Purification and Determination of Intact Molecular Mass by Electrospray Ionization Mass Spectrometry of the Photosystem II Reaction Center Subunits*

Jyoti Sharma, Maria Panico, James Barber, and Howard R. Morris‡

From the Wolfson Laboratories, Department of Biochemistry, Imperial College, London, SW7 2AY, United Kingdom

A reverse phase high pressure liquid chromatography purification system for the rapid separation of photosystem II reaction center proteins free of salts and detergents is described. This procedure results in the isolation of the three small subunits: α- and β-subunits of cytochrome b559 and PsbI protein, with near base-line resolution between each peak, although the D1 and D2 proteins were partially deconvoluted. The molecular masses obtained by electrospray ionization mass spectrometry for the purified β-subunit of cytochrome b559, α-subunit of cytochrome b559, and the PsbI protein, 4,394.8 ± 0.4, 9,283.7 ± 0.8, and 4,209.5 ± 0.4 Da, respectively, are in excellent agreement with values obtained from previous characterization studies (Sharma, J., Panico, M., Barber, J., and Morris, H. R. (1997) J. Biol. Chem. 272, 3935–3943). Direct electrospray analysis of the D1 and D2 proteins suggests that these components exist in heterogeneous forms. The molecular mass ascribed to a predominant form of the D1 protein, 38,040.9 ± 6.5 Da, and the D2 protein, 39,456.1 ± 7.7, are also in agreement with those expected for the mature nonphosphorylated states of these subunits.

Photosystem II (PSII) is a pigment-protein complex that catalyzes the unique reactions resulting in the splitting of water molecules into dioxygen and reducing equivalents. This complex is embedded in the thylakoid membrane of plants, algae, and cyanobacteria and is made up of more than 20 different subunits. It is now accepted that the photosynthetic reactions of PSII take place in a reaction center comprised of two proteins, D1 and D2 (4), which show many characteristics similar to those of the L and M subunits of purple photosynthetic bacteria. These subunits bind all of the redox factors required for primary electron transfer.

In higher plants the D1 and D2 subunits, encoded by the chloroplast psbA and psbD genes, respectively, are synthesized on the 70S ribosomes that are attached to the stromal thylakoids. Evidence from several biochemical and molecular biological studies has shown that upon translation both proteins undergo a variety of structural modifications. It is believed that most of these covalent changes are involved in the development and assembly of active PSII complexes or in controlling the triggering and degradation of the photodamaged PSII components (for review, see Ref. 5).

Mass spectrometry is a highly valuable technique in the field of structural biochemistry. Electrospray ionization mass spectrometry (ESI-MS), with an accuracy of about 0.01%, provides an extremely sensitive method for determining the precise molecular mass of biological molecules >100 kDa. In many cases, the measurement of the intact molecular mass of a protein and comparison with the predicted value have been used to verify gene sequences, locate mRNA processing and editing events (1), identify mutations (6), detect post-translational modifications, and provide confirmation of start and stop signals of a gene, which, in some instances such as that of the psbC gene encoding the CP43 protein, have been difficult to define by other methods (7). In fact, now a substantial number of publications, concerned mainly with hydrophilic proteins, describe the successful application of this technique.

ESI-MS of very hydrophobic species such as membrane proteins has, however, proved significantly more challenging than related studies on hydrophilic proteins. This characteristic can be attributed mainly to the incompatibility between most mass spectrometric techniques and the presence of detergents and/or salts required to retain the analytes in solution. Thus, the successful analysis of hydrophobic proteins and peptides relies upon developing successful sample handling protocols that are compatible with the technique. Recently, we reported the use of MS to elucidate the complete primary structures of the small subunits of the isolated PSII reaction center complex (1). In the studies reported here, we have employed ESI-MS together with a novel purification method to analyze the intact D1 and D2 subunits isolated from pea plants (Pisum sativum L.). Detailed characterization of the modification sites and further verification of the D1 and D2 protein sequences were obtained by mapping studies reported in the accompanying paper (12).

MATERIALS AND METHODS

Isolation of PSII Reaction Centers—The PSII reaction center preparations (RCII), consisting of the D1 and D2 proteins, the α- and β-subunits of cytochrome b559, and the product of the psbI gene, were isolated from pea thylakoid membranes as detailed previously (8).

Reverse Phase-High Performance Liquid Chromatography for the Separation of the Reaction Center Proteins—Partial separation of the PSII reaction center proteins was achieved by using a Spherisorb Aquapore RP-300 (220 × 4.6 mm) column fitted to a Kontron HPLC system (Datsystem 450, HPLC pump 420, Detector 430 and Mixer M800). RCII were dialyzed against 2 × 2,000 ml of aqueous 5% acetic acid at 4 °C for 16–24 h and were loaded directly onto the column. The subunits making up the isolated reaction center were eluted with a solvent system of A = aqueous 5% acetic acid and B = propan-1-ol, using a linear gradient from 100% A to 100% B over 60 min at a flow rate of 0.5
Elution was monitored at 254 nm and 280 nm, and fractions were collected at 1-min intervals.

**Analytical Polyacrylamide Gel Electrophoresis and Immunodetection**—The fractions obtained from HPLC purification of the reaction center complex were analyzed by discontinuous SDS-polyacrylamide gel electrophoresis, using the LKB 2001 vertical electrophoresis apparatus (9). Linear gradient, 10–17% polyacrylamide, gels containing 6 M urea of 1-mm thickness were used during analytical polyacrylamide gel electrophoresis. The buffer system employed was 0.2 M glycine, 25 mM Tris-HCl, and 0.1% SDS. The reaction center samples were solubilized in an equal volume of 250 mM Tris-HCl, 4% SDS, and 2% β-mercaptoethanol (pH 8.9) before application to the gel. After electrophoresis the proteins were electroblotted onto polymeric membranes, nitrocellulose (Sartorius 0.45 mm), using Towbin buffer (Tris glycine, pH 8.3) according to a modification of the protocol first devised by Towbin et al. (10). Transfer was performed at 4 °C using a plate electrode blotting system. The antibodies used to detect the D1 and D2 proteins were a kind gift from Dr. P. Nixon. An alkaline phosphatase conjugation procedure, developed by Sigma, was then employed followed by appropriate chromogenic substrates to visualize the products.

**ESI-MS Analysis**—ESI mass spectra of the intact reaction center proteins were obtained by direct injection of 10-μl aliquots of HPLC-

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**Fig. 1.** Reverse phase HPLC UV (254 nm) elution profile obtained from fractionating preparations of dark-treated PSII reaction centers with conditions specified under "Materials and Methods."

**Fig. 2.** Western blot analysis using D1- and D2-specific antibodies, across the main UV-absorbing fractions obtained from the HPLC purification of dark-treated RCII.

**Fig. 3.** Mass-to-charge ESI mass spectrum of HPLC fraction 34, showing some of the charge states observed for the D1 protein. The signal at m/z 1,404.2 corresponds to residual three-charge component of the psbI gene product.
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 90% acetonitrile 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) ratio at a flow rate of 5 μl min⁻¹. The mass spectrometer was calibrated using a solution of horse heart myoglobin (1 pmol).

RESULTS

Two mass spectrometric techniques, ESI-MS and matrix-assisted laser desorption ionization-time of flight-MS (MALDI-TOF-MS), are now used routinely for elucidating the intact molecular masses of large biological molecules. The main advantage of ESI-MS is that it offers a much higher mass accuracy than MALDI-TOF-MS; on the other hand, the latter procedure is thought to benefit from an ability to tolerate the presence of moderate quantities of salts and detergents. However, despite the anticipated utility of MALDI-MS for analyzing the hydrophobic proteins studied here, our initial studies to characterize partially purified D1 and D2 proteins by this technique were unsuccessful.

A key factor in the structural studies reported in this paper has been the ability to develop appropriate sample handling and purification strategies. After attempting numerous liquid chromatographic separations, including ion exchange and gel permeation methods, all of which failed, an effective reverse phase chromatographic method for isolating the D1 and D2 proteins in media compatible with ESI-MS was obtained. The first step of our protocol involved the dialysis of the RCII samples against 5% aqueous acetic acid. We found that this step assisted in the slow removal of some of the salts and detergents that are present in the preparations while maintaining the solubility of the protein complex. The RCII subunits were then separated from the dialysis sample by employing a propan-1-ol and 5% aqueous acetic acid solvent mix in a gradient HPLC elution system. We found that a flow rate of 0.5 ml min⁻¹ and linear 60-min elution gradient (increasing organic solvent) gave the best protein separation with this system while again avoiding precipitation of the protein on the column.

The optimized HPLC profile generated for dark-treated RCII using the devised protocol is presented in Fig. 1. Apart from the broad UV-absorbing peak at approximately 36 min which shows virtually no separation at the peaks (but in later studies were shown to be deconvoluted), the rest of the UV-absorbing components at retention times of 27.0, 30.4, 32.8, and 47.0 min have been isolated to near base-line resolution. Subsequently, the HPLC fractions corresponding to these regions of UV absorption were analyzed by immunoblotting using antibodies against the D1 and D2 (Fig. 2). It can be seen that the earlier HPLC fractions corresponding to the UV absorbance region at 33–43 min are dominated by the D1 subunit, whereas in the later ones, corresponding to the tail of this broad peak, D2 is the main component. Thus, this chromatographic procedure provides a partial separation of the two highly similar D1 and D2 proteins. The last UV absorbance peak, eluting at 47.0 min, which appeared as a green solution, has been attributed to the free pigments of the PSII samples. The three smaller UV peaks at 27.0, 30.4, and 32.8 min, were presumed, and later shown, to correspond to small subunits of the reaction center complex.

HPLC fractions corresponding to all of the regions of UV absorbance were then analyzed by direct injection into the ESI-MS, avoiding a drying or concentration step that we have shown leads to irreversible protein adsorption. After deconvolution of the ESI mass spectra the components corresponding to the first three UV-absorbing peaks, 27.0, 30.4, and 32.8 min, were attributed average molecular masses of 4,394.8 ± 0.4,
9,283.7 ± 0.8, and 4209.5 ± 0.4 Da, respectively. These masses are in excellent agreement with values obtained from previous characterization studies (1) and can be assigned to the β-subunit of cytochrome b_{559}, the α-subunit of cytochrome b_{559}, and the psbl gene product, respectively. In contrast to these components, the ESI mass spectra generated from the HPLC fractions corresponding to the D1 and D2 proteins were complicated; signal heterogeneity was observed within the peaks corresponding to distinct charge states (Fig. 3). This characteristic is indicative of structural heterogeneity within the D1 and D2 protein populations. However, by using relatively large scale reaction center preparations we have been able to determine accurate molecular masses for principal forms of both the D1 and D2 proteins (Fig. 4 and 5).

The average chemical molecular mass expected for the D1 protein of peas, calculated from its nucleotide-derived protein sequence and utilizing the known COOH- (11) and NH₂-terminal modifications (7), is 38,033.6 Da for the nonphosphorylated form and 38,113.6 Da for the phosphorylated form. In comparison, we obtained an average experimental molecular mass of 38,040.9 ± 6.5 Da for one of the principle components in HPLC fraction 34. This species is in relatively good agreement with the expected molecular mass of the nonphosphorylated form of the D1 subunit. However, the transformed ESI mass spectrum in Fig. 4 also clearly indicates the presence of heterogeneous forms of the D1 protein. The higher molecular mass products probably correlate with D1 subunits that have been modified in a way different from the nonphosphorylated protein. Subsequent mapping studies on the D1 protein using cyanogen bromide and trypsin digestion with MS analyses, reported in the accompanying paper (12), have identified oxidations and phosphorylations as sources of the structural diversity observed.

The calculated molecular mass for the D2 protein of pea, including NH₂-terminal processing, is 39,463.5 Da for the nonphosphorylated form and 39,543.5 Da for the phosphorylated form. An average chemical molecular mass of 39,456.1 ± 7.7 Da was obtained by deconvolution of the ESI mass spectrum of HPLC fraction 38. Thus, based on the close correlation of molecular masses, the protein characterized is assigned as the nonphosphorylated, NH₂-terminally processed form of the D2 subunit. Once again, heterogeneous forms for this component can also be observed in the transformed spectrum (Fig. 5), possibly corresponding to oxidations reported in the accompanying paper (12).

**DISCUSSION**

A classical protein purification technique used regularly in the preparation of samples for MS analysis is column chromatography. Two forms of this methodology, affinity chromatography and gel filtration chromatography, had previously been applied during the characterization of the NH₂-terminal (7) and COOH-terminal (11) structures of the D1 protein. The former protocol was utilized to isolate phosphorylated polypeptides specifically and thus was of limited use in this study. On the other hand, the gel filtration protocol was found to provide a means for partially separating PSII reaction center proteins and pigments (11). This purification system, however, could not be applied directly for MS studies as it employed the use of SDS, which is incompatible with most MS ionization methods. Omitting the use of detergent in the gel filtration procedure was one of the first methods explored to separate the RCII components for MS studies. Unfortunately, the modified protocol was found to result in reduction of resolution and significant decrease in yield, thus this system was also unsuitable for
application in these structural investigations.

Despite the difficulties of purifying hydrophobic proteins for MS studies, we have developed an effective method for isolating the five RCII protein subunits in a salt- and detergent-free state and in a single chromatographic step. This is an important achievement because these hydrophobic polypeptides and proteins have previously only been separated using systems containing salts and detergents. Our protocol with its combined dialysis and HPLC purification steps gives a near base-line separation of the three small subunits and partial resolution of the D1 and D2 proteins. Moreover, we found it essential to analyze the purified components directly by ESI MS, thus allowing rapid characterization following their isolation.

The precise molecular masses obtained for the three small subunits, the α-subunit of cytochrome b$_{559}$, β-subunit of cytochrome 559, and the psbI gene product, are 9,283.7 ± 0.8, 4,394.8 ± 0.4, and 4,209.5 ± 0.4 Da, respectively. These values are in good agreement with the values predicted from their protein sequences, after making allowances for the structural modifications reported in our previous paper (1).

During the HPLC purification, we noticed that the D1 and D2 proteins eluted over an unusually broad concentration range of propan-1-ol, between approximately 51 and 66%. Because the characteristics of this elution profile cannot be attributed solely to the extremely high hydrophobicity of these components, we believed that additional factors such as sample heterogeneity were present. This was substantiated further by the complexity of the ESI mass spectra for the D1 and D2 subunits; nevertheless we have made reasonably accurate assignments of the main forms of these proteins. The molecular mass ascribed to the D1 protein is in good agreement with that expected for the nonphosphorylated, NH$_2$- and COOH-terminally processed component. Molecular ions corresponding to that anticipated for the nonphosphorylated, NH$_2$-terminally processed form of the D2 subunit could also be identified in these studies. In addition, our data show for the first time that there is a degree of molecular diversity on the D1 and D2 proteins present in mature PSII reaction center complexes, a topic that is addressed in the following paper (12).

REFERENCES

1. Sharma, J., Panico, M., Barber, J., and Morris, H. R. (1997) J. Biol. Chem. 272, 3935–3943
2. Barber, J. (1989) in Oxford Surveys of Plant Molecular and Cell Biology (Miflin, B., ed) Vol. 6, pp. 113–162, Oxford University Press, Oxford, U. K.
3. Hankamer, B., Barber, J., and Boekema, E. J. (1997) Annu. Rev. Plant Physiol. 48, 641–671
4. Babcock, G. T. (1996) in Photosynthesis: From Light to Biosphere (Mathis, P., ed) Vol. II, pp. 209–215, Kluwer Academic Publishers, Amsterdam
5. Aru, E.-M., Virgin, I., and Andersson, B. (1993) Biochim. Biophys. Acta 1143, 113–134
6. Lindo, V. S., Kakkar, V. V., Learmonth, M., Melissa, E., Zappacosta, F., Panico, M., and Morris, H. R. (1995) Br. J. Haematol. 89, 589–601
7. Michel, H., Hunt, D. F., Shabanowitz, J., and Bennett, J. (1988) J. Biol. Chem. 263, 1129–1130
8. Chapman, D. J., Gounaris, K., and Barber, J. (1990) in Methods in Plant Biochemistry (Rogers, L. J., ed) Vol. 5, pp. 171–193, Academic Press, London
9. Laemmli, U. K. (1970) Nature 227, 680–685
10. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
11. Takahashi, M., Shiraiishi, T., and Asada, K. (1988) FEBS Lett. 240, 6–8
12. Sharma, J., Panico, M., Shipton, C. A., Nilsson, F., Morris, H. R., and Barber, J. (1997) J. Biol. Chem. 272, 33158–33166