# Interaction of CPa-1 with the Manganese-Stabilizing Protein of Photosystem II: Identification of Domains Cross-Linked by 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide†

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ABSTRACT: The structural organization of photosystem II proteins has been investigated by use of the zero-length protein cross-linking reagent 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide and monoclonal and polyclonal antibody reagents. Photosystem II membranes were treated with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide which cross-links amino groups to carboxyl groups which are in van der Waals contact. This treatment did not affect the oxygen evolution rates of these membranes and increased the retention of oxygen evolution after $CaCl_2$ washing. Analysis of the proteins cross-linked by this treatment indicated that two cross-linked species with apparent molecular masses of 95 and 110 kDa were formed which cross-reacted with antibodies against both the 33-kDa manganese-stabilizing protein and the chlorophyll protein CPa-1. Cleavage of the 110-kDa cross-linked species with cyanogen bromide followed by N-terminal sequence analysis was used to identify the peptide fragments of CPa-1 and the manganese-stabilizing protein which were cross-linked. Two cyanogen bromide fragments were identified with apparent molecular masses of 50 and 25 kDa. N-Terminal sequence analysis of the 50-kDa cyanogen bromide fragment indicates that this consists of the C-terminal 16.7-kDa fragment of CPa-1 and the intact manganese-stabilizing protein. This strongly suggests that the manganese-stabilizing protein is cross-linked to the large extrinsic loop domain of CPa-1. N-Terminal analysis of the 25-kDa cyanogen bromide fragment indicates that this consists of the C-terminal 16.7-kDa peptide of CPa-1 and the N-terminal 8-kDa peptide of the manganese-stabilizing protein. These results indicate that the domain $^{364}E$–$^{440}D$ of CPa-1 is cross-linked to the domain $^{1}E$–$^{76}K$ of the manganese-stabilizing protein. The large extrinsic loop domain of CPa-1 thus appears to anchor the manganese-stabilizing protein to the photosynthetic membrane via a charge-pair interaction.

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 ($\alpha$ and $\beta$ subunits of cytochrome $b_{559}$), and 4 kDa (*psb*I gene product) appear to form the minimum complex capable of photosynthetic oxygen evolution (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 (Akerlund et al., 1982; 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 and chloride (Ghanotakis et al., 1984; Andersson et al., 1984). These proteins appear to play an important role in the regulation of calcium and chloride concentrations within the PS II complex. 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 (One & 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 complete absence of the manganese-stabilizing protein (Bricker, 1992). The presence of the manganese-stabilizing protein is, however, required for high rates of oxygen evolution to be observed.

Reconstitution studies with native and chymotrypsin-modified manganese-stabilizing protein have shown that 16 amino acid residues located at the N-terminus of this protein are required for the stable and functional association of the

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[1] Abbreviations: Chl, chlorophyll; CPa-1, CP-47; DCBQ, 2,6-dichloro-*p*-benzoquinone; DTSP, dithiobis(succinimidyl propionate); EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; Mes, 2-(*N*-morpholino)ethanesulfonic acid; NHS-biotin, *N*-hydroxysuccinimidobiotin; PS II, photosystem II; PVDF, poly(vinylidene difluoride); Tris, tris(hydroxymethyl)aminomethane.

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manganese-stabilizing protein with PS II membranes (Eaton-Rye & Murata, 1989). Indirect evidence has suggested that carboxyl groups on the manganese-stabilizing protein are involved in electrostatic interactions with PS II (Shen & Inoue, 1991). The sites of these interactions have not been identified.

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 site of PS II. First, the removal of the chloride-independent manganese from PS II membranes leads to a structural change which exposes a region of CPa-1 recognized by a monoclonal antibody (Bricker & Frankel, 1987). Second, the manganese-stabilizing protein shields CPa-1 from proteolytic attack (Bricker & Frankel, 1987). Third, the presence of the manganese-stabilizing protein shields lysyl residues located on CPa-1 from labeling with NHS-biotin (Bricker et al., 1988). Finally, protein cross-linking data from three independent groups 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 (E. L. Camm, personal communication). It should be noted that proteins must be in van der Waals contact to be cross-linked with EDC and such proteins are usually thought to be interacting via a charge-pair mechanism (Hackett & Strittmatter, 1984). These cross-linking data strongly suggest that CPa-1 provides *a binding site* for the manganese-stabilizing protein's association with PS II. Other binding sites may be present on other PS II core components (Gounaris et al., 1988; Mei et al., 1989).

In this paper, we have used CNBr cleavage and N-terminal sequence analysis to identify the domains on CPa-1 and the manganese-stabilizing protein which are cross-linked with the protein cross-linker EDC. We have found that the N-terminal region of the manganese-stabilizing protein is cross-linked to the large extrinsic loop domain of CPa-1.

## Materials and Methods

Chloroplasts were isolated from market spinach as described previously (Bricker et al., 1985). Oxygen-evolving PS II membranes were isolated as described by 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. Chl concentration was determined by the method of Arnon (1949). Electrophoresis (Delepelaire & Chua, 1979), "Western blotting" to either nitrocellulose or PVDF membranes (Towbin et al., 1979), and antibody probing were performed as previously described (Bricker et al., 1988). The polyclonal anti-manganese-stabilizing protein antibody used in this study was produced in this laboratory by immunization of mice with manganese-stabilizing protein purified by the procedure of Kuwabara et al. (1985). This purified protein also cross-reacts with a monoclonal antibody, FCC4, which recognizes the manganese-stabilizing protein (Frankel & Bricker, 1990). The polyclonal anti-CPa-1 antibody used in this study was kindly supplied by Dr. N.-H. Chua and was produced by immunization of rabbits with purified peptide "5" from *Chlamydomonas* (Chua & Blomberg, 1979). CNBr fragments were resolved in the electrophoretic system of Schagger and von Jagow (1987). Proteins and protein fragments were also labeled "in situ" on the PVDF membranes with NHS-biotin and detected with an avidin–peroxidase conjugate and 4-chloro-1-naphthol (LaRochelle & Froehner, 1986) when high sensitivity was required.

Oxygen evolution assays were performed polarographically with a Hansatech oxygen electrode in a volume of 1.0 mL at a light intensity of 1500 $\mu$mol of photons $m^{-2}$ $s^{-1}$ of copper sulfate-filtered white light at room temperature. The reaction buffer contained 50 mM Mes–NaOH, pH 6.0, 0.4 M sucrose, and 15 mM $CaCl_2$. All assays were performed at a Chl concentration of 10 $\mu$g/mL with 250 $\mu$M DCBQ as an electron acceptor.

For protein cross-linking, PS II membranes were washed twice with 50 mM Mes–NaOH, pH 6.0, 300 mM sucrose, 15 mM NaCl, and 10 mM $MgCl_2$. The membranes were then washed once with 50 mM sodium–potassium phosphate buffer, pH 6.0, 300 mM sucrose, 15 mM NaCl, and 10 mM $MgCl_2$, pelleted at 38000$g$, and resuspended to 1.0 mg/mL Chl in the same buffer. The membrane suspension was brought to 15 mM EDC and incubated at 4 °C for 20 min in the dark. The EDC-modified PS II membranes were washed twice with 50 mM Mes–NaOH, pH 6.0, 300 mM sucrose, 15 mM NaCl, and 10 mM $MgCl_2$ and resuspended to a Chl concentration of 2.0 mg/mL.

Preparative quantities of CPa-1, the manganese-stabilizing protein, and a 110-kDa cross-linked protein which reacted with both anti-CPa-1 and anti-manganese-stabilizing protein antibodies were isolated by electrophoresis followed by electroelution. After electrophoresis (Delepelaire & Chua, 1979), the polyacrylamide gels were stained with acid-free Coomassie blue [0.1% Coomassie blue (w/v) in 25% methanol] followed by a brief destaining in 25% methanol. The relevant protein bands were excised, incubated for 30 min in 25 mM Tris–glycine buffer, pH 8.8, and 1.0% LDS, and then electroeluted in 25 mM Tris–glycine buffer, pH 8.8, and 0.1% LDS. After electroelution, the samples were washed several times with deionized water and concentrated to 250 $\mu$L in a Centricon 30 (Polysciences, Inc.) centrifugal ultrafiltration apparatus and then stored at −80 °C prior to cleavage witn CNBr.

The isolated CPa-1, manganese-stabilizing protein, and the 110-kDa cross-linked species were subjected to cleavage with CNBr (Gross, 1969). All procedures were performed in a chemical safety hood with proper ventilation. CNBr cleavage was performed at 120 mg of CNBr/mL in 50% formic acid for 4 h at 25 °C. After cleavage, samples were lyophilized and then solubilized in 50 mM Tris-HCl, pH 6.8, 1.4% LDS, 3.6% 2-mercaptoethanol, 6.0% sucrose, and 3.6% saturated bromophenol blue prior to electrophoresis. After electrophoresis, the peptide fragments were electrophoretically transferred to a PVDF membrane and were then either processed for detection by antibody reagents or prepared for sequencing. For sequencing, the PVDF membranes were stained with acid-free Coomassie blue, destained with 25% methanol, and rinsed extensively with deionized water and then dried. N-Terminal sequence analysis was performed on an Applied BioSystems Model 477A sequencer at Baylor University.

## Results and Discussion

Figure 1 shows the result of a typical EDC cross-linking experiment. At 15 mM EDC, two major cross-linked products with apparent molecular masses of 95 and 110 kDa are formed which cross-react with antibodies directed against CPa-1 and the manganese-stabilizing protein. We estimate, from Coomassie blue staining, that about 10–15% of CPa-1 and the manganese-stabilizing protein are present in these cross-linked products. Upon heating (70 °C for 5 min), these cross-linked protein complexes migrate at 65 and 76 kDa, respectively (Bricker et al., 1988). We have previously shown that in the absence of the manganese-stabilizing protein no cross-linked products are formed which can be recognized by anti-CPa-1 antibodies (Bricker et al., 1988). The cross-linked product

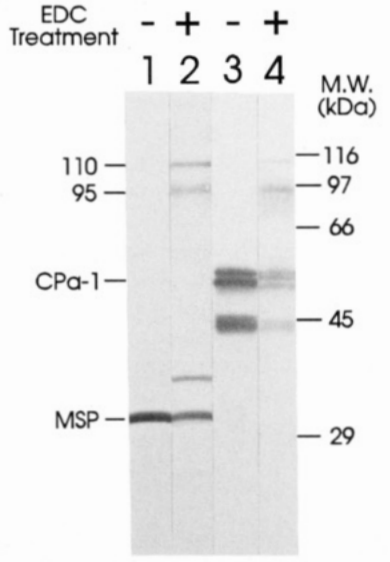


FIGURE 1: Identification of the 110- and 95-kDa EDC cross-linked products by antibodies which recognize the manganese-stabilizing protein and CPa-1. After being cross-linked with 15 mM EDC, the PS II proteins were resolved by LDS–PAGE in a 12.5–20% linear polyacrylamide gel. The proteins were "Western blotted" and probed with antibodies against either the manganese-stabilizing protein (lanes 1 and 2) or CPa-1 (lanes 3 and 4). The proteins in lanes 1 and 3 were not treated with EDC. Split lane experiments confirmed that both antibodies recognized the same 110- and 95-kDa bands (data not shown). It should be noted that in lane 3, the labeled band at about 45 kDa is an alternative, more highly denatured, migrational form of CPa-1 which we have described previously (Bricker & Frankel, 1987). Molecular masses of standard proteins are shown to the right.

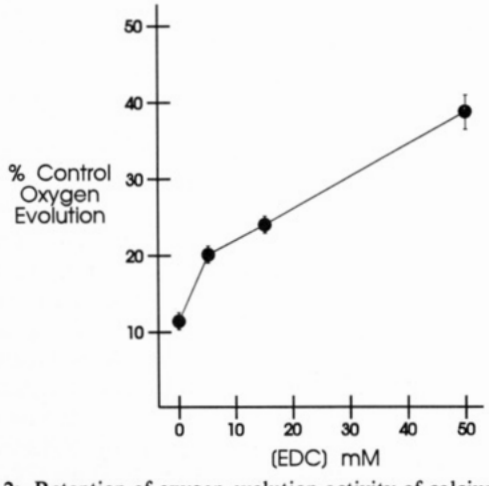


FIGURE 2: Retention of oxygen evolution activity of calcium chloride-washed PS II membranes after treatment with EDC. PS II membranes were cross-linked with various concentrations of EDC, washed with 1.0 M $CaCl_2$ to remove the non-cross-linked manganese-stabilizing protein from the cross-linked membranes, and assayed for oxygen evolution activity. This graph represents five independent experiments with five replicates per experiment. The control oxygen evolution rate was 550 $\mu$mol of $O_2$ (mg of Chl)$^{-1}$ h$^{-1}$. Error bars = 1 SD.

which exhibited an apparent molecular mass of 110 kDa was chosen for further study.

Figure 2 demonstrates that the manganese-stabilizing protein which is cross-linked to PS II membranes is capable of supporting oxygen evolution. In this experiment, the PS II membranes were treated with varying quantities of EDC, washed with 1.0 M $CaCl_2$, and then assayed for oxygen evolution activity; 1.0 M $CaCl_2$ washing removes essentially all of the extrinsic proteins associated with the oxygen-evolving site of PS II (Ono & Inoue, 1983; Bricker, 1992) and severely

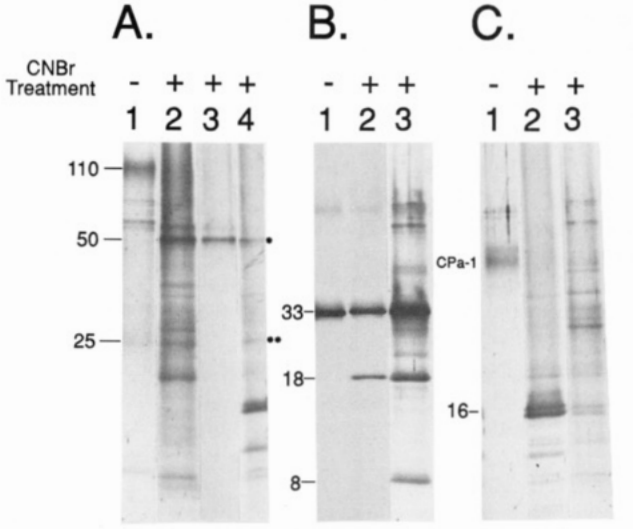


FIGURE 3: CNBr cleavage of the 110-kDa cross-linked product, the manganese-stabilizing protein, and CPa-1. The 110-kDa cross-linked product (panel A), the manganese-stabilizing protein (panel B), and CPa-1 (panel C) were isolated, cleaved with cyanogen bromide, "Western blotted" after electrophoresis, and detected either by "in situ" NHS-biotinylation or by specific antibodies. Panel A: lane 1, uncleaved 110-kDa cross-linked product; lane 2, CNBr-cleaved 110-kDa cross-linked product. The proteins in lanes 1 and 2 were detected by "in situ" NHS-biotinylation. The proteins in lane 3 were detected with an anti-manganese-stabilizing antibody while the proteins in lane 4 were detected with an anti-CPa-1 antibody. The 50- and 25-kDa CNBr fragments of the 110-kDa cross-linked product are indicated with either one or two stars, respectively, to the right of panel A. Panel B: lane 1, uncleaved manganese-stabilizing protein (labeled 33 kDa); lane 2, cleaved manganese-stabilizing protein. The proteins in lanes 1 and 2 were detected with an anti-manganese-stabilizing protein antibody. Lane 3, detection of cleaved manganese-stabilizing protein by "in situ" NHS-biotinylation. The 18- and 8-kDa CNBr fragments are labeled. Note that the 8-kDa fragment is not detected by the polyclonal antibody used in this study. Panel C: lane 1, uncleaved CPa-1; lane 2, cleaved CPa-1. The proteins in lanes 1 and 2 were detected with an anti-CPa-1 antibody. Lane 3, detection of cleaved CPa-1 by "in situ" NHS-biotinylation. The 16-kDa CNBr fragment is labeled.

inhibits oxygen evolution activity at low calcium and chloride concentrations. Increasing the concentration of EDC increases the amount of the manganese-stabilizing protein which remains associated with the PS II membranes (data not shown) and increases the rate of oxygen evolution activity observed in $CaCl_2$-washed membranes. These findings confirm the observations of Enami et al. (1991). Using EDC, these authors observed essentially complete cross-linking of the manganese-stabilizing protein to PS II membranes. They were able to demonstrate that all of the cross-linked manganese-stabilizing protein appeared to be present in a cross-linked complex with CPa-1. Additionally, these membranes retained full oxygen-evolving activity. The findings of Enami et al. (1991) coupled with our results presented in this paper strongly suggest that the manganese-stabilizing protein which is cross-linked to the PS II membranes by EDC remains organized in a structurally and functionally relevant manner.

Figure 3 shows the results obtained from cyanogen bromide cleavage of the 110-kDa cross-linked product, the manganese-stabilizing protein, and CPa-1. In panel A, the 110-kDa cross-linked product is shown before (lane 1) and after (lane 2) CNBr cleavage. The protein and peptide fragments shown in these lanes were detected by in situ NHS-biotinylation. A large number of peptide fragments are generated by the CNBr treatment. One of these fragments, with an apparent molecular mass of 50 kDa, is detected by both an anti-manganese-stabilizing protein antibody (lane 3) and an anti-CPa-1

antibody. This peptide was not detected immunologically after cleavage of the manganese-stabilizing protein (panel B, lane 2) or after cleavage of CPa-1 (panel C, lane 2). A second CNBr fragment of the 110-kDa cross-linked product, which had an apparent molecular mass of 25 kDa, was detected with the anti-CPa-1 antibody but was not detected with the anti-manganese-stabilizing protein antibody. This fragment was also not detected after cleavage of the manganese-stabilizing protein (panel B, lane 2) or after cleavage of CPa-1 (panel C, lane 2). Since these two CNBr fragments (50 and 25 kDa) are detected after cleavage of the 110-kDa cross-linked product but are not detected after cleavage of either the manganese-stabilizing protein or CPa-1, it is probable that these fragments are derived from regions of CPa-1 and the manganese-stabilizing protein which are cross-linked.

In Figure 3, panel B, isolated manganese-stabilizing protein is shown both before (lane 1) and after (lanes 2 and 3) CNBr cleavage. The protein in lanes 1 and 2 was detected with an anti-manganese-stabilizing protein antibody while the protein in lane 3 was detected by in situ NHS-biotinylation. The manganese-stabilizing protein is 247 amino acid residues in length and contains 1 methionine ($^{78}$Met). Cleavage with CNBr yields two fragments with apparent molecular masses of about 18 and 8 kDa (panel B, lane 3). The partial cleavage observed is due to the presence of a threonine residue ($^{79}$Thr) following $^{78}$Met. It has been shown that Met-Thr residues are cleaved with low yield by CNBr (Schroeder et al., 1969). It should also be noted that the antibody which recognizes the manganese-stabilizing protein only recognizes the 18-kDa CNBr fragment. Any CNBr fragment of the 110-kDa cross-linked product which involved the 8-kDa fragment of the manganese-stabilizing protein would not be recognized by this antibody.

Figure 3, panel C, illustrates the result of CNBr cleavage of CPa-1. Lanes 1 and 2 are probed with a polyclonal antibody which recognizes CPa-1 while the peptide fragments of lane 3 were detected by in situ staining with NHS-biotin. CPa-1 is normally observed as a doublet (Bricker & Frankel, 1987) in this gel system. Cleavage with CNBr produces a large number of fragments. The principal fragments detected by the polyclonal anti-CPa-1 antibody are a doublet located at about 16 kDa (panel C, lane 2). These fragments are also recognized by the monoclonal antibody FAC2 (Bricker & Frankel, 1987) (data not shown) and are derived from the C-terminal CNBr fragment of CPa-1 (CNBr peptide K, see Table I). A number of other peptide fragments are also detected with the polyclonal antibody.

The 50- and 25-kDa CNBr fragments of the 110-kDa cross-linked product were isolated in preparative quantities and subjected to N-terminal sequence analysis. Since these putative cross-linked fragments should contain peptides which are derived from both the manganese-stabilizing protein and CPa-1, it was expected that two N-termini would be present and that two amino acid residues would be released at each sequencing cycle. This is precisely what was observed. Additionally, equimolar amounts of the two residues were released at each sequencing cycle, indicating a 1:1 stoichiometry of the two sequenced peptides. These results are summarized in Table I. Approximately 3 pmol of the 50-kDa fragment and somewhat less than 1 pmol of the 25-kDa fragment were present during the sequencing runs. Both the 50- and 25-kDa CNBr fragments yielded the N-terminal sequence of the manganese-stabilizing protein and the N-terminus of the CNBr peptide K of CPa-1. These results suggest that the 50-kDa CNBr fragment of the 110-kDa cross-linked product

Table I: N-Terminal Sequence Analysis of the 50- and 25-kDa CNBr Fragments of the 110-kDa Cross-Linked Product with Comparison to the Predicted CNBr Fragments of CPa-1 and the Manganese-Stabilizing Protein[a]

(A) Sequences of Isolated Peptides

| peptide fragment | sequencing cycle 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| 50-kDa CNBr fragment (3 pmol) | E | G | G | K | R | L | T | Y | D | E |
| | P | T | F | F | E | T | F | P | V | V |
| 25-kDa CNBr fragment (<1 pmol) | E | G | X | K | R | L | T | Y | D | E |
| | P | T | F | X[b] | X | T | F | P | V | X |

(B) Predicted CNBr Peptides

| peptide | N-terminal sequence | molecular mass (kDa) |
|---|---|---|
| CPa-1 peptides | | |
| A | $^{2}$GLPWYRVHTV...$^{25}$M | 2.7 |
| B | $^{26}$HTALVAGWAG...$^{37}$M | 1.2 |
| C | $^{38}$ALYELAVFDP...$^{55}$M | 2.0 |
| D | $^{56}$MRQG$^{60}$M | 0.7 |
| E | $^{61}$FVIPF$^{66}$M | 0.7 |
| F | $^{67}$TRLGITNSWG...$^{102}$ | 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...$^{506}$V** | **16.7** |
| manganese-stabilizing protein peptides | | |
| **A** | **$^{2}$EGGKRLTYDE...$^{78}$M** | **8.5** |
| B | $^{79}$TRLTYTLDEI...$^{247}$Q | 18.1 |

[a] The standard single-letter codes for the amino acid residues are used. The peptide fragments which were observed to be cross-linked are shown in boldface type. [b] X indicates positions where unequivical amino acid assignments could not be made.

consists of intact manganese-stabilizing protein cross-linked to CNBr peptide K of CPa-1 while the 25-kDa CNBr fragment of the 110-kDa cross-linked product consists of CNBr fragment A of the manganese-stabilizing protein cross-linked to CNBr peptide K of CPa-1. This assignment is supported by the lack of cross-reactivity of the 25-kDa CNBr fragment of the 110-kDa cross-linked product with the anti-manganese-stabilizing protein antibody. Figure 3, panel B, shows that this antibody does not recognize the 8-kDa CNBr fragment of the manganese-stabilizing protein while Figure 3, panel A, shows that this antibody also does not recognize the 25-kDa CNBr fragment of the 110-kDa cross-linked product.

Hydropathy plots of the derived amino acid sequence of Cpa-1 predict that this protein possesses 6 transmembrane $\alpha$ helixes (Vermaas et al., 1987; Bricker, 1990) with a large, 190 amino acid residue, extrinsic loop domain located between the fifth and sixth transmembrane helixes. Analysis of the charged amino acid distribution in this protein (Bricker, 1990) using the method of von Heinje and Gavel (1990) predicts that the N- and C-termini of CPa-1 are exposed to the stromal face of the thylakoid membrane while the large extrinsic loop is exposed at the lumenal face. $^{359}$Met-$^{360}$Pro, the CNBr cleavage site that generates peptide K, is located approximately in the middle of the large extrinsic loop of CPa-1. Since the manganese-stabilizing protein is located in the lumen of the thylakoid membrane, our data demonstrate that this protein is cross-linked to the large extrinsic loop domain of CPa-1. Our findings additionally show that residues located in the 8.0-kDa, N-terminal domain of the manganese-stabilizing protein are involved in the formation of the protein–protein association with CPa-1. These results indicate that the domain $^{364}$E–$^{440}$D of CPa-1 is cross-linked to the domain $^{1}$E-$^{76}$K of the manganese-stabilizing protein. Our results are consistant with the findings of Eaton-Rye and Murata (1989), who demonstrated, through reconstitution studies, that chymotrypsin-treated manganese-stabilizing protein was unable to form a stable

association with PS II. This protease treatment cleaved 16 amino acid residues from the N-terminus of the manganese-stabilizing protein. These authors concluded that the N-terminus of the manganese-stabilizing protein was required for the binding of this protein to PS II.

These results strongly support the hypotheses that the chlorophyll protein CPa-1, in addition to its role as an interior light-harvesting antenna for PS II, also interacts with components required for normal oxygen evolution in this photosystem. More specifically, it appears that the large extrinsic domain of this protein is closely associated with the oxygen-evolving site. We have shown previously that, upon removal of the two chloride-independent manganese which are associated with the active site of oxygen evolution, a conformational change occurs in PS II which leads to the exposure of the epitope of the monoclonal antibody FAC2 on CPa-1 (Bricker & Frankel, 1987). This epitope is located in the region between $^{360}$Pro and $^{391}$Ser in the extrinsic loop domain (Frankel & Bricker, 1989). We have also shown that the manganese-stabilizing protein shields domains on CPa-1 from biotinylation with the amino group-reactive reagent NHS-biotin (Bricker et al., 1987). Recently, mapping studies have been completed (Frankel and Bricker, unpublished results) which have determined that the only biotinylated regions present on CPa-1 lie in the large extrinsic loop region (specifically, $^{304}$Lys–$^{321}$Lys and $^{389}$Lys–$^{438}$Lys) of CPa-1. Additionally, site-directed mutagenesis studies on CPa-1, in *Synechocystis* 6803, have demonstrated that replacement of the conserved residues $^{384}$Arg-$^{385}$Arg with glycyl residues leads to a 50% loss of PS II oxygen evolution activity without a concomitant loss of PS II proteins (Putnam-Evans and Bricker, unpublished results). 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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