Interaction of the 33-kDa Extrinsic Protein with Photosystem II: Identification of Domains on the 33-kDa Protein That Are Shielded from NHS-Biotinylation by Photosystem II[†]

Laurie K. Frankel and Terry M. Bricker*

Department of Plant Biology, Louisiana State University, Baton Rouge, Louisiana 70803

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ABSTRACT: The structural association of the spinach 33-kDa extrinsic protein of photosystem II with the membrane-bound components of the photosystem was investigated by labeling the 33-kDa extrinsic protein with the amino group-specific reagent *N*-hydroxysuccinimidobiotin both on NaCl-washed photosystem II membranes and free in solution. After quenching of the labeling reagent and isolation of the biotinylated molecules, the biotinylation sites were identified by *Staphylococcus* V8 protease digestion and analysis of the resultant peptide fragment mixture by matrix-assisted laser desorption/ionization mass spectrometry. When the 33-kDa extrinsic protein was modified on PS II membranes, three domains were biotinylated: ^{14}K, ^{41}K-^{76}K, and ^{190}K-^{236}K. When the 33-kDa extrinsic protein was modified in solution, four additional domains were biotinylated: ^{1}E-^{4}K, ^{20}K, ^{101}K-^{105}K, and ^{159}K-^{186}K. These additional modified domains reside in portions of the 33-kDa protein that are not accessible to the bulk solvent when the protein is associated with PS II and may define regions of interaction with the photosystem.

Photosystem II (PS II)[1] is a multi-subunit thylakoid membrane protein complex that catalyzes the light-driven oxidation of water to molecular oxygen and the reduction of plastoquinone to plastoquinol. This complex consists of both intrinsic and extrinsic protein subunits. Intrinsic polypeptides with apparent molecular masses of 49 (CP 47), 45 (CP 43), 34 (D1), 32 (D2), 9 and 4.5 (α- and β-subunits of cytochrome b_{559}), and 4 kDa (*psb*I gene product) appear to form the minimum complex capable of photosynthetic oxygen evolution (Burnap & Sherman, 1991; Bricker, 1992).

In higher plants, three additional extrinsic protein components with apparent molecular masses of 33, 24, and 17 kDa are associated with the oxygen-evolving complex. Removal of the 24- and 17-kDa components by salt-washing (usually 1.0 M NaCl) dramatically lowers the oxygen-evolving capacity of PS II vesicles (Akerlund et al., 1982) and PS II membranes (Kuwabara & Murata, 1982). Much of the lost activity can be recovered by reconstitution with the 24- and 17-kDa proteins (Akerlund et al., 1982) or by the addition of moderate concentrations of calcium (Ghanotakis et al., 1984) and chloride (Andersson et al., 1984). These proteins are assumed to play a role in the regulation of calcium and chloride concentrations within the PS II complex. In cyanobacteria, no proteins homologous to the 24- and 17-kDa proteins have been identified, although analogous proteins may be present (Shen & Inoue, 1993).

The extrinsic 33-kDa protein is much more tightly associated with the intrinsic PS II proteins than are the 24- and 17-kDa proteins. Removal of this protein requires treatment with high concentrations of alkaline Tris (Yamamoto et al., 1981), $CaCl_2$ (Ono et al., 1983), or NaCl−urea (Miyao & Murata, 1984). Treatment with alkaline Tris also leads to the loss of the manganese cluster associated with the active site of PS II (Kuwabara & Murata, 1982). This was initially interpreted as evidence that the manganese cluster was associated with this extrinsic protein. $CaCl_2$ and NaCl−urea washes, however, efficiently remove the 33-kDa protein without the concomitant loss of the manganese cluster. In the absence of the 33-kDa protein, high concentrations of chloride are required to maintain the integrity of the manganese cluster (Miyao & Murata, 1984). At chloride concentrations below 100 mM, two of the four manganese associated with PS II rapidly become paramagnetically uncoupled and then dissociate from PS II membranes (Mavankal et al., 1986). These studies indicate that the extrinsic 33-kDa protein acts as a manganese-stabilizing protein for PS II. Additionally, this protein appears to lower the calcium requirement for optimal PS II activity (Philbrick et al., 1991; Bricker, 1992). It has been suggested that the 33-kDa component may act as a calcium-binding protein within PS II (Wales et al., 1989; Yocum, 1991). However, no direct evidence supporting this hypothesis has been presented. Recently, we have confirmed and extended previous studies (Ono & Inoue, 1984; Miyao & Murata, 1984; Kuwabara et al., 1985; Miyao et al., 1987) which demonstrated that significant rates of oxygen evolution can occur in the absence of the manganese-stabilizing protein. The presence of this protein is, however, required for the high rates of oxygen evolution observed *in vivo* and in isolated PS II preparations (Burnap & Sherman, 1991; Bricker, 1992).

In cyanobacteria, deletion of the *psb*O gene, which encodes the 33-kDa extrinsic protein, leads to a reduction in steady-

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* Corresponding author phone: (504) 388-8556; e-mail address: BTBRIC@LSUVM.SNCC.LSU.EDU.

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[1] Abbreviations: Chl, chlorophyll; DTSP, dithiobis(succinimidyl propionate); EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; MALDI, matrix-assisted laser desorption/ionization; MES, 2-(*N*-morpholino)ethanesulfonic acid; NHS-biotin, *N*-hydroxysuccinimidobiotin; PAGE, polyacrylamide gel electrophoresis; PS II, photosystem II; PVDF, polyvinylidene fluoride; TCA, trichloroacetic acid; Tris, tris-(hydroxymethyl)aminomethane.

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state oxygen evolution activity (Philbrick et al., 1991; Burnap & Sherman, 1991), a loss of photoautotrophic growth at low calcium concentrations (Philbrick et al., 1991), and an increased susceptibility to photoinhibition and dark photoinactivation (Philbrick et al., 1991; Burnap & Sherman, 1991). Additionally, increased dampening of the oxygen yield during trains of saturating flashes and altered thermoluminescence properties of PS II are observed (Burnap et al., 1992). These results are very similar to observed alterations in the PS II parameters of higher plant PS II membranes that have been depleted of the 33-kDa protein *in vitro* (Miyao et al., 1987; Vass et al., 1987). Interestingly, in *Chlamydomonas*, mutations which lead to a loss of the 33-kDa protein yield a PS II-minus phenotype (Mayfield et al., 1987). These results suggest that the *assembly* of PS II differs dramatically in cyanobacteria and *Chlamydomonas* (and possibly higher plants) while the *functional* attributes of the photosystem appear to be quite similar.

While this protein obviously is an important component of the oxygen-evolving complex, relatively little is known of its structural organization. Studies examining the stoichiometry of this protein indicate that there are either one (Miyao & Murata, 1989; Enami et al., 1991) or two copies (Xu & Bricker, 1992; Betts et al., 1994) present per PS II reaction center. Additionally, the presence of an intramolecular disulfide bridge has been demonstrated (Camm et al., 1987; Tanaka & Wada, 1988). We have examined the secondary structure of the 33-kDa protein in solution by far-UV circular dichroism spectroscopy. Our results suggested that the 33-kDa protein contains a large proportion of β-sheet structure and a relatively small amount of α-helical structure (Xu et al., 1994). Previous studies have demonstrated that the 33-kDa extrinsic protein is associated with CP 47 (Bricker et al., 1988; Frankel & Bricker, 1992; Odom & Bricker, 1992) and perhaps other intrinsic components of PS II (Mei et al., 1989; Enami et al., 1992). The N-terminal domain of the 33-kDa extrinsic protein appears to be important in its interaction with PS II (Eaton-Rye & Murata, 1989; Odom & Bricker, 1992).

In this paper, we have used NHS-biotin to modify the 33-kDa extrinsic protein either associated with PS II on NaCl-washed oxygen-evolving membranes or as a free protein in solution. After modification, the biotinylated molecules were purified and digested with *Staphylococcus* V8 protease, and the resultant peptide mixture was analyzed by MALDI mass spectrometry. When the 33-kDa extrinsic protein was modified on PS II membranes, three domains are biotinylated: ^{14}K, ^{41}K-^{76}K, and ^{190}K-^{236}K. When the 33-kDa extrinsic protein was modified in solution, four additional domains were biotinylated: ^{1}E-^{4}K, ^{20}K, ^{101}K-^{105}K, and ^{159}K-^{186}K. These additional modified domains reside in portions of the 33-kDa protein that are not accessible to the bulk solvent when the protein is associated with PS II and may define regions of interaction with the photosystem.

MATERIALS AND METHODS

Chloroplasts were isolated from market spinach as previously described (Bricker et al., 1985). Chl concentration was measured by the method of Arnon (1949). Oxygen-evolving PS II membranes were prepared by the method of Berthold et al. (1981) with the modifications described by Ghanotakis and Babcock (1983). Typical preparations had a Chl *a*/*b* ratio of 1.9−2.0 and oxygen evolution rates in excess of 450 μmol of O_2 (μg of Chl)$^{-1}$ (h)$^{-1}$. The extrinsic 24- and 17-kDa proteins were removed from the PS II membranes by washing twice for 1 h with 1.0 M NaCl, 300 mM sucrose, 10 mM $MgCl_2$, and 50 mM Mes−NaOH, pH 6.0, at 1.0 mg of Chl/mL followed by two washes with and resuspension in 300 mM sucrose, 10 mM $MgCl_2$, 15 mM NaCl, and 50 mM Tes−NaOH, pH 7.0, at 1.0 mg of Chl/mL. All washes were performed at 0−4 °C. Exposed lysyl residues on the 33-kDa extrinsic protein were labeled with NHS-biotin as described previously (Bricker et al., 1988) except that the NHS-biotin concentration during labeling was 150 μM and the labeling time was 1.5 h. The labeling reaction was stopped by the addition of Tris-HCl, pH 6.8, to a concentration of 50 mM, and residual NHS-biotin was removed by washing the membranes twice with 300 mM sucrose, 10 mM $MgCl_2$, 15 mM NaCl, and 50 mM Mes-NaOH, pH 6.0. The 33-kDa protein was then isolated as described previously (Xu & Bricker, 1992). Labeling of the purified 33-kDa extrinsic protein in solution was performed in a similar manner at a protein concentration of 236 μg/mL in 300 mM sucrose, 10 mM $MgCl_2$, 15 mM NaCl, and 50 mM Tes−NaOH, pH 7.0. The labeling reaction was quenched by the addition of Tris-HCl, pH 6.8, to a concentration of 50 mM, and the residual NHS-biotin was removed by centrifugal ultrafiltration in a Centricell 30 device (Polysciences, Inc.). The purified protein was quantified using an extinction coefficient of 16 mM^{-1} cm^{-1} at 276 nm (Oh-oka et al., 1986; Xu & Bricker, 1992).

After modification, the biotinylated protein solution was brought to 10% TCA, the protein precipitate was collected by centrifugation and then washed twice with cold 100% acetone, dried under vacuum, dissolved in 8 M urea and 400 mM ammonium bicarbonate, and carboxyamidomethylated with iodoacetamide (Stone et al., 1989) prior to purification on a monomeric avidin column (Pierce Chemical Co.). Briefly, the carboxyamidomethylated protein was diluted to 0.5 M urea and passed over a 2.0-mL monomeric avidin column which had been preequilibrated with 50 mM sodium−potassium phosphate, pH 7.4, 150 mM NaCl. After washing extensively with 50 mM sodium−potassium phosphate, pH 7.4, and 150 mM NaCl to remove any unmodified protein, the biotinylated protein was eluted with 5 mM biotin in 50 mM sodium−potassium phosphate, pH 7.4, and 150 mM NaCl. The biotinylated protein was concentrated by centrifugal ultrafiltration, precipitated, washed as described above, and resolubilized in 8 M urea and 400 mM ammonium bicarbonate. The solubilized protein was then diluted to 2 M urea and 100 mM ammonium bicarbonate and digested overnight at 37 °C with *Staphylococcus* V8 protease. The final 33-kDa protein:protease ratio was 25:1.

Analytical PAGE of the biotinylated PS II proteins was performed under conditions described by Delepelaire and Chua (1979) in gradient 12.5−20% acrylamide gels. The resolved proteins were electroblotted onto PVDF membranes (Immobilon-P, Millipore Co.). Panels of the blot were either stained with Coomassie Blue stain or were blocked for 4 h with 5% nonfat dry milk in 10 mM Tris-HCl, pH 7.4, and 150 mM NaCl; washed extensively with 10 mM Tris-HCl, pH 7.4, and 150 mM NaCl; and probed with 1:1000 dilution of an avidin−peroxidase conjugate (Sigma Chemical Co.) in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1% BSA.

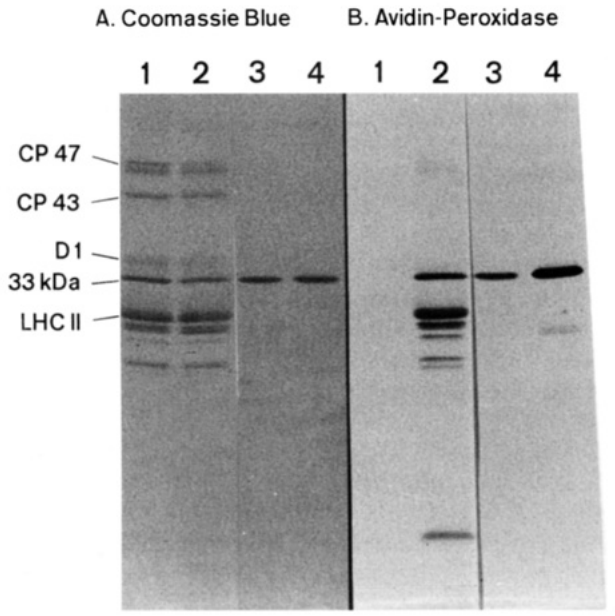

FIGURE 1: NHS-biotinylation patterns of NaCl-washed PS II membranes and purified biotinylated 33-kDa extrinsic protein. Proteins were separated electrophoretically in gradient 12.5−20% acrylamide gels (Delepelaire & Chua, 1981) and were electroblotted onto PVDF membranes and either stained with Coomassie Blue (panel A) or probed with an avidin−peroxidase conjugate and developed with 4-chloro-1-naphthol + H_2O_2 (panel B) to identify biotinylated protein bands as described in Materials and Methods. Lane 1, control NaCl-washed PS II membranes (5 μg of Chl); lane 2, NHS-biotinylated NaCl-washed PS II membranes (5 μg of Chl); lane 3, 33-kDa extrinsic protein that was biotinylated on the NaCl-washed PS II membranes and then purified (2 μg of protein); lane 4, 33-kDa extrinsic protein that was biotinylated in solution (2 μg of protein). Several PS II components are labeled to the left.

Biotinylated bands were visualized by color development using 4-chloro-1-naphthol and H_2O_2.

MALDI mass spectrometry was performed on intact and protease-digested 33-kDa extrinsic protein at the Wistar Protein Microchemistry Laboratory (Philadelphia, PA). The protein samples were diluted to about 1−5 pmol/μL with 0.1% TFA, mixed with the matrix (saturated solutions of cyano-4-hydroxycinnamic acid for samples less than 20−30 000 Da and sinapinic acid for samples more than 20 000 Da), and analyzed on a PerSpective Biosystems Voyager Biospectrometry Workstation. The intact 33-kDa extrinsic protein was analyzed using internal mass calibration (ovalbumin) while the protease-digested protein was analyzed using external mass calibration. MALDI mass spectra were analyzed using the GPMAW program (Lighthouse Data, Denmark).

RESULTS AND DISCUSSION

Figure 1 illustrates the result of a typical NHS-biotinylation experiment. Panel A shows the Coomassie Blue staining of Western blots of non-biotinylated NaCl-washed PS II membranes (lane 1), biotinylated NaCl-washed PS II membranes (lane 2), and purified 33-kDa protein that had been either biotinylated on the NaCl-washed membranes (lane 3) or were free in solution (lane 4). Panel B illustrates the result of probing this Western blot with an avidin−peroxidase conjugate followed by development with 4-chloro-1-naphthol to identify the biotinylated proteins. NaCl-washed PS II membranes exhibit biotinylation of the 33-kDa extrinsic protein, the light-harvesting chlorophyll *a*/*b* protein (LHC II), and several other proteins. The 33-kDa protein that was biotinylated in solution exhibited a greater apparent degree of modification than that which was biotinylated on the NaCl-washed PS II membranes.

Table 1: Assignments for Peptides Produced from a *Staphylococcus* V8 Protease Digest of the 33-kDa Extrinsic Protein Which Was NHS-Biotinylated on NaCl-Washed PS II Membranes[a]

observed mass	predicted mass	Δmass	peptide assignment
5597.1	5597.3	−0.2	^{88}I-^{139}E
5023.2	5022.4	0.8	^{140}S-^{187}E
4767.1	4764.5	2.6	^{19}V-^{62}E + 2 acetamide
4667.4	4673.3	−5.9	^{19}V-^{62}E + Na^+
4524.2	4523.8	0.4	^{140}S-^{183}E
4016.7	4018.5	1.8	^{210}T-^{246}E
3746.1	3745.3	0.8	^{105}K-^{139}E
3551.7	3549.0	2.7	^{33}G-^{62}E + acetamide + biotin
3366.6	3361.0	5.6	^{33}G-^{62}E + acetamide + Na^+
3324.3	3321.9	2.4	^{33}G-^{62}E + acetamide
3244.3	3238.4	5.9	^{213}V-^{238}E + 2 biotin
3072.2	3072.4	−0.2	^{63}G-^{89}E
3058.8	3057.2	1.6	^{63}G-^{87}E + biotin
3011.9	3011.3	0.6	^{213}V-^{238}E + biotin
2831.4	2830.2	1.2	^{63}G-^{87}E
2785.1	2784.2	0.9	^{213}V-^{238}E
2511.8	2512.6	−0.8	^{188}N-^{209}E + biotin + Na^+
2263.8	2262.5	1.3	^{188}N-^{209}E
2031.3	2030.4	0.9	^{122}R-^{139}E
1871.5	1870.0	1.5	^{88}I-^{104}E
1736.0	1732.9	3.1	^{105}K-^{121}E
1666.0	1666.8	−0.8	^{90}G-^{104}E + K^+
1249.1	1247.3	1.8	^{11}I-^{18}E + biotin + K^+
1094.7	1093.2	1.5	^{239}G-^{247}Q

[a] The observed ions were the $[M + H]^+$ species, unless otherwise noted.

In these experiments, the concentration of NHS-biotin was adjusted to minimize the number of biotin residues introduced into each individual 33-kDa protein molecule. This minimizes possible deleterious effects that could arise from the simultaneous replacement of a large number of positively charged lysyl residues with neutral biotin moieties within a given protein molecule. The fewer the number of biotins introduced into the protein, the lower the probability of artifactual protein unfolding precipitated by the introduction of the biotin modification. MALDI mass spectrometry of the intact modified proteins indicated that one biotin residue was introduced into the 33-kDa protein which was modified on the NaCl-washed PS II membranes while one to three biotin residues were introduced into the protein which was modified in solution (data not shown). This was consistent with the observed apparent biotinylation levels shown in Figure 1B. One consequence of the use of these relatively low levels of NHS-biotin is that within a given experiment biotinylated and non-biotinylated versions of the same peptide are often observed. In Table 1, for instance, the peptide ^{63}G-^{89}E was observed at both 3074.2 Da (unmodified) and 3302.4 Da (biotinylated). This did not present any difficulties in either the analysis or the interpretation of the mass spectral data.

The overnight digestion of the 33-kDa extrinsic protein with *Staphylococcus* V8 protease yielded a mixture of completely and partially digested peptide fragments. The simultaneous analysis of the peptides in this mixture by MALDI mass spectrometry provided a highly accurate, sensitive, rapid, and cost effective method for the examination of such multicomponent peptide systems (Bevis & Chait,

1990; Hillenkamp et al., 1991; Henzel et al., 1993). There were some tradeoffs however. Since numerous peptides are present in this mixture, suppression of some peptide fragment ions may occur. This, however, tends to be significantly less pronounced in MALDI mass spectrometry than with other ionization techniques such as fast atom bombardment or plasma desorption (Bevis & Chait, 1990). Additionally, the simultaneous collection of mass data over a relatively wide range (1000−10 000 Da) and the inability to use internal calibration with these peptide mixtures lead inherently to a degradation in the mass accuracy obtained for individual peptides. In this paper, peptide assignments were made within a 0.2% mass error envelope surrounding the theoretical peptide mass, which was determined from the known amino acid sequence (Oh-oka et al., 1986; Tyagi et al., 1987). Under the digestion conditions used in this study, *Staphylococcus* V8 protease was highly selective and cleaved at the C-terminal side of glutamyl residues that are not followed by prolyl or additional glutamyl residues. The program GPMAW examined the known sequence of the 33-kDa extrinsic protein for *all* possible peptides that lie within the 0.2% mass error envelope; only those peptides that are preceded by a glutamyl residue (or the N-terminus of the intact protein) and that end with a glutamyl residue (or the C-terminus of the intact protein) are candidates for peptide assignments. In all instances, only one peptide was identified which fit these criteria.

Two protein modifications were introduced into the 33-kDa extrinsic protein during the course of these experiments. First, the protein was labeled with NHS-biotin either on NaCl-washed PS II membranes or free in solution. This modification resulted in the addition of 227.1 mass units for each incorporated biotin residue. Second, prior to purification of the biotinylated protein by affinity chromatography, the 33-kDa protein was reduced with DTT and carboxyamidomethylated with iodoacetamide. This modification resulted in the addition of 57.1 mass units to each cystyl-containing peptide. Carboxyamidomethylation allowed for a more efficient affinity purification on the monomeric avidin column and for a more complete subsequent digestion of the 33-kDa extrinsic protein with the *Staphylococcus* V8 protease (Stone et al., 1989).

Table 1 summarizes the results obtained from the *Staphylococcus* V8 protease digestion of the 33-kDa extrinsic protein that had been biotinylated on NaCl-washed PS II membranes (three experiments). Twenty-four peptides were identified ranging in mass from 5597.1 to 1094.7 Da. The majority of these peptides were observed as their $[M + H]^+$ ions, although a few $[M + Na]^+$ and $[M + K]^+$ ions were observed. These additional ions arise principally from the use of sodium potassium phosphate buffer in the purification of the modified 33-kDa protein. Five biotinylated peptides were observed: ^{11}I-^{18}E, ^{33}G-^{62}E + acetamide, ^{63}G-^{87}E, ^{188}N-^{209}E, and ^{213}V-^{238}E. The latter peptide was observed to contain either one or two biotins. Since NHS-biotin modifies lysyl residues (and free N-termini), these results suggest that three domains (^{14}K, ^{41}K-^{76}K, and ^{180}K-^{236}K) of the 33-kDa protein are accessible to modification by this reagent on NaCl-washed PS II membranes. A word of caution in interpreting these results. It is possible that the removal of the 24- and 17-kDa components by 1.0 M NaCl treatment could induce conformational changes in the 33-kDa protein, which remains associated with PS II. This could lead to alterations in the labeling pattern of the membrane-bound protein. It should be noted, however, that no such conformational changes have ever been documented.

Table 2: Assignments for Peptides Produced from a *Staphylococcus* V8 Protease Digest of the 33-kDa Extrinsic Protein Which Was NHS-Biotinylated in Solution[a]

observed mass	predicted mass	Δmass	peptide assignment
5604.6	5597.3	7.3	^{88}I-^{139}E
5254.0	5249.5	−5.5	^{140}S-^{187}E + biotin
5026.9	5022.4	4.5	^{140}S-^{187}E
4527.2	4523.8	3.4	^{140}S-^{183}E
3553.2	3549.0	4.2	^{33}G-^{62}E + acetamide + biotin
3748.5	3745.3	3.2	^{105}K-^{139}E
3367.0	3364.1	2.9	^{33}G-^{62}E + acetamide + K^+
3324.1	3325.0	−0.9	^{33}G-^{62}E + acetamide
3302.4	3299.5	2.9	^{63}G-^{89}E + biotin
3135.8	3139.6	−3.8	^{94}V-^{121}E + 1 biotin
3115.2	3111.5	3.7	^{63}G-^{89}E + K^+
3074.2	3072.4	1.8	^{63}G-^{89}E
3058.6	3057.2	1.4	^{63}G-^{87}E + biotin
3012.9	3011.3	1.6	^{213}V-^{238}E + biotin
2996.2	2988.1	8.1	^{184}L-^{209}E + 1 biotin
2909.8	2912.2	−2.4	^{94}V-^{121}E
2832.4	2830.2	2.2	^{63}G-^{87}E
2786.0	2784.2	1.8	^{213}V-^{238}E
2553.3	2549.8	3.5	^{188}N-^{212}E
2514.3	2512.6	1.7	^{188}N-^{209}E + biotin + Na^+
2494.7	2489.6	5.1	^{188}N-^{209}E + biotin
2452.1	2455.4	−3.3	^{2}G-^{18}E + 2 biotin
2352.5	2357.4	4.9	^{11}I-^{32}E
2264.9	2262.5	2.4	^{188}N-^{209}E
2066.0	2069.5	3.5	^{122}R-^{139}E + K^+
2031.9	2030.4	1.5	^{122}R-^{139}E
1871.4	1870.0	1.4	^{88}I-^{104}E
1734.7	1732.9	1.8	^{105}K-^{121}E
1673.2	1669.7	3.5	^{19}V-^{32}E + biotin + K^+
1665.0	1666.8	1.8	^{94}V-^{104}E + K^+
1621.2	1621.4	−0.2	^{1}E-^{10}E + 2 biotin
1432.4	1433.4	−1.0	^{1}E-^{10}E + biotin + K^+
1395.6	1394.3	1.3	^{1}E-^{10}E + biotin
1265.4	1265.2	0.2	^{2}G-^{10}E + biotin
1249.7	1247.3	2.4	^{11}I-^{18}E + biotin + K^+
1168.0	1167.2	0.8	^{1}E-^{10}E
1094.2	1093.2	1.0	^{239}G-^{247}Q
1079.0	1077.2	1.8	^{2}G-^{10}E + K^+

[a] The observed ions were the $[M + H]^+$ species, unless otherwise noted.

Table 2 presents similar results obtained for digestion of the 33-kDa extrinsic protein that had been biotinylated in solution (two experiments). Thirty-eight peptides were identified which ranged in mass from 5604.6 to 1079.0 Da. Twelve biotinylated peptides were observed: ^{1}E-^{10}E, ^{2}G-^{10}E, ^{11}I-^{18}E, ^{19}V-^{32}E, ^{33}G-^{62}E + acetamide, ^{63}G-^{87}E, ^{63}G-^{89}E, ^{94}V-^{121}E, ^{184}L-^{209}E, ^{140}S-^{187}E, ^{188}N-^{209}E, and ^{213}V-^{238}E. Peptide ^{1}E-^{10}E was observed to contain either one or two biotins. This is quite interesting since this peptide contains only one lysyl residue (^{4}K). The presence of two biotins strongly suggests that the N-terminus of this peptide was modified. It had been shown previously that the N-terminus of the 33-kDa protein was not blocked (Oh-oka et al., 1986) and, thus, would be susceptible to NHS-biotinylation. These results demonstrate that seven domains of the 33-kDa extrinsic protein (^{1}E-^{4}K, ^{14}K, ^{20}K, ^{41}K-^{76}K, ^{101}K-^{105}K, ^{159}K-^{186}K, and ^{190}K-^{236}K) are accessible to NHS-biotin when the protein is modified in solution. The results presented in Tables 1 and 2 are summarized in Figure 2.

The four domains which were only biotinylated when the 33-kDa protein was modified in solution (^{1}E-^{4}K, ^{20}K, ^{101}K-

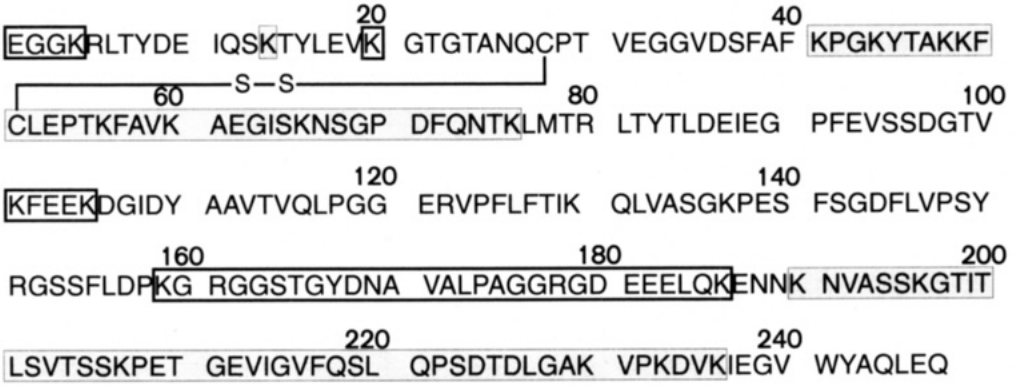

FIGURE 2: Summary of the location of biotinylated domains on the spinach 33-kDa extrinsic protein of photosystem II. Boxed regions are domains that are biotinylated only in solution while shaded regions are domains biotinylated both in solution and on NaCl-washed PS II membranes.

^{105}K, and ^{159}K-^{186}K) represent regions of the protein that become exposed when the 33-kDa protein is released from PS II. Two hypothetical mechanisms could explain the differential modification observed for these regions. First, the 33-kDa extrinsic protein could undergo a significant conformational change upon release from PS II. This could lead to the exposure of lysyl residue-containing domains, which are not normally present on the surface of the protein. A second hypothesis is that no large conformational change is elicited by release of the 33-kDa protein from PS II and that the lysyl residue-containing domains are normally present on the surface of the 33-kDa protein. These domains, however, are shielded from the modification reagent by interaction with other PS II components. No direct evidence is available which allows the differentiation between these two hypotheses. Indirect evidence, however, suggests that the second hypothesis may be more favorable. First, lysyl residues in general are located at a protein's surface (however, many specific examples exist of buried lysyl residues). Second, in solution, the 33-kDa protein exhibits a significant amount of secondary structure (38% β-sheet and 9% α-helix; Xu et al., 1994) and, thus, does not appear to be randomly unfolded. It is unclear, however, if the secondary structure observed in solution is representative of the protein's structure when it is associated with PS II. Finally, it is apparent that no irreversible denaturation and unfolding of the 33-kDa protein occur upon its release from PS II since the isolated protein is capable of restoring high rates of oxygen-evolving activity to PS II membranes depleted in this component (Betts et al., 1994).

The observed biotinylation of the domain ^{1}E-^{4}K only in solution is quite interesting. Previously, other workers have presented evidence implicating the N-terminus of the 33-kDa extrinsic protein in the binding of this protein to PS II. Eaton-Rye and Murata (1989) isolated the 33-kDa protein and used chymotrypsin to remove the N-terminal 16 amino acid residues of this protein. This modification abolished rebinding of the protein to NaCl−urea-washed PS II membranes and prevented the restoration of oxygen-evolving activity. Odom and Bricker (1992) demonstrated that residues in the N-terminal domain, ^{1}E-^{76}K, of the 33-kDa protein were crosslinked with the water-soluble carbodiimide EDC to the domain ^{364}E-^{440}D of CP 47. This result indicated that residues within these domains were interacting via a salt bridge (Hackett & Strittmatter, 1984). Our demonstration that the N-terminal domain of the 33-kDa extrinsic protein is biotinylated only in solution confirms and extends these earlier observations.

The homobifunctional crosslinking reagent DTSP has also been used to crosslink the 33-kDa protein to CP 47 (Enami et al., 1987; Bricker et al., 1988). DTSP crosslinks lysyl residues that lie within a 1.2-nm radius. Queirolo (1992) presented evidence which suggested that the domain ^{159}K-^{236}K of the 33-kDa extrinsic protein was crosslinked to the domain ^{418}K-^{423}K on CP 47. Our observation that the domain ^{159}K-^{186}K of the 33-kDa protein was biotinylated only in solution further supports the hypothesis that this region may also be associated with CP 47.

It has been suggested that the 33-kDa protein may interact with proteins other than CP 47 within the PS II core complex. Other crosslinking reagents of variable length have been used to crosslink the 33-kDa extrinsic protein to the 4.8-kDa *psb*I gene product and the α-subunit of cytochrome b_{559} (Enami et al., 1992) and the D1 and D2 proteins (Mei et al., 1989). Unfortunately, the sites of interaction between the 33-kDa protein and these other components have not been determined. It is possible that the domains ^{20}K and ^{101}K-^{105}K may define regions of interaction of the 33-kDa extrinsic protein with these and perhaps other integral PS II components.

In an earlier study (Bricker et al., 1988), we examined the patterns of NHS-biotinylation of the proteins associated with PS II membranes. The 33-kDa extrinsic protein and a number of other PS II components were biotinylated on both control and NaCl-washed PS II membranes. When the 33-kDa protein was removed by the $CaCl_2$ or alkaline Tris washing, CP 47 became accessible and was observed to be biotinylated. No other PS II proteins were observed to be differentially biotinylated in the presence and absence of the 33-kDa protein. This was interpreted as being evidence that the extrinsic 33-kDa protein and CP 47 interact. Subsequently, we identified two domains on CP 47 that were biotinylated only in the absence of the 33-kDa extrinsic protein, ^{304}K-^{321}K and ^{389}K-^{419}K (Frankel & Bricker, 1992). These results, of course, do not preclude the possibility that other PS II components interact with the 33-kDa extrinsic protein. Other interacting components may not label well with NHS-biotin. Indeed, the other proteins that have been hypothesized to interact with the 33-kDa protein (D1, D2, the α- and β-subunits of cytochrome b_{559}, and the *psb*I gene product) all would label poorly with this reagent due to the

low number of lysyl residues and/or the presence of blocked N-termini.

Finally, it has been suggested that domains on the 33-kDa extrinsic protein may interact with the 24- and 17-kDa extrinsic components of PS II. Indeed, it has been shown that the 33-kDa protein is required for high affinity binding of the 24- and 17-kDa species (Miyao & Murata, 1989). We hypothesize that domains within the regions ^{41}K-^{76}K and ^{190}K-^{236}K, which we observe to be biotinylated both in solution and on NaCl membranes, may be involved in the interaction of the 33-kDa protein with the 24- and 17-kDa components. We are currently testing this hypothesis using the techniques that we have described.

CONCLUSIONS

The use of NHS-biotin as a site-specific labeling reagent coupled with endoproteinase digestion and analysis of the peptide fragments by MALDI mass spectrometry has proved a simple and useful technique in the examination of the interaction of the 33-kDa extrinsic protein with PS II. Our results strengthen the hypothesis that the N-terminus of the 33-kDa protein is associated with the photosystem. Additionally, other domains of the 33-kDa protein that may also interact with PS II have been identified. It should also be noted that the techniques which we have used in this study should be generally applicable to the examination of protein–protein interactions in other multi-subunit complexes.

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