Polypeptide D1 of the photosystem II reaction center of oxygenic photosynthesis is expressed in precursor form (pre-D1), and it must be proteolytically processed at its C terminus to enable assembly of the manganese cluster responsible for photosynthetic water oxidation. A rapid and highly sensitive enzyme-linked immunosorbent assay-based microtiter plate method is described for assaying this D1 C-terminal processing protease. A protocol is described for the isolation and purification to homogeneity of the enzyme from the green alga, <i>Scenedesmus obliquus</i>. Amino acid sequence information on the purified protease was used to clone the corresponding gene, the translated sequence of which is presented. A comparison of the gene product with homologous proteases points to a region of conserved residues that likely corresponds to the active site of a new family of processing proteases. These observations led to the conclusion that proteolytic processing of precursor D1 is required for assembly of the manganese cluster. The LF-1 strain has been shown to be incapable of processing pre-D1 (13, 21, 22). This lack of processing has been attributed to the absence of the D1 Ctp-protease (13, 20–22). These observations led to the conclusion that proteolytic processing of precursor D1 is required for assembly of the manganese cluster (13, 21, 22) either because proteolytic cleavage frees the C-terminal carboxyl group of D1 to coordinate manganese (6) or because the presence of the C-terminal extension blocks the C-terminal region of D1 from adopting a conformation compatible with manganese coordination. This conclusion has been further reinforced by two experimental observations. A site-directed mutant of <i>Synechocystis</i> 6803, in which D1-Ser-345 was replaced by proline, lost the ability to process the D1 polypeptide and to assemble at least some of the coordinating ligands to this complex (5–8).

The photosystem II reaction center contains two homologous polypeptides, D1 and D2, that are responsible for the coordination of the primary photoreactants (1, 2). D1 has also been shown to harbor the redox-active tyrosine, Tyr<sub>2</sub> (3, 4), that links the oxidized primary electron donor of photosystem II to the oxygen-evolving manganese cluster and is thought to provide at least some of the coordinating ligands to this complex (5–8). D1 is expressed in precursor form (9–11), inserted into the thylakoid membrane, and processed at its C terminus (6, 12–15) by a proteolytic enzyme, D1 C-terminal processing protease (Ctp-protease).<sup>1</sup> In cyanobacteria, 16 residues are cleaved from precursor D1 (6), 9 in higher plants (15, 16), and 8 in <i>Chlamydomonas</i> with processing occurring in all cases at the carboxyl side of D1-Ala-344. <i>Euglena</i> is the sole oxygenic photosynthetic organism that is believed not to undergo C-terminal processing (1), although, in this case, no C-terminal extension is present. Based on a comparison of 43 psbA genes, the amino acid sequence on the amino side of the processing site is strictly conserved within the 8 residues immediately upstream of the processing site (1). On the carboxyl side of the processing site, no residue is conserved throughout all 43 sequences. While position 345 is normally occupied by either an alanine or a serine, replacement of Ser-345 in <i>Synechocystis</i> 6803 with arginine or alanine (6) or in <i>Chlamydomonas</i> with glycine, cysteine, valine, or phenylalanine (17) still allows processing to proceed normally, <i>in vivo</i>. The x-ray-induced, non-oxygen evolving LF-1 mutant strain of <i>Scenedesmus obliquus</i> was originally characterized by Metz and Bishop (20) as having little variable fluorescence, being unable to evolve oxygen and containing on a per chlorophyll basis in thylakoid membranes less than half the manganese of wild type. All of these characteristics imply an inability to assemble the manganese cluster. The LF-1 strain has been shown to be incapable of processing pre-D1 (13, 21, 22). This lack of processing has been attributed to the absence of the D1 Ctp-protease (13, 20–22). These observations led to the conclusion that proteolytic processing of precursor D1 is required for assembly of the manganese cluster (13, 21, 22) either because proteolytic cleavage frees the C-terminal carboxyl group of D1-Ala-344 to coordinate manganese (6) or because the presence of the C-terminal extension blocks the C-terminal region of D1 from adopting a conformation compatible with manganese coordination. This conclusion has been further reinforced by two experimental observations. A site-directed mutant of <i>Synechocystis</i> 6803, in which D1-Ser-345 was replaced by proline, lost the ability to process the D1 polypeptide and to assemble at least some of the coordinating ligands to this complex (5–8). D1 is expressed in precursor form (9–11), inserted into the thylakoid membrane, and processed at its C terminus (6, 12–15) by a proteolytic enzyme, D1 C-terminal processing protease (Ctp-protease).<sup>1</sup>

<sup>1</sup> The abbreviations used are: Ctp-protease, C-terminal processing protease; CHAPS, 3-[3-cholamidopropyl]dimethylammoniom-1-propanesulfonate; ELISA, enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatography; MALDI, matrix-assisted laser desorption ionization; MES, 2-(N-morpholino)ethanesulfonic acid; PCR, polymerase chain reaction; pre-D1, precursor form of the D1 polypeptide following N-terminal processing but prior to C-terminal processing; PSII, photosystem II; RACE, rapid amplification of cDNA ends; PAGE, polyacrylamide gel electrophoresis; bp, base pair.

<sup>2</sup> B. Diner, unpublished data.
Scenedesmus acid sequence information, obtained from the purified enzyme, and amino acid sequencing of its D1 Ctp-protease. This first definitive proof that an enzyme that functions as D1 can be explained by a single base pair deletion in the coding (LF-1-RVT-1). We show that the loss of D1 processing in LF-1 processing of pre-D1.

Euglena and as D1 Ctp-protease, has as yet described C-terminal extension of reac-

zyme from pea and wild type Scenedesmus that catalyze C-terminal processing of the D1 polypeptide in thylakoid membranes isolated from the Scenedesmus LF-1 strain, rendering the thylakoid membranes photosensitizable for water oxidation. Satoh and co-workers (26), in a very thorough effort, have recently isolated and purified D1 Ctp-protease from spinach. This enzyme was partially sequenced and the gene that encodes it cloned and fully sequenced (27). The luminal location of this protease is indicated by the need for sonication (26) or detergent treatment (see below) to free the water-soluble enzyme from the thylakoid membranes and by the presence of a luminal transit peptide (27). This localization and the substrate specificity (28) of the enzyme are both consistent with its being the D1 Ctp-protease, the substrate of which is also located in the lumen (29). However, no mutational test, showing this enzyme to function in vivo as D1 Ctp-protease, has as yet been reported. While the translated spinach gene does show homology (42.2% identity, (27)) to the ctpA gene mentioned above, there exists no direct demonstration that the ctpA gene product of Synechocystis has D1 Ctp-protease activity.

In this paper, we report the development of a sensitive microtiter plate ELISA assay for D1 Ctp-protease. By using this assay to monitor the activity of this enzyme from the green alga, Scenedesmus obliquus, we report the isolation, purification, and amino acid sequencing of its D1 Ctp-protease. This enzyme shows the expected cleavage site specificity using a synthetic peptide as substrate. We have exploited the amino acid sequence information, obtained from the purified enzyme, to clone and sequence the D1 Ctp-protease genes from the Scenedesmus wild type, from the non-photoautotrophic LF-1 strain, and from a photoautotrophic LF-1 suppressor strain (LF-1-RVT-1). We show that the loss of D1 processing in LF-1 can be explained by a single base pair deletion in the coding region of the gene, causing a frame shift and a premature translational termination. Restoration of D1 processing and photoautotrophy in the suppressor strain is correlated with a nearby single base pair insertion. We present, therefore, the first definitive proof that an enzyme that functions as D1 Ctp-protease in vitro is also responsible for in vivo proteolytic processing of pre-D1.

EXPERIMENTAL PROCEDURES

Algal Strains

The algal strains used in this study were the wild type of S. obliquus, strain D3, and a non-photosynthetic low fluorescent mutant (LF-1), derived from wild type by x-ray mutagenesis (20). A photoautotrophic suppressor strain, derived from LF-1 (LF-1-RVT-1) (31), was also examined. All strains were kindly provided by Dr. Norman Bishop (Oregon State University, Corvallis, OR). Except where fermentors were used, the cells were grown in 20-liter carboys on NYG medium (30) in the light at 25 °C.

Preparation of Primary Antibody

A synthetic peptide (EVMHERNAHFPLDLRA), identical to the final 16 residues of practically all known sequences of polypeptide D1 (1), was synthesized (>95% pure, Multiple Peptide Systems) and coupled to keyhole limpet hemocyanin by using glutaraldehyde at a 1:1 ratio of peptide to keyhole limpet hemocyanin (w/w). New Zealand White rabbits were immunized using the peptide-keyhole limpet hemocyanin complex dispersed in phosphate-buffered saline buffer (3.1 mg/ml) and emulsified by mixing with an equal volume of Freund’s adjuvant and injected into 5–6 subcutaneous dorsal sites for a total volume of 0.6 ml. The initial immunization was followed by three booster injections 21 days apart. The antisemur that was used as primary antibody in the assays was obtained from one rabbit 10 days after the second booster injection. The animals were bled from the ear vein. The blood was heated to 37 °C for 1 h, chilled to 0 °C for 15 h, and centrifuged. Further purification of the immunoglobulins was not attempted. The serum was frozen and stored at −80 °C.

Microtiter Plate Assay of D1 Protease

The ELISA based assay for the detection of D1 Ctp-protease activity detects product formation very specifically, by the use of the above-mentioned primary antibody that associates to at least 30-fold greater extent to the product than to the substrate. In practice, the ratio of signals for processed PSII cores of wild type to unprocessed PSII cores of LF-1 is usually 15–30. Substrate core complexes are linked to a 96-well microtiter plate; D1 Ctp-protease is added to the wells for a fixed time, and the product is analyzed by using the primary antibody followed by an enzyme-linked secondary antibody. The quantity of primary antibody attached to the product of the D1 Ctp-protease reaction is detected through the alkaline phosphatase conjugate of the secondary antibody, and the alkaline phosphatase activity is measured colorimetrically to quantify protease activity.

Preparation of Microtiter Plates—PSII core complexes were isolated from Scenedesmus wild type and LF-1 as described by Diner et al. (13) and stored at −80 °C. Immediately before use, they were diluted to 2 ng of chlorophyll/μl using TBS (20 mM Tris-HCl, pH 8.0, 150 mM NaCl). Each well requires 5 μl of chlorophyll or 2.5 ml of diluted LF-1 cores and 0.2 μg of chlorophyll or 100 μl of wild type cores. Ten plates were typically prepared at the same time.

Fresh glutaraldehyde (25% solution, Electron Microscopy Sciences, EM grade, Fort Washington, PA) was diluted to 0.5% with TBS immediately before use, and 25 μl of the diluted glutaraldehyde was pipetted into each well of a 96-well microtiter plate (Nunc Immunoplate Maxi-sorp, catalog number 439035). 25 μl of the diluted LF-1 cores were pipetted per well into all but three wells of the plate, and the plate was shaken to mix the glutaraldehyde and the core complexes. 25 μl of the diluted wild type cores were pipetted per well into each of the remaining three wells, and the plates were shaken for 1–2 h at room temperature.

At the end of the incubation period, the wells were washed to remove unbound core complexes and blocked to prevent nonspecific protein binding. Both were accomplished with four rinses with TTBS (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% Tween 20) and then filling each well with TTBS. The plates were then incubated for 1–2 h at room temperature, after which the wells were again rinsed. The remaining TTBS was aspirated and shaken out of the wells. The plates were then sealed in plastic bags containing a moistened paper towel. The plates were then stored at −20 °C and could be used for several months. However, the background signal in blank wells lacking enzyme gradually increased with storage time.

Assay of Enzyme Activity—D1 Ctp-protease preparations were diluted to 50 μl with 20 mM HEPES-KOH, pH 7.25, 20% glycerol (assay buffer) and placed in individual assay wells. Usually only rows B–G and columns 2–11 were used for assaying enzyme activity because the wells at the periphery of the plate often gave slightly higher signals. The control wells (located in column 12) were not incubated with protease and thereby show the maximum (wild type wells) and minimum (LF-1 wells) signals attainable with the D1 ELISA assay. The samples of D1 protease were typically allowed to incubate for 1 h at room temperature after which each well was given three quick rinses with TTBS and then allowed to soak for 2 min. The TTBS was aspirated, and the wells were
refilled without rinsing for another 2 min incubation. This wash/incubation was repeated one more time. The plate was then aspirated dry and turned over to tap any remaining buffer onto a paper towel.

5 μl of primary antibody serum was diluted with 5 ml of phosphate-buffered saline (1.44 g of Na2HPO4, 0.24 g of KH2PO4, pH 7.2, 8 g of NaCl, 0.2 g of KCl, and 20 g of bovine serum albumin per liter), previously filter sterilized through a 0.2-μm membrane. 50 μl of diluted primary antibody solution was added to each well. The plates were covered and allowed to incubate on a rotary shaker at 37 °C. After 30 min, the plate was washed as above with three cycles of soak and aspiration using TTBS.

10 μl of goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma Immunochemicals A-7539) were diluted into 5 ml of TTBS (3 g of Tris-HCl, pH 8.0, 8 g of NaCl, 0.2 g of KCl, and 20 g of bovine serum albumin per liter), previously filter sterilized through a 0.2-μm membrane. 50 μl of diluted secondary antibody solution was added to each well, and the covered plates were allowed to incubate on a rotary shaker at 37 °C. After 30 min, the plate was washed three times with TTBS as above.

The colorimetric substrate consisted of one 5-mg tablet of p-nitrophenyl phosphate (Kirkegaard & Perry Laboratories) dissolved in 5 ml of 5× diluted DEA Buffer (Kirkegaard & Perry Laboratories). 50 μl of this solution was added to each well, and the plate was incubated at 37 °C in a microtiter plate reader (Molecular Devices Thermomax) interfaced to a Macintosh SE/30 computer. The increase in absorption at 405 minus 650 nm for 10 min. Activity is expressed as the percentage of the signal obtained with a concentration of wild type PSI cores equal to that of LF-1. 100% corresponds to fully processed cores.

FIG. 1. Enzyme activity versus enzyme concentration. Purified Scenedesmus enzyme was diluted with assay buffer (20 mM HEPES-KOH, pH 7.25, 20% glycerol) at the indicated relative concentrations and incubated in the microtiter plate wells for 1 h at room temperature. The most concentrated enzyme solution used was approximately 13 nM. At 50 μl/well this is equivalent to 650 fmol of enzyme/well. The ordinate is expressed as the percentage of the signal obtained with a concentration of wild type PSI cores equal to that of LF-1. 100% corresponds to fully processed cores.

An example of the dependence of the assay on enzyme concentration is shown in Fig. 1 where the intensity of the color reaction is shown to be proportional to enzyme concentration up to approximately 20% pre-D1 cleaved. The assay is also linear with time (Fig. 2) up to approximately 30 min.

FIG. 2. Time course of D1 protease assay. Purified Scenedesmus enzyme was diluted to a concentration of 810 pm or 40 fmol per well (50 μl) with assay buffer. The enzyme was incubated in the microtiter plate wells at room temperature for the indicated times. The ordinate has the same significance as in Fig. 1.

FIG. 3. pH dependence of the purified Scenedesmus enzyme using the standard ELISA assay.

FIG. 3. pH dependence of the purified Scenedesmus enzyme using the standard ELISA assay.

Purification of Scenedesmus D1 Protease

Protease Extraction—Wild type cells of S. obliquus cells were grown on NY medium in a 200-liter fermentor to an A600 of about 6 (2–4 days) at which point the culture was harvested. Cells from the fermentor were suspended in 1 volume per weight of 20 mM HEPES-KOH, 10 mM KCl, 10% glycerol, and pH 7.25 (buffer H), and centrifuged 5 min at 5000 rpm (4200 × g) in a Sorvall GSA-3 rotor. The pelleted cells were resuspended in 1 volume per weight in buffer H and stored at −80 °C until use. For a typical protease isolation, 1 liter of cell suspension was thawed and processed through a microfluidizer (model 110Y; Microfluidics Corp., Newton, MA) by using four passes (18,000–25,000 p.s.i.) with cooling in a wet ice bath between passes. The homogenate was centrifuged at 10 min at 16,000 × g in a Sorvall GSA rotor to remove cell debris. The pellet was washed with buffer H to resuspend sedimented thylakoids and was added to the supernatant. The combined slurry was homogenized by stirring 30 min or by using a Teflon/glass homogenizer. Thylakoids were collected by centrifuging 2 h (or overnight) in Beckman 45Ti rotors at 235,000 × g. The pelleted thylakoids were resuspended in buffer H by using a Teflon/glass homogenizer and brought to a concentration of 2 mg of chlorophyll/ml and 0.5% by volume Triton X-100 from a 20% aqueous stock. After stirring for 30 min, the thylakoids were centrifuged for 2 h at 235,000 × g in a 45 Ti rotor. The Triton X-100 supernatant was collected and stored on ice. The thylakoid pellet was resuspended in buffer H and brought to 2 mg/ml chlorophyll and 0.5% Triton X-100. After stirring 30 min it was centrifuged 2 h in a 45 Ti rotor at 45,000 rpm (235,000 × g).

Hydroxyapatite Column—The second Triton X-100 supernatant was combined with the first and loaded onto a 5 × 35-cm column of hydroxylapatite (Fast flow, Calbiochem) that had been previously equilibrated with 10 mM K2HPO4/KH2PO4, pH 7.0, 10% glycerol. After loading (10 ml/min), the column was washed with 500 ml of equilibration buffer, and 250-ml fractions were collected. This was followed by 1 liter of 100 mM K2HPO4/KH2PO4, pH 7.0, and 10% glycerol, and 50-ml fractions were collected and assayed undiluted using the microtiter plate assay. All of the above steps, following cell breakage, were performed at 4 °C.

The active fractions (200–300 ml) were concentrated over an Amicon YM-10 ultrafilter to about 50 ml and then diluted and recentrifuged twice in 20 mM HEPES-KOH, pH 7.25, 20% glycerol to drop the phosphate concentration about 10-fold. The sample was brought to a final volume of 21.75 ml to which there was added 6.25 ml of saturated-
neutralized ammonium sulfate to give a final concentration of 1 M (NH₄)₂SO₄. The suspension was left on ice for 0.5–1 h and then spun at 10,000 × g to remove precipitate. The supernatant was then filtered by using a 0.45-μm Acrodisc membrane (Gelman Sciences).

Hydrophobic Interaction Column—The hydrophobic interaction column (TSK-Gel Phenyl-5PW, 20-mm inner diameter × 15 cm, Tosoh Haas) was preceded by a guard column of the same material (20-mm inner diameter × 2 cm). The column was first washed at 3 ml/min for 20–30 min with buffer B (20 mM HEPES-KOH, pH 7.25, and 20% glycerol) and then equilibrated at 10 °C with 60% buffer A (20 mM HEPES-KOH, pH 7.25, and 20% glycerol plus 2 M (NH₄)₂SO₄, 40% buffer B) at the same flow rate for 30–40 min. The buffers had been previously filtered through 0.45-μm membranes. The sample was loaded onto the hydrophobic interaction column at 3 ml/min by using a Pharmacia Superloop. The buffer mixture was maintained at 60% A:40% B for 25 min, sufficient to empty the Superloop and to wash out unbound protein (Fig. 4). A linear gradient was then applied at 3 ml/min which went from 60% A to 80% B over the next 90 min. The run was completed by ramping up to 100% B over the following 20 min and maintained at that level for another 15 min. Three-mL fractions were collected. The eluant was assayed by diluting aliquots of the column fractions 1:4 to 1:10 with buffer B (assay buffer). The peak of enzyme activity appeared at a point where 67% buffer B was entering the column (0.67 M (NH₄)₂SO₄, 85 min into the run). After each use the hydrophobic interaction column was equilibrated with water, given four 1-ml pulses with 0.2 M NaOH, and then reequilibrated with water.

Active fractions from the hydrophobic interaction column were pooled and concentrated by using a Centriprep 10 (Amicon) to ≤3 ml and passed through an Econo-Pac10 DG desalting column (Bio-Rad) previously equilibrated with buffer B and used according to the manufacturer’s instructions.

MonoQ Column—The approximate 4-ml sample was then loaded onto an HR10/10 MonoQ column (Pharmacia Biotech Inc.) at a flow rate of 1 ml/min, previously equilibrated with buffer B. The column was subsequently washed for 10 min with 100% buffer B followed by a linear gradient from 100% buffer B to 50% buffer C (buffer B plus 0.5 M NaCl) over a period of 100 min and maintained at 100% buffer C for 10 min, all at a flow rate of 1 ml/min (Fig. 5). 2-ml fractions were collected. Aliquots of each fraction were diluted 1:4 with buffer B and assayed. The peak of activity typically appeared at a position in the gradient where 28% buffer C (0.14 M NaCl) was entering the column (38 min into the run).

Isoelectric Focusing—Preparative isoelectric focusing was carried out in a Bio-Rad Rotofor cell using 45 ml of 20% glycerol, 0.1% CHAPS, 1% Servalyte 4–6, and 0.25% Servalyte 3–10. The cell was prewet for 1 h at 12 watts to establish the pH gradient and following the addition of 4 ml of sample to the well subsequently run for 3–4 h at 5 °C at 12 watts. Peak protease activity corresponded to fractions with an isoelectric point of 5.0 ± 0.2.

Gel Filtration Column—The active Rotofor fractions were concentrated to about 50 μl by using a Centricon 10 (Amicon) and injected onto a TSK-Gel G4000SWXL column (8-μm particle size, 7.8 mm inner diameter × 60 cm) previously equilibrated with buffer D (buffer B plus 100 mM NaCl). The column was run at 0.25 ml/min, and 0.5-ml fractions were collected (Fig. 6). Aliquots of each fraction were diluted 1:4 or 1:6 with buffer B and assayed with peak activity eluting at 92 min. A TSK G3000SW column (7.5-mm inner diameter × 60 cm) was also used on occasion and run under the same conditions. The peak of activity in this case appeared at 66 min into the run. The active fractions were pooled and concentrated by using a Centricon 10 (Amicon) to about 50 μl and stored at −80 °C.

All of the above column steps were performed at 10 °C.

Preparation of Purified Enzyme for Amino Acid Sequencing

SDS-PAGE and blotting were carried out according to Ref. 32. Solubilization buffer (0.2 M sucrose, 6% SDS, 125 mM Tris, 4 mM EDTA, 0.04% bromphenol blue, and 2% β-mercaptoethanol (v/v) adjusted to pH 8.9) was added to an equal volume of concentrated D1 Ctp-protease and loaded onto the sample well of the polyacrylamide gel. SDS-PAGE with 12% polyacrylamide gels were run at room temperature according to Laemmli (42) with the upper buffer chamber containing 0.2% SDS and 0.1% thiglycolate. Gels were then soaked for 25 min in transfer buffer composed of 10 × Tris buffer (250 mM Tris, 1.92 mM glycine) + 400 ml of MeOH diluted to 4 liters with Milli-Q water. Bio-Rad Trans-Blot polyacrylamide diffusional membrane (0.2 μm) was soaked 10 s in methanol and then 20 min in transfer buffer. Blotting was carried out in a Bio-Rad liquid Trans-Blot Cell using the above indicated transfer buffer and according to the manufacturer’s instructions at 240 mA for 3 h at room temperature. The blot was washed three times for 5 min each with Milli-Q water and then stained for 1 min with 0.1% amido black in 10% acetic acid in Milli-Q water. The blot was destained for 1 min with 5% aqueous acetic acid and washed thoroughly with Milli-Q water. The blot was air-dried and sent for sequencing to the Wistar Protein Microchemistry Laboratory (Philadelphia, PA). Tryptic digest of the protein on the blot and subsequent HPLC purification of the tryptic fragments were performed as described in Ref. 32. Edman sequencing was conducted at both the N terminus and on selected HPLC-purified tryptic fragments. In the latter case the MALDI (matrix-assisted laser desorption ionization) mass spectra were also obtained on the sequenced fragments as a confirmation of the sequence assignments. The amino acid sequences obtained are shown in Fig. 8.

Isolation of Nucleic Acid

Total RNA was extracted from Scenedesmus by the following procedure. 35 g of frozen cell paste was ground with 80 ml of buffer G (8 M guanidine HCl, 20 mM EDTA, 20 mM MES, pH 7.0, and 50 mM β-mercaptoethanol) in a PowerGen 125 tissue homogenizer (Fisher) for 60 s on set high. The homogenate was extracted with 150 ml of phenol/chloroform/isooamyl alcohol (25:24:1) and then spun in a Servall GSA rotor for 45 min at 8000 rpm (10,400 × g) at 25 °C. The supernatant was recovered and the RNA precipitated with 0.2 volumes of 1 M acetic acid and 0.7 volumes of ethanol while at −20 °C, overnight. The RNA was
Pelleted by centrifuging in a Sorvall GSA rotor at 16,300 × g for 15 min at 4 °C. The pellet was washed twice with 10 ml of 3 M sodium acetate, pH 5.2, with a final rinse of 15 ml of 70% ethanol. The pellet was resuspended in 1 ml of RNase-free water and stored at −70 °C until use. Poly(A) containing messenger RNA was recovered from undiluted total RNA using the Poly(A)tract system from Promega, according to the manufacturer's instructions. The integrity of the RNA was confirmed by electrophoresis in 1% Tris acetate agarose gel. Total chromosomal DNA was recovered from Scenedesmus by the following procedure. About 500 mg of cells were harvested from the surface of agar plates and resuspended in 500 μl of TSE (5 mM Tris-HCl, pH 8.5, 50 mM NaCl, and 5 mM EDTA). The resuspended cells were frozen dry in liquid nitrogen and then ground while frozen by using a mortar and pestle. Ground cells were suspended in 1.4 ml of 0.1 volume of 3 M sodium acetate with 2 ml of chloroform and spun as above. Nucleic acids were precipitated in 1 ml of TE. Typical concentrations were 400 μg/ml of TSE, 40 μg/ml of proteinase K (2.5 mg/ml), 100 μl of 20% SDS, and 100 μl of 20% Sarkosyl. The mixture was incubated at 65 °C for 2 h, then extracted with 2 ml of buffer-saturated phenol, and centrifuged for 15 min at 9400 × g in a Sorvall HS-4 rotor. The supernatant was extracted with 2 ml of chloroform and spun as above. Nucleic acids were precipitated from the extracted supernatant with 0.1 volume of 3 M sodium acetate and an equal volume of isopropyl alcohol. The precipitate was dried in a Speedvac (Savant) and resuspended in 900 μl of TE (5 mM Tris-HCl, pH 7.5, 1 mM EDTA) in microcentrifuge tubes. RNA was pelleted by spinning at 9400 × g for 20 min in the HS-4 rotor and then dried at 70 °C until use. The DNA was resuspended by adding 1.8 ml of isopropyl alcohol to the 900 μl of TSE (5 mM Tris-HCl, pH 8.5, 50 mM NaCl, and 5 mM EDTA). The resuspended cells were frozen dry in liquid nitrogen and then ground while frozen by using a mortar and pestle. Ground cells were suspended in 1.4 ml of 0.1 volume of 3 M sodium acetate with 2 ml of chloroform and spun as above. Nucleic acids were precipitated from the extracted supernatant with 0.1 volume of 3 M sodium acetate and an equal volume of isopropyl alcohol. The precipitate was dried in a Speedvac (Savant) and resuspended in 900 μl of TE (5 mM Tris-HCl, pH 7.5, 1 mM EDTA) in microcentrifuge tubes. RNA was digested with 20 units of RNase-it (Stratagene). Starch was removed by adding 300 μl of 7.8 M ammonium acetate and centrifuging 30 min at 12,000 × g. The DNA was precipitated by adding 1.8 ml of isopropyl alcohol and centrifuging 30 min at 12,000 × g. The DNA was resuspended in 1 ml of TE. Typical concentrations were 400 μg/ml.

Cloning and Sequencing

Oligo(dT)-primed cDNA was prepared from Scenedesmus poly(A) mRNA by using a SuperScript preamplification system from Life Technologies, Inc. This cDNA was used as template for a polymerase chain reaction primed with the appropriate oligonucleotides. The amplification employed a “touchdown” cycle sequence (33) with the annealing temperature decreased in increments of 0.5 °C every cycle, from 60 to 50 °C, followed by 15 cycles at 47 °C. The reaction product was electrophoresed in a Tris acetate low melting point agarose gel and excised from the gel. The gel was melted at 70 °C and a 10-μl aliquot was used as template in an identical repeat amplification reaction, except that the amplification was followed by a 30-min incubation at 72 °C to enhance the 3′ addition of single deoxyadenosines by Taq polymerase. This second amplification reaction produced a concentrated band of DNA that was excised from a low melt gel and ligated directly into a pGEM-T vector (Promega, Madison, WI), according to the supplier's instructions. E. coli XL1-Blue (Stratagene) cells were transformed with the ligated products and plated on LB medium containing ampicillin.

Clones containing inserts of the expected size were sequenced on an ABI 377 automated sequencer. The nucleotide sequences of the cloned fragments were used to design exact gene-specific primers. The primers were then used for the rapid amplification of cDNA ends (RACE) in conjunction with a 5′ RACE kit and a 3′ RACE kit from Life Technologies, Inc. and used according to the manufacturer’s instructions, except for the substitution of expand high fidelity polymerase (Boehringer Mannheim) in place of Taq polymerase. Reaction products of each were run on low melt agarose gels. Both bands were excised. As the expand high fidelity polymerase generates blunt-ended fragments, the following step was included to add single deoxyadenosine tails. 20 μl aliquots of each band were incubated at 72 °C for 30 min in the presence of 10 mM Tris-HCl, 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.33 mM dATP, and 0.5 units of Taq polymerase. The tailored fragments were then ligated into a pGEM-T vector. E. coli cells were transformed with the ligation products and selected as above.

A minimum of four independent clones were sequenced (both strands) for each of the 5′ RACE and 3′ RACE products of the LF-1 and wild-type cDNAs. Clones from the 5′ end overlapped clones from the 3′ end by 440 bases. Sequencing was performed as above, and the data managed using the Lasergene SeqMan program (DNASTAR Inc.).

HPLC of Synthetic Peptides

HPLC was run at 45 °C on a Vydac C-18 column (218TP54, Sum, 4.6 mm inner diameter × 25 cm) plus a C-18 guard column with a gradient run from 100% 0.1% trifluoroacetic acid in H2O to 30% 0.1% trifluoroacetic acid in H2O, 70% 0.1% trifluoroacetic acid in acetonitrile as described in Fig. 10. RESULTS

Protease Purification—The D1 protease is associated with the thylakoid fraction as described under the “Experimental Procedures.” The liberation of the enzyme upon treatment of the thylakoids with Triton X-100 is consistent with its localization within the thylakoid lumen where it has access to its substrate, the C terminus of the D1 polypeptide. Hydroxyapatite chromatography (Table I) is the first major purification step following protease extraction and is able to accommodate large amounts of protein. It removes carotenoid and chlorophyll pigments as well as about 80% total protein. The fractions containing D1 protease elute very near a red cytochrome band upon washing with 100 mM K2HPO4/KH2PO4, pH 7.0, and 10% glycerol.

The protease is bound to the hydrophobic interaction column at 1.2 M (NH4)2SO4 and then eluted using a decreasing concentration (NH4)2SO4 salt gradient. The elution profile, shown in Fig. 4, shows the protease eluting at 85 min as 0.67 M (NH4)2SO4 enters the column.

MonoQ anion exchange chromatography (Fig. 5) provides the largest increase in specific activity (Table I), with the protease eluting at a point in the elution profile where 0.14 mM NaCl is entering the column and where the background protein concentration is low.

Preparative isoelectric focusing shows the enzyme migrating with an isoelectric point of 5.0 ± 0.1. This value agrees fairly well with a calculated (43) isoelectric point of 5.34, based on the translated sequence of the mature D1 protease (see below).

Table I

| Step                          | Activity rel. units | Protein rel. units |
|-------------------------------|--------------------|--------------------|
| Membrane extract              | ND                 | ND                 |
| Hydroxylapatite pooled fractions | 168,000            | 210 mg             |
| Hydrophobic Interaction pooled fractions | 49,000            | 20 mg              |
| MonoQ pooled fractions         | 52,000             | 1.1 mg             |
| Isoelectric focusing pooled fractions | 15,600            | ND                 |
| Gel filtration pooled fractions | 9,100              | 5–10 μg            |

a rel. units, relative units.

ND, not determined.
Gel filtration chromatography (Fig. 6 and Table I) shows peak activity appearing at about 92 min from the start of the run. A comparison with the elution times of a collection of standard proteins, run under the same conditions, gives an estimated molecular mass ranging from 36 to 42 kDa. This agrees favorably with a molecular mass of 42.6 kDa based on SDS-PAGE (Fig. 7) and a calculated (43) mass of 40,578 Da, based on the translated sequence of mature Ctp-protease (see below). These results indicate clearly that the protease is monomeric.

The yields of the various steps and their respective specific activities are listed in Table I.

Cloning and Sequencing—The HPLC-purified tryptic peptides of the purified D1 protease were sequenced as under “Experimental Procedures” and were ordered by matching them to translated D1 protease genes from wheat and spinach (27). Degenerate oligonucleotide primers “A” and “B” (Fig. 8) based on these sequences were designed to prime regions of minimum degeneracy in the cDNA at sites corresponding to the peptide fragments shown in Fig. 8. The spacing of the primers predicted a reverse transcriptase-PCR product of about 770 bp. Oligo(dT)-primed cDNA was prepared from Scenedesmus poly(A) mRNA as described under “Experimental Procedures.” This cDNA was used as template for PCR using touchdown cycle sequence and using as primers the oligonucleotides A and B (Fig. 8). Electrophoresis of the reaction product produced a faint band of about 770 bp. This band was then used as template for a second round of a repeat amplification reaction as described above. The 770-bp product was 3’-extended with a single deoxyadenosine, ligated into a pGem-T vector (Promega, Madison, WI) and cloned and sequenced. The nucleotide sequence was then used to design gene-specific primers for RACE as described above. The 5’ RACE procedure produced an amplified DNA product that was 1 kilobase pairs in length. The 3’ RACE procedure yielded a product that was 1.4 kilobase pairs in length. These were purified, 3’-tailed, and ligated into pGEM-T vectors.

Multiple independent clones were sequenced as a precaution against mutations introduced during the course of DNA amplification. We reasoned that a true mutation, present in LF-1, would appear in all LF-1 clones and in none of the wild type clones, whereas amplification mutants would appear randomly. A mutation rate of 1 error per 2000 bases sequenced was still observed despite the use of a “high fidelity” polymerase.

Once the nucleotide sequence difference between the D1 protease genes of LF-1 and wild type was determined by sequencing the RACE clones, the mutation was verified in the Scenedesmus genome by PCR amplification and sequencing of the homologous region. 2 mg each of chromosomal DNA from the wild type, LF-1, and the suppressor strain, LF-1-RVT-1, were used as template. A pair of gene-specific primers 507 bp apart, according to the cDNA sequence, was used to prime the reaction, which used the touchdown method described above. The reaction products were run on a standard agarose gel, and the predominant band, running at 1200 bp, was excised. The fact that the genomic PCR product was 700 bp larger than expected indicates the presence of one or more introns. The DNA was recovered using GeneClean (Bio 101, La Jolla, Ca) and sequenced as above using the same primers used for PCR.

Comparison of Amino Acid and Nucleotide Sequencing—The 5’ end of the mRNA from wild type was determined by sequencing the RACE clones. A total of 21 was sequenced, and the three longest showed a consistent start point. The remaining clones were shorter and had random start points, presumably the
result of incomplete cDNA synthesis. The first in-frame methionine was designated the start codon.

Nucleotide sequencing of the wild type Scenedesmus cDNA predicts a protein of 464 amino acids. Of these the first 77 comprise a leader sequence as N-terminal amino acid sequencing of the mature protein indicated the first residue to be valine 78 (Fig. 8). A thylakoid transit sequence can be discerned with basic residues (Lys-39 and Arg-40), 38 and 37 residues, respectively, upstream from the mature N terminus, followed by a region of hydrophobic residues and terminating in a typical AXA lumenal processing site (34, 35). Upstream of the transit sequence is a region, enriched in serines and threonines, that is characteristic of a chloroplast signal sequence (36).

Nucleotide sequencing of the cDNA isolated from the LF-1 mutant revealed a single base deletion in the glycine 387 codon that shifts the reading frame, causing a translation stop after 2 amino acids (Fig. 9).

The point mutation was confirmed in genomic DNA by using PCR to directly sequence a 500-bp region of the LF-1 mutant and wild type genomes. In addition, the same region was sequenced from the LF-1 suppressor strain (LF-1-RVT-1, Ref. 31), and wild type genomes. In addition, the same region was sequenced from an LF-1 mutant strain (LF-1-RVT-1, Ref. 31), which was shown to have a single base pair insertion 7 bases downstream of the LF-1 deletion. This insertion restores the proper reading frame and gives rise to three amino acid replacements with respect to wild type (Fig. 9).

Specificity of Proteolytic Cleavage—The correspondence in vivo between the ability to express the full-length gene product and D1 processing activity demonstrates a clear cut role of the gene product in in vivo D1 processing. That this gene product is indeed the D1 C-terminal processing protease and not a cofactor in proteolytic processing is indicated by the demonstration of in vitro processing of D1. The use of an antibody in the ELISA assay that is directed against the mature C terminus would argue that processing occurs as expected at Ala-344. The specificity of proteolytic cleavage by the purified enzyme was further examined using a synthetic 19-mer peptide corresponding to the last 19 residues of the D1 polypeptide of Scenedesmus (NAHNFPFLDLASVEAPSVNA). Shown in Fig. 10 is the HPLC chromatographic elution profile run as described under the “Experimental Procedures” of the 19-mer standard (NAHNFPFLDLASVEAPSVNA) as well as the 9-mer (SVEAPSVNA) and 10-mer (NAHNFPFLDLA) standards that are expected to result from D1 proteolytic cleavage of the 19-mer. Also shown is the 19-mer alone incubated for 4.25 h at room temperature with Scenedesmus D1 protease purified according to the complete procedure described above. The products are observed to migrate with the same mobility as the 9-mer and 10-mer standards indicating that cleavage by the enzyme occurs between Ala-10 and Ser-11 of the 19-mer, corresponding to Ala-344 and Ser-345, respectively, of the D1 polypeptide as observed in vivo.

Properties of the Enzyme—The molecular mass, isoelectric point, and extinction coefficient of the mature Scenedesmus enzyme (387 residues) were calculated using the Peptidesort program of the Wisconsin Sequence Analysis Package (43).

These 40,577 Da and 5.34 and 21,740 m⁻¹ cm⁻¹ (280 nm), respectively. These compare favorably with a measured molecular mass of 40,635 ± 248 Da as determined by MALDI (accurate to 0.1%) and an experimental isoelectric point of 5.0 ± 0.2 as determined by isoelectric focusing. The measured mass in-

FIG. 9. The translated nucleotide sequence for a 60-base region of the D1 protease gene from three strains of Scenedesmus. The circled lysine is a likely component of the active site of the protease (41) and Tang et al., in preparation. The parentheses show the site of the single G deletion in the LF-1 and suppressor strains. An arrow identifies the site of a single T insertion in the LF-1 suppressor strain, LF-1-RVT-1.

FIG. 10. A, reverse phase (C-18) HPLC separation of an equimolar mixture of standard synthetic peptides that mimic the last 19 amino acid residues of Scenedesmus polypeptide D1 and each of the 9- and 10-mer cleavage products that result from proteolytic processing at Ala-344. In order of elution: SVEAPSVNA, NAHNFPFLDLA, and NAHNFPFLDLASVEAPSVNA. Injected were 40 μl of a solution containing 0.5 mM each of the three polypeptides in 25 mM HEPES-KOH, pH 7.5. B, reverse phase (C-18) HPLC separation following 255 min room temperature incubation of the 19-mer (0.5 mM in 40 μl of 25 mM HEPES-KOH, pH 7.5) with purified Scenedesmus D1 protease (approximately 0.2 μg of enzyme used). The time table for the gradient elution at 45 °C is as follows: 0–0.5 min, 100% 0.1% trifluoroacetic acid in H₂O; 0.5–5 min, linear gradient from 100% 0.1% trifluoroacetic acid in H₂O to 90% 0.1% trifluoroacetic acid in H₂O, 10% 0.1% trifluoroacetic acid in acetonitrile; 5–30 min, linear gradient from 90% 0.1% trifluoroacetic acid in H₂O, 10% 0.1% trifluoroacetic acid in H₂O, 10% 0.1% trifluoroacetic acid in acetonitrile to 67%, 0.1% trifluoroacetic acid in acetonitrile; 30–40 min, linear gradient from 67%, 0.1% trifluoroacetic acid in H₂O, 33%, 0.1% trifluoroacetic acid in acetonitrile to 30%, 0.1% trifluoroacetic acid in H₂O, 70%, 0.1% trifluoroacetic acid in acetonitrile. For further conditions see “Experimental Procedures.”
indicates that the enzyme is processed only at its N terminus.

Assuming the initial extraction of enzyme to be quantitative, the amounts of enzyme recovered in the purification (Table I) imply a ratio of enzyme to PSI reaction center to be on the order of 1/100–1/1000. The t1/2 of in vivo processing of the D1 polypeptide in Scenedesmus by pulse-chase labeling was estimated to be 1–2 min (13). A comparison of the two numbers gives an in vivo turnover rate for the enzyme of between 1 and 10 s⁻¹.

Protease Inhibitors—The purified D1 protease was tested against a collection of classical protease inhibitors at concentrations that were 10 times the usual upper limits needed for inhibition in their respective classes. These included antipain (500 μg/ml, serine and cysteine proteases), 4-amidinophenylmethylene sulfon fluoride (400 μg/ml, serine protease), aprotinin (500 μg/ml, serine protease), chymostatin (1 mg/ml, chymotrypsin), 3,4-dichloroisocoumarin (5 mM, serine protease), diisopropyl fluorophosphate (5 mM, serine protease), and metalloprotease. Nearly 50% protein was depleted from the LF-1 mutant was unable to do so. We have shown that while a cell extract from the wild type was able to render thylakoid membranes from the LF-1 mutant provides an explanation for the loss in D1 processing activity. A deletion in LF-1 of a base pair (within codon 387) results in the expression of a truncated translation product. (Figs. 8 and 9) produces a frameshift and a premature stop, resulting in the expression of a truncated translation product.

DISCUSSION

The LF-1 mutant strain of Scenedesmus was isolated by Dr. Norman Bishop following x-ray mutagenesis (20, 30). This mutant has been extensively studied by a number of groups and shown to be unable to process the precursor form of the D1 protoporphyrin (13, 29), and the specific protease to the thylakoid lumen where the C terminus of the enzyme is removed at the carboxyl side of the characteristic processing site of lumenally directed transit peptides. The consensus is shown for amino acids that are identical in the first three of those that are underlined identical in all four.

In the photoautotrophic LF-1-RTV-1 suppressor strain that is once again able to process pre-D1, the insertion of a base 7 nucleotides downstream of the deletion restores in-frame translation, thus clearly linking pre-D1 processing activity to the gene present in vivo.

The correlation of in-frame translation of the present gene with the ability to process pre-D1 in vivo, the localization of the protease to the thylakoid lumen where the C terminus of pre-D1 is located (13, 29), and the specific in vitro cleavage by the enzyme at the pre-D1 processing site in PSII core complexes and in the pre-D1 mimic synthetic peptide all contribute to the conclusion that the enzyme described here is indeed the D1 Ctp-protease. That the mutation in LF-1 completely inactivates the enzyme at the pre-D1 processing site in PSII core complexes and in the pre-D1 mimic synthetic peptide all contribute to the conclusion that the enzyme described here is indeed the D1 Ctp-protease.
protease (40,635 ± 248 Da) and the mass calculated from the translated gene sequence (40,577 Da) means that processing of the enzyme only occurs at the AXA N-terminal processing site. The presence of the protease in the thylakoid lumen (see also Refs. 27 and 28) is consistent with the localization in the lumen of its substrate, the C terminus of pre-D1 (29). The lumenal localization of the enzyme also implies that pre-polyepitope D1 is processed only after it is inserted into the membrane and folded such that the unprocessed C terminus extends into the lumen.

Many of the inhibitors that have proven effective against individual classes of proteases show little propensity for inhibition in the case of the D1 protease isolated from either spinach (37) or Scenedesmus (25, this work), contributing to the idea that this enzyme belongs to a new class of protease. Pakrasi and collaborators (23) have pointed out the homology that exists between Synechocystis ctpA and Escherichia coli tail-specific protease ( tsp 38), also called prc (39), responsible for C-terminal processing of penicillin-binding protein. Like D1 protease, Tsp protease is resistant to the common serine protease inhibitors, phenylmethylsulfonyl fluoride and diisopropyl fluorophosphate (41). A comparison of the sequences of D1 proteases isolated from Scenedesmus as well as from cyanobacteria and higher plants (Fig. 11, see also (27, 40)), a partial list of which is included here, reveals very few conserved residues. A clustering of conserved residues from 299 to 336 (PLVVLV to RARRRG) and Ser-372 in Fig. 8) and a lysine (homologous to D1 protease Ser-310 in Fig. 11) assuring the expression of inactive protease.

Proteins of the photosynthetic reaction center of Synechocystis sp. PCC 6803 (Scprc) and PsII from spinach (Spinacia oleracea) contain an N-terminal processing site (Fig. 1, A and B, respectively). The presence of the protease in the thylakoid lumen (see also 21, 22, 27) as well as from cyanobacteria and higher plants (Fig. 11, see also 27, 40)) is consistent with the localization in the lumen. The presence of these residues is particularly significant because the stop codon that appears in the mutated D1 protease gene of LF-1 lies within the active site region (codon 389 from start, Fig. 8; codon 327 from mature N terminus, Fig. 11) assuring the expression of inactive protease.

Given the elevated pHK of lysine in solution (10.8) and the low pH (4–5) of the thylakoid lumen, there would need to be a substantial lowering of its pHK for lysine to act as a proton acceptor in the active site. Such a pHK lowering can come either from the enzyme itself or from the enzyme-substrate complex. Consistent with the former is a concentration of residues with cationic side chains in the region of the putative active site (e.g. Lys-320, Lys-323, Arg-324, and Arg-331, numbering from the mature N terminus, Fig. 11). Nonetheless, the pH optimum of the isolated enzyme in the ELISA assay (Fig. 3, pH 6.3) is similar to what has been observed by others and higher than what is predicted for the pH of the thylakoid lumen. It is likely, therefore, that there are other factors in vivo that shift the pH dependence of the enzyme so that it is active in the physiological pH range.

Acknowledgments—We are grateful to Dr. Norman Bishop for providing the Scenedesmus wild type, LF-1, and LF-1-RVT-1 strains. We also thank Dr. Xiao-Song Tang for helpful discussion, Barbara Larsen for MALDI mass spectrometry, Thomas Miller for N-terminal sequence data, Rand Schwartz for excellent technical assistance, Silvia Stack for nucleotide sequencing data, Winona Wagner for help with large scale algal growth, and James Metz for introducing us to Scenedesmus.
The D1 C-terminal Processing Protease of Photosystem II from \textit{Scenedesmus obliquus}: PROTEIN PURIFICATION AND GENE CHARACTERIZATION IN WILD TYPE AND PROCESSING MUTANTS

Jeffrey T. Trost, Dexter A. Chisholm, Douglas B. Jordan and Bruce A. Diner

\textit{J. Biol. Chem.} 1997, 272:20348-20356.
doi: 10.1074/jbc.272.33.20348

Access the most updated version of this article at http://www.jbc.org/content/272/33/20348

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 41 references, 12 of which can be accessed free at http://www.jbc.org/content/272/33/20348.full.html#ref-list-1