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Epitope mapping of the monoclonal antibody FAC2 on the apoprotein of CPa-1 in photosystem II

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Using a combination of cyanogen bromide cleavage and endoproteinase digestion we have shown that the putative epitope for the monoclonal antibody FAC2 lies in the region ^{360}Pro–^{391}Ser on the apoprotein of CPa-1. This region lies entirely within the large extrinsic loop of this protein. We have shown previously that the epitope of FAC2 becomes exposed in oxygen-evolving membranes upon treatment with alkaline Tris which releases all four of the manganese associated with the oxygen-evolving site of photosystem II. The epitope is not exposed, however, after $CaCl_2$ treatment and exposure to low concentrations of chloride, conditions which lead to the release of two of the four manganeses associated with the oxygen-evolving site [(1987) Arch. Biochem. Biophys. 256, 295–301]. These results suggest that, upon release of the chloride-insensitive manganese from photosystem II membranes, a conformational change occurs which leads to the exposure of ^{360}Pro–^{391}Ser on CPa-1 to the monoclonal antibody FAC2.

Photosystem II; Protein, CPa-1; Monoclonal antibody; Epitope mapping

1. INTRODUCTION

At least seven proteins appear to be required for photosynthetic oxygen evolution within the photosystem II (PS II) membrane protein complex [1]. These include the 47 kDa protein CPa-1 (CP47), the 45 kDa protein CPa-2 (CP43), the 34 kDa D1 protein, the 32 kDa D2 protein, the extrinsic 33 kDa manganese-stabilizing protein, and the 9 and 4.5 kDa subunits of cytochrome *b*-559. Additionally, several low molecular mass polypeptides have recently been identified which appear to be associated with PS II [2]. At least one of these, the 4.1 kDa product of the *psb*I gene, appears to be associated with the PS II reaction center [3]. The oxygen-evolving complex also contains a number of inorganic cofactors which are absolutely necessary for water oxidation. These cofactors include bound manganese, calcium and chloride [4–6]. In vivo, the proteins D1, D2 and cytochrome *b*-559 may form the reaction center of PS II and bear as prosthetic groups the primary and secondary electron acceptors and donors of the photosystem (Y_z, P680, pheophytin, Q_A, and, probably, Q_B). D1-D2-cytochrome *b*-559 complexes have been isolated from both higher plants [7–9] and recently from cyanobacteria [10]. These complexes, however, do not contain Q_A and cannot maintain a stable charge separation. Additionally, this complex does not contain bound manganese and is non-functional with respect to oxygen evolution. The proteins which bind the inorganic cofactors involved in oxygen evolution and the extrinsic 33 kDa protein have not been unambiguously identified. Interestingly, all PS II submembrane complexes which have been isolated to date which have the ability to evolve oxygen contain the two chlorophyll-proteins CPa-1 and CPa-2.

In a previous communication [11] we described the properties of the monoclonal antibody FAC2, which recognized the chlorophyll-protein CPa-1. Interestingly, alkaline Tris-treatment, which removes the strongly bound pool of manganese associated with the oxygen-evolving site, increases the exposure of the antigenic determinant of FAC2. In this communication we have localized the putative antigenic determinant of the monoclonal antibody FAC2 to a 32 amino acid residue region located in the large extrinsic loop region of CPa-1. These findings suggest that this region of CPa-1 may interact structurally with the PS II component(s) responsible for manganese ligation.

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2. MATERIALS AND METHODS

A hybridoma cell line which secretes the monoclonal antibody FAC2 was isolated after immunization of Balb/C mice with purified PS II core [12] as previously described [13,14]. The 49 kDa apoprotein of CPa-1 was isolated by preparative electrophoresis of $CaCl_2$-washed PS II oxygen-evolving membranes isolated by the procedure of Ghanotakis and Babcock [14] using the buffer system of Delepelaire and Chua [15]. 2.0 mg Chl of the $CaCl_2$-washed membranes were separated on a 15% polyacrylamide gel with the dimensions 1.5 mm × 12 cm × 14 cm. Electrophoresis conditions were 1.0 W for 20 h at 4°C. The preparative gels were stained with acid-free Coomassie blue (0.1% Coomassie brilliant blue in 25% MeOH) for 1 h and destained with 25% MeOH. Cyanogen bromide (CNBr) treatment of the CPa-1

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apoprotein within gel fragments was carried out by the procedure of Nikodem and Fresco [15] at a CNBr concentration of 55 mg/ml for 24 h. After CNBr cleavage, the peptide fragments were separated in the electrophoretic system of Shagger and Von Jagow [17] which clearly resolves peptides of low apparent molecular mass (1–30 kDa). The 15.7 kDa CNBr fragment which bears the epitope of FAC2 (see below) was excised from these gels after staining with acid-free Coomassie blue as previously described, and electroeluted in a buffer containing 0.025 M Tris, 0.19 M glycine, pH 8.3, and 0.1% lithium dodecyl sulfate overnight at room temperature at 1 W. After electroelution, the 15.7 kDa peptide was transferred to a buffer containing 100 mM Tris-HCl, pH 6.8, and treated with 0.63 mg/ml carboxypeptidase Y (Sigma Chemical Co., catalog no. C-3888, lot no. 37F-8135) in the presence of freshly prepared phenylmethylsulfonyl fluoride (PMSF) at a concentration of 5 mM for various times at 37°C. The resulting peptides were then electrophoresed in the low molecular mass resolving gel system noted above. After electrophoresis, peptide fragments were transferred to Immobilon PVDF membrane (Millipore Co.) by electroblotting and either stained wich acid-free Coomassie blue (destained with 50% MeOH), or blocked with 5% non-fat dry milk in TS buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.2). After blocking, the blots were then probed with FAC2 ascites fluid at a 1:2000 dilution in 1% BSA in TS buffer overnight at room temperature. The blots were then developed by incubation with goat anti-mouse IgG + IgM-alkaline phosphatase conjugate (1:2000 dilution in 1% BSA in TS) and incubation with BCIP and NBT. For sequencing, Coomassie blue stained bands were excised from the Immobilon and subjected to automated Edman degradation with either an Applied Biosystems 470A or Porton PI 2090 sequencer.

3. RESULTS AND DISCUSSION

Fig.1 shows the results of CNBr cleavage of the apoprotein of CPa-1. With Coomassie blue staining, nine major CNBr fragments ranging in apparent molecular mass between 15.7 and 2 kDa are observed (fig.1). Several other minor fragments are also visible and are probably the result of incomplete cleavage. Upon probing with the monoclonal antibody FAC2, a single major CNBr fragment is observed with an apparent molecular mass of 15.7 kDa (fig.1). The N-terminal amino acid sequence of this peptide is PTFFE. Examination of the derived amino acid sequence of the spinach *psb*B gene which encodes CPa-1 [26] indicates that the predicted CNBr fragment K (^{360}Pro-^{508}Val) contains this N-terminal sequence (see table 1). The predicted mass of CNBr peptide K is 16.7 kDa which corresponds well with the observed apparent molecular mass. Hydropathy plot analysis [18] indicated that CNBr peptide K (149 amino acid residues) contains about ½ of the predicted large extrinsic loop of CPa-1 as well as the sixth transmembrane helix and the hydrophilic C-terminus of CPa-1 (see fig.3).

In an effort to define further the location of the antigenic determinant of FAC2, the 15.7 kDa CNBr-peptide was isolated by preparative electrophoresis, electroeluted, and treated with a variety of proteolytic enzymes (data not shown). The majority of these treatments either destroyed the antigenic determinant of FAC2 or produced large fragments of the 15.7 kDa peptide which were of little use for further defining the location of the antigenic determinant. Experiments with carboxypeptidase Y purchased from Sigma Chemical Co. (catalog no. C-3888, lot no. 37F-8135), however, proved to be very interesting. Preliminary experiments

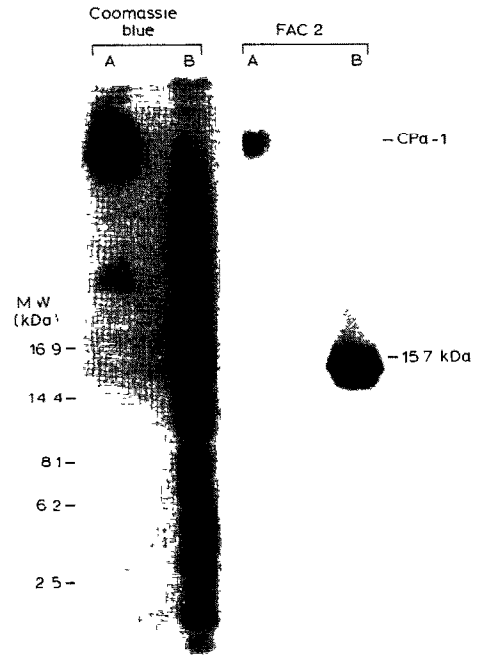

Fig.1. Western blot analysis of cyanogen bromide cleavage of CPa-1. After cyanogen bromide treatment, electrophoresis and transfer to Immobilon, lanes A (−CNBr) and B (+CNBr) were either stained with Coomassie blue or blocked and then probed with the monoclonal antibody FAC2. A single major band at 15.7 kDa which binds the monoclonal antibody is observed after cyanogen bromide treatment.

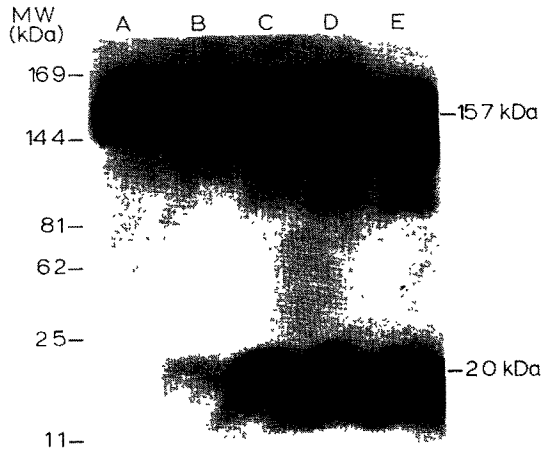

Fig.2. Western blot analysis of a timecourse of treatment of the isolated 15.7 kDa cyanogen bromide fragment with Sigma carboxypeptidase Y (catalog no. C-3888, lot no. 37F-8135) in the presence of 5 mM PMSF. After enzyme treatment and electrophoresis, the proteins were transferred to Immobilon, blocked, and probed with the monoclonal antibody FAC2. Lanes: A, 0 min incubation, no enzyme; B, 0 min + enzyme; C, 15 min + enzyme; D, 30 min + enzyme; E, 60 min + enzyme. One major fragment was observed which bound the monoclonal antibody FAC2. This is located at about 2 kDa.

Table 1

Predicted cyanogen bromide fragments from the spinach *psb*B gene product [26] (CPa-1) and sequences of isolated peptides bearing the epitope of the monoclonal antibody FAC2

Predicted cyanogen bromide fragments		
Peptide	N-terminal sequence	Molecular mass (kDa)
A	^{2}GLPWYRHTV.....^{25}M	2.7
B	^{26}HTALVAGWA.....^{37}M	1.2
C	^{38}ALYELAVFD.....^{55}M	2.0
D	^{56}WRQG^{60}M	0.7
E	^{61}FVIPF^{66}M	0.7
F	^{67}TRLGITNSW.....^{102}M	3.8
G	^{103}FSGLCFLAA.....^{231}M	14.1
H	^{232}GNIETVLSS.....^{256}M	2.5
I	^{257}WYGSATTPI.....^{330}M	8.5
J	^{331}DNGDGIAVG.....^{359}M	3.3
K	^{360}PTFFETFPV.....^{508}V	16.7

Sequences of isolated peptides which bear the epitope of FAC2		
Peptide	N-terminal sequence	Apparent molecular mass (kDa)
15.7 kDa CNBr fragment	PTFFE	15.7
2kDa endoproteinase fragment of 15.7 kDa peptide	PTFFETFPVVLIDGDGIVRADV	2.0

demonstrated that this carboxypeptidase Y preparation was contaminated with an unidentified, PMSF-insensitive endoproteinase activity. Carboxypeptidase Y obtained from Calbiochemical Co. did not exhibit this endoproteolytic activity. The results obtained from treating the 15.7 kDa CNBr fragment with Sigma carboxypeptidase Y in the presence of 5 mM PMSF are shown in fig.2. A major fragment with an apparent molecular mass of 2.0 kDa was observed which reacted with the monoclonal antibody FAC2. Upon extended incubation (data not shown) the 2.0 kDa fragment was further digested and could not be detected by either immunoprobing or Coomassie blue staining. N-terminal sequencing of the 2.0 kDa fragment yields the following sequence: PTFFETFPVVLIDGDGIVRADV, which corresponds to the N-terminal 22 amino acid residues of

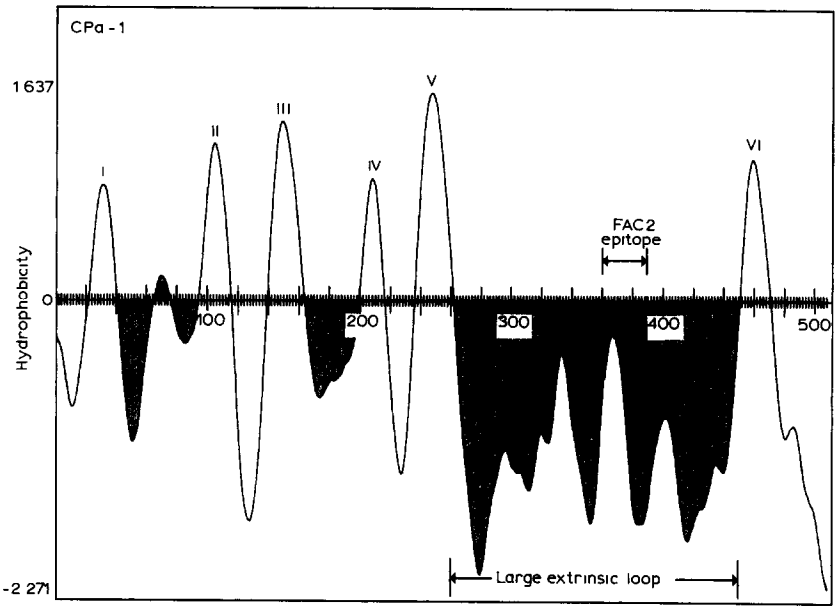

Fig.3. Hydropathy plot of the predicted amino acid sequence [26] of the *psb*B gene product, CPa-1. Rao-Argos membrane-spanning parameters [18] were used and smoothed twice over a span of seven amino acid residues. Putative membrane-spanning alpha helixes are shown by roman numerals I–VI. Hydrophilic regions which are predicted to be located on the lumenal side of the membrane by Von Heijne and Gavel analysis [25] are shaded. The location of the large extrinsic loop region of CPa-1 is indicated. The location of the putative epitope of the monoclonal antibody FAC2 is labeled and also indicated by the bold line. Both the N- and C-termini of this protein are predicted to be located on the stromal side of the membrane.

CNBr-peptide K. Because short peptides often migrate anomalously in SDS-PAGE and because of the difficulties in sequencing short peptides (primarily loss of sample Immobilon), we conservatively suggest that the epitope for FAC2 lies within the N-terminal 32 amino acid residues (^{360}Pro–^{391}Ser) of the N-terminus of the CNBr peptide K and is located entirely in the large extrinsic loop region of CPa-1. These results have been summarized in table 1.

Our results indicate that the putative epitope for the monoclonal antibody FAC2 lies within the large extrinsic loop of the apoprotein of CPa-1. In an earlier study, we had shown that this antibody not only recognizes CPa-1 on 'Western blots' but also on PS II membranes which had been attached to ELISA plates [11]. We observed that the accessibility of FAC2 to its antigenic determinant was greatly increased by alkaline Tris-treatment of PS II membranes. This treatment removes the extrinsic 33 kDa manganese-stabilizing protein as well as the four strongly bound manganeses associated with the oxygen-evolving site of PS II. The antigenic determinant was not exposed after $CaCl_2$ treatment followed by incubation of the PS II membranes in low chloride buffer. This treatment removes the manganese-stabilizing protein and leads to a loss of two of the four manganeses associated with the oxygen-evolving site. We concluded that removal of the two chloride-insensitive manganeses led to a conformational change in components associated with PS II which influenced the exposure of the antigenic determinant of FAC2 on CPa-1. This conformational change leads to the exposure of the region ^{360}Pro–^{391}Ser on CPa-1. It is tempting to speculate on the nature of this conformational change. Several lines of indirect evidence appear to indicate that the manganese associated with the oxygen-evolving site is associated with the D1 and/or D2 proteins of the PS II reaction center [19,20]. Additionally, results from our laboratory and others have strongly suggested that CPa-1 is closely associated with the extrinsic manganese-stabilizing protein. The manganese-stabilizing protein shields CPa-1 from tryptic attack [11], the presence of the manganese-stabilizing protein prevents labeling of lysyl residues on CPa-1 by NHS-biotin [21], and Cpa-1 can be crosslinked to the manganese-stabilizing protein by a number of protein crosslinkers ([21,22]; Edith Camm, personal communication). After crosslinking of CPa-1 with the manganese-stabilizing protein, the strongly bound manganese is not released by alkaline pH treatment [23]. We have suggested that CPa-1, in addition to its role as an interior chlorophyll-*a* antenna protein for PS II, may provide a binding site for the manganese-stabilizing protein [21]. Additional binding sites may be located on the D1-D2-cytochrome *b*-559 complex [24]. With these data in mind, it is easy to imagine a model in which release of the strongly bound manganese from the PS II core leads to structural changes within the D1-D2-cytochrome *b*-599 complex. These changes could lead to an increased exposure of ^{360}Pro–^{391}Ser on CPa-1 to the external environment. It should be noted that no unequivocal evidence is available demonstrating the ligation of manganese directly to the D1-D2-cytochrome *b*-599 complex. If the manganese is associated with other, as yet unidentified PS II components, then conformational changes in these other proteins upon manganese removal would lead to an exposure of the antigenic determinant of FAC2 on CPa-1.

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