Interaction of CPa-1 with the Manganese-Stabilizing Protein of Photosystem II: Identification of Domains on CPa-1 Which Are Shielded from *N*-Hydroxysuccinimide Biotinylation by the Manganese-Stabilizing Protein†

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ABSTRACT: The structural organization of photosystem II proteins has been investigated by use of the amino group-labeling reagent *N*-hydroxysuccinimidobiotin (NHS-biotin) and calcium chloride-washed photosystem II membranes. We have previously shown that the presence of the extrinsic, manganese-stabilizing protein on photosystem II membranes prevents the modification of lysyl residues located on the chlorophyll protein CPa-1 (CP-47) by NHS-biotin [Bricker, T. M., Odom, W. R., & Queirolo, C. B. (1988) *FEBS Lett. 231,* 111–117]. Upon removal of the manganese-stabilizing protein by calcium chloride-washing, CPa-1 can be specifically modified by treatment with NHS-biotin. Preparative quantities of biotinylated CPa-1 were subjected to chemical cleavage with cyanogen bromide. Two major biotinylated peptides were identified with apparent molecular masses of 11.8 and 15.7 kDa. N-terminal sequence analysis of these peptides indicated that the 11.8-kDa peptide was ^{232}G–^{330}M and that the 15.7-kDa peptide was ^{360}P–^{508}V. The 15.7-kDa CNBr peptide was subjected to limited tryptic digestion. The two smallest tryptic fragments identified migrated at apparent molecular masses of 9.1 (nonbiotinylated) and 7.5 kDa (biotinylated). N-terminal sequence analysis and examination of the predicted amino acid sequences of these peptides suggest that the 9.1-kDa fragment was ^{422}R–^{508}V and that the 7.5-kDa fragment was ^{360}P–^{421}A. These results strongly suggest that two NHS-biotinylated domains, ^{304}K–^{321}K and ^{389}K–^{419}K, become exposed on CPa-1 when the manganese-stabilizing protein is removed by $CaCl_2$ treatment. Both of these domains lie in the large extrinsic loop E of CPa-1.

Photosystem II (PS II)[1] is a multisubunit thylakoid membrane protein complex which 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 (CPa-1), 45 (CPa-2), 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 analogous to the 24- and 17-kDa proteins have been identified.

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 taken 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. 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).

CPa-1 forms an interior light-harvesting antenna for PS II [reviewed in Bricker (1990)]. In addition to this role, a number of lines of evidence suggest that this protein structurally interacts with the oxygen-evolving complex of PS II. The removal of the chloride-independent manganese from PS II membranes leads to a conformational change which exposes

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[1] Abbreviations: BSA, bovine serum albumin; chl, chlorophyll; DTSP, dithiobis(succinimidyl propionate); EDC, 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide; MES, 2-(*N*-morpholino)ethanesulfonic acid; NHS-biotin, *N*-hydroxysuccinimidobiotin; PAGE, polyacrylamide gel electrophoresis; PS II, photosystem II; PVDF, poly(vinylidene difluoride); Tris, tris(hydroxymethyl)aminomethane; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

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a region of CPa-1 recognized by a monoclonal antibody (Bricker & Frankel, 1987). Protein cross-linking data from three independent groups have documented the formation of cross-linked products between CPa-1 and the manganese-stabilizing protein with DTSP (Enami et al., 1987; Bricker et al., 1988), EDC (Bricker et al., 1988; Enami et al., 1991), and 2-iminothiolane (C. B. Queirolo and T. M. Bricker, unpublished observations; E. L. Camm, personal communication). Recently, site-directed mutagenesis studies have demonstrated that alteration of a highly conserved basic residue pair located in the large extrinsic loop E domain of CPa-1 leads to a defect in the oxygen-evolving complex of PS II. This mutation also affects the in vitro stability of the oxygen-evolving complex (Putnam-Evans and Bricker, in press). Finally, we have determined that the presence of the manganese-stabilizing protein shields lysyl residues located on CPa-1 from labeling with NHS-biotin (Bricker et al., 1988).

In this paper, we have used CNBr cleavage and limited tryptic digestion coupled with N-terminal sequence analysis to identify the domains on CPa-1 which are shielded from NHS-biotinylation by the manganese-stabilizing protein. Our results indicate that two domains on CPa-1, ^{304}K–^{321}K and ^{389}K–^{419}K, are NHS-biotinylated in the absence of the manganese-stabilizing protein. Both of these domains are located in the large extrinsic loop E of CPa-1.

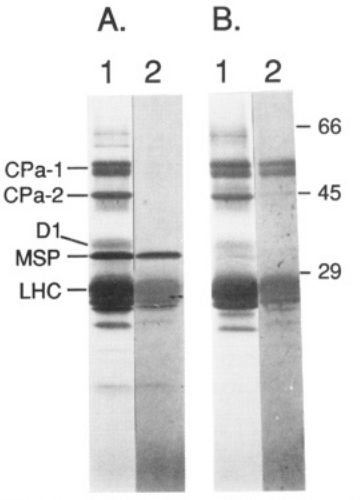

FIGURE 1: NHS-biotinylation patterns of control and $CaCl_2$-washed PS II membranes. Proteins were separated electrophoretically in gradient 12.5–20% acrylamide gels (Delepelaire & Chua, 1981) and were electroblotted onto PVDF membranes and either stained with Coomassie blue (lane 1) or probed with an avidin–peroxidase conjugate (lane 2) to identify biotinylated protein bands as described under Materials and Methods. (Panel A) Control PS II membranes. (Panel B) $CaCl_2$-washed PS II membranes. A number of major PS II protein components are labeled to the left. Molecular mass standards (in kilodaltons) are shown to the right.

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. The manganese-stabilizing protein was removed from the PS II membranes by washing twice for 1 h with 1.0 M $CaCl_2$, 300 mM sucrose, 10 mM $MgCl_2$, 15 mM NaCl, 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 MES–NaOH, pH 6.0, at 1.0 mg of chl/mL. All washes were performed at 0–4 °C. Exposed lysyl residues on CPa-1 were labeled with NHS-biotin as described previously (Bricker et al., 1988) except that the NHS-biotin concentration during labeling was 40 μg/mL.

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. Preparative electrophoresis of these proteins was performed with the same gel system in 15% acrylamide gels. After preparative electrophoresis, the gels were stained with acid-free Coomassie blue [0.1% Coomassie blue R (w/v) in 25% methanol] for 1 h, and the CPa-1 protein doublet was excised from the preparative gels. CNBr cleavage of CPa-1 in the gel strips was performed as described by Nikodem and Frescoe (1979). The cleaved protein was electroeluted from the gel strips as described previously (Odom & Bricker, 1992) and the peptide mixture separated on preparative gels using the system of Schagger and von Jagow (1987) to resolve low molecular weight fragments. In some experiments, the CNBr fragments were electroblotted onto PVDF membranes (Immobilon-P, Millipore Co.) and processed for N-terminal sequence analysis. In other experiments, a peptide of interest, a portion of the extrinsic loop E of CPa-1 having an apparent molecular mass of 15.7 kDa, was excised from the preparative gels after acid-free staining and then electroeluted from the gel strips as described above. This peptide was then prepared for cleavage with trypsin by exchanging buffers into 0.05% LDS and 5 mM NH_4HCO_3 and concentration, both performed in a centrifugal ultrafiltration device (Centricon 10, Amicon Co.). The protein fragment was precipitated by incubation in 90% cold acetone and 1 mM HCl at –20 °C for 3 h. The pellet was centrifuged briefly and washed twice with cold acetone. The pellet was allowed to air-dry completely before being dissolved n 8 M urea and 400 mM NH_4HCO_3, pH 8.0. The peptide solution was then diluted 4-fold with deionized water, and 60 μg of sequencing-grade TPCK-treated trypsin (Calbiochem Co.) was added to yield an approximate peptide: trypsin ratio of 25:1. Incubation at 37 °C proceeded for 3 h, and the reaction was terminated by the addition of 5 mM PMSF in 2-propanol. The tryptic fragments were resolved with the low molecular weight polyacrylamide electrophoresis system described above, and the peptides were then electroblotted onto PVDF membranes (Immobilon-P). Portions of the blot were stained with acid-free Coomassie blue stain and processed for N-terminal sequence analysis, while other portions 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 a 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. Biotinylated bands were visualized by color development using 4-chloro-1-naphthol and H_2O_2. N-terminal sequence analysis was performed on an Applied BioSystems Model 477A sequencer at Baylor University.

RESULTS AND DISCUSSION

Figure 1 illustrates the result of a typical NHS-biotinylation experiment. In panel A, control PS II membranes exhibit strong biotinylation of the manganese-stabilizing protein (MSP) and the light-harvesting chlorophyll *a*/*b* protein (LHC) with several other proteins exhibiting relatively low levels of labeling. No labeling of CPa-1 was observed. In panel B, the PS II membranes were first washed with 1.0 M $CaCl_2$ to

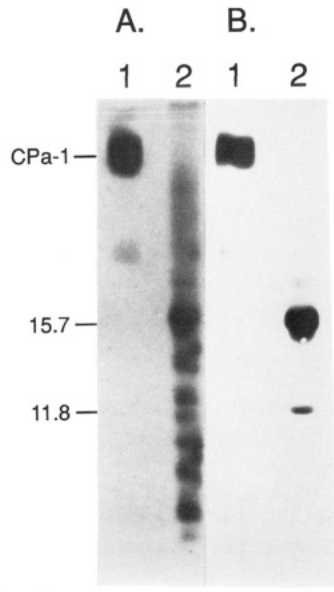

FIGURE 2: CNBr cleavage patterns of biotinylated CPa-1. CNBr fragments were separated electrophoretically (Shagger & von Jagow, 1987) and were electroblotted onto PVDF membranes and either stained with Coomassie blue (panel A) or probed with an avidin–peroxidase conjugate (panel B) to identify biotinylated peptide bands as described under Materials and Methods. Lane 1, uncleaved CPa-1; lane 2, CNBr-cleaved CPa-1. CPa-1 and the biotinylated 15.7- and 11.8-kDa CNBr fragments are labeled to the left of the figure.

remove the extrinsic proteins associated with the oxygen-evolving complex (MSP, 24- and 17-kDa proteins). In this experiment, CPa-1 and the light-harvesting chlorophyll *a*/*b* protein are strongly labeled. We have shown previously (Bricker et al., 1988) that the removal of the manganese-stabilizing protein specifically leads to the exposure of NHS-biotinylatable residues on CPa-1. It should be noted that CPa-1 migrates as a closely spaced doublet in this PAGE system (Bricker & Frankel, 1987). This doublet is believed to represent two migrational forms of CPa-1.

In the experiments described in this paper, $CaCl_2$-washing was used to remove the manganese-stabilizing protein. Similar results are obtained when alkaline Tris washes are used to remove the manganese-stabilizing protein (Bricker et al., 1988). We have shown recently that one $CaCl_2$ wash is sufficient to remove greater than 99% of the manganese-stabilizing protein from PS II membranes (Bricker, 1992). It should be noted that under the conditions used in this experiment, two of the four manganese associated with the manganese cluster of PS II are lost. In the absence of the manganese-stabilizing protein, chloride concentrations of 100 mM are required to maintain an intact manganese cluster (Miyao & Murata, 1984). We have not observed any bulk differences of the biotinylation pattern of manganese-stabilizing protein-depleted PS II membranes containing four, two, or zero manganese (data not shown).

In order to map the location of the biotinylated residues on CPa-1, a combination of CNBr cleavage and limited tryptic digestion was used. Figure 2 illustrates the results obtained by CNBr cleavage of biotinylated CPa-1. Approximately 10 Coomassie blue-stainable CNBr fragments were resolved with molecular masses below 16.7 kDa, which is the largest predicted limit CNBr fragment of CPa-1 (Table IA). Of these, only two biotinylated CNBr peptides were resolved. These were located at apparent molecular masses of 15.7 and 11.8 kDa. Analysis of the derived amino acid sequence of spinach CPa-1 (Morris & Herrman, 1984) indicates that this protein contains 10 methionyl residues. This suggests that complete CNBr cleavage of this protein should yield 11 CNBr peptides which would range in predicted molecular masses from 16.7 to 0.7 kDa. The sequences of these predicted peptides and their predicted molecular masses are summarized in Table IA. We hypothesized that the observed 15.7-kDa CNBr fragment corresponded to the predicted CNBr fragment K. It was difficult, however, to assign a specific predicted CNBr peptide to the observed 11.8-kDa peptide since its apparent molecular mass did not correspond well to any of the predicted fragments.

The 15.7- and 11.8-kDa CNBr peptides were subjected to N-terminal sequencing, the results of which are summarized in Table IB. The N-terminus of the 15.7-kDa CNBr fragment was P-T-F-F-E-T. Only one N-terminus was detected. This sequence identifies the 15.7-kDa CNBr fragment as the predicted CNBr peptide K. The predicted mass of CNBr fragment K is 16.7 kDa. This mass corresponds well to the observed mass of the 15.7-kDa biotinylated peptide of CPa-1. CNBr peptide K contains five lysyl residues which could be biotinylated with NHS-biotin: ^{389}K, ^{418}K, ^{419}K, ^{438}K, and ^{497}K.

The N-terminus of the 11.8-kDa CNBr fragment was X-X-I-E-T-V-L-S; again, only a single N-terminus was detected. This sequence corresponds to predicted CNBr fragment H. However, a problem was immediately apparent with this identification. The predicted mass of CNBr fragment H is 2.5 kDa. Additionally, this fragment contains no lysyl residues and, thus, should not exhibit labeling with NHS-biotin. We hypothesize that the observed 11.8-kDa biotinylated CNBr fragment results from the failure of CNBr to cleave at ^{256}M of peptide H. This would result in the formation of a CNBr peptide with a predicted mass of 11 kDa (peptide H-I) which would correspond well with the observed apparent molecular mass of 11.8 kDa. Peptide H-I contains three lysyl residues which could be biotinylated with NHS-biotin: ^{304}K, ^{308}K, and ^{321}K. It should be noted that we have never observed even a trace of any biotinylated peptide at any apparent molecular mass less than 11.8 kDa even following extended CNBr cleavage. At least two hypotheses can explain the apparent failure of CNBr to cleave at ^{256}M. First, the cleavage of M-X residues can be influenced by the surrounding amino acid residues, particularly in membrane proteins (Ozols & Gerard, 1977). The methionine residues may not be fully accessible to CNBr or able to adopt a conformation necessary for the cleavage to occur (Allen, 1981). In this regard, it should be noted that ^{256}M is located within putative transmembrane helix V, a site that would be predicted to possess significant secondary structure even under the rather harsh conditions employed during CNBr cleavage. Second, ^{256}M may be oxidized to methionine sulfoxide either in a native protein or as an artifact of isolation. CNBr cannot cleave at methionine sulfoxide residues.

To further define the location of the biotinylated residues on the 15.7-kDa CNBr peptide, preparative quantities of this fragment were isolated and subjected to limited tryptic digestion. Figure 3 illustrates the result of this experiment. In this figure, a single lane of resolved tryptic pepides was bisected with half being stained with acid-free Coomassie blue (lane 1) and the second half being probed with an avidin–peroxidase conjugate to visualize the biotinylated fragments (lane 2). Approximately nine peptides were resolved. It should be noted that prolonged digestion with trypsin yielded essentially complete digestion of the 15.7-kDa CNBr fragment with no peptides being resolved in the electrophoretic system used (Schagger & von Jagow, 1987). The two smallest

Table I: N-Terminal Sequence Analysis of the 11.8- and 15.7-kDa CNBr Peptides of CPa-1 and 7.5- and 9.1-kDa Tryptic Fragments of the 15.7-kDa CNBr Peptide: Comparison with the Predicted CNBr Fragments of CPa-1[a]

(A) Predicted CNBr Peptides of CPa-1

peptide	N-terminal sequence	molecular mass (kDa)
A	^{2}GLPWYRVHTV...^{25}M	2.7
B	^{26}HTALVAGWAG...^{37}M	1.2
C	^{38}ALYELAVFDP...^{55}M	2.0
D	^{56}WRQG^{60}M	0.7
E	^{61}FVIPF^{66}M	0.7
F	^{67}TRLGITNSWG...^{102}M	3.8
G	^{103}FSGLCFLAAI...^{231}M	14.1
H	^{232}GNIETVLSSS...^{256}M	2.5
I	^{257}WYGSATTPIE...^{330}M	8.5
J	^{331}DNGDGIAVGW...^{359}M	3.3
K	^{360}PTFFETFPVV...^{508}V	16.7

(B) Sequences of Isolated Peptides

peptide fragment	sequencing cycle no. 1	2	3	4	5	6	7	8	9	10
15.7-kDa CNBr peptide of CPa-1 (5 pmol)	P	T	F	F	E	T				
11.8-kDa CNBr peptide of CPa-1 (5 pmol)	X[b]	X	I	E	T	V	L	S		
7.5-kDa tryptic fragment of 15.7-kDa CNBr peptide (10 pmol)	P	T	F	F	E	T	F	P	V	V
9.1-kDa tryptic fragment of 15.7-kDa CNBr peptide (major) (30 pmol)	R	A	Q	L	G	E	I	F	E	L
9.1-kDa tryptic fragment of 15.7-kDa CNBr peptide (minor) (20 pmol)	A	Q	L	G	E	I	F	E	L	D

[a] The standard single-letter codes for the amino acid residues are used. [b] X indicates positions where unequivocal amino acid assignments could not be made.

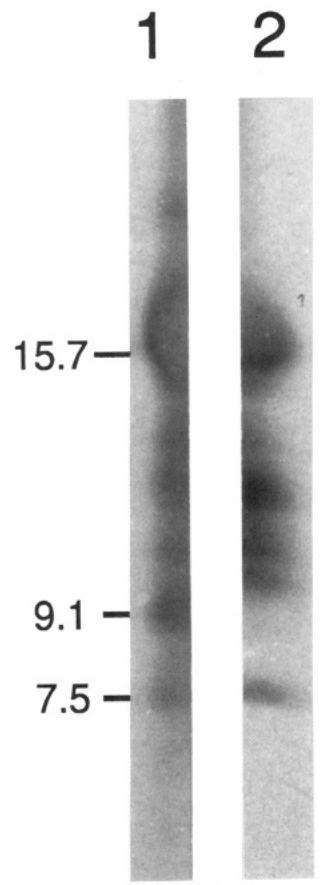

FIGURE 3: Limited tryptic digestion of the biotinylated 15.7-kDa CNBr fragment of CPa-1. Tryptic peptides of the biotinylated 15.7-kDa CNBr fragment were separated electrophoretically (Shagger & von Jagow, 1987) and were electroblotted onto PVDF membranes and either stained with Coomassie blue (lane 1) or probed with an avidin–peroxidase conjugate (lane 2) to identify biotinylated peptide bands as described under Materials and Methods. The 15.7-kDa CNBr fragment of CPa-1, the 9.1-kDa nonbiotinylated tryptic fragment, and the 7.5-kDa biotinylated tryptic fragment are labeled to the left of the figure.

resolved peptides were of particular interest: a 7.5-kDa biotinylated peptide and a 9.1-kDa nonbiotinylated peptide. All of the other higher apparent molecular mass peptides were biotinylated. These two peptides were subjected to N-terminal sequence analysis.

Table IB shows the results of this analysis. The N-terminal sequence of the 7.5-kDa biotinylated tryptic fragment was P-T-F-F-E-T-F-P-V-V; this sequence corresponds to the N-terminus of the 15.7-kDa CNBr fragment of CPa-1. N-terminal sequence analysis of the 9.1-kDa nonbiotinylated tryptic fragment yielded two N-terminal sequences: a major (30 pmol) sequence of R-A-Q-L-G-E-I-F-E-L and a minor (20 pmol) sequence of A-Q-L-G-E-I-F-E-L-D. These sequences correspond to tryptic fragments generated by cleavage after ^{422}R and ^{423}R, respectively. We hypothesize that the observed 7.5-kDa biotinylated tryptic fragment consists of a mixture of the peptides ^{360}P–^{422}R and ^{360}P–^{423}R. These peptides would have a predicted molecular mass of 7.2 and 7.3 kDa, respectively, which corresponds well to the observed apparent molecular mass of 7.5 kDa. Additionally, these peptides contain three lysyl residues which could be biotinylated by NHS-biotin: ^{389}K, ^{418}K, and ^{419}K. We also hypothesize that the observed 9.1-kDa nonbiotinylated tryptic fragment consists of a mixture of the peptides ^{423}R–^{508}V and ^{424}A–^{508}V. These peptides would have a predicted molecular mass of 9.6 and 9.4 kDa, respectively, which corresponds well to the observed apparent molecular mass of 9.1 kDa. The lysyl residues present in these peptides, ^{438}K and ^{497}K, are not biotinylated. It should be noted that the combined apparent molecular mass of the two analyzed peptides (biotinylated 7.5 kDa and nonbiotinylated 9.1 kDa) is 16.6 kDa. This is very close to the predicted mass of CNBr peptide K (16.7 kDa). This result strongly supports the sequence assignments we have made.

The findings which we report here are summarized in Figure 4. Two biotinylated domains have been identified on CPa-1: ^{304}K–^{321}K and ^{389}K–^{419}K. These domains are only assessible to the labeling reagent when the 33-kDa, manganese-stabilizing protein is removed from PS II membranes and define regions on CPa-1 which are shielded by the manganese-stabilizing protein.

The identification of the protein components of PS II which interact with the oxygen-evolving site has been the subject of

A. Predicted Sequence of the 11.0 kDa CNBr Fragment H-I of CPa-1

232 ⁑
GNIETVLSSSIAAVFFAAFVVAGTMWYGSATTPIE

LFGPTRYQWDQGYFQQEIYRRVSAGLAENQSF

304 321 330
SEAWSKIPEKLAFYDYIGNNPAKGGLFRAGSM

B. Predicted Sequence of the 16.7 kDa CNBr Fragment K of CPa-1:

360 389
PTFFETFPVVLIDGDGIVRADVPFRRAESKYSVE

419
QVGVTVEFYGGELNEVSYSDPATVKKYARRAQL

GEIFELDRASLKSDGVFRSSPRGWFSFGHASFA

LLFFFGHIWHGSRSLFRDVFAGIDPLDVQVEFGA

508
FQKIGDPTTRRQGV

FIGURE 4: Location of the NHS-biotinylated domains on CPa-1. The derived amino acid sequences (Morris & Herrmann, 1984) for CNBr peptides H-I and K are shown. Biotinylated domains are shaded. Small crossed arrow, ^{256}Met-^{257}Trp site which exhibits no apparent cleavage with CNBr. Large arrows, sites of tryptic digestion observed to yield the 9.1-kDa nonbiotinylated and 7.5-kDa biotinylated peptides.

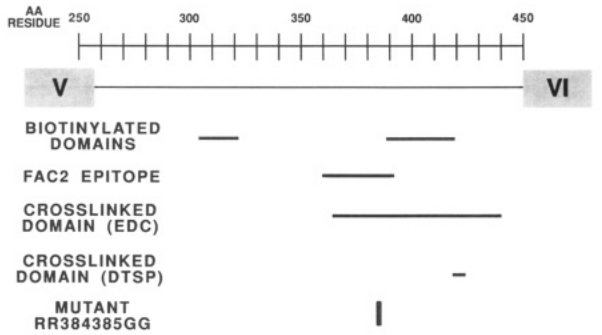

FIGURE 5: Summary of the sites of interaction of the large extrinsic loop of CPa-1 with components involved in oxygen evolution. The locations of the biotinylated domains (this work), EDC-cross-linked domains (Odom & Bricker, 1992), and DTSP-cross-linked domains (Queirolo and Bricker, unpublished results) define sites of interaction of CPa-1 with the manganese-stabilizing protein. The location of the FAC2 epitope (Frankel & Bricker, 1989) defines a region of CPa-1 which is sensitive to a conformational change which occurs in PS II upon removal of the chloride-insensitive manganese. The location of the site-directed mutant RR384385GG defines a site which influences oxygen evolution activity and the in vitro stability of the oxygen-evolving complex. Putative transmembrane helixes V and VI are shaded. Amino acid residue numbers are shown above. For a full topological model of CPa-1, see Bricker (1990).

much current research and debate. While the D1 and D2 proteins undoubtedly contribute significantly to the architecture of the oxygen-evolving site, important roles for other PS II proteins cannot be excluded at this time based on the experimental evidence which is currently available. The data which we have presented in this paper strongly support the hypothesis that the large extrinsic loop E of the chlorophyll protein CPa-1 interacts with components involved in oxygen evolution (Bricker, 1990). A number of other lines of evidence also support this hypothesis. These results are summarized in Figure 5. First, we have isolated a monoclonal antibody directed against CPa-1 which only recognizes its antigenic determinant when the chloride-insensitive manganese associated with the oxygen-evolving site of PS II are removed (Bricker & Frankel, 1987). The epitope for this antibody is located on the domain ^{360}P–^{391}S (Frankel & Bricker, 1989) which is located in the large extrinsic loop E of CPa-1. Second, a variety of protein cross-linkers are capable of cross-linking CPa-1 to the manganese-stabilizing protein in spinach PS II membranes. These include DTSP (Enami et al., 1987; Bricker et al., 1988), 2-iminothiolane (E. Camm, personal communication; C. B. Queirolo and T. M. Bricker, unpublished observations), and the water-soluble carbodiimide EDC (Bricker et al., 1988; Enami et al., 1991). EDC is particularly interesting since it cross-links amino groups to carboxyl groups which are in van der Waals contact (Hackett & Strittmatter, 1984). Proteins cross-linked by this reagent are assumed to be interacting via a salt bridge. We have mapped the domains on both the manganese-stabilizing protein and CPa-1 which are cross-linked with EDC. The domain ^{364}E–^{440}D of CPa-1 is cross-linked to the N-terminal domain (^{2}E–^{76}K) of the manganese-stabilizing protein (Odom & Bricker, 1992). Additionally, we have recently completed mapping of the DTSP cross-linking domains on CPa-1 and the manganese-stabilizing protein (Queirolo and Bricker, unpublished results). DTSP cross-links lysyl residues which are located within 12 nm of each other. The domain ^{418}K–^{423}K on CPa-1 is cross-linked to the domain ^{159}K–^{236}K on the manganese-stabilizing protein. Thus, both of these cross-linkers, EDC and DTSP, cross-link regions located on the large extrinsic loop E of CPa-1 to the manganese-stabilizing protein. Finally, site-directed mutagenesis studies within the large extrinsic loop E of CPa-1 suggest a possible role for this protein in water oxidation (Putnam-Evans and Bricker, submitted for publication). Substitution of glycyl residues for the basic residue pair ^{384}R^{385}R in CPa-1 leads to a 50% loss of oxygen-evolving capability in vivo and a marked loss in the stability of the oxygen-evolving complex in vitro. It should be noted that this mutation lies within the epitopic domain of the monoclonal antibody FAC2. These studies have demonstrated that CPa-1 strongly interacts with the oxygen-evolving site and have yielded the most complete picture currently available of the interaction of the manganese-stabilizing protein with an intrinsic component of PS II.

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