Volume 259, number 1, 161-164 FEB 07945 December 1989

Lumen-side topography of the α-subunit of the chloroplast cytochrome *b*-559

G.-S. Tae and W.A. Cramer

Department of Biological Sciences, Purdue University, West Lafayette, IN 47907, USA

Received 31 October 1989

Removal of the extrinsic 33 kDa polypeptide increased the accessibility to trypsin of a COOH-terminal tridecapeptide epitope of the α subunit of cytochrome *b*-559 (*psb*E gene product). The sensitivity of the cytochrome epitope to trypsin was not measurably affected by removal of the 16 and 23 kDa extrinsic polypeptides, nor increased by removal of the OEC manganese along with the 33 kDa protein. While protecting α-cytochrome *b*-559 against trypsin, the 33 kDa protein is also proteolyzed, suggesting the possibility of an additional protein component involved in the shielding of the cytochrome. Shielding of the COOH-terminal epitope of α-cytochrome *b*-559 by the OEC 33 kDa protein implies that these COOH-terminal chains of the cytochrome are part of a protein network in the lumen space near the photosystem II reaction center. This network may contain residues that are involved in the binding of essential OEC metal ions.

Manganese; Oxygen-evolving complex; Thylakoid protein topography

1. INTRODUCTION

The photosystem II reaction center core consists of the D1, D2, and cytochrome *b*-559 α and β polypeptides [1], and at least one other small polypeptide [2]. The orientation of the D1 [3] and α-cytochrome *b*-559 [4,5] polypeptides has been determined. The NH_2- and COOH-termini of both proteins are on the stromal and lumenal sides of the membrane, respectively, the *b*-559 polypeptide spanning the membrane once and the D1 protein five times as presumed α-helices. The arrangement of peptide mass from these proteins on the lumenal side of the membrane is of interest because of the location of the site of oxygen evolution on this side. The membrane-bound oxygen-evolving complex is capped by the extrinsic 33 kDa, 23 kDa, and 17 kDa proteins [6]. Of the three extrinsic polypeptides, the 33 kDa is in closest proximity to the membrane surface, as shown by its association with PS II/OEC core particles that have high rates of O_2 evolution [7,8]. The 33 kDa protein was used as a ligand on an affinity column to purify a D1-D2/cyt *b*-559 reaction center complex [9], and can be cross-linked to the CP47 protein of the minimal O_2 evolving PS II complex [10]. Removal of all three extrinsic polypeptides by Tris resulted in increased accessibility of the D1 protein to antibody probes [3]. The binding site of the Mn involved in O_2 evolution is thought to be contained in the PS II proteins on the lumen side of the thylakoid membrane. It is shown in the present work that the COOH-terminal domain of the α-cytochrome *b*-559 subunit, containing a number of potential metal-binding amino acids, is shielded by the 33 kDa extrinsic polypeptide.

Correspondence address: W.A. Cramer, Department of Biological Sciences, Purdue University, West Lafayette, IN 47907, USA

Abbreviations: Chl, chlorophyll; Hepes, 4-12-hydroxyethyl-1-piperazine ethanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; OEC, oxygen-evolving complex; PS II, photosystem II; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

The procedures involved in preparation of PS II complex [11], exposure of membrane to trypsin, peptide synthesis, preparation of antibody, and immunoblot analysis have been described [4]. The removal of (i) the 15 and 23 kDa or (ii) 16, 23, and 33 kDa OEC extrinsic polypeptides with ($CaCl_2$ extraction) or without (Tris extraction) the OEC manganese utilized the procedures of (i) Kuwabara and Murata [12] and (ii) Seibert et al. [13], respectively.

2.1. *NaCl washing to remove extrinsic 16 and 23 kDa polypeptides*

PS II complex (0.5 mg Chl/ml) was incubated with 0.3 M sucrose, 40 mM MES-NaOH, pH 6.5, and 1 M NaCl for 30 min in the light at 4°C and centrifuged at 35 000 × *g* for 10 min. The pellet was washed and resuspended in 50 mM sucrose, 10 mM NaCl, 50 mM Hepes, pH 7.5.

2.2. *$CaCl_2$ washing to remove extrinsic 16, 23, and 33 kDa polypeptides*

PS II complex (1 mg Chl/ml) was incubated in 0.3 M sucrose, 40 mM MES-NaOH, pH 6.5, and 0.9 M $CaCl_2$ for 30 min in the dark at 4°C and centrifuged at 35 000 × *g* for 10 min. The pellet was washed and resuspended in 50 mM sucrose, 10 mM NaCl, 50 mM Hepes, pH 7.5.

2.3. *Tris washing to remove extrinsic 16, 23, 33 kDa polypeptide, and OEC manganese [13]*

PS II complex (0.5 mg Chl/ml) was incubated in 0.8 M Tris-HCl buffer, pH 8.2, and 0.3 M sucrose for 30 min in the light at 4°C and

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

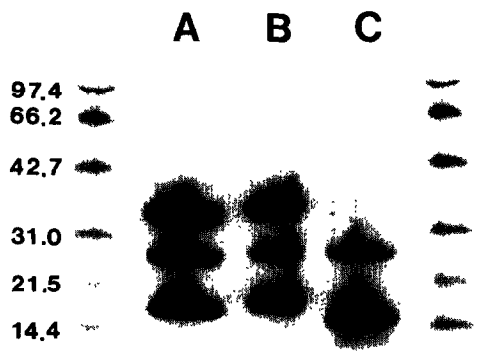

Fig.1. SDS-PAGE analysis (8–25% gradient) of extrinsic OEC polypeptides from PS II particles extracted with 0.8 M Tris-HCl, pH 8.2 (A), 0.9 M $CaCl_2$ (B), and 1 M NaCl (C). After washing, the polypeptides were dialyzed against 10 mM Hepes, pH 7.4, containing 140 mM NaCl, lyophilized, and solubilized in 1% SDS, 8 M urea, 0.14 M 2-mercaptoethanol, and 0.11% phosphoric acid. Molecular weight standards: rabbit muscle phosphorylase *b* (97 400); bovine serum albumin (66 200); hen egg white ovalbumin (42 700); bovine carbonic anhydrase B (31 000); soybean trypsin inhibitor (21 500); hen egg white lysozyme (14 400).

centrifuged at 35 000 × *g* for 10 min. The sediment was washed and resuspended in 50 mM sucrose, 10 mM NaCl, 50 mM Hepes, pH 7.5.

3. RESULTS

The 82 residue *b*-559 α-subunit in the PS II reaction center core complex is known to span the membrane once through a 26 residue hydrophobic domain, and to be oriented with the NH_2- and COOH-termini on the stromal and lumenal sides of the membrane [4]. The polar COOH-terminal half of the protein consists of 39 residues extending from Asp^{44} to Phe^{82} COOH. The accessibility of the α-cytochrome *b*-559 COOH-terminal domain on the lumen side can be probed with protease. The resulting intactness of the COOH-terminus could be assayed with an antibody to a tridecapeptide epitope extending from Arg^{68} to Arg^{80} [4]. Two effects of trypsin on the α-polypeptide could be discerned in previous studies [4]. From the stromal side, trypsin cleaved after Arg^7, generating a proteolysis product slightly smaller ($\Delta M_r \approx -750$) than the parental molecule (confirmed by sequencing, A. Trebst, pers. comm.). From the lumen side, trypsin removed the COOH-terminal tridecapeptide epitope. The effect of the OEC extrinsic polypeptides in shielding the α-polypeptide COOH-terminal domain from trypsin can be tested by removing (i) the extrinsic 16 and 23 kDa proteins, (ii) the three extrinsic OEC polypeptides, or (iii) the three extrinsic polypeptides together with the OEC manganese by incubation in 1.0 M NaCl [13], 0.9 M $CaCl_2$ [14], or 0.8 M Tris-HCl [14], respectively. The extrinsic polypeptides extracted by these procedures are shown in lanes C, B, and A, respectively, of fig.1. O_2 evolution could be restored to a level of 74% by addition of 15 mM $CaCl_2$ to the membranes from which the 16 and 23 kDa polypeptides were removed (initial rate of O_2 evolution in control and NaCl-treated sample, 945 and 244 μmol O_2/mg Chl per h, respectively). The effect of trypsin incubation for 15, 30, and 60 min on the integrity of the *b*-559 α-subunit is shown in PS II-membranes containing all OEC polypeptides (fig.2, lanes B–D), membranes depleted in the 16, 23 kDa proteins (fig.2, lanes F–H), and membranes from which the 16, 23, and 33 kDa polypeptides have been removed (fig.2, lanes J–L), together with most of the OEC manganese (fig.2, lanes N–P). Almost complete cleavage near the NH_2-terminus of the *b*-559 α-subunit is seen in the control, but most of the COOH-terminal epitope is shielded from trypsin incubated for 15 and 30 min. Depleting the membranes of the 16 and 23 kDa proteins hardly affects the accessibility to the protease. However, removal of the extrinsic 33 kDa protein greatly increased the accessibility and/or sensitivity to trypsin, and the additional removal of the manganese appeared to have no additional effect. It was concluded that the 33 kDa extrinsic protein, and not the 16 and 23 kDa proteins, is

Fig.2. Effect of removal of OEC extrinsic polypeptides on the accessibility to trypsin of the COOH-terminus of α-cytochrome *b*-559. Untreated (A–D), NaCl-washed (E–H), $CaCl_2$-washed (I–L), and Tris-washed (M–P) PS II complexes were treated with trypsin (trypsin : Chl = 1 : 20) for 15 (B, F, J. and N), 30 (C, G, K, and O), and 60 min (lanes D, H, L, and P). Each lane of the 18% double cross-linked SDS gel containing 4 M urea was loaded with the membrane equivalent of 10 μg Chl. Chl *a*/*b* ratios of untreated NaCl-washed, $CaCl_2$-washed and Tris-washed PS II complex were 1.98, 1.98, 1.97, and 1.97, respectively. Polypeptides were transferred to a nitrocellulose filter (Hybond-C) with semi-dry transfer blotter (130 mA, constant current, 20 min).

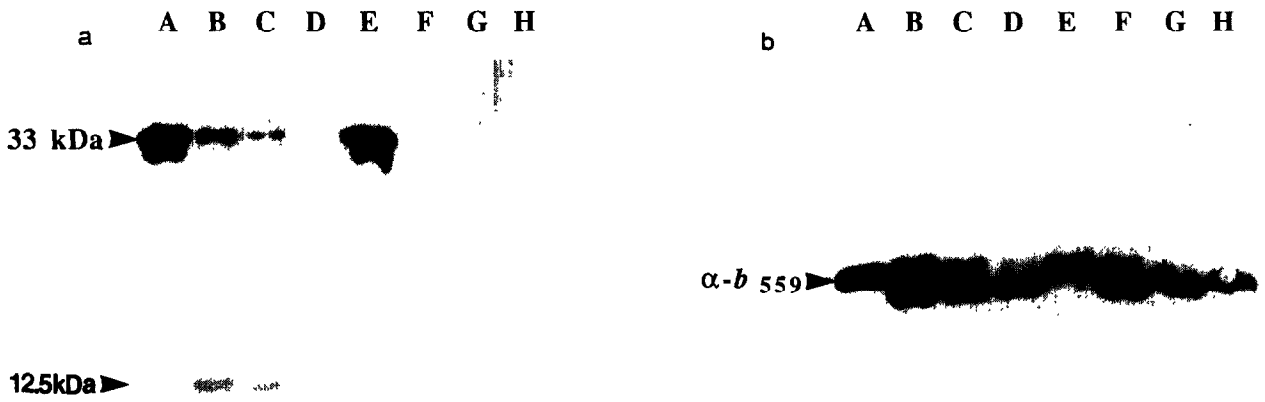

Fig.3. Effect of removal of 16 kDa and 23 kDa extrinsic polypeptides on the accessibility to trypsin of (a) the extrinsic 33 kDa polypeptide and (b) α-cytochrome *b*-559. Untreated (A–D) and NaCl-washed (lanes E–H) PS II complex. Control lanes (A and E); trypsinolysis for 15 (lanes B, F), 30 (lanes C, G), and 60 min (lanes D, H). Lanes were loaded with the equivalent of 10 μg Chl.

part of a proteinaceous shield of the COOH-terminal domain of α-cytochrome *b*-559.

The effect of trypsin on the integrity of the 33 kDa polypeptide was examined while it acts as a shield of cytochrome *b*-559 (fig.3a). Although the presence of the 33 kDa protein shielded the α-subunit, the 33 kDa was itself almost completely degraded in control membranes (fig.3a, lanes B–D), and was completely degraded in the 15 min incubation with trypsin in membranes depleted of the 16 and 23 kDa proteins (fig.3a, lanes F–H). As part of the same experiment, it was determined that the COOH-terminal domain of the α-subunit was far more resistant to trypsin and essentially intact except for the longest trypsin incubation time with membranes depleted of the 16 and 23 kDa polypeptides (fig.3b). The 16 kDa protein was completely degraded when the control membranes were exposed to trypsin for the shortest (15 min) of the trypsin incubation times used in these experiments (data not shown).

The shielding of the *b*-559 α-subunit in the presence of trypsin could be a result of peptide fragments of the 33 kDa protein that are generated by the trypsinolysis. Peptide fragments of M_r 12 500 can be seen in lanes B–D of fig.3a. Alternatively, the shielding of cytochrome *b*-559 in the presence of the 33 kDa protein may be due to the presence of another PS II protein or peptide that is also shielded by the 33 kDa protein. The latter explanation is suggested by the resistance of the α-subunit COOH-terminus to trypsin when the 33 kDa protein is thoroughly fragmented by the protease.

4. DISCUSSION

The binding of the OEC manganese may involve residues of D1–D2 that are in the membrane bilayer or in interhelix loops I–II and III–IV [11], and/or lumen-side domains of other PS II proteins such as cytochrome *b*-559 and CP43/47. The present data imply that the α-cytochrome *b*-559 COOH-terminal domain, containing as many as 39 residues (spinach protein), is in close proximity to the extrinsic 33 kDa protein and possibly other protein components of the O_2-evolving PS II core. Of the residues in the COOH-terminal domain that could be involved in the binding of manganese or calcium, the following are conserved in the α-cytochrome *b*-559 sequence deduced from the nucleotide sequence in three plants and two cyanobacteria (summarized in [5]): Asp^{44}, Ser/Thr^{48}, Asn/Asp^{52}, Glu^{53}, Thr^{56}, Glu/Gln^{57}, Gln^{60}, and Asp/Ser^{70} (numbering from sequence of spinach protein). All residues except the last pair are well upstream of the minimum cleavage event, at Arg^{68}, that would result in loss of the COOH-terminal epitope. Therefore, the absence of an effect of manganese removal on the sensitivity to trypsin does not imply anything about a role of these residues in ligation of manganese. Positively charged residues that could be involved in the binding of Cl^- are Arg^{50}, Arg^{59}, and Arg^{68}, conserved in all five sequences. The residues after position 72 are not considered because this position is the COOH-terminus in *C. paradoxa*. The β-subunit of cytochrome *b*-559 is

unlikely to contribute to ligation on the lumen side because preliminary data indicate that it is also oriented with its NH_2-terminus on the stromal side of the membrane (Tae and Cramer, unpublished), and the length of its COOH-terminus exposed on the lumen side is then expected to be very short [15]. If photosystem II contains two copies of cytochrome *b*-559, for which there is substantial (summarized in [4,16]), but not universal [17] agreement, then there are two copies of the α-cytochrome *b*-559 COOH-terminal domain and of the above residues on the lumen side of the thylakoid membrane.

Acknowledgements: We thank C.F. Yocum for helpful discussions; B. Andersson, and R. Burnap and L.A. Sherman for antibody to the OEC 16 and 33 kDa polypeptides, respectively, and Janet Hollister for patient and precise collaboration on the manuscript. This research was supported by NIH GM-38323.

REFERENCES

[1] Nanba, O. and Satoh, K. (1987) Proc. Natl. Acad. Sci. USA 84, 109–112.
[2] Ikeuchi, M. and Inoue, Y. (1988) FEBS Lett. 241, 99–104.
[3] Sayre, R.T., Andersson, B. and Bogorad, L. (1986) Cell 47, 601–608.
[4] Tae, G.-S., Black, M.T., Cramer, W.A., Vallon, O. and Bogorad, L. (1988) Biochemistry 27, 9075–9080.
[5] Vallon, O., Tae, G.-S., Cramer, W.A., Simpson, D., Hoyer-Hansen, G. and Bogorad, L. (1989) Biochim. Biophys. Acta 975, 132–141.
[6] Åkerlund, H.E., Jansson, C. and Andersson, B. (1982) Biochim. Biophys. Acta 681, 1–10.
[7] Ghanotakis, D.F., Demetriou, D.M. and Yocum, C.F. (1987) Biochim. Biophys. Acta 891, 15–21.
[8] Babcock, G.T. (1987) in: Photosynthesis (Amesz, J. ed.) pp. 125–158, Elsevier, Amsterdam.
[9] Gounaris, K., Chapman, D.J. and Barber, J. (1988) FEBS Lett. 234, 374–378.
[10] Bricker, T.M., Odom, W.R. and Queirolo, C.B. (1988) FEBS Lett. 231, 111–117.
[11] Kuwabara, T. and Murata, N. (1982) Plant Cell Physiol. 23, 533–539.
[12] Kuwabara, T. and Murata, N. (1983) Plant Cell Physiol. 24, 741–747.
[13] Seibert, M., Dewit, M. and Staehelin, A. (1987) J. Cell Biol. 105, 2257–2265.
[14] Trebst, A. (1986) Z. Naturforsch. 41c, 240–245.
[15] Cramer, W.A., Theg, S.M. and Widger, W.R. (1986) Photosyn. Res. 10, 393–403.
[16] Dekker, J.P., Bowlby, N.R. and Yocum, C.F. (1989) FEBS Lett., in press.
[17] Miyazaki, A., Shina, T., Toyoshima, Y., Gounaris, K. and Barber, J. (1989) Biochim. Biophys. Acta 975, 142–147.