Characterization of the Light-induced Cross-linking of the \( \alpha \)-Subunit of Cytochrome \( b_{559} \) and the D1 Protein in Isolated Photosystem II Reaction Centers*

(Received for publication, May 22, 1995, and in revised form, July 18, 1995)

Roberto Barbato, Giulia Friso, Markella Ponticos, and James Barber§

From the Photosynthesis Research Group, Biochemistry Department, Wolfson Laboratories, Imperial College of Science, Technology & Medicine, London SW7 2AY, United Kingdom

Illumination of the isolated reaction center of photosystem II generates a protein of 41 kDa molecular mass. Using immunoblotting, it is confirmed that the protein is an adduct of the D1 protein and the \( \alpha \)-subunit of cytochrome \( b_{559} \). Its formation seems to be photochemically induced, being independent of temperature between 4 and 20 \( ^\circ \)C and unaffected by a mixture of protease inhibitors. The maximum levels are detected when the pH is in the region 6.5-8.5 and when illumination intensities are moderate. Although higher light intensities induce a higher rate of formation, the accumulation of the adduct is not affected due to light-induced degradation. This degradation is also affected by the presence of protease inhibitors. Proteolytic mapping and N-terminal sequencing indicates that the cross-linking process involves the N-terminal serine of the \( \alpha \)-subunit of cytochrome \( b_{559} \) and D1 residues in the 239-244 FGQEE motif close to the QB binding site. In conclusion, the results indicate that the \( \alpha \)-subunit of cytochrome \( b_{559} \) and D1 protein are a consequence of the vulnerability of PSII to photodamage and that this effect is the basis of the physiological phenomenon of photoinhibition (12, 13).

Photocross-linking of the reaction center proteins, D1 and D2, have been generated using 23-kDa N-terminal fragments of isolated PSII reaction centers, it was shown that a 23-kDa N-terminal fragment of the D1 protein is observed when they are illuminated under aerobic conditions in the absence of exogenous electron acceptor (17). Under such conditions, recombination occurs between the radical pair state consisting of the oxidized primary electron donor (P680) and reduced primary electron acceptor pheophytin \( a \) (pheo \( a \)). This recombination forms a triplet state of P680 (18), which is able to generate singlet oxygen (19, 20). It is likely, therefore, that this highly reactive form of oxygen causes damage to the D1 protein. The damage itself does not seem to be directly involved in the cleavage process but triggers the D1 protein for degradation (21) possibly via a conformational change (22). Because this isolated complex consists of only the D1 and D2 proteins, the \( \alpha \)- and \( \beta \)-subunits of cytochrome \( b_{559} \) (Cyt \( b_{559} \)) and the product of the psbI gene (9, 23), it has been concluded that the mechanism of cleavage is contained within the reaction center and that no external proteases are involved. For this reason, we have undertaken a study to elucidate the nature of the autocatalytic cleavage, which generates the 23-kDa N-terminal fragments. Previous studies have indicated that the cleavage occurs on the C-terminal side of residue 238 in the D1 sequence (14, 24). As emphasized by Greenberg et al. (14), this cleavage site is lo-
lated in the loop joining putative transmembrane segments IV and V, which is in the vicinity of the Qb binding region (16).

The investigation that we report here was stimulated by the observation that in addition to the appearance of the light-induced 23-kDa N-terminal fragment, a band was induced at about 41 kDa when isolated reaction centers were exposed to strong illumination. This band was reported by Shipton and Barber (25) and subsequently shown, by immunological blotting, to consist of D1 protein and the α-subunit of Cyt b559 (26).

Here, we further characterize the properties of this D1 protein/α-subunit Cyt b559 adduct and consider how the formation of a covalent linkage between these two reaction center subunits could be involved in events leading to D1 protein cleavage and the generation of the 23-kDa N-terminal fragment.

MATERIALS AND METHODS

Isolation of PSII Reaction Centers and Irradiation Conditions—Photosystem II reaction center (RCII) complexes were isolated from pea and wheat chloroplast membranes as described by Namba and Satoh (9) with modifications described by Chapman et al. (27). Irradiation of RCII complexes was performed, if not otherwise stated, in a thermostatic cuvette at 4°C at a chlorophyll concentration of 50 μg ml⁻¹, using a Flexilux 650 lamp able to give fluence rates up to 2000 μmol m⁻² s⁻¹. Irradiation was carried out in 50 mM Tris-HCl, pH 8.0, containing 2 mM n-dodecyl β-D-maltoside. For pH-dependence experiments, Bis-Tris propan, MES, and Tris were used as buffer systems.

SDS-PAGE and Limited Proteolysis—SDS-PAGE in the presence of 6 M urea was carried out as described by Barbotto et al. (28) using a 12–18% linear acrylamide gradient. The stacking gel did not contain urea. Gels were stained with acid-free Coomassie Brilliant Blue R-250 in 40% methanol and 1% acetic acid and destained in 40% methanol. The investigation that we report here was stimulated by the observation that in addition to the appearance of the light-induced 23-kDa N-terminal fragment, a band was induced at about 41 kDa when isolated reaction centers were exposed to strong illumination. This band was reported by Shipton and Barber (25) and subsequently shown, by immunological blotting, to consist of D1 protein and the α-subunit of Cyt b559 (26).

Here, we further characterize the properties of this D1 protein/α-subunit Cyt b559 adduct and consider how the formation of a covalent linkage between these two reaction center subunits could be involved in events leading to D1 protein cleavage and the generation of the 23-kDa N-terminal fragment.

MATERIALS AND METHODS

Isolation of PSII Reaction Centers and Irradiation Conditions—Photosystem II reaction center (RCII) complexes were isolated from pea and wheat chloroplast membranes as described by Namba and Satoh (9) with modifications described by Chapman et al. (27). Irradiation of RCII complexes was performed, if not otherwise stated, in a thermostatic cuvette at 4°C at a chlorophyll concentration of 50 μg ml⁻¹, using a Flexilux 650 lamp able to give fluence rates up to 2000 μmol m⁻² s⁻¹. Irradiation was carried out in 50 mM Tris-HCl, pH 8.0, containing 2 mM n-dodecyl β-D-maltoside. For pH-dependence experiments, Bis-Tris propan, MES, and Tris were used as buffer systems.

SDS-PAGE and Limited Proteolysis—SDS-PAGE in the presence of 6 M urea was carried out as described by Barbotto et al. (28) using a 12–18% linear acrylamide gradient. The stacking gel did not contain urea. Gels were stained with acid-free Coomassie Brilliant Blue R-250 in 40% methanol and 1% acetic acid and destained in 40% methanol. The investigation that we report here was stimulated by the observation that in addition to the appearance of the light-induced 23-kDa N-terminal fragment, a band was induced at about 41 kDa when isolated reaction centers were exposed to strong illumination. This band was reported by Shipton and Barber (25) and subsequently shown, by immunological blotting, to consist of D1 protein and the α-subunit of Cyt b559 (26).

Here, we further characterize the properties of this D1 protein/α-subunit Cyt b559 adduct and consider how the formation of a covalent linkage between these two reaction center subunits could be involved in events leading to D1 protein cleavage and the generation of the 23-kDa N-terminal fragment.
subunit (compare lanes 2C and 4C in Fig. 2). Therefore, the above experiments show unequivocally that the site for light-induced cross-linkage is located at an amino acid residue lying on the C-terminal side of lysine-238 of the D1 protein.

V8 Proteolysis Indicates That the Cross-linking Site Is Located on the N-terminal Side of Residue 244—A second series of experiments were conducted using Staphylococcus aureus V8 protease. Fig. 3 shows the effect of treatment of the C-subunit of Cytb559 with two concentrations of this enzyme. In both cases, a main digestion band (SaCyta) was detected, by either Coomassie staining (panel A) or immunoblotting with anti-Cyta (panel B), which had a molecular mass of about 1 kDa lower than the native band. N-terminal sequencing of both bands (see Table I) gave the same sequence, indicating that the V8-induced proteolytic cleavage had occurred toward the C-terminal end of the C-subunit.

The effect of V8 proteolysis of the isolated D1 protein and the 41-kDa adduct was also investigated, as shown in Fig. 4. Immunoblotting with anti-D1C antiserum (panel A) showed that the digestion of D1 with V8 (lane 2) resulted in the appearance of two or, more often, three bands in the range of 8–10 kDa. These bands probably correspond to Sa8 and Sa10 doublet described by Marder et al. (37) and Greenberg et al. (14). As these bands were recognized by the C-terminal specific antibody (raised to the last 19 amino acid residues of the protein), they must contain the C terminus of D1. In particular, it was found by N-terminal sequencing (see Table I) that the Sa8 fragment is derived from a cleavage at glutamate 244, as suggested previously by Marder et al. (37). In the case of V8 proteolysis of the 41-kDa adduct (lane 4 of panel A), the anti-D1C antibody recognizes three fragments in the range of 15–20 kDa and a 8-kDa fragment. As this last fragment has the same electrophoretic mobility and immunological reactivity as the Sa8 fragment from D1, it is reasonable to assume that they are the same proteolytic fragment (i.e. from 245 to the C terminus of the D1 protein (see Fig. 9)).

When anti-Cyta was used to probe V8 proteolytic digestion products of the 41-kDa adduct (panel B, lane 1), a number of bands were detected in the 15–20-kDa range. Of note is that two of them are the same as those detected by the anti-D1C antibody (marked by circles in lane 4 of panel A and lane 1 of panel B). Since the Sa8 fragment was detected after treatment of the 41-kDa adduct with V8, it seems likely that cross-linking of the C-subunit and the D1 protein occurs on the C-terminal side of residue 244. This conclusion, taken together with the result derived from the Lys-C experiments (Fig. 2), suggests that the linkage takes place between 239 and 244 of D1 protein, i.e. in the FGQEE motif. Detection of the V8-induced doublet of the 41-kDa band by both anti-D1C and anti-Cyta indicates that these contain the branched peptide formed by the C-sub-

### Table I

| N-terminal sequencing of some Lys-C and Sa8 fragments from D1 and cytoa |
|--------------------------|--------------------------|
| 41 kDa D1-cytoa adduct   | No sequence              |
| Lys-C-D1C                | FGQEE TYNIVAAH           |
| Sa8-D1C                  | SGSTGERSFADII            |
| cytoa                    | SGSTGERSF                |
| Sa8-cytoa                | SGSTGERSF                |

---

**Fig. 2.** Immunoblotting with antibodies anti-D1N (panel A), anti-D1C (panel B), and anti-cytoa (panel C) of undigested 41 kDa-band (lanes 1A, 1B, and 1C), undigested D1 (lanes 2A and 2B), undigested cytoa (lane 2C), Lys-C-digested 41-kDa band (lanes 3A, 3B, and 3C), Lys-C-digested D1C (lanes 4A and 4B), and Lys-C-digested cytoa (lane 4C). Wheat RCII complexes were irradiated at a chlorophyll concentration of 50 μg/ml for 15 min with 600 μmol m-2 s-1 white light to induce the formation of the 41-kDa adduct. After SDS-PAGE and acid-free Coomassie staining, the corresponding band was cut out from the gel and loaded on the stacker of a second identical gel. For proteolysis, acrylamide bands were overlaid with 20 μl of Lys-C (10 units/ml), and electrophoresis was started. When the tracking dye reached the bottom of the stacking gel, power was switched off for 3 h to allow proteolysis; gels were then run as usual. See Table I for N-terminal sequence of the C-terminal fragment of D1 induced by Lys-C treatment (arrow on lane 4B). Bands marked with a circle in lanes 2C and 4C are aggregation products of cytoa. Arrows on lanes 3B and 3C emphasize the identity of the fragments detected by anti-D1C and anti-cytoa from Lys-C proteolysis of the 41 kDa. Arrow on lane 3A marks the N-terminal fragment from Lys-C proteolysis of the 41-kDa adduct and D1, which represents the 2-238 segment of the protein.

**Fig. 3.** Limited proteolysis of cytoa with V8 protease. A. Coomassie staining; B, immunoblotting with anti-cytoa, antiserum. Lane 1, no protease was added. Lanes 2 and 3 contain, respectively, 1.0 and 5.0 μg of protease. N-terminal sequencing of cytoa and Sa-cytoa is reported in Table I.

**Fig. 4.** Immunoblotting with antibodies anti-D1C (panel A), anti-cytoa (panel B), and anti-D1N (panel C) of undigested D1 (lanes 1A and 1C), undigested 41 kDa (lanes 3A and 3C), undigested cytoa (lane 3B), digested D1 (lanes 2A and 3B), digested 41 kDa (lanes 1B), and digested cytoa (lane 2B). RCII complexes were treated as described in the legend to Fig. 1. Bands were isolated by SDS-PAGE and subjected to proteolysis for 1 h using 1 μg of V8 protease.
pattern is observed, confirming that the adduct is also severely inhibited. In contrast, the light-
induced 41-kDa band is readily detected at pH values 6.5, 7.5, and 8.5. When a sample that had been irradiated at pH 7.5 (i.e. containing the 41-kDa band) was incubated in the dark at pH 5.5 or 9.5, no effect on the level of the adduct was noted, an observation that rules out the possibility that the band is not observed in Fig. 7 because of its instability toward pH 5.5 or 9.5.

**DISCUSSION**

The immunological blotting data presented above are consistent with the previous conclusion that the 41-kDa band generated by illuminating isolated PSII RCs is a cross-linked adduct of the D1 protein and the α-subunit of Cyt b_{559}. Treatment of this adduct, isolated from illuminated RCIs of wheat with Lys-C, generated a 21-kDa fragment that consisted of the C-terminal portion of the D1 protein and the α-subunit of Cyt b_{559}. Thus, the cross-linking occurs on the C-terminal side of residue 238 of the D1 protein. The C-terminal portion of the D1 protein (from 239 to 344) was found to have an apparent molecular mass of about 10 kDa, while the α-subunit of Cyt b_{559} has about the same mass, thus accounting for the observed 21-kDa value for the Lys-C-induced fragment of the adduct.

Experiments using *S. aureus* V8 protease further suggest that the cross-linking site is on the N-terminal side of the tyrosine residue 245 of the D1 protein. This conclusion is based on the fact that the SaV8 fragment formed due to V8 cleavage at glutamate 244 on the D1 protein is also generated when the 41-kDa adduct is digested with this enzyme (e.g. Fig. 3, lane A4). Thus, these results lead to the overall conclusion that the adduct is formed by a cross-linking of the α-subunit of Cyt b_{559} with a residue in the 239–244 motif FGQEEE (see Fig. 9).

The N terminus of the α-subunit of Cyt b_{559} is not blocked and can be sequenced by Edman degradation as indicated in Table I. In contrast, the N terminus of the D1 protein is blocked and cannot be readily sequenced by the Edman procedure.

**FIG. 8.** The effect of different pH values on the formation of the 41-kDa adduct. RCII complexes were diluted to 20 μg of chlorophyll/ml in 0.1 M Bis-Tris propane at the indicated pH value. 2 mM n-dodecyl β-D-maltoside was also added. Irradiation was carried out at 4°C with 600 μmol quanta m^{-2} s^{-1} for the indicated period of time. Each gel lane contained 0.2 μg of chlorophyll. Lane M contains prestained molecular markers. The blot was probed with anti-cytosin serum.

**FIG. 9.** Representation of the proposed cross-linkage site between the D1 protein and the cytochrome b_{559} α-subunit, resulting in the generation of the 41-kDa light-induced band. The N-terminal serine residue of the cytochrome b_{559} α-subunit seems to cross-link with the FGQEEE motif on the D1 protein (shaded).
have also found that the 41-kDa adduct is not amenable to N-terminal sequencing using Edman degradation. The reason for this is unclear, but it could be that the N terminus of the α-subunit is not available in the adduct as it is in the free protein. This therefore suggests that the cross-linking to the FGQEEE motif involves the N-terminal residue of the α-subunit, which is a serine. The action of V8 on the 41-kDa adduct is also consistent with this since the products of this proteolysis did not indicate an attachment of the C-terminal end of the α-subunit to the D1 protein where the Cyt subunit has a V8 cleavage site (e.g. Fig. 4C).

The identified cross-linking site on the D1 protein has the unusual feature of having three glutamic acid residues adjacent to each other. Such a motif is not typical and occurs in only a few proteins (24). The pKa value for a single glutamic acid residue is 4.3 and thus at neutral pH would be unprotonated. However, the existence of three adjacent carboxyl groups of glutamic acid would be expected to significantly shift the pKa to a higher value so that protonation would occur at neutral pH. This shift of pKa could aid a condensation reaction with the N-terminal serine of the α-subunit, thus forming a peptide linkage. Such a reaction is endothermic and would not be expected to happen spontaneously. Indeed, the cross-linking is induced by illumination. The mechanism involved does not seem to be enzymic (lack of effect of protease inhibitors and temperature) but involves a physically induced reaction driven by the absorption of light energy. Moreover, we have also shown that oxygen needs to be present for the adduct to appear. It is difficult to perceive how the proposed condensation reaction is driven. Under anaerobic conditions the heme of Cyt b559 becomes photoreduced, but no such reaction occurs when oxygen is present (38). It is conceivable that the formation of the adduct is dependent on the redox state of the heme, which affects the conformation of the α-subunit. Alternatively, light-induced oxidation processes could aid the cross-linking.

Although the precise mechanism for the formation of this adduct is not clear, the identification of the linkage site has at least two important implications. The first is that the N terminus of the α-subunit must be exposed on the stromal side of PSII, as suggested by Tae et al. (39) and Vallon et al. (40). Moreover, our data indicate that Cyt b559 may be more closely linked to the histidine residues 22 and 17 on the α-subunit itself in line with other observations (41). However, further experiments are required to give credence to this idea. Among the experiments to be carried out will be those that identify whether the linkage and cleavage processes involve the same specific residues.

Acknowledgment—We thank Ian Blench for conducting the N-terminal sequencing.

REFERENCES

1. Debus, R. (1992) Biochim. Biophys. Acta 1102, 269–282
2. Anderson, B., and Franzen, L.-G. (1992) in Molecular Mechanisms in Bioenergetics (Ernst, L., ed.) pp. 121–143, Elsevier Publishers, Amsterdam, The Netherlands
3. Mi, M., and de Haas, I. (1988) Biochemistry 27, 1–7
4. Deisenhofer, J., P., Epp, O., Miki, K., Huber, R., and Michel, H. (1984) J. Mol. Biol. 180, 385–395
5. Deisenhofer, J., P., Epp, O., Miki, K., Huber, R., and Michel, H. (1985) Nature (Lond.) 315, 618–624
6. Trebst, A. (1987) Z. Naturforsch. 42, 742–750
7. Barber, J. (1987) Trends Biochem. Sci. 12, 321–326
8. Ruffner, S. V., Donnelly, M., Blundell, T. L., and Nugent, J. H. A. (1992) Photosyn. Res. 34, 287–300
9. Namba, O., and Satoh, K. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 109–112
10. Barber, J., Chapman, D. J., and Teffer, A. (1987) FEBS Lett. 220, 67–72
11. Mattoh, A. K., Pick, U., Hoffman-Falk, H., and Edelmann, M. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 1572–1576
12. Barber, J., and Anderson, B. (1992) Trends Biochem. Sci. 17, 61–66
13. Pradet, A., Adir, N., and Ophir, J. (1992) in Topics in Photosynthesis (Barber, J., ed) Vol. 11, pp. 220–250, Elsevier, Amsterdam, The Netherlands
14. Greenberg, B. M., Gaba, V., Mattoh, A.-K., and Edelmann, M. (1987) EMBO J. 6, 2865–2869
15. Salter, A. H., Vigny, I., Hageman, A., and Anderson, B. (1992) Biochemistry 31, 3990–3998
16. De las Rivas, J., Andersson, B., and Barber, J. (1992) FEBS Lett. 301, 241–242
17. De las Rivas, J., Shipton, C. A., Ponticco, M., and Barber, J. (1993) Biochemistry 32, 6944–6950
18. Durrant, R. J., Giorgi, L. B., Barber, J., Klug, D. R., and Porter, G. (1990) Biochim. Biophys. Acta 1017, 167–175
19. Teffer, A., Bishop, S. M., Phillips, D., and Barber, J. (1994) J. Biol. Chem. 269, 13422–13425
20. Teffer, A., Dhami, S., Bishop, S. M., Phillips, D., and Barber, J. (1994) Biochemistry 33, 14469–14474
21. Aro, E.-M., Hvidal, T., Carlberg, I., and Andersson, B. (1990) Biochim. Biophys. Acta 1019, 269–275
22. He, W.-Z., Newell, W. R., Haris, P. I., Chapman, D., and Barber, J. (1991) Biochemistry 30, 4552–4559
23. Weber, A., Packman, L., Chapman, D. J., Barber, J., and Gray, J. C. (1989) FEBS Lett. 242, 259–262
24. Shipton, C. A., Marder, J. B., and Barber, J. (1990) Z. Naturforsch. 45, 765–771
25. Shipton, C. A., and Barber, J. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 6591–6595
26. Barbato, R., Grisi, F., Rigoni, F., Frizzo, A., and Giacomelli, G. M. (1992) FEBS Lett. 309, 165–169
27. Chapman, D. J., Gounaris, K., and Barber, J. (1991) in Methods in Plant Biochemistry (Rogers, L. J., ed) Vol. 5, pp. 171–193, Academic Press, London
28. Barbato, R., Shipton, C. A., Giacomelli, G. M., and Barber, J. (1991) FEBS Lett. 290, 162–166
29. Cleveland, D. W., Fischer, S. G., Kirscher, M. W., and Laemmli, U. (1977) J. Biol. Chem. 252, 1102–1106
30. Dunn, S. D. (1986) Anal. Biochem. 157, 144–153
31. Matsuoka, T. (1987) J. Biol. Chem. 262, 10035–10038
32. Barbato, R., Grisi, F., Giard, M. T., Rigoni, F., and Giacomelli, G. M. (1991) Biochemistry 30, 10220–10226
33. Arnon, D. I. (1949) Plant Physiol. 24, 1–15
34. Frizzo, A., Giacomelli, G. M., Barber, J., and Barbato, R. (1993) Biochim. Biophys. Acta 1184, 265–270
35. Teffer, A., Hird, S. M., Packman, L., Dyer, T. A., and Gray, J. C. (1989) Plant Mol. Biol. 12, 141–151
36. Marder, J. B., Teffer, A., and Barber, J. (1988) Biochim. Biophys. Acta 932, 362–365
37. Marder, J. B., Goloubinoff, P., and Edelmann, M. (1984) J. Biol. Chem. 259, 3900–3908
38. Barber, J., and De las Rivas, J. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 10942–10946
39. Tae, G.-S., Black, M. T., Cramer, W. A., Vallon, O., and Bogorad, L. (1998) Biochemistry 37, 9075–9080
40. Vallon, O., Tae, G.-S., Cramer, W. A., Simpson, D., Hoyer-Hansen, G., and Bogorad, L. (1989) Biochim. Biophys. Acta 975, 132–141
41. Chapman, D. J., Gounaris, K., and Barber, J. (1989) Photosynthetica 23, 411–426

Protein Cross-linking in Photosystem II

24037