Characterization of the Low Molecular Weight Photosystem II Reaction Center Subunits and Their Light-induced Modifications by Mass Spectrometry*

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A sensitive and simple reverse phase HPLC purification scheme was developed for the rapid separation of the small protein subunits from photosystem II reaction center preparations. The precise molecular masses of the α- and β-subunits of cytochrome b₅₅₉ and the psbI gene product from pea plants, found to be 4394.6 ± 0.6, 9283.6 ± 0.7, and 4209.5 ± 0.5 Da, respectively, were then successfully determined for the first time by electrospray- and fast atom bombardment-mass spectrometry. Discrepancies between the molecular weights assigned and those calculated from the respective DNA sequences were observed for α- and β-subunits of cytochrome b₅₅₉. Currently, the nucleotide sequence of the psbI gene product from pea plants is not available. Application of novel mapping and sequencing strategies has assured the elucidation of full primary structures of all of the purified subunits. The modifications identified here include the post-translational processing of the initiating methionine on both subunits of cytochrome b₅₅₉, N-terminal acetylation and an mRNA editing site at residue 26 (Ser → Phe) on the β-subunit, and retention of the N-terminal formyl-Met on the psbI gene product. In addition, specific oxidation of a single amino acid residue was identified on the psbI gene product and the β-subunit purified from light-treated reaction center preparations. Overall, these studies provide the first detailed primary structural characterization of the small subunits of the reaction center complex and their associated light-induced modifications.

Photocatalysis (PSII) catalyzes the photoinduced splitting of water into molecular oxygen and reducing equivalents. This complex is embedded in the thylakoid membrane of plants, algae, and cyanobacteria and is composed of a large number of subunits (1, 2). At the heart of the complex is the reaction center where primary and secondary electron transfer processes occur. The cofactors that support these reactions are bound to the D1 and D2 proteins. These proteins are the products of the psbA and psbD genes, respectively, and are comparable with the L and M subunits of the reaction center of purple photosynthetic bacteria (3).

In its isolated form, the reaction center of PSII contains, in addition to the D1 and D2 proteins, the α- and β-subunits of cytochrome b₅₅₉, which are the products of the psbE and psbF genes, and the PSII-I protein, which is encoded by the psbI gene. In a recent paper (4), a sixth component has been claimed, which has an apparent molecular mass of about 6.5 kDa and is the product of the nuclear gene recently named psbW.

Sites in the D2 and D1 proteins, named Q₅ and Q₆, respectively, bind plastoquinones that facilitate secondary electron flow. However, in the case of the isolated reaction center complex the plastoquinone binding affinity is significantly reduced, and no secondary electron flow occurs unless appropriate acceptors are added (5). In their absence, the photochemical reaction is restricted to the formation of the radical pair state P680‘Pheo−’, where P680 is the primary donor, consisting of chlorophyll a, and Pheo is pheophytin a. The radical pair has a lifetime of about 35 ns and recombines by singlet and triplet routes. The latter creates the P680 triplet, distinguished by its EPR spin polarized signal (6). Under aerobic conditions, this triplet signal is quenched by oxygen, giving rise to singlet oxygen (7, 8) and a triplet lifetime of about 33 μs (9). The singlet oxygen generated under these conditions damages the reaction center, leading to the loss of chlorophyll (10), degradation of the D1 and D2 proteins (11), and inhibition of electron transport activity (12).

In this paper we report experiments to study the primary structures of the low molecular weight proteins of the isolated PSII reaction center complex with a view to identifying post-translational modifications, including the effect of illumination under conditions when singlet oxygen is being produced. We have employed a combination of fast atom bombardment-mass spectrometry and electrospray-mass spectrometry. These methodologies have not been used extensively for characterizing membrane proteins because of the problem of contaminating detergents. During the course of this work we have therefore developed procedures to overcome this problem.

EXPERIMENTAL PROCEDURES

Isolation of PSII Reaction Centers and Photoinhibitory Illumination—The PSII reaction center, consisting of the D1 and D2 proteins, the α and β subunits of cytochrome b₅₅₉, and the product of the psbI gene, was isolated from pea thylakoid membranes as described by Nanba and Satoh (13) with modifications described by Chapman et al. (14). Irradiation of RCII complexes was performed in a thermostatic cuvette maintained at 4 °C, using heat-filtered white light at 4000 microeinsteins m⁻² s⁻¹ for 15 min. The sample was suspended in 50 mM MES, 0.4 mM sucrose, and 2 mM n-dodecyl-β-maltoside (pH 6.0) at a chlorophyll concentration of 100 μg ml⁻¹.

Reverse Phase HPLC Separation of the PSII Reaction Center Proteins—Partial separation of the PSII reaction center proteins was effected using a Spherisorb Aquapore RP-300 (220 × 4.6-mm) column...
fitted to a Kontron HPLC system (Datasystem 450, HPLC pump 420, detector 430, and mixer M800). Reaction center preparations were dialyzed against 2 × 2.0 liters of aqueous 5% acetic acid at 4 °C for 16–24 h and were injected directly onto the column. The column was held at 100% A (aqueous 0.1% trifluoroacetic acid) followed by a linear increase to 100% B (0.05% acetic acid in aqueous 0.1% trifluoroacetic acid) over 60 min at a flow rate of 1.0 ml min⁻¹. Elution was monitored at 214 and 280 nm, and fractions were collected at 1-min intervals.

Reverse Phase HPLC Separation of Peptide Mixtures—Separation of the polypeptides produced by enzymatic digestion was effected using the above system and conditions except that the Aquapore RP-300 × 4.6-mm column was replaced by an Aquapore OD-300.

Tryptic Digestion—Lyophilized protein samples were redissolved in 200 μl of 50 mM ammonium bicarbonate (pH 8.5) and digested with trypsin for 6 h at 37 °C, using an enzyme:substrate ratio of 1:50 (w/w). The reaction was terminated by freezing followed by lyophilization.

Chymotryptic Digest—Lyophilized protein samples were resuspended in 200 μl of 50 mM ammonium bicarbonate (pH 8.5) and digested with chymotrypsin for 3 h at 37 °C, using an enzyme:substrate ratio of 1:50 (w/w). The reaction was terminated by freezing followed by lyophilization.

Cyanogen Bromide Digest—Lyophilized protein samples were resuspended in 100 μl of a solution of CNBr in 70% formic acid and incubated in the dark at room temperature for 5 h. The reaction was terminated by adding 5 volumes of water followed by freezing and lyophilization. In the case of samples to be analyzed by gas phase sequencing, the solution of CNBr in 70% formic acid was applied to protein present on polybren-coated glass fiber discs and incubated overnight. The acid and CNBr were removed under a stream of nitrogen, and the glass fiber disc was submitted to gas phase sequence analysis (see below).

Carboxyesterification—Manethic HCl reagent was prepared by bubbling HCl gas into methanol until the solution became warm. After cooling, a 100-μl aliquot of this reagent was added to the dried sample, which was then incubated at room temperature for 30 min. The reaction was terminated by drying under a stream of nitrogen.

Acetylation—Acetylation of free amino groups was carried out using a solution of 3 volumes of methanol, 1 volume of acetic anhydride. The samples to be derivatized were dried in glass tubes and resuspended in 10 μl of milli-Q water (purified by Millipore® system), and 40 μl of the acetylation reagent was added to each tube and left for 1 min at room temperature. The reaction was terminated by drying under a stream of nitrogen.

Manual Edman Degradation—The samples for manual sequencing were dried down in a glass tube and redissolved in 100 μl of a 5% solution of phenylisothiocyanate in pyridine was added to each sample, and the coupling reaction was flushed with nitrogen prior to its incubation at 45 °C. After 1 h, this reaction was terminated by removing excess reagents under a stream of nitrogen. To each of the dried samples 100 μl of trifluoroacetic acid was added, and the mixtures were incubated for a further 10 min at 45 °C, causing the cleavage of the phenylthiocarbamyl-labeled terminal amino acid as the thiocarbamyl derivative. The remaining truncated peptide products were subsequently dried under nitrogen and analyzed by MS.

Gas Phase Edman Sequencing—Peptides were Edman-degraded using a CI 4000 gas phase protein sequencer, and the standard protein sequencing method was used (15). Samples which failed to give sequence information were digested on the glass fiber disc by CNBr as described above.

FAB-MS Analysis—FAB mass spectra were acquired using a Fisons VG ZAB 2SE 2FPD mass spectrometer fitted with a cesium ion gun operating at 30 kV. The instrument was calibrated using CsI clusters. Data acquisition and processing were performed using VG Analytical Opus software. Dried peptide samples were dissolved in 60% propan-1-ol in 5% aqueous acetic acid in acetonitrile and 2 methoxyethanol in a 1:1:1 ratio at a flow rate of 5 μl min⁻¹. The mass spectrometer was calibrated using a solution of horse myoglobin (1 pm ρ mol⁻¹), to give average chemical molecular masses for the signals observed.

MALDI-MS Analysis—MALDI mass spectra were acquired using a Fisons VG ZAB 2SE 2FPD mass spectrometer, fitted with a UV laser (337 nm). The instrument was calibrated using CsI clusters. Data acquisition and processing were performed using VG Analytical Opus software. The samples were dissolved in 60% propan-1-ol in 5% aqueous acetic acid and 1-μl aliquots were loaded onto a probe that had been treated with a saturated solution of 2,5-dimethoxy-4-hydroxycinnamic acid made up in a 35:65 (w/v) mixture of 90% acetonitrile in aqueous 0.1% trifluoroacetic acid and aqueous 0.1% trifluoroacetic acid.

**RESULTS**

Purification and Molecular Weight Determination by ES-MS of the Three Small Protein Subunits Present in Dark PSII Reaction Center Preparations—The successful application of MS in protein sequencing largely depends on the availability of purified sample, relatively free of salt and detergents. We have developed a highly efficient reverse phase HPLC protocol to isolate the three small subunits present in PSII reaction centers in conditions amenable for MS analysis. The first step of the procedure involves the dialysis of reaction center preparations into 5% aqueous acetic acid using a 50-kDa cut-off membrane (see “Experimental Procedures” for details). This crucial step assists in slowly removing salts and detergents introduced during the original isolation of the PSII reaction center complexes while avoiding precipitation of the extremely hydrophobic transmembrane proteins under investigation. The smaller subunits of the PSII reaction center complex were then separated from the diazylated samples to near base-line resolution by using aqueous 0.1% trifluoroacetic acid and 0.1% trifluoroacetic acid in acetonitrile in a gradient HPLC elution system.

The HPLC profile generated for dark (control) reaction center preparations using the above protocol is shown in Fig. 1A. At a wavelength of 214 nm, three main absorbance peaks, at retention times of 44.5, 55.4, and 64.4 min, were detected. The HPLC fractions corresponding to these regions of UV absorbance were analyzed by direct injection into the ES-mass spectrometer over a period of 2 min. After transformation, the ES mass spectra of fractions 45, 55, and 64 gave molecular masses of 4394.6 ± 0.6, 9283.6 ± 0.7, and 4209.5 ± 0.5 Da, respectively. The calculated masses of the α- and β-subunits of cytochrome b_559 and the PSII-I protein based on their DNA sequences are 9414.6 Da (pea), 4424.2 Da (potato), and 4167.8 Da (tomato) column was replaced by a probe that had been previously treated with a 1:1 mixture of glycerol and monothioglycerol.

**ES-MS Analysis—**ES-MS spectra of the intact reaction center proteins and their digestion products were obtained by direct injection of 10-μl aliquots of HPLC-purified samples into the ion source of a VG Bio-Q triple quadrupole electrospray mass spectrometer. The instrument was operated using a carrier flow buffer of 0.1% formic acid in aqueous 0.1% trifluoroacetic acid, aqueous 0.1% trifluoroacetic acid, propan-1-ol, and 2 methoxyethanol in a 1:1:1:1 (v/v/v/v) ratio at a flow rate of 5 μl min⁻¹. The mass spectrometer was calibrated using a solution of horse myoglobin (1 pm μmol⁻¹), to give average chemical molecular masses for the signals observed.

MALDI-MS Analysis—MALDI mass spectra were acquired using a...
the psbI gene from pea plants has not been obtained, although the nucleotide sequences from other organisms including rice, wheat, mustard, liverwort, and tobacco are available. A partial amino acid sequence (residues 2–16) has been determined for the pea PSII-I subunit (17); however, the lack of a complete psbI sequence for pea and the nature of the NH2-terminal post-translational modification make it impossible to calculate an expected molecular mass for this polypeptide. Similarly, NH2-terminal modification of the \( \beta \)-subunit also prevents the theoretical calculation of its exact molecular mass. The unambiguous assignment and characterization of the structural changes attributable to the mass discrepancies of the three main isolated components was achieved by subsequent MS mapping studies. In addition to the major UV absorbance peaks discussed above, there are also two relatively small UV peaks observed in the chromatogram, at 42.4 and 48.0 min. These components have been identified by a combination of polyacrylamide gel electrophoresis and MS studies as minor contaminants of dodecyl maltoside and CP proteins (chlorophyll-binding proteins), respectively.

**Characterization of the Primary Structure of the \( \beta \)-Subunit of Cytochrome \( b_{559} \)—** Characterization of the HPLC-purified component eluting at 44.8 min, having an intact molecular mass of approximately 4.4 kDa, was carried out using FAB mapping studies after trypsin digestion. Two main quasimolecular ions (\( M + H \)) were observed at \( m/z \) 996.4 and 2890.4 in the spectrum. The exact mass correlation of the signal at \( m/z \) 996.4 with that calculated for the tryptic peptide Thr6–Arg13 (Table I legend) of the \( \beta \)-subunit provided the first evidence identifying this polypeptide as a cytochrome \( b_{559} \) subunit rather than the PSII-I protein, which does not contain a tryptic peptide of this mass. Three peptides in total are expected to be generated by tryptic digestion of the \( \beta \)-subunit. The anticipated molecular mass for the C-terminal peptide is \( m/z \) 2830.4, and the absence

![Fig. 2. Electrospray mass spectra of HPLC fractions 45 (A), 55 (B), and 64 (C), isolated from dark PSII reaction center preparations (see Fig. 1A). These components were subsequently assigned to the \( \beta \)-subunit of cytochrome \( b_{559} \), \( \alpha \)-subunit of cytochrome \( b_{559} \), and PSII-I protein, respectively.](image)

**Table I**

Cytochrome psbF gene product from *Pisum sativum* (pea) (31)

The DNA sequence and the protein structure characterized from these MS studies are as follows (tryptic cleavage sites indicated in boldface type).

| DNA sequence | protein sequence |
|--------------|-----------------|
| MTIDRTYPF    | Acetyl-TIDRTYPF |
| RVRLAVHGL    | RVRLAVHGL      |
| AVPTVSFLGS   | AVPTVSFLGS     |
| ISAMFIQ      | ISAMFIQ        |

FAB mapping of tryptic peptides from \( \beta \)-subunit of cytochrome \( b_{559} \) purified from dark PSII reaction center preparations is shown below.

| Elution time | Measured (\( M + H \)^+) | Expected (\( M + H \)^+) | Peptide |
|--------------|--------------------------|------------------------|---------|
| min          | Da                        | Da                     |         |
| 20.99        | 996.4                     | 996.5                  | 6–13    |
| 30.74        | 2890.4                    | 2830.4                 | 14–39   |
| 41.86        | 4396.0                    | 4425.2                 | Intact  |

**Table II**

Cytochrome psbE gene product from *P. sativum* (pea) (31)

The DNA sequence and the protein structure characterized from these MS studies are as follows (tryptic cleavage sites indicated in boldface type).

| DNA sequence | protein sequence |
|--------------|-----------------|
| MSGSTGERSF   | SGSTGERSF       |
| ADIITSIRYW   | ADIITSIRYW      |
| IHSITIPSFL   | IHSITIPSFL      |
| FIAGWLFSVT   | FIAGWLFSVT      |
| GLAYDVGSP    | GLAYDVGSP       |
| RPNEYFETTR   | RPNEYFETTR      |
| QGIPPLITGRF  | QGIPPLITGRF     |
| DSLEQLDEFS   | DSLEQLDEFS      |
| RSF          | RSF             |

Electrospray and FAB mapping of tryptic peptides from \( \alpha \)-subunit of cytochrome \( b_{559} \) purified from dark PSII reaction center preparations is shown below.

| Elution time | Measured mass | Expected mass | Peptide |
|--------------|---------------|---------------|---------|
| min          | Da            | Da            |         |
| 20.78        | 692.2         | 692.3         | 2–8     |
| 29.15        | 1121.7        | 1121.6        | 9–18    |
| 31.47        | 953.6         | 953.6         | 61–69   |
| 36.87        | 1484.8        | 1484.6        | 70–81   |
| 57.12        | 6645.4        | 6645.5        | 2–60    |
| 59.94        | 5970.8        | 5970.8        | 9–60    |
of this signal and the concomitant detection of a molecular ion with a 60-atomic mass unit increment, m/z 2890.4, suggests the presence of molecular modifications between Trp14 and Arg57. The molecular ion corresponding to the NH2-terminal tryptic product, Thr2–Arg57 (initiation of this protein at the second residue was suggested by previous sequencing studies (17)) was not observed in the FAB map possibly due to the suppression of the molecular ion corresponding to this peptide.

To generate a complete map of the protein, the individual tryptic peptides of the β-subunit of cytochrome b559 were isolated by reverse phase HPLC. The four main UV absorbance peaks observed were screened by FAB-MS, and molecular masses were determined for three of these components (Table I). Subsequently, the purified products were subjected to automated gas phase sequencing. The NH2-terminal sequence obtained for the component eluting at 30.7 min (996.4 Da) was Thr-Tyr-Pro-Ile-Phe-Thr-Val-Arg, while that derived from the component eluting at 41.9 min (2890.4 Da) was Trp-Leu-Ala-Val-His-Gly-Leu-Ala-Val-Pro-Val-Phe-Phe. These results confirm the assignments of these components as tryptic peptides of the β-subunit of cytochrome b559, Thr2–Arg13 and Trp14–Arg57, respectively. Importantly, the sequence generated from the smaller peptide (Thr2–Arg13) matches exactly with the DNA sequence, while the sequence obtained from the larger peptide (Trp14–Arg57) diverges from the DNA derived amino acid sequence at residue 26, where the expected serine is in fact a phenylalanine. The nucleic acid codons of these two amino acids differ only in one base position. The DNA sequence may thus be in error, or as has recently been described in the sequence of spinach β-subunit, mRNA editing could have taken place (20). This substitution would result in a 60-atomic mass unit increase on the molecular weight of this peptide and thus corresponds to the mass discrepancy observed.

FAB-MS analysis of the absorbance peak at 21.0 min in the chromatogram of the tryptic digestion on the β-subunit of cytochrome b559 did not yield any significant mass signals. This component was believed to be the as yet unidentified NH2-terminal tryptic peptide, Thr2–Arg6. It is well known that small hydrophilic peptides can often escape detection by FAB-MS due to suppression effects (35). This phenomenon can usually be overcome by using chemical derivatization to increase the hydrophobicity of analytes. Carboxyesteryfication, which results in the modification of the free carboxyl groups to esters, was used in this study. From its predicted sequence it is anticipated that there are two free carboxyl groups on the peptide Thr2–Arg6, one at the C terminus and the other due to Asp5. The quasimolecular ion derived from esterification of this peptide would thus be expected at m/z 532.2. Only one molecular ion, m/z 574.2, was detected in the spectrum derived from the derivatized sample. The 42-atomic mass unit difference between these signals implies the presence of an acetyl group on the peptide. This modification is believed to correspond to the NH2-terminal blocking group that had been detected but not identified during amino acid sequencing of the β-subunit (17, 18) and therefore is most probably located on the amino group of residue Thr2.

Further confirmation of the structure ascribed to the β-subunit was accomplished by a chymotryptic FAB map of this component (Fig. 3). The amino acid substitution at residue 26 was suggested by the molecular ions at m/z 803.3, 1100.3, 1223.4, and 1497.5. These signals have been assigned to Gly58–Phe26, Phe27–Phe36, Leu15–Phe26, and Phe27–Arg39. They each correspond to chymotryptic digestion products created by cleavage at Phe26. The presence of the second modification on the β-subunit, the NH2-terminal acetylation, was also confirmed by the mass shift of the chymotryptic product giving rise to the molecular ion at m/z 1167.3. This signal is 42 atomic mass units larger than that expected for peptide Thr2–Phe26. Corroboration of the other mass signals, m/z 1237.4 and 1350.5, with those expected from peptides Gly58–Arg39 and Leu26–Arg39, respectively, indicates that there is no other modifications in these regions. The map of the β-subunit of cytochrome b559 generated by chymotryptic digestion covers the whole protein molecule.

Characterization of the Primary Structure of the α-Subunit of Cytochrome b559—The α-subunit of cytochrome b559 is the only reaction center constituent that is expected in the 9-kDa mass range. Based on its DNA sequence, an average chemical mass of 9414.6 Da was calculated for this component (Table II legend); however, amino acid sequencing studies have indicated that the initiating methionine residue of this protein is proteolytically removed (17), leading to an expected molecular mass of 9283.5 Da. The close correlation (0.1 Da) between the predicted and experimentally determined molecular mass leads to the putative assignment of the HPLC-purified component eluting at 55.4 min as the α-subunit of cytochrome b559 (des-Met). Confirmation of this assignment was obtained by mapping studies on the HPLC-purified component.

Direct FAB-MS analysis of the products generated by tryptic digestion shows four molecular ions at m/z 954.6, 1122.7, 1485.8, and 2422.5. All these masses can be assigned to expected trypsin-induced peptides of the α-subunit of cytochrome b559: Gln61–Arg69, Ser9–Arg18, Phe70–Arg81, and Gln61–Arg81, respectively (Table II legend). Following reverse phase purification of the tryptic digest, fractions containing UV absorbances were screened by FAB-MS and ES-MS, and the results are presented in Table II. The UV absorbances can be related to expected tryptic peptides that map the entire α-subunit (residues 2–81) except for residues 82 and 83, and all of these assignments have been confirmed by automated gas phase
sequencing.

Characterization of the Primary Structure of the PSII-I Protein—Direct FAB-MS analysis of the products generated by tryptic digestion on the HPLC-purified component eluting at 64.4 min indicates the presence of two main digestion products, at m/z 3528.2 and 3952.6, and the intact polypeptide, at m/z 4210.7 (Table III). Since neither of these signals can be directly correlated to expected tryptic products from the β-subunit of cytochrome b559, it seemed likely that the component under investigation was the psbI gene product. This suggestion was further verified by the fact that mass differences between the molecular weight of the undigested product (m/z 4210.7) and the two digestion products (m/z 3528.2 and 3952.6) correspond to the molecular weights of the tryptic peptides that would be generated by cleavage at either of the two arginine residues in the C-terminal domain (residues 30 and 34) of the psbI gene product predicted from tobacco, mustard, and wheat DNA sequences (Table III legend). On this basis, the observed molecular ions at m/z 3952.6 can be provisionally assigned to the modified PSII-I peptide residues equivalent to MetC–ArgD, while m/z 3528.2 would correspond to the modified PSII-I peptide equivalent to MetC–ArgD.

The tryptic digestion products of the PSII-I protein were then carboxyesterified prior to FAB-MS analysis. The results shown in Table III indicate that there are a total of four carboxy groups on the undigested protein. The two tryptic products, however, each contained only two esterifiable groups. Since one of the esters on each peptide corresponds to the carboxyl group at the C-terminal of the peptide, it can be deduced that there are a total of three acidic amino acids on the PSII-I protein, only one of which is located NH2-terminal to ArgD. The region between residues 35 and 36 must therefore be composed of two acidic residues. These results, derived from mass shifts before and after esterification are consistent with the proposal that the C-terminal sequence of the psbI gene product from pea plants is identical to that of its tobacco, wheat, and mustard counterparts, being Glu-Glu.

To determine the number of free amino groups present on the psbI gene product, an aliquot of the purified protein was acetylated in acetic anhydride/methanol, and the products were analyzed directly by FAB-MS (Fig. 4A). The molecular ion observed at m/z 4252.6 is 42 atomic mass units larger than the molecular ion measured for the psbI gene product (m/z 4210.7). This result shows that there is a single amino group present on this polypeptide. Amino acid sequencing data obtained by Webber et al. (17) as well as the information obtained in the Edman reaction reported below suggest that the NH2-terminal amino group of the psbI gene product is blocked. The acetylation site observed here must thus be on a lysine residue, since arginine is not blocked quantitatively under the conditions used. In most other plant PSII-I proteins there appears to be a conserved lysine residue at position 5. In contrast, the partial amino acid sequence obtained for the pea by Webber et al. (17) has proline assigned at this site. Our results suggest that there is in fact a lysine residue in the PSII-I protein of pea plants.

The Edman degradation reaction was performed on an aliquot of the purified PSII-I protein, and the products were analyzed directly by FAB-MS. The spectra obtained (Fig. 4B) show a molecular ion at m/z 4345.7. The +135-Da mass shift on the molecular ion corresponding to the intact PSII-I protein (m/z 4210.7) can be attributed to the formation of a phenylthiocarbamyl derivative, which again is only observed with lysine-containing peptides. These data not only confirm that the NH2 terminus of the psbI gene product is blocked (16, 17), but they also provide additional evidence that there is a lysine residue present in the sequence of the pea component.

The presence of an NH2-terminal modification on the psbI gene product was confirmed by the absence of phenylthiohydantoin-derivatives in automated gas phase sequencing analysis. However, sequence information was successfully generated after treatment of the glass fiber sequencing disc containing the sample with CNBr, a reagent that cleaves proteins on the C-terminal side of methionine. The amino acid sequence obtained for residues 2–27 (Leu-Thr-Leu-Lys-Leu-Phe-Val-Tyr-Thr-Ile-Val-Ile-Val-Leu-Phe-Phe-Ile-Phe-Gly-Phe-Leu-Ser-Asn-Asp) establishes a major part of the sequence, including the presence of lysine at position 5. In fact, the pea PSII-I protein sequence found here is highly homologous to the sequences of the other plant psbI gene products (Table III legend). There is only a single amino acid difference among the tobacco, mustard, and wheat in comparison to the pea sequence, where valine 11 has been substituted by isoleucine here. This sequence change has also been reported by Webber et al. (17).

Fragmentation data acquired by FAB-MS analysis provided confirmation of the proposed primary structure for the PSII-I protein. The NH2-terminal sequence ions afford the a, b, and c series of signals, while C-terminal sequences were derived from x, y, and z ions. Mass differences between two consecutive sequence ions in a series correspond to amino acid residue differences. The fragment ions detected covered the region between Leu4 and Arg34 (data not shown), and, using the tobacco gene sequence as a template, fragment ion masses were detected confirming the Edman sequence derived above with lysine in position 5 and extending the sequence from position 27 to 34 in correspondence with the gene sequence as Pro-Gly-Arg-Asn-Pro-Gly-Arg. Based on a combination of all of the above experiments, the full amino acid sequence for the psbI gene product from pea plants can be inferred. The expected molecular mass calculated for this protein sequence is 4181.8 Da. This is 28 atomic mass units smaller than the experimentally determined molecular mass for HPLC-purified PSII-I protein, 4209.5 ± 0.5 Da (Fig. 2C). The mass increment observed implies the presence of a formyl group. From the Edman data, this modification must be located at the NH2-terminal amino group and thus corresponds to the blocking group.

Evidence verifying the nature of the NH2-terminal modification on this component was obtained by cyanogen bromide digestion of the HPLC-purified sample. The main molecular ion observed in the MALDI-high field-MS spectrum (Fig. 4C), at m/z 4051.8, differs by a mass of 159 atomic mass units from the undigested psbI gene product (observed here at m/z 4210.8).
This mass shift is equivalent to the molecular weight of a formyl-methionine residue. Collectively, these studies have not only provided the first report of the complete amino acid sequence for the psbI gene product present in the PSII reaction center complex of pea plants, but they have also revealed the nature of its NH2-terminal blocking group.

Characterization of the Primary Structural changes Induced on the Three Small Subunits during Illumination of the Isolated PSII Reaction Centers—In order to characterize the effects of irradiation on the smaller subunits of the reaction center, the α- and β-subunits of cytochrome b559 and the PSII-I protein were isolated from light-treated reaction center preparations (4000 microeinsteins m⁻² s⁻¹ at 4 °C for 15 min). The HPLC profile obtained for the light-treated sample is presented in Fig. 1B. As can be seen, the UV absorbances identified as β-subunit of cytochrome b559 (44.9 min) and α-subunit of cytochrome b559 (55.5 min) in the dark samples were also detected in the chromatogram of the illuminated reaction center preparations. ES-MS analysis of the relevant HPLC fractions has confirmed these assignments. In contrast to these two components, the absorbance peak attributed to the PSII-I protein, 64.7 min, was much reduced in size compared with its dark counterpart. This change indicates the possibility of light-induced modification on this component. Further differences between the dark and irradiated samples were the presence of two additional absorbance peaks, at 43.0 min (fraction 43) and 59.9 min (fraction 60), in the light-treated samples. We have defined these in the studies reported below as light-induced modified products of the β-subunit of cytochrome b559 and the PSII-I protein.

Characterization of the Light-induced Modification on the β-Subunit of Cytochrome b559—ES-MS analysis of the component eluting at 43.0 min in the irradiated sample gave a precise molecular mass of 4410.6 ± 0.5 Da (Fig. 5A). The molecular mass of this molecule is 16 atomic mass units higher than that obtained in the dark experiment (Fig. 2A) or for the residual β-subunit of cytochrome b559 eluting at 44.9 min. This mass shift can be attributed to a single oxidation event.

Localization of the oxidation site was attempted by mapping studies. Direct FAB-MS analysis of an aliquot from the tryptic digestion mixture of the oxidized β-subunit shows three main molecular ions in the high mass region at m/z 2906.6, 3884.4, and 4412.0. All of these ions are 16 atomic mass units larger than the molecular weights expected (calculated from the protein sequence in the Table II legend) for the peptides of the β-subunit. They can be attributed to the oxidized peptides of Trp14–Arg39, Thr4–Arg39 and the undigested protein, respectively. Collectively, these results not only confirm the assignment of the light-induced component purified in fraction 43 from illuminated reaction center preparations as the oxidized form of the β-subunit, but they also indicate that the modified amino acid is located between Trp14 and Arg39.

The chymotryptic FAB map of the oxidized β-subunit (Fig. 6) indicates the presence of four main products; these correlate to the molecular ions at m/z 803.2, 1116.4, 1167.3, and 1223.5. Three of these signals, m/z 803.2, 1167.3, and 1223.5, were also present in the chymotryptic map obtained from unmodified β-subunit and thus can be assigned to peptides Gly19–Phe36, Ac-Thr2–Phe10, and Leu15–Phe26, respectively. The molecular ion at m/z 1100.3 that had been attributed to Phe27–Phe36 in the spectrum of unmodified β-subunit was not present in the spectrum of the light-induced component. Instead, a signal at m/z 1116.4 was detected. From the 16-atomic mass unit mass shift on this component it is evident that the oxidized amino acid is located within the chymotryptic peptide corresponding to Phe27–Phe36. The primary structure deduced from these studies establish that the component eluting in HPLC fraction 43 corresponds to an oxidized form of the β-subunit of cytochrome b559. Location of the modified residue has been restricted to the region between Phe27 and Phe36. Previous studies on model systems have shown that tyrosine, tryptophan, histidine, methionine, and cysteine are the most readily oxidizable amino acid residues (36). The presence of only a single amino acid from this group, Met34, in the region of
As the oxidized form of the PSII-I and also indicates that the result verifies the assignment of this light-induced component. This reaction center sample shows that after illumination, unlike FAB-MS analysis of the purified product (data not shown). Suggestion is supported by fragmentation data obtained by direct cleavage at Met1 in the modified PSII-I compared with the normal bromide digestion of the oxidized PSII-I protein. The failure to sequence information could be obtained even after cyanogen bromide treatment, contrasting with an experiment on normal (dark) β-subunit in which the expected digestion products were observed in MALDI-MS experiments.

Characterization of the Light-induced Modification on the PSII-I Protein. — The deconvoluted ES mass spectrum of the component eluting at 59.9 min gave a molecular mass of 4225.7 ± 0.6 Da (Fig. 5B). This molecule has a slightly earlier retention time than the PSII-I protein, which elutes at approximately 64 min, and is 16 atomic mass units heavier than this protein. Furthermore, the increase in the UV absorbance of the 59.9-min signal is concomitant with the reduction in the UV absorbance of the unmodified PSII-I. All of these characteristics suggest that the component in fraction 60 represents a singly oxidized form of the psbI gene product.

A comparison of the tryptic FAB map generated by analysis of the light-modified PSII-I protein (Fig. 7) with that obtained from the unmodified protein (Table III) indicated a +16-atomic mass unit shift of all three molecular ions detected in the spectrum of the light-specific molecule. The mass signals at m/z 3544.1, 3968.5, and 4226.7 have therefore been assigned to the oxidized peptides corresponding to formyl-Met1–Arg30, formyl-Met1–Arg34, and undigested protein, respectively. This result verifies the assignment of this light-induced component as the oxidized form of the PSII-I and also indicates that the modified amino acid is located in the NH2-terminal fragment of the protein, between formyl-Met1 and Arg30.

Further characterization of the oxidized psbI gene product was initiated by application of automated gas phase sequencing. As with the psbI gene product isolated from dark reaction center preparations, no sequence was obtained by direct analysis of this light-induced PSII-I component. However, in contrast to the nonoxidized sample (discussed previously), no sequence information could be obtained even after cyanogen bromide digestion of the oxidized PSII-I protein. The failure to cleave at Met1 in the modified PSII-I compared with the normal sample strongly implicates Met1 as the modified residue. This suggestion is supported by fragmentation data obtained by direct FAB-MS analysis of the purified product (data not shown).

Inspection of the HPLC profile obtained from the irradiated reaction center sample shows that after illumination, unlike the β-subunit of cytochrome b559, where a relatively smaller proportion is modified, most of the psbI gene product has been oxidized. It would therefore appear that oxidation of the PSII-I is a relatively extensive process. In contrast to the β-subunit and the PSII-I protein, no light-induced modification of the α-subunit of cytochrome b559 was detected under the conditions employed.

DISCUSSION

The analysis of protein components in membrane multisubunit complexes, such as PS II, has traditionally been performed by SDS-polyacrylamide gel electrophoresis. This technique has generated a vast amount of information, but its application has been limited due to poor resolution, especially for proteins below 15 kDa. One of the aims of the study presented here was to develop a complementary purification protocol to fractionate the low molecular weight subunits of the reaction center of PSII for mass spectrometric analysis, thereby leading to their detailed structural characterization, and to provide a means for monitoring the effects of illumination on these components.

Reverse phase HPLC is a purification system that offers the ability to separate protein components on the basis of their hydrophobicity, and thus this technique can often ensure the separation of subunits that are difficult to resolve by SDS-polyacrylamide gel electrophoresis. In addition, this chromatographic procedure does not require the use of salts and detergents but instead removes these compounds from the sample, thus making it highly compatible with mass spectrometric techniques. Unfortunately, the application of this effective analytical strategy to membrane proteins has been hampered by their extreme hydrophobicity; these molecules are very difficult systems to work with, especially under conditions in which detergents are not permitted. Our preliminary purification studies revealed that it is crucial to maintain the solubility of the PSII complex prior to isolation of its components. We found that dialysis of the reaction center preparations into 5% aqueous acetic acid preserved the solubility of the transmembrane proteins and acted as a partial purification step. The dialyzed reaction center samples were then applied directly to reverse phase HPLC, and components purified using 0.1% trifluoroacetic acid and acetonitrile as the gradient elution system. This protocol resulted in the successful isolation of the three small subunits of the isolated PSII reaction center. It should be noted that the D1 and D2 proteins cannot be eluted from the column under the same conditions. Their purification requires a differ-
ent solvent system. In addition, we note the absence of any evidence for the 6.5-kDa PSII-W protein (4) in our PSII preparations using the HPLC purification and mass spectrometric mapping methods described here.

The ES-MS technique is presently unsurpassed in its accuracy for the determination of whole protein molecular mass. In this study, a precise molecular mass of 9283.6 ± 0.7 Da was attributed to the α-subunit of cytochrome b$_{559}$. This result is in agreement with the sequence derived from the pea DNA except for NH$_2$-terminal processing. Proteolytic cleavage of the initiating formyl methionine on the pea α-subunit of cytochrome b$_{559}$ had previously been characterized by classical protein sequencing methods (17). Our FAB mapping studies verify the presence of this post-translational modification and also provide confirmation of the rest of the primary structure of the pea protein.

It is of interest to note that the α-subunit is the only reaction center protein in which we find the absence of an NH$_2$-terminal blocking group. This characteristic is important in view of the suggestion that the α-subunit is cross-linked, via its NH$_2$-terminus, to the D1 protein during photoinhibitory light treatments of photosystem II complexes (18, 19). The formation of the corresponding 41-kDa cross-linked product early in photoinhibition was suggested to be a tag marking the D1 protein for degradation (19). Thus, it would seem that the structure of the amino terminus of the α-subunit may be of particular relevance in the molecular processes of photoinhibition.

The precise molecular mass we attributed to the β-subunit of cytochrome b$_{559}$, 4394.6 ± 0.6 Da, was found to be 30 mass units smaller than that calculated from its corresponding DNA sequence. This mass discrepancy was resolved by further structural studies, which identified an NH$_2$-terminal modification event, entailing the removal of the initiating formyl-methionine residue followed by an acetylation of the amino terminus of the second threonine residue and a sequence change at residue 26, where a serine has been replaced by a phenylalanine. A comparison of the gene sequences of various β-subunit components indicates that Phe$_{26}$ is conserved in most higher plants except for spinach and pea. However, the results of some recent molecular biology investigations on spinach plants have revealed the presence of an mRNA editing process (20), during which a single C to U base conversion in the 26th codon restores the code for a phenylalanine residue. The data reported in this paper document the first direct protein sequencing information indicating the occurrence of a similar Ser → Phe transformation in the β-subunit of pea plants and thus provides evidence for an analogous mRNA editing event for the psbF transcript of this species. The functional relevance of the mRNA editing is currently being investigated by several groups (21). It has been found that the editing of the psbF and a second gene also on the same codon, psbL, in spinach plants occurs in a tissue- and stage-specific manner (20) but appears not to be conserved in other plant species (22). Moreover, in spinach plants it was reported that the processing step is essential for function of the β-subunit of cytochrome b$_{559}$. The inability to restore the correct sequence was associated with a slower growth, lowered chlorophyll content, and high fluorescence: characteristics that are usually associated with inactive reaction centers.

The presence of the second modification on the β-subunit, the NH$_2$-terminal processing, had only been characterized previously as a "blocking group" during protein sequencing studies (17), the structural details of which had remained elusive until now; this modification has been identified by our structural studies as the co-translational cleavage of the NH$_2$-terminal formyl-methionine followed by NH$_2$-terminal acetylation of Thr$_2$. Generally, the NH$_2$-terminal processing (the removal of the formyl-methionine residue) is considered to be a relatively common modification; however, in comparison, amino acetylations are only frequently observed in eukaryotic cytoplasmic proteins (23). Only three of the PSII components, D1, D2, and CP43, are now known to have this modification (24). In all of these cases, the acetylation is conserved to a threonine residue (Thr$_2$ in D1 and D2 and Thr$_{15}$ in CP43). It is thought that the structure of the NH$_2$ terminus of a protein may be of significance in controlling its stability and in the organization of membrane proteins (25).

Unlike the other PSII reaction center proteins, the psbl gene of pea chloroplasts had not been fully sequenced. However, a partial sequence (residues 2–16) for this component had been obtained by NH$_2$-terminal amino acid sequencing (17). It revealed that despite the extremely high homology of this protein between higher plants, there were two sites of difference; one was a conservative substitution, Val$_{11}$ → Ile, while the other was a nonconservative change, Lys$_{5}$ → Pro. Direct ES-MS analysis of the purified pea PSII-I protein gave a precise molecular mass of 4209.5 ± 0.5 Da. This mass did not appear to be in agreement with the available partial sequence information. It thus became necessary to generate a complete primary structure for this molecule. Application of a tryptic mapping strategy confirmed that the C-terminal sequence of the PSII-I protein in peas was homologous to that of tobacco, mustard, and wheat. In contrast, the structure in the NH$_2$-terminal region, achieved by automated gas phase sequencing, diverged from sequences of these three species at the 11th residue, where Val is substituted by Ile. Also we have shown residue 5 to be lysine and not proline as reported by Webber et al. (17). The work of these authors and of Ikeuchi and Inoue (16) indicated the presence of an unidentified NH$_2$-terminal blocking group on the methionine of the PSII-I protein. The characterization of the NH$_2$-terminal blocking group has been revealed in our work by a combination of the data generated by MS fragmentation and mapping experiments after CNBr digestion. The NH$_2$-terminal blocking group was assigned to be formyl located on the amino group of the first amino acid residue, Met$_1$.

All proteins synthesized in chloroplast, mitochondria, and prokaryotic systems are initiated with a formyl-methionine residue. However, this group is removed by a ribosome-bound enzyme shortly after its synthesis. It therefore follows that amino-terminal formylated proteins have only rarely been detected. In fact, analyses of the primary structures of other proteins involved in photosynthesis indicates that they are all post-translationally processed except for the H subunit of the bacterial reaction center (26) and the α-subunit of light-harvesting antenna complex II from Rhodopseudomonas acidophila (27). The presence of the formyl group on the psbl gene product found here is thus unusual. Formyl groups are often detected on proteins that are rapidly turning over, to the extent that their rate of production saturates the processing enzyme (28). At present there is no evidence that this is the case for the PSII-I protein. Alternatively, the function of the formyl group could be analogous to that of the o-subunit of LH2 in R. acidophila in that it may provide a ligand to a chlorophyll molecule. This suggestion cannot immediately be verified due to the dearth of the desired detailed three-dimensional structural information. Nevertheless, direct evidence emerging from a recent molecular biology study on a Chlamydomonas reinhardtii mutant lacking the psbl gene has suggested that the PSII-I protein may assist in maintaining the stability of the PSII complex or may modulate electron transport activity. In

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*J. Sharma, M. Panico, J. Barber, and H. R. Morris, manuscript in preparation.*
these investigations the function of the PSII-I protein appeared to be especially important under stressful high light conditions (29). These characteristics may be understood if the psbI gene product did in fact serve as a chlorophyll-binding protein.

Comparison of the HPLC profile for dark and illuminated reaction center samples revealed the presence of two additional components after light treatment, at 43.0 and 59.9 min, and reduction in the UV absorbance of the component eluting at 64.7 min in the latter samples. The intact precise molecular mass attributed to the light-specific product eluting at 43.0 min was 4410.6 ± 0.5 Da. Both the slightly earlier retention time and 16-atomic mass unit increment attributed to this molecule as compared with the β-subunit of cytochrome b₅₅₉ have suggested that this light-induced component is a singly oxidized form of the β-subunit. This assignment was subsequently confirmed by the tryptic and chymotryptic mapping. These studies also revealed that the oxidized amino acid residue was located in the region of Phe²⁷–Phe³⁶. The precise location of the oxidized amino acid was deduced from the inability to generate CNBr digestion products, as had been done for the unmodified protein. There is only a single methionine residue (Met³⁴) in the sequence of the β-subunit, and its conversion to a sulfoxide upon oxidation would render it inaccessible to cleavage by CNBr treatment.

The dramatic reduction in absorbance of the UV peak corresponding to the psbI gene product appeared to be accompanied by the emergence of a large UV peak at the slightly earlier retention time of 59.9 min. The precise molecular mass, 4225.7 ± 0.6 Da, attributed to this subunit is 16 mass units larger than that of the PSII-I protein. Subsequent, tryptic FAB mapping of this light-specific component confirmed it as the oxidized psbI gene product. The precise location of the modification came from two lines of evidence: first, the inability to generate sequence information after cyanogen bromide digestion as had been done in the unmodified PSII-I counterpart, and second, fragmentation data generated by FAB-MS. From this it was clear that the oxidation site was at the NH₂ terminus and probably located on the initiating methionine residue.

In contrast, despite careful analyses of all HPLC fractions, no modified form of the α-subunit of cytochrome b₅₅₉ was detected in the illuminated sample. This finding is interesting, since the α-subunit has six readily oxidizable residues (Met, Cys, His, Trp, and Tyr) and yet does not seem to be modified, while both the β-subunit and PSII-I protein, which have four and two potentially oxidizable residues, respectively, are singly oxidized during the light treatment studied here. Furthermore, under these illumination conditions, a larger and major portion of the PSII-I protein appears to be modified as compared with the β-subunit. The absence of any direct correlation between the level of oxidation and the number of potentially oxidizable residues leads to the conclusion that there is a degree of specificity in these light-induced modifications. Indeed, it should be noted that the oxidation of the β-subunit and the PSII-I protein appears to be restricted to methionine residues, and that the α-subunit does not have any methionine residues in the mature protein.

Under the conditions used for the illumination treatment it is known that the isolated reaction center acts as a singlet oxygen producer (7, 8). The light-induced radical pair P680 'Pheo' recombines to generate the P680 triplet (P680⁺) state with a yield of about 30% at room temperature. Under aerobic conditions, this triplet state interacts with dioxygen (which is also a triplet) to form singlet oxygen.

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P680^+ + O_2 \rightarrow P680 + O_2^+ \]

**REACTION 1**

Although this reaction is greatly enhanced in isolated PSII reaction centers because of the lack of secondary electron acceptors and donors (which compete with the recombination reaction), it is likely to occur in vivo, albeit at a very low frequency (30). It seems, therefore, that the oxidation of the β-subunit and the PSII-I protein is due to singlet oxygen attack at the suggested methionine groups determined in the work presented here. The oxidation is therefore very specific and probably occurs in vivo during photoinhibitory damage of PSII. Indeed, we have found that when reaction centers were isolated from plants suffering from photoinhibitory stress there was an increase in the oxidation levels of the two components without any further illumination applied.

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