins were eluted from SDS−PAGE gel slices using a Schleicher & Schuell EluTrap system. Blotting of proteins onto a polyvinylidene fluoride membrane (Immobilon P, Millipore) was done with a Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell. N-terminal sequencing of blotted proteins stained with Coomassie Blue was performed by the W. M. Keck Foundation Biotechnology Resource Laboratory (Yale University, School of Medicine).

For peptide mapping, proteins were digested in gels according to Stone and Williams (1996). SDS−PAGE-separated proteins were stained with Coomassie Blue for 1 h prior to destaining for a minimum of 2 h. Excised gel pieces were incubated for 15 min in 50% CH_3CN/0.1 M Tris, pH 8.0. Modified trypsin (Promega), diluted in a 1:2 ratio with 0.1 M Tris, pH 8.0, and 0.1% Tween 20, was added to semi-dried gel pieces (final enzyme ratio 0.5 μg/15 mm^3 gel). After 24 h of incubation at 37 °C, the sample volume was doubled with 0.1 M Tris/60% CH_3CN, and 1 mM dithiothreitol was added, followed by a 20 min incubation at 50 °C. Then 200 mM methyl-4-nitrobenzene sulfonate was added and the samples were incubated at 37 °C for 40 min. Peptides were extracted with 0.1% trifluoroacetic acid/60% CH_3CN. Extracts were speed-vac dried and redissolved in H_2O to the final volume of 110 μL. 0.2 mL of the digests containing 2 M urea were loaded in onto a reverse-phase HPLC column for automated peptide mapping. Fractions were detected at 210 nm. HPLC runs of protein tryptic

digests were done by the W. M. Keck Foundation Biotechnology Resource Laboratory. Samples for amino-acid analysis were presented to the W. M. Keck Foundation Biotechnology Resource Laboratory in 20 mM MES, pH 6.5.

All liquid chromatography experiments were performed by using a Pharmacia FPLC system. A Mono-Q column was used for anion-exchange chromatography. The eluent buffer contained 0.5% betaine, 20 mM MES, pH 6.55, and 30% ethylene glycol. Gel-filtration experiments were done with a HiPrep Sephacryl S-100 column (for catalase purification and PPO molecular-weight determination) or Superose 12 HR 10/30 column (for catalase molecular-weight determination). The gel-filtration buffer contained 0.5% betaine, 20 mM MES, pH 6.55, 0.15 M NaCl, and 30% ethylene glycol. FPLC flow rates were 0.3 and 0.5 mL/min for anion-exchange and gel-filtration runs, respectively. Fractions were detected at 280 nm. For molecular-weight determinations, the gel-filtration columns were calibrated by using the low and high molecular-weight gel-filtration calibration kits from Pharmacia which included the following standards: cytochrome *c* (12.4 kDa), chymotrypsinogen (25 kDa), ovalbumin (43 kDa), albumin (67 kDa), aldolase (158 kDa), and dextran (2,000 kDa). For batch adsorption, a sample was nutated for 20 min with anion-exchange beads (Q Sepharose FF, Pharmacia) which were previously washed with the sample buffer. The supernatant was decanted after the sample was spun with a hand centrifuge for 1 min.

Assays. All O_2-evolution and consumption measurements were made on a YSI Model 53 O_2 monitor, equipped with a Teflon-membrane-covered Clark-type O_2 electrode. A Neslab RTE-9DD circulator bath was used to maintain the sample cell's water jacket at 25 °C and to equilibrate the temperature of the electrode and buffers before an assay. The electrode was calibrated with air-saturated water for which the O_2 concentration was taken as 0.25 mM.

Catalase activity was measured as the initial O_2-evolution rate from 1 mM H_2O_2. Unless otherwise stated, the assay buffer consisted of 0.5% betaine, 20 mM Tris, pH 8.0, and 30% ethylene glycol. The assay was initiated by injecting a solution of hydrogen peroxide through a side inlet into the sample cell containing 4 mL of assay buffer with an aliquot of the sample.

The PPO activity was measured as the initial O_2-consumption rate following injection of 1 mM dopamine into the assay media. The assay media was the same as that used for measurement of catalase activity.

Anion or thiol inhibition of PSII membrane-associated catalase was carried out by addition of inhibitor at various concentrations directly into the catalase assay media. Inhibition studies with 3-amino-1,2,4-triazole were performed according to Margoliash et al. (1960); prior to assay, the sample (purified PSII membrane-associated catalase or 0.3 mg of Chl/mL of PSII-30 °C) was incubated with 4 mM ascorbate and 20 mM 1,2,4-aminotriazole for 2 h at 30 °C. For measurement of the effect of copper chelation, PPO was incubated with 50 mM 3,3′,5,5′-diethyldithiocarbamate on ice for 1 h.

For the determination of K_m values, O_2-evolution/consumption rates were measured at different substrate concentrations and then plotted as rate vs substrate concentration. Each plot was fit by the Michaelis−Menten equation to obtain the K_m value. The final value for K_m was taken as an average of values determined using two different enzyme concentrations.

Table 1: Relative Catalase Activities and O_2-Evolution Rates of PSII Samples before and after 90 min Incubation at 30 °C[a]

	catalase activity, %			O_2-evolution rate, %	
experiment	PSII	PSII-30 °C	snt-30 °C	PSII	PSII-30 °C
1	100	12	95	100	97
2	100	17	89	100	84
3	100	26	87	100	102

[a] Assay buffer: 20 mM MES, pH 6.5, 15 mM NaCl, 10 mM $CaCl_2$, and 30% ethylene glycol.

Spectroscopy. The UV−visible absorption spectrum of PSII membrane-associated catalase was recorded with a Varian Cary 3e spectrophotometer. The sample buffer contained 0.5% betaine, 20 mM MES, pH 6.5, 0.15 M NaCl, and 30% ethylene glycol. Other UV−visible measurements were done on a Perkin Elmer Lambda 3B spectrophotometer. A Bio-Rad model 1650 densitometer was used to record the protein profiles of polyacrylamide gels. Both absorption and densitometry data were digitized by using a program written in Labview (National Instrument Corporation) on a Macintosh SE computer.

A Varian SpectrAA atomic absorption spectrometer was used for determination of copper content. The snt-30 °C was boiled to reduce the volume to 0.5 mL. Then 2 mL each of concentrated $HClO_4$ and HNO_3 were added and the sample again was boiled to reduce the volume to 0.5 mL. The final volume of the sample was brought to 1 mL by 0.25% HNO_3. Copper content was calibrated with an atomic-absorption standard solution from Aldrich. Estimation of the Cu/PSII ratio was made based on the initial volume of snt-30 °C which was from heat-treated PSII membranes of known concentration.

RESULTS

Mild Heat Treatment of PSII Samples. PSII membrane samples showed high catalase activity in the dark. The measured catalase activity was directly proportional to the PSII membrane concentration and typical O_2-evolution rates from hydrogen peroxide were 400−600 mM O_2/(min·μg of Chl·mM H_2O_2) when measured at pH 6.5 in the dark. When the samples were incubated 90 min at 30 °C, most of the catalase activity was found in the supernatant (Table 1). The catalase activity in snt-30 °C and the residual catalase activity of PSII-30 °C were both inhibited by 3-amino-1,2,4-triazole, a specific inhibitor of heme catalases. Repeated resuspension of the inhibited PSII-30 °C pellet in buffer did not restore catalase activity. After both heat and 3-amino-1,2,4-triazole treatment, the PSII membranes retained less than 5% of the initial catalase activity, but the light-induced O_2-evolution activity was not significantly changed.

In addition to the catalase activity, a polyphenol oxidase activity was observed in the PSII membrane samples. O_2-consumption rates due to PPO were in the range of 450−500 mM O_2/(min·μg of Chl·mM dopamine) when measured at pH 6.5. Mild heat treatment of the PSII samples resulted in release of the PPO into the supernatant (Table 2). Solubilization of PPO was accompanied by its activation: PPO activity of the snt-30 °C was about 7 times higher than the activity of nontreated PSII (Table 2). Since the PSII membranes showed 67% of the original PPO activity after the first heat treatment, an additional heat treatment of PSII-

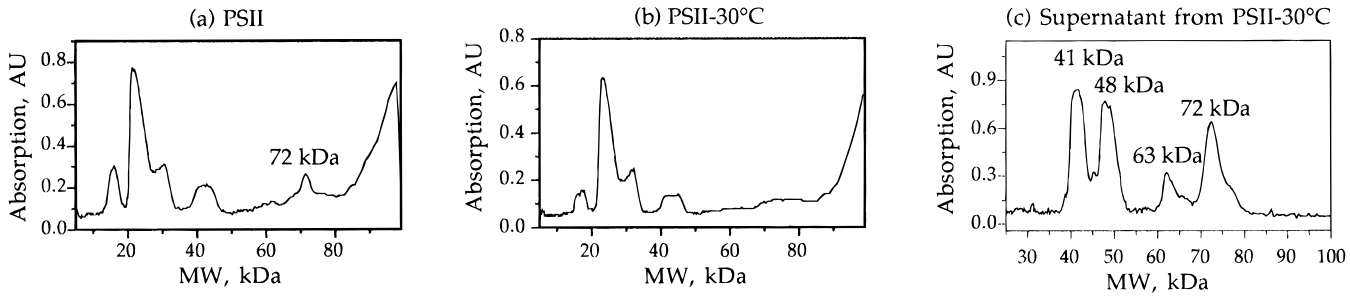

FIGURE 1: SDS−PAGE densitometry profiles of (a) PSII; (b) PSII-30 °C; and (c) snt-30 °C. 12.5% (a, b) and 9% (c) polyacrylamides were used for the SDS−PAGE.

Table 2: Relative Polyphenol Oxidase Activities of the PSII Samples before and after 90 min Incubations at 30 °C[a]

sample	PPO activity, %
PSII	100
PSII-30 °C	
first heat treatment	67
second heat treatment	23
snt-30 °C	684

[a] Assay buffer: 20 mM MES, pH 6.5, 15 mM NaCl, 10 mM $CaCl_2$, and 30% ethylene glycol.

30 °C was carried out to further deplete PPO. The PPO activity of PSII-30 °C was decreased from 67% to 23% after the additional heat treatment (Table 2). Considering the high PPO activity of snt-30 °C, one can see that two heat treatments depleted PPO from the PSII membranes almost completely. It should be noted that two consecutive heat treatments of PSII membranes did not inactivate light-induced O_2 evolution.

To obtain some information on the location of the PSII membrane-associated catalase and PPO in thylakoid membranes, we checked the catalase and PPO activities of thylakoid membranes and compared these results with data for PSII membranes. Both thylakoid and PSII membrane samples had high catalase and PPO activities. However, mild heat treatment of thylakoid membranes did not release significant amounts of either activity, indicating that both enzymes are bound to the luminal surface of the thylakoid membrane.

SDS−PAGE densitometry profiles of PSII membranes before and after heat treatment and of the supernatant after heat treatment are shown in Figure 1. The most significant difference between the PSII and the PSII-30 °C densitometry profiles is the absence of a 72 kDa band in the PSII-30 °C profile. The lower molecular-weight region of the PSII samples had overlapping bands, due to the intrinsic proteins of the PSII complex, which made it difficult to observe any changes. The densitometer profile of the supernatant from PSII-30 °C showed four distinct bands with apparent molecular weights 72 ± 5, 63 ± 3, 48 ± 2, and 41 ± 1 kDa (data are based on 20 SDS−PAGE runs). The 63 kDa protein was the least abundant protein. The intensities of the 41 and 48 kDa bands from snt-30 °C were similar to the intensities of the extrinsic PSII polypeptides from 1 M $CaCl_2$ extraction of PSII membranes. Sometimes, in addition to the above-mentioned proteins, SDS−PAGE of snt-30 °C showed the presence of small amounts of the extrinsic PSII proteins with apparent molecular weights of 39 ± 1 and 26 ± 1 kDa (data not shown). It is interesting that no release of the 18 kDa extrinsic PSII protein was observed upon heat treatment of PSII membranes.

Atomic-absorption measurements of the snt-30 °C showed significant levels of copper. The ratio Cu/PSII was estimated to be 0.2−0.3.

Identification of the 72, 63, 48, and 41 kDa Proteins. To identify the heat-released proteins, their N-terminal sequences were determined. Both the 72 and 48 kDa proteins had the same N-terminus, APILPDVEKST-, as the known thylakoid 64 kDa protein (Hind et al., 1995). The 63 and 41 kDa proteins had blocked N-termini.

In order to characterize further the four heat-released polypeptides, we ran HPLC profiles of tryptic digests of each of the four proteins. As was expected, the HPLC peptide profiles from the 72 and 48 kDa polypeptides matched very well. The HPLC peptide profiles from the 63 and 41 kDa proteins did not match with each other or with those of the 72 and 48 kDa polypeptides.

To determine which protein is responsible for the catalase reaction, the catalase activity was measured for bands on a native gel. First, the native polypeptides in the snt-30 °C were separated by electrophoresis on a gel containing 5% polyacrylamide. Then, the gel was immersed in 3% H_2O_2. In this solution, the catalase-active band was detected by the formation of bubbles of O_2 and marked on the gel. Next, the gel was stained with Coomassie Blue which allowed the catalase-active band to be excised precisely. The protein from the excised gel piece was eluted, concentrated, and identified by SDS−PAGE, which showed a single protein band at 63 kDa.

The function of the 41 kDa protein, which had a blocked N-terminus and was distinct from other heat-released proteins, remains to be determined. In this study, we did not conduct specific investigations to answer this question.

Purification of the PSII Membrane-Associated Catalase. The PSII membrane-associated catalase was purified from snt-30 °C by a simple two-step procedure. First, snt-30 °C was treated with anion-exchange beads in a buffer containing 0.5% betaine, 20 mM MES, pH 6.5, and 30% ethylene glycol. After a 20 min incubation at ambient temperature, the decanted supernatant (snt-Q) retained 100% of the original catalase activity, and no PPO activity was detected. SDS−PAGE of this fraction showed an intense 63 kDa band and a very faint band at 72 kDa. The A_{404}/A_{278} ratio of snt-Q, a parameter of catalase purity, was low and equal to 0.14. Second, gel-filtration chromatography was used to separate the PSII membrane-associated catalase from the 72 kDa protein. When non-concentrated snt-Q was put through the gel-filtration column, the catalase-active fractions were eluted first, corresponding to a molecular weight of about 130 kDa, and then the 72 kDa protein was eluted. This purification step improved the A_{404}/A_{278} ratio from 0.14 to 1.1.

Table 3: Amino-Acid Composition of the PSII Membrane-Associated Catalase Calculated for a Single Subunit of 63 kDa

amino acid	no. of residues	amino acid	no. of residues
Asx	72.88	Ileu	26.00
Thr	36.65	Leu	39.77
Ser	38.76	Tyr	20.57
Gly	40.12	Phe	37.19
Glx	40.68	His	20.80
(Pro + Cys)[a]	41.70	Lys	25.86
Ala	35.92	Arg	35.49
Val	29.05	Trp	ND
Met	19.55		

[a] Not quantitative.

Although the PSII membrane-associated catalase was stable in the snt-30 °C, which contains the other heat-released proteins, it was found to be highly susceptible to inactivation during manipulations. This problem was most likely due to irreversible aggregation of the enzyme. Addition of nonionic detergents and/or sugars did not prevent this inactivation. However, betaine significantly improved the stability of the enzyme. When 0.5% betaine was added to the buffers, the catalase samples could be passed through a gel-filtration FPLC column essentially without deactivation. However, betaine did not prevent loss of activity during concentration. Attempts to use ion-exchange chromatography instead of gel filtration for further purification and concentration of the PSII membrane-associated catalase also failed due to its irreversible deactivation on binding to a column. Typically, concentration of samples containing the PSII membrane-associated catalase resulted in 80−90% loss of catalase activity. Thus, to purify the PSII membrane-associated catalase, steps that required its concentration or binding to a column were avoided. It should be noted that, during all purification steps, the concentration of the PSII membrane-associated catalase was very low. The catalase-enriched fractions were only concentrated for UV−visible absorption measurements or precipitated with TCA for SDS−PAGE.

Characterization of the PSII Membrane-Associated Catalase. The purified PSII membrane-associated catalase exhibits a single band on a denaturing SDS polyacrylamide gel with an apparent molecular weight of 63 kDa (Figure 6a). Its native molecular weight was determined by gel filtration to be about 130 kDa, which is consistent with a dimer. The amino-acid composition calculated for a 63 kDa subunit is presented in Table 3. As was reported above, the catalase has a blocked N-terminus.

The enzyme's pH optimum is at 8.2 (Figure 2). The catalase activity is inhibited by 3-amino-1,2,4-triazole, thiols, including 2-mercaptoethanol (I_{50} = 0.15 mM), and by the anions cyanide (I_{50} = 3.1 μM), azide (I_{50} = 0.6 μM) and formate (I_{50} = 119 mM).

The dependence of catalase activity on the hydrogen peroxide concentration is shown in Figure 3. No inhibition was observed at high concentrations of hydrogen peroxide. The apparent K_m was calculated to be 47 mM, which is in agreement with typical K_m values for heme catalases (Hochman & Goldberg, 1991; van Eyk et al., 1992).

The absorption spectrum of the PSII membrane-associated catalase exhibited peaks at 278, 404, 500, and 620 nm and shoulders at 538 and 572 nm in the oxidized form (Figure 4). The enzyme was not reduced upon addition of sodium dithionite. The pyridine ferrohemochrome of the enzyme showed an absorption spectrum typical of protoheme IX: peaks were observed at 417, 522, and 556 nm. Addition of peracetic acid to the PSII membrane-associated catalase resulted in a significant decrease of the Soret peak at 404

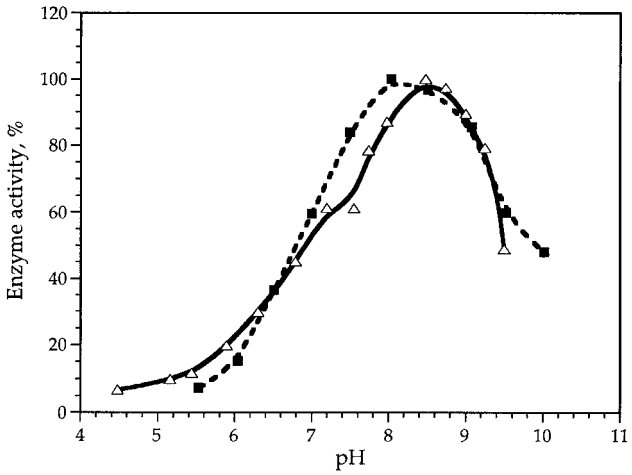

FIGURE 2: pH dependence of PSII membrane-associated catalase (■) and PPO (△). Assay buffer: 0.5% betaine, 20 mM bis-Tris propane, and 30% ethylene glycol.

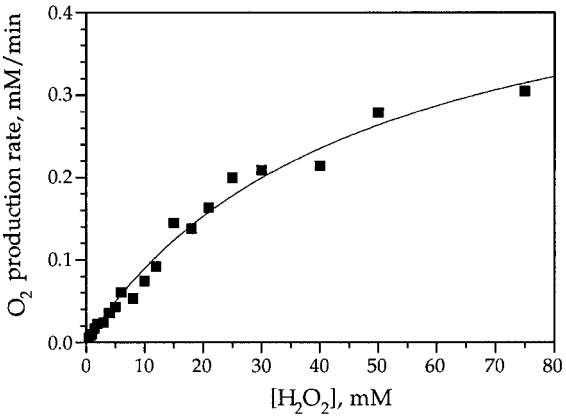

FIGURE 3: Dependence of catalase activity on H_2O_2 concentration. The assay sample contained a 5 μL aliquot of enzyme and 4 mL of assay buffer (0.5% betaine, 20 mM Tris, pH 8.0, and 30% ethylene glycol). The solid line is a fit of the Michaelis−Menten equation to the data.

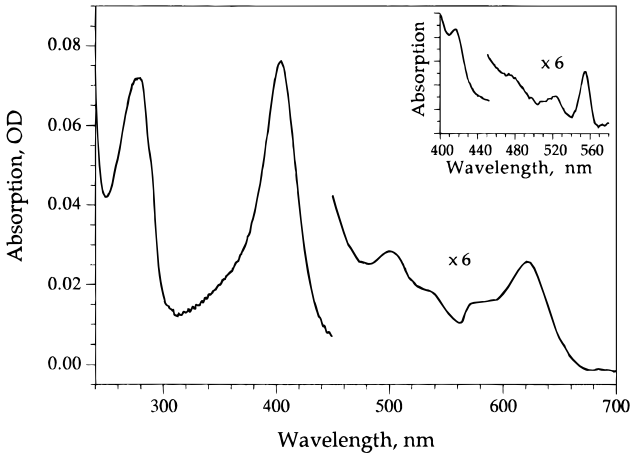

FIGURE 4: Absorption spectrum of 0.4 μM PSII membrane-associated catalase (A_{404}/A_{278} = 1.1). The insert shows the absorption spectrum of the pyridine ferrohemochrome of the PSII membrane-associated catalase, obtained by adding 0.5 M NaCl, 10% pyridine, and a small amount of $Na_2S_2O_4$ to the enzyme solution.

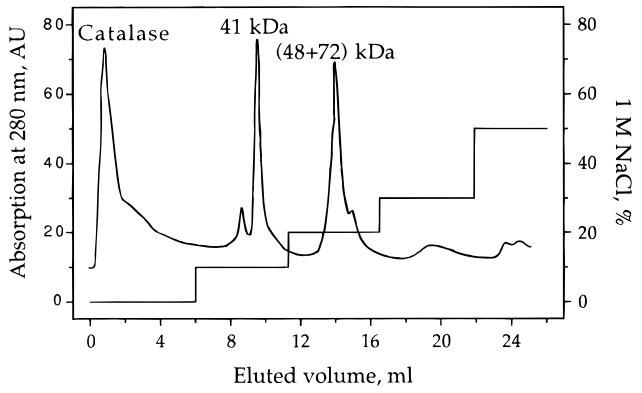

FIGURE 5: FPLC profile of the snt-30 °C on a Mono-Q anion-exchange column eluted with a NaCl step gradient. Buffer: 0.5% betaine, 20 mM MES, pH 6.55, and 30% ethylene glycol.

nm, which is characteristic of formation of compound I (Schonbaum & Chance, 1976).

The catalase also could be dissociated from PSII membranes, together with the extrinsic PSII polypeptides, by 1 M $CaCl_2$. 1 M NaCl did not prove to be effective in removing the catalase from PSII membranes. However, high-salt treatments significantly inactivated the PSII membrane-associated catalase. We also measured the catalase activity at different steps during the preparation of PSII membranes from thylakoid membranes. When thylakoid membranes were incubated with Triton X-100-containing buffer followed by centrifugation, high catalase activity was found both in the pellet, enriched in PSII, and in the supernatant which contained mostly PSI.

Purification of PPO (48 and 72 kDa Proteins). The PSII membrane-associated PPO was purified from snt-30 °C by a single run through an anion-exchange column (Figure 5). A NaCl step gradient was used for elution of the proteins. All fractions were checked for catalase and PPO activities. SDS−PAGE densitometry profiles of the three main peaks are shown in Figure 6.

The PPO-active fraction was eluted with a 0.2 M NaCl step gradient. This fraction contained the 48 kDa protein with some contamination of the 72 kDa protein (Figure 6). The PSII membrane-associated catalase was found in the void volume ($A_{404}/A_{278} = 0.28$). The 41 kDa polypeptide was eluted as the main peak at 0.1 M NaCl, and it was neither catalase- nor PPO-active.

Sometimes prior to the FPLC step, the snt-30 °C was incubated with anion-exchange beads in the presence of 0.1 M NaCl. In this case, more than 90% of the PPO was bound to the beads; the PSII membrane-associated catalase and most of the 41 kDa protein were left in the solution.

Table 4: Thylakoid PPO Specificity Data

substrate	phenol type	PPO activity, arb. units	$K_m \pm 1\%$ (mM)
dopamine	*ortho*	17.4	0.33
4-methylcatechol	*ortho*	10.5	6.58
catechol	*ortho*	4.88	8.87
D,L-dopa	*ortho*	3.04	ND
(−)-epicatechin	*ortho*	1.34	ND
(+)-catechin	*ortho*	1.14	ND
chlorogenic acid	*ortho*	0.50	ND
resorcinol	*meta*	0	−
hydroquinone	*para*	0	−
L-tyrosine	mono	0	−
p-coumaric acid	mono	0	−
gallic acid	trihydroxy	0	−

PPO was eluted from the beads with a 0.2 M NaCl buffer. SDS−PAGE of this fraction showed an intense band at 48 kDa and faint bands at 41 and 72 kDa.

Purification of the 72 kDa protein alone was not successful for the following reasons. When the 48 kDa protein was present in a sample, the 72 kDa protein was progressively converted into the 48 kDa protein. Thus, by the end of the purification procedure the final amount of the 72 kDa protein was very small. On the other hand, some of 72 kDa was found to be present in fractions enriched with the PSII membrane-associated catalase. No cleavage or PPO activity was observed in this case. However, when these fractions were run through a gel-filtration column, the eluted 72 kDa protein was contaminated with the 63 kDa monomer of the catalase.

Characterization of PPO. Mild heat treatment of PSII membranes released PPO into solution, and simultaneously activated it by about 7-fold. Two forms of thylakoid PPO were identified in the supernatant after heat treatment. As determined by SDS−PAGE, their molecular weights were 48 and 72 kDa. Both of these proteins had the same N-terminal sequence, APILPDVEKST-. The FPLC experiments showed that PPO activity was associated only with the 48 kDa protein. The 72 kDa protein did not exhibit any PPO activity, and in the presence of the 48 kDa protein it was converted into the 48 kDa PPO-active form. Gel-filtration experiments showed that the active form of PPO was a monomer (the protein eluted at a molecular weight of 45 kDa).

The pH dependence of PPO activity is presented in Figure 2. The optimal pH was about 8, and there was practically no activity below pH 6.0. The PPO activity was inhibited by 3,3′,5,5′-diethyldithiocarbamate, a copper chelating agent. As seen in Table 4, the thylakoid PPO showed specificity for *ortho*-dihydroxyphenols, among which dopamine was the

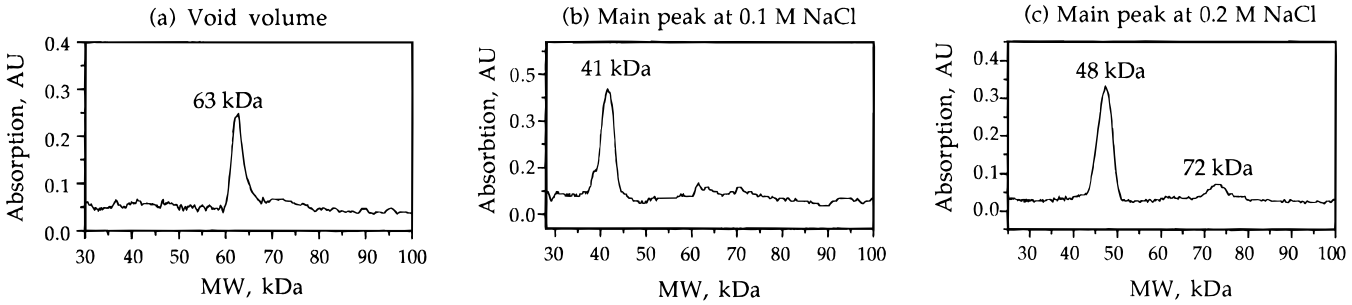

FIGURE 6: SDS−PAGE densitometry profile of the each of the three main peaks in the FPLC run of the snt-30 °C shown in Figure 5. 9% polyacrylamide was used for the SDS−PAGE.

Table 5: Properties of PSII Membrane-Associated Catalase, Purified in This Study, and Two Other Spinach Catalases Reported Previously

property	PSII membrane-associated catalase	spinach leaf catalase[a]	green catalase[b]
molecular mass (kDa)			
subunit	63	NA	60
native form	130	300	125
pH optimum	8.5	5.3−8.0	6.0
UV−visible spectra			
maxima, nm	278, 404, 500, 620	278, 404, 502, 620	279, 403, 542, 592, 723
shoulders, nm	538, 572	533	290, 500, 630
dithionite	no reduction	NA	no reduction
protoheme IX	yes	yes	yes
novel heme	no	no	yes
K_m, mM	47	NA	NA
inhibition, I_{50}			
a. 2-mercaptoethanol	0.15 mM	NA	no inhibition
b. cyanide	3.1 μM	5 μM	inhibition
c. azide	0.6 μM	20 μM	inhibition
d. formate	119 mM	NA	inhibition
e. 3-amino-1,2,4-triazole	inhibition	NA	NA
stability	highly aggregated	highly aggregated	NA

[a] Galston et al. (1951) and Gregory (1968). [b] Hirasawa et al. (1987, 1989).

best substrate. PPO was not active toward any of the tested *meta*-, *para*-, and trihydroxyphenols. The K_m values for dopamine, catechol, and 4-methylcatechol were calculated by fitting data directly to the Michaelis−Menten equation. We were not able to determine K_m values for other *ortho*-dihydroxyphenols due to their limited solubility.

DISCUSSION

In the present study, we show that mild heat treatment of PSII membranes releases several polypeptides into the supernatant. Two of these heat-released proteins are identified as membrane-bound catalase and polyphenol oxidase. Both the catalase and PPO have been purified and characterized.

Catalase. The purified PSII membrane-associated catalase contains protoheme IX and its native form is a dimer of a 63 kDa polypeptide. It is useful to compare the properties of the PSII membrane-associated catalase with the properties of two known spinach catalases. A spinach leaf catalase was reported by Galston et al. (1951) and Gregory (1968); 90% of the catalase activity was found not to be associated with any subcellular particle. An unusual green catalase was also extracted from spinach leaves (Hirasawa et al., 1987, 1989), although its cellular distribution was not studied. The characteristics of the PSII membrane-associated, spinach leaf, and green catalases are presented in Table 5. The PSII membrane-associated and green catalases have major differences in UV−visible absorbance, pH optimum, inhibitor sensitivity, sensitivity to 2-mercaptoethanol, and heme content, whereas the PSII membrane-associated and spinach leaf catalases have somewhat similar properties. Their absorption spectra are very similar, and both enzymes are unstable due to irreversible aggregation at high concentrations. However, there are also major differences between these two catalases. These include differences in their native molecular weights, the pH ranges for optimal catalase activity, and inhibitor sensitivity. A similarity in the absorption spectra and susceptibility to inactivation due to aggregation are quite typical for plant catalases (Beaumont et al., 1990). Thus, we conclude that the PSII membrane-associated catalase and spinach leaf catalase are distinct from each other and that the PSII membrane-associated catalase characterized in this study is a new spinach catalase. Based on the observation that mild heat treatment of thylakoid membranes did not result in release of catalase into the supernatant, as it was the case with PSII-membrane samples, the PSII membrane-associated catalase appears to be bound to the luminal surface of the thylakoid membrane.

It is known that PSII preparations from cyanobacteria do not exhibit any catalase activity (Dubinin et al., 1992). As a result, cyanobacteria form hydrogen peroxide in the light and secrete it into the medium at a high rate. The concentration of H_2O_2 may sometimes be as high as 55 mM. In the presence of DCMU or under anaerobic conditions, H_2O_2 production virtually ceases. It was proposed that hydrogen peroxide is formed in the electron-transport chain at the step of ferredoxin oxidation by the generation of O_2^- and its subsequent dismutation. Up to 40% of the total electron flow in the electron-transport chain can be used for hydrogen peroxide formation.

In higher plants, all of the protein complexes responsible for the photosynthetic light reactions are organized into the thylakoid membrane. Several research groups have found that hydrogen peroxide is formed during the water-oxidation reaction of PSII (Ananyev et al., 1992; Fine & Frasch, 1992; Hillier & Wydrzynski, 1993; Klimov et al., 1992, 1993). There are also other possible sources of hydrogen peroxide formation in chloroplasts (Gerasimenko et al., 1989). For example, hydrogen peroxide may be produced from the autoxidation of heme proteins, plastoquinols, or NADH or from secondary reactions of superoxide. If the production of hydrogen peroxide in thylakoid membranes has the same rate as in cyanobacteria, the concentration of hydrogen peroxide inside the chloroplast during O_2 evolution could become quite high. For this reason, the association of a catalase with thylakoid membranes might be crucial in order to protect chloroplast proteins from damage.

Removal of the extrinsic polypeptides from PSII membranes results in production of hydrogen peroxide during light-induced O_2 evolution (Schröder & Åkerlund, 1986; Berg & Seibert, 1987). We have found that the 1 M $CaCl_2$ treatment, used to remove the extrinsic polypeptides from PSII membranes, depletes the PSII membrane-associated catalase as well. Because high-salt treatments also significantly deactivate the catalase, the residual catalase activity of 1 M $CaCl_2$-treated PSII is negligibly low.

Table 6: Properties of PPO Purified in This Study and the Previously Reported PPO

PPO characteristic	48 kDa	spinach thylakoid PPO[a]
isolation method	heat treatment of PSII membranes	sonication of chloroplasts
molecular weight		
SDS−PAGE	48 kDa	42.5 kDa
gel-filtration	45 kDa	158 and 42.5 kDa
PPO activity	highly active	latent
activation mode	heat (?)	C_{18} fatty acids, aging
pH optimum	8.0	7.0−8.0
substrate specificity	*o*-dihydroxyphenols	*o*-dihydroxyphenols
K_m, mM		
a. dopamine	0.33	0.74
b. catechol	8.87	3.13
c. D,L-dopa	ND	8.3
d. chlorogenic acid	ND	11.6

[a] Golbeck and Cammarata (1981).

Therefore, the loss of PSII membrane-associated catalase activity in extrinsic-polypeptide-depleted PSII may explain why it is possible to detect light-induced hydrogen peroxide production by PSII membranes only after high-salt treatments.

The disproportionation of hydrogen peroxide in the dark by a reaction cycle involving the S_{-1} and S_1 states of the O_2-evolving center proposed by Velthuys and Kok (1978) was studied by several research groups (Frasch & Mei, 1987; Mano et al., 1987, 1993; Taoka et al., 1993). High pH was necessary to observe this catalase-like reaction. The high catalase activity of thylakoid or PSII membranes due to the presence of the PSII membrane-associated catalase raises the question of whether any of the dark rate of hydrogen peroxide disproportionation is actually due to an S_1/S_{-1} cycle. The residual catalase activity of PSII-30 °C was irreversibly inhibited by 3-amino-1,2,4-triazole, which is known to bind covalently to the compound I intermediate of catalase, without loss of light-induced O_2-evolution activity. This result indicates that little, if any, of the catalase activity from PSII membranes in the dark is catalyzed by the O_2-evolving center of PSII.

Polyphenol Oxidase. The PSII membrane-associated PPO is present in a latent form with a molecular weight of 72 kDa. Heat treatment releases this latent form from the membrane and also causes it to be processed into an active form with a molecular weight of 48 kDa. Both the 48 and 72 kDa PPO proteins have the same N-terminus which is also identical that of a known thylakoid 64 kDa protein (Hind et al., 1995). The apparent molecular weights of proteins determined in the present study are 5−10 kDa higher than previously reported molecular weights. For example, the 24 and 33 kDa extrinsic PSII proteins ran at 26 ± 1 and 39 ± 1 kDa in our SDS−PAGE experiments. Thus, taking this into consideration, we conclude that the 72 kDa protein we have characterized is identical to the known thylakoid 64 kDa polypeptide. Our results provide strong support for the proposal by Hind et al. (1995) that the 64 kDa thylakoid protein previously thought to be a LHCII kinase is, in fact, the latent form of PPO.

The properties of the active 48 kDa PPO purified in this study and the spinach thylakoid PPO characterized by Golbeck and Cammarata (1981) are summarized in Table 6. The properties of the two enzymes are very similar, especially with regard to the pH dependence and substrate specificity. There are, however, a few differences. For example, the enzyme purified by Golbeck and Cammarata (1981) consisted of two interconvertible forms: a tetramer and monomer with a subunit molecular weight of 42.5 kDa. Both of these forms had latent PPO activity, but they could be activated either by C_{18} fatty acids or spontaneously, but slowly, with time. On the other hand, our experiments did not reveal any tetrameric PPO and the PPO activity of the 48 kDa protein was high and did not require additional activation. These minor differences in properties can be accounted for by differences in experimental conditions, isolation method or activation mode. Therefore, we conclude that the active PPO we have characterized is identical to the 42.5 kDa spinach PPO reported by Golbeck and Cammarata (1981).

During mild heat treatment and further manipulations, most of the latent 72 kDa PPO protein was converted into an active 48 kDa PPO protein. Although no specific studies on the cleavage mechanism were conducted in this work, FPLC experiments revealed that the 72 kDa protein was cleaved only in the presence of the 48 kDa protein. This observation agrees with the finding that active PPO also has protease activity (Kuwabara & Hashimoto, 1990). Although the physiological role of the thylakoid membrane-bound PPO remains to be elucidated, it may have a proteolytic function.

The presence of high levels of copper in the snt-30 °C (Cu/PSII = 0.2−0.3), together with SDS−PAGE experiments, indicates that PPO is quite abundant in PSII membrane preparations, although most of the PPO exists in a latent form. It is known that PSII membrane preparations contain Cu(II) which has been found to interfere with EPR spectroscopic measurements (Miller & Brudvig, 1991). It seems likely that PPO may be the source of this Cu contamination. If so, a mild heat treatment of PSII membranes could be a useful method to remove Cu(II).

Both the PSII membrane-associated catalase and PPO have pH optima near 8.0. In addition, both enzymes appear to bind to the luminal surface of the thylakoid membrane. The pH dependence and the luminal location of the PSII membrane-associated catalase and PPO have interesting implications on their function under physiological conditions. Because the pH of the lumen is acidic during light-induced O_2 evolution, these enzymes would not be expected to be active in the light. Oppositely, in the dark, the luminal pH is close to the optimum for the catalase and PPO. It appears that the PSII membrane-associated catalase and PPO are thylakoid bound luminal enzymes which are important mostly for dark processes in the thylakoid lumen.

REFERENCES

Ananyev, G., Wydrzynski, T., Renger, G., & Klimov, V. (1992) *Biochim. Biophys. Acta 1100*, 303−311.

Andersson, B., & Styring, S. (1991) *Curr. Top. Bioenerg. 16*, 1−81.

Arnon, D. I. (1949) *Plant Physiol. 24*, 1−15.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., & Struhl, K., Eds. (1995) *Current Protocols in Molecular Biology*, Vol. 2, Chapter 10, Wiley-Interscience, New York.

Beaumont, F., Jouve H.-M., Gagnon, J., Gaillard, J., & Pelmont, J. (1990) *Plant Sci. 72*, 19−26.

Beck, W. F., de Paula, J. C., & Brudvig, G. W. (1985) *Biochemistry 24*, 3035−3043.

Berg, S. P., & Seibert, M. (1987) *Photosynth. Res. 13*, 3−17.

Berthold, D. A., Babcock, G. T., & Yocum, C. F. (1981) *FEBS Lett. 134*, 231−234.

Coughlan, S. J., & Hind, G. (1986) *J. Biol. Chem. 261*, 11378−11385.
Dubinin, A. V., Zastrizhnaya, O. M., & Gusev, M. V. (1992) *Microbiology 61*, 261−266.
Fine, P. L., & Frasch, W. D. (1992) *Biochemistry 31*, 12204−12210.
Frasch, W. D., & Mei, R. (1987) *Biochim. Biophys. Acta 891*, 8−14.
Galston, A. W., Bonnichsen, R. K., & Arnon, D. I. (1951) *Acta Chem. Scand. 5*, 781−790.
Gerasimenko, V. V., Goldfeld, M. G., Khangulov, S. V., Andreeva, N. E., Barynin, V. V., & Grebenko, A. I. (1989) *Biofizika 34*, 618−622.
Golbeck, J. H., & Cammarata, K. V. (1981) *Plant Physiol. 67*, 977−984.
Gregory, R. P. F. (1968) *Biochim. Biophys. Acta 159*, 429−439.
Hillier, W., & Wydrzynski, T. (1993) *Photosynth. Res. 38*, 417−423.
Hind, G., Marshak, D. R., & Coughlan, S. J. (1995) *Biochemistry 34*, 8157−8164.
Hirasawa, M., Gray, K. A., Shaw, R. W., & Knaff, D. B. (1987) *Biochim. Biophys. Acta 911*, 37−44.
Hirasawa, M., Gray, K. A., Ondrias, M. R., Larsen, R. W., Shaw, R. W., Morrow, K. J., & Knaff, D. B. (1989) *Biochim. Biophys. Acta 994*, 229−234.
Hochman, A., & Golberg, I. (1991) *Biochim. Biophys. Acta 1077*, 299−307.
Irrgang, K. D., Kablitz, B., Vater, J., & Renger, G. (1993) *Biochim. Biophys. Acta 1143*, 173−182.
Klimov, V. V., Ananyev, G. M., & Zastrizhnaya, O. M. (1992) in *Research in Photosynthesis II* (Murata, N., Ed.) pp 441−444, Kluwer Academic Publishers, The Netherlands.
Klimov, V., Ananyev, G., Zastrizhnaya, O., Wydrzynski, T., & Renger, G. (1993) *Photosynth. Res. 38*, 409−416.
Kuwabara, T. (1995) *FEBS Lett. 371*, 195−198.
Kuwabara, T., & Hashimoto, Y. (1990) *Plant Cell Physiol. 31*, 581−589.
Lax, A. R., & Vaughn, K. C. (1991) *Plant Physiol. 96*, 26−31.
Mano, J., Takahashi, M., & Asada, K. (1987) *Biochemistry 26*, 2495−2501.
Mano, J., Kawamoto, K., Dismukes, C. G., & Asada, K. (1993) *Photosynth. Res. 38*, 433−440.
Margoliash, E., Novogrodsky, A., & Schejter, A. (1960) *Biochem. J. 74*, 339−348.
Miller, A.-F., & Brudvig, G. W. (1991) *Biochim. Biophys. Acta 1056*, 1−18.
Ono, T., & Inoue, Y. (1983) *FEBS Lett. 164*, 255−260.
Race, H. L., Eaton-Rye, J. J., & Hind, G. (1995) *Photosynth. Res. 43*, 231−239.
Schonbaum, G. R., & Chance, B. (1980) in *The Enzymes* (Boyer, P. D., Ed.) 3rd ed., Vol. 13, pp 363−408, Academic Press, New York.
Schröder, W. P., & Åkerlund, H.-E. (1986) *Biochim. Biophys. Acta 848*, 359−363.
Stone, K. L., & Williams, K. R. (1996) in *The Protein Protocols Handbook* (Walker, J. M., Ed.) pp 415−427, Humana Press, Totowa, NJ.
Taoka, S., Jursinic, P. A., & Seibert, M. (1993) *Photosynth. Res. 38*, 425−431.
Trebst, A., & Depka, B. (1995) *Photosynth. Res. 46*, 41−44.
van Eyk, A. D., Litthauer, D., & Oelofsen, W. (1992) *Int. J. Biochem. 24*, 1101−1109.
Velthuys, B., & Kok, B. (1978) *Biochim. Biophys. Acta 502*, 211−221.
Yu, S. G., Stefansson, H., & Albertsson, P. A. (1992) in *Research in Photosynthesis I* (Murata, N., Ed) pp 283−286, Kluwer Academic Publishers, The Netherlands.
Zhang, L. X., Wang, J., Wen, J. Q., Liang, H. G., & Du, L. F. (1995) *Physiol. Plant. 95*, 591−595.

BI9613842