ELSEVIER

Biochimica et Biophysica Acta 1187 (1994) 296–300

BBA Biochimica et Biophysica Acta

Exposure of a Photosystem II complex to chemically generated singlet oxygen results in D1 fragments similar to the ones observed during aerobic photoinhibition

Neelam P. Mishra, Demetrios F. Ghanotakis *

Department of Chemistry, University of Crete, P.O. Box 1470, 71409 Iraklion, Crete, Greece and Institute of Molecular Biology and Biotechnology, FORTH, Iraklion, Crete, Greece

Received 23 February 1994

Abstract

Exposure of a PS II core complex to singlet oxygen, generated either chemically in the dark or through the photodynamic action of rose bengal, caused a rapid degradation of D1 protein. Photoinhibitory illumination of the PS II complex, either at 25°C or at 4°C, resulted in D1 protein fragments similar to those observed upon exposure of the PS II complex to singlet oxygen. Histidine, a singlet oxygen scavenger, provided a significant protection of D1 protein against degradation. We therefore suggest that singlet oxygen, which is generated during acceptor-side induced photoinhibition, is responsible for the in vitro fragmentation of D1 protein.

Key words: D1 protein degradation; Photoinhibition; Photosystem II complex; Singlet oxygen

1. Introduction

The D1 polypeptide of Photosystem II (PS II) turns over rapidly in the light [1,2]. This property of D1 protein has often been linked to the phenomenon of photoinhibition [3–5]. Photoinhibition of photosynthetic activity leads to the inactivation of electron transport in PS II and subsequently to degradation of the D1 reaction center protein (for reviews see Refs. [6,7]). This damage is rectified by a repair mechanism involving synthesis and incorporation of new D1 protein into the complex [8,9]. Over the last few years, a number of studies have approached the problem of D1 protein damage and degradation in vitro. Although the molecular mechanism of light-induced degradation of D1 protein is not yet established, it is generally described as a two-step reaction [10,11]. The oxidative damage of the PS II reaction center induces conformational changes which lead to the degradation of D1 protein [12]. The majority of experimental evidence favours an all-enzymatic digestion of D1 protein, particularly in connection with acceptor-side induced photoinhibition. The temperature dependency of light-induced D1 degradation [10,12] has been taken as evidence that the in vitro D1 fragmentation is of an enzymatic nature rather than a direct light-induced chemical peptide cleavage. Moreover, it has been shown that D1 degradation in vitro can be blocked by inhibitors acting against serine proteinases [13–15].

On the other hand, there is experimental evidence that reactive oxygen species are involved in the irreversible oxidative damage of D1 protein [11,16–19]. By using various oxygen radical scavengers (superoxide dismutase, catalase, glutathione, ascorbate or propylgallate) a partial protection of the D1 polypeptide against degradation has been demonstrated [11,17,20]. Toxic oxygen species are known to cause a variety of structural changes to proteins, including direct fragmentation and cross-linking [21–23]. In the presence of

Abbreviations: Chl, chlorophyll; MES, 2-(*N*-morpholino)ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; PS II, Photosystem II; SDS, sodium dodecyl sulfate.

* Corresponding author. Department of Chemistry, University of Crete, P.O. Box 1470, 71409 Iraklion, Crete, Greece. Fax: +30 81 233669/210951.

SSDI 0005-2728(94)00062-A

oxygen, ^{3}P680 formed during strong illumination of PS II complex reacts readily to form singlet oxygen, which is extremely reactive and capable of causing oxidative damage in the vicinity of its formation [24].

Although it is recognized that singlet oxygen causes oxidative damage to D1 protein, which can adversely affect its activity, stability and function, no direct singlet oxygen-induced fragmentation of D1 protein has been demonstrated. Recently, we proposed that singlet oxygen, which is generated during aerobic illumination of the PS II complex, is responsible for the photobleaching of photosynthetic pigments, D1 protein degradation and protein cross-linking [23]. In the present communication, we provide experimental evidence that exposure of a PS II core complex to singlet oxygen, generated either chemically in the dark or through the photodynamic action of rose bengal, results in D1 protein fragments similar to the ones generated during photoinhibition.

2. Materials and methods

An oxygen-evolving PS II core complex was isolated from spinach by solubilization of PS II membranes with octyl glucoside as described earlier [25]. The PS II complex, at 50 μg Chl ml^{-1}, resuspended in 0.4 M sucrose, 50 mM MES (pH 6.0), and 10 mM NaCl (SMN), was illuminated under aerobic conditions with heat-filtered white light (3000 μE m^{-2} s^{-1}). The samples were illuminated either at 25°C (5 min) or 4°C (7 min) under constant stirring. In a series of experiments, the samples which were illuminated at 4°C for 7 min were transferred to 25°C and incubated at that temperature for 30 min.

In certain experiments, the PS II core complex at 50 μg Chl ml^{-1} in SMN, was exposed to weak light (350 μE m^{-2} s^{-1}) at 25°C or 4°C for 1 min, either in the presence or absence of 2 μM rose bengal. Controls containing the same amounts of rose bengal were incubated in the dark for the same period.

Exposure of the PS II complex to singlet oxygen in the dark was performed by incubating the isolated PS II core complex, resuspended in SMN at 50 μg Chl ml^{-1}, for 1 min either at 25°C or 4°C, in a reaction mixture containing hydrogen peroxide (0.5 mM) and sodium hypochlorite (0.5 mM). Wherever indicated, the samples which were treated with NaOCl/H_2O_2 at 4°C were subsequently transferred to 25°C and incubated at that temperature for 7 min. Controls containing the same amount of either hydrogen peroxide or sodium hypochlorite alone were run simultaneously under similar conditions.

The singlet oxygen scavenger histidine (25 mM) was added prior to either photoinhibitory illumination or treatment with NaOCl/H_2O_2 or rose bengal wherever indicated. Proteinase inhibitors (PMSF (40 μM), leupeptin (2 μM) or aprotinin (0.6 μM)) were added to the PS II core complex prior to either photoinhibitory illumination or exposure to chemically generated singlet oxygen as described in Ref. [15].

Following the treatments, the samples were washed twice with SMN and finally resuspended in SMN prior to analysis.

SDS-PAGE was carried out on a 12–18% gradient in the presence of 6 M urea according to Laemmli [26]. The proteins were transferred on to PVDF membranes and were probed with D1-antiserum as described earlier [23]. The antiserum specific to the N-terminal of the D1 protein was a kind gift from Dr. Autar K. Mattoo. The molecular mass of protein bands in the immunoblots were estimated according to the relative mobility of polypeptides on SDS-PAGE using standard methods of determining the molecular mass of protein in the gel. Chlorophyll concentration was determined in 80% acetone according to Arnon [27].

3. Results

The PS II core complex was either treated with chemically generated singlet oxygen or exposed to strong white light. Exposure of the PS II core complex to singlet oxygen, generated by NaOCl/H_2O_2 in the dark at 25°C, caused rapid degradation of the reaction center D1 protein as revealed by the appearance of several immunodetectable fragments (Fig. 1). At least seven fragments having apparent molecular masses of approximately 24, 23, 20, 16, 14, 13 and 11 kDa were detected (lane 5). Other lower molecular mass D1 breakdown products at about 9 and 7 kDa were also present (lane 5). In addition, a species having an apparent molecular mass of approximately 41 kDa also appeared in the singlet oxygen-treated PS II core complex (lane 5). On the basis of the cross-reactivity of this band with anti-D2 antiserum, it is suggested to represent a D1–D2 heterodimer truncated in D1 [28]. By using an antibody raised against a synthetic peptide homologous to the N-terminal of the D1 protein, it was shown that all D1 fragments were of N-terminal origin (Fig. 1). Exposure of the PS II core complex to hydrogen peroxide or hypochlorite alone did not result in degradation of D1 protein (lanes 2 and 3, respectively).

As shown in Fig. 1, exposure of the PS II complex to singlet oxygen generated chemically in the dark at 4°C, also resulted in D1 fragmentation (lane 6). The D1 fragments detected during exposure of PS II to singlet oxygen at 4°C were similar to the ones observed at 25°C. Moreover, no further degradation of D1 protein was observed when the temperature of the PS II core complex, which had been exposed to singlet oxygen at 4°C, was raised to 25°C (lane 7). Degradation of D1

protein was significantly inhibited in the presence of histidine (lane 11). These results clearly demonstrate that D1 protein cleavage is due to the effect of singlet oxygen.

The involvement of singlet oxygen in the mechanism of D1 protein fragmentation was further verified by using singlet oxygen generated by the photodynamic action of the xanthene dye, rose bengal. Rose bengal is known to generate singlet oxygen in the presence of light and oxygen [24]. As shown in Fig. 1, weak light illumination at 25°C in the presence of rose bengal also led to rapid fragmentation of D1 polypeptide (lane 12). The D1 degradation pattern during weak light illumination in the presence of rose bengal was similar to that observed after treatment with singlet oxygen generated chemically in the dark. Illumination of the PS II core complex at 4°C in the presence of rose bengal under weak light also produced similar D1 fragments (data not shown). These observations further strengthen the concept of an involvement of singlet oxygen in D1 protein fragmentation. The presence of histidine during rose bengal treatment significantly protected D1 protein against degradation (Fig. 1, lane 13).

In order to compare the degradation of D1 protein induced by chemically generated singlet oxygen with that caused by strong illumination, the PS II core complex was subjected to photoinhibitory light either at 25°C or 4°C. Fig. 1 clearly shows that strong illumination at both temperatures results in D1 protein fragmentation accompanied by the appearance of various breakdown products. The immunoblot in Fig. 1 shows that the anti-$D1_N$ antibody detects seven different breakdown products of D1 polypeptide having apparent molecular masses of about 24, 23, 20, 16, 14, 13 and 11 kDa (lanes 4 and 8). There was also an appearance of a 41 kDa protein band in the light-treated PS II core complex at both temperatures (lanes 4 and 8). It is interesting to note that all fragments which appeared following photoinhibition of the PS II core complex at 25°C and 4°C resembled those obtained after exposure to singlet oxygen generated either chemically by $NaOCl/H_2O_2$ in the dark or through the photodynamic action of rose bengal. However, there was an important difference between the photoinhibitory illumination and exposure to externally generated singlet oxygen. In the PS II complex exposed directly to singlet oxygen, fragmentation of D1 protein occurred very fast (less than 1 min of dark incubation). On the other hand, prolonged exposures to photoinhibitory light were required in order to achieve a similar extent of damage. Approximately 7 min were required during light treatment at 4°C (lane 8), while 5 min were sufficient to achieve the same degree of D1 degradation at 25°C (lane 4). Similar D1 fragments under photoinhibitory conditions have been reported previously by several workers [14,28–30].

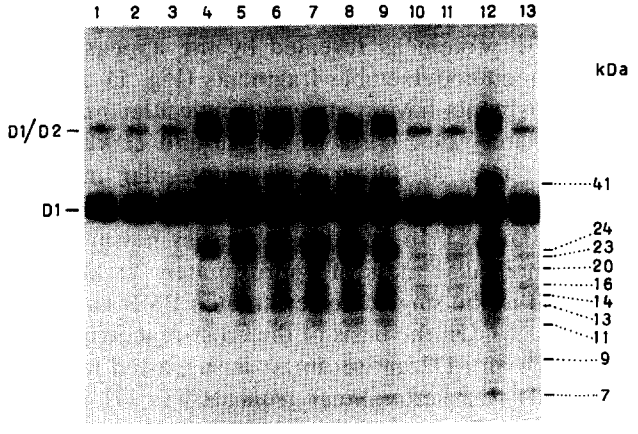

Fig. 1. Western blot analyses of D1 protein degradation in a PS II core complex exposed to either photoinhibitory light or chemically generated singlet oxygen. Lanes: 1, no addition, dark control; 2, treated with H_2O_2 at 25°C; 3, treated with NaOCl at 25°C; 4, illuminated with photoinhibitory light for 5 min at 25°C; 5, dark-incubated in the presence of H_2O_2 and NaOCl at 25°C; 6, dark-incubated in the presence of H_2O_2 and NaOCl at 4°C; 7, dark-incubated in the presence of H_2O_2 and NaOCl at 4°C and subsequently transferred to 25°C in the dark; 8, illuminated with photoinhibitory light at 4°C; 9, illuminated at 4°C and then transferred to 25°C in the dark; 10, illuminated with photoinhibitory light in the presence of histidine at 25°C; 11, dark-incubated with H_2O_2 and NaOCl in the presence of histidine at 25°C; 12, illuminated with weak light in the presence of rose bengal at 25°C; 13, illuminated with weak light in the presence of rose bengal and histidine at 25°C. The blots were immunodecorated with anti-$D1_N$ protein serum (see Section 2 for details).

In a series of experiments, the PS II core complex which had been photoinhibited at 4°C, was subsequently transferred to 25°C in the dark. As shown in Fig. 1 (lane 9), no significant changes were observed in the pattern of D1 fragmentation upon transfer of a low temperature photoinhibited PS II core complex to room temperature. In contrast to our data, a large number of recent studies have inferred that D1 protein degradation is temperature-sensitive and does not occur or is retarded at low temperature. The protective role of histidine, a singlet oxygen scavenger, observed during photoinhibition of the PS II complex at 25°C (Fig. 1, lane 10) was also observed during low temperature photoinhibition (data not shown). These findings imply that singlet oxygen is involved in the mechanism of light-induced D1 degradation both during room temperature as well as low temperature photoinhibition. Singlet oxygen has been previously implicated in chilling-induced damage of PS II proteins and lipids [31,32].

It has been reported that proteinase inhibitors which act against serine proteinases can partially protect D1 protein against degradation which occurs during in vitro photoinhibition [13–15]. In order to investigate the involvement of any proteolytic reaction in D1 degradation under our experimental conditions, the PS II core complex was incubated in the dark in the

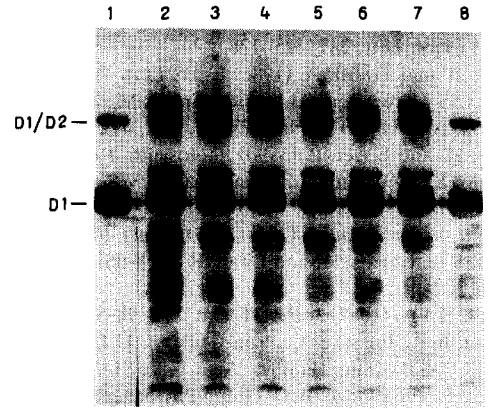

Fig. 2. Immunoblot demonstrating D1 protein degradation in the presence or absence of proteinase inhibitors. The PS II core complex was either dark-incubated in the presence of $NaOCl/H_2O_2$ or light-treated at 25°C (unless otherwise indicated) in the presence or absence of PMSF. Lanes: 1, no addition, dark control; 2, dark-incubated with H_2O_2 and NaOCl; 3, illuminated with weak light in the presence of rose bengal and PMSF; 4, dark-incubated with H_2O_2 and NaOCl in the presence of PMSF; 5, illuminated with photoinhibitory light; 6, illuminated with photoinhibitory light in the presence of PMSF at 4°C; 7, illuminated with photoinhibitory light in the presence of PMSF; 8, illuminated with photoinhibitory light in the presence of histidine. The blots were immunodecorated with anti-$D1_N$ protein serum (see Section 2 for details).

presence of PMSF prior to exposure to either photoinhibitory light or chemically generated singlet oxygen. The results presented in Fig. 2 show that the presence of PMSF, during strong illumination or exposure to singlet oxygen, did not protect D1 against degradation (lanes 5, 6, 7 and 2, 3, 4, respectively). In addition to PMSF we also tried the proteinase inhibitors aprotinin and leupeptin, but again we observed no protection against D1 degradation (data not shown). However, the addition of histidine prior to photoinhibitory illumination at room temperature significantly protected D1 protein against degradation (Fig. 2, lane 8).

4. Discussion

Recent studies have focused on the possible role(s) of singlet oxygen in D1 protein damage and degradation observed during photoinhibition [33,34]. In the present communication, we have been able to demonstrate in vitro D1 protein fragmentation caused by exposure of the PS II core complex to singlet oxygen generated either chemically in the dark or through the photodynamic action of rose bengal at 25°C and 4°C. Exposure of the PS II complex to singlet oxygen resulted in rapid degradation of D1 protein and the appearance of several breakdown products. The fact that the direct action of singlet oxygen gives rise to fragments similar to those detected during the in vitro photoinhibition of the PS II core complex, strongly suggests an involvement of singlet oxygen in the molecular mechanism of D1 degradation. Moreover, the protection of D1 protein against degradation observed in the presence of histidine, a singlet oxygen quencher, further supports the involvement of singlet oxygen in the D1 cleavage mechanism in vitro. A similar effect of singlet oxygen in the in vivo degradation of D1 protein is very possible.

Although the data presented in this communication suggest that chemically generated singlet oxygen induces cleavage of D1 protein in a manner similar to that observed during photoinhibition, we cannot say with certainty whether the fragments are identical. Since during exposure of the PS II core to $NaOCl/H_2O_2$ in the dark, singlet oxygen is generated in the solution and not at specific sites, as is probably the case during photoinhibition, it is possible that additional fragments are generated during exposure to chemically generated singlet oxygen. As we showed in Ref. [23], D1 degradation is followed (or accompanied) by cross-linking and thus it is difficult to accumulate the fragments in high quantities in order to isolate and sequence them.

Protein cleavage by reactive oxygen species is a well-known process [21,22,35]. Protein fragmentation and cross-linking have been previously reported following exposure to sodium hypochlorite/hydrogen peroxide mediated formation of singlet oxygen [36,37]. The process of fragmentation was suggested to involve the oxidative scission [36] and/or hydrolysis of the peptide bond [38]. Moreover, several amino acid residues, like histidine, tryptophan and methionine, were found to be vulnerable to modification by oxidative stress. Recently, Tang et al. [39] have reported that histidine residues are damaged during photoinhibitory illumination of an isolated reaction center complex from spinach. At present, it is difficult to envisage the reaction mechanism involved in the singlet oxygen-mediated D1 protein fragmentation. Although the results presented in this work provide experimental evidence that D1 protein cleavage is initiated by singlet oxygen itself, the possibility that products of singlet oxygen-initiated free radical cascade are involved in the process cannot be excluded. Further experiments are in progress in order to elucidate the mechanism of action of singlet oxygen in the process of D1 protein degradation.

Acknowledgements

The kind gift of the D1 antibody from Dr. Autar K. Mattoo is gratefully acknowledged. The authors would like to thank Dr. H. Van Gorkom for helpful discussions. This work has been supported by a grant from the Greek Secretariat for Research and Technology

(PENED91-444) and a grant from the United Nations Industrial Development Organization (UNIDO-CRP/GRE90-01).

References

[1] Mattoo, A.K., Hoffman-Falk, H., Marder, J.B. and Edelman, M. (1984) Proc. Natl. Acad. Sci. USA 81, 1380–1384.
[2] Edelman, M., Mattoo, A.K. and Marder, J.B. (1984) in Chloroplast Biogenesis (Ellis, J., ed.), pp. 283–302, Cambridge University Press.
[3] Kyle, D.J., Kuang, T.Y., Watson, J.L. and Arntzen, C.J. (1987) Biochim. Biophys. Acta 765, 89–96.
[4] Andersson, B., Salter, A.H., Virgin, I., Vass, I. and Styring, S. (1992) J. Photochem. Photobiol. B: Biol. 15, 15–31.
[5] Prasil, O., Adir, N. and Ohad, I. (1992) in Topics in Photosynthesis (Barber, J., ed.), Vol. 11, pp. 220–250, Elsevier, Amsterdam.
[6] Andersson, B. and Styring, S. (1991) in Current Topics in Bioenergetics (Lee, C.P., ed.), Vol. 16, pp. 2–81, Academic Press, San Diego.
[7] Aro, E.-M., Virgin, I. and Andersson, B. (1993) Biochim. Biophys. Acta 1143, 113–134.
[8] Kyle, D.J., Ohad, I. and Arntzen, C.J. (1984) Proc. Natl. Acad. Sci. USA 81, 4070–4074.
[9] Ohad, I., Kyle, D.J. and Arntzen, C.J. (1984) J. Biol. Chem. 99, 481–485.
[10] Aro, E.-M., Hundal, T., Carlberg, I. and Andersson, B. (1990) Biochim. Biophys. Acta 1019, 269–275.
[11] Richter, M., Rhule, W. and Wild, A. (1990) Photosynth. Res. 24, 229–235.
[12] Van Wijk, K.J., Andersson, B. and Styring, S. (1992) Biochim. Biophys. Acta 1100, 207–215.
[13] Shipton, C.A. and Barber, J. (1992) Biochim. Biophys. Acta 1099, 85–90.
[14] Salter, A.H., Virgin, I., Hagman, A. and Andersson, B. (1992) Biochemistry 31, 3990–3908.
[15] Virgin, I., Salter, A.H., Ghanotakis, D.F. and Andersson, B. (1991) FEBS Lett. 287, 125–128.
[16] Arntz, B. and Trebst, A. (1986) FEBS Lett. 194, 43–49.
[17] Sopory, S., Greenberg, B.M., Roshini, A.M., Edelman, M. and Mattoo, A.K. (1989) Z. Naturforsch. 45C, 412–417.
[18] Kuhn, M. and Boger, P. (1990) Photosynth. Res. 23, 291–296.
[19] Casano, L.M., Gomez, L.D. and Trippi, V.S. (1990) Plant Cell Physiol. 31, 377–382.
[20] Barenyi, B. and Krause, G.H. (1985) Planta 163, 218–226.
[21] Wolff, S.P., Garner, A. and Dean, R.T. (1986) Trends Biochem. Sci. 11, 27–31.
[22] Davies, K.J.A. (1987) J. Biol. Chem. 262, 9895–9901.
[23] Mishra, N.P., Francke, C., Van Gorkom, H.J. and Ghanotakis, D.F. (1994) Biochim. Biophys. Acta 1186, 81–90.
[24] Knox, J.P. and Dodge, A.D. (1985) Phytochemistry 24, 889–896.
[25] Ghanotakis, D.F., Demetriou, D.M. and Yocum, C.F. (1987) Biochim. Biophys. Acta 891, 15–21.
[26] Laemmli, U.K. (1970) Nature 227, 680–685.
[27] Arnon, D.I. (1949) Plant Physiol. 24, 1–15.
[28] Virgin, I., Ghanotakis, D.F. and Andersson, B. (1990) FEBS Lett. 269, 45–48.
[29] Shipton, C.A. and Barber, J. (1991) Proc. Natl. Acad. Sci. USA 88, 6691–6695.
[30] Barbato, R. Shipton, C.A., Giacometti, M. and Barber, J. (1991) FEBS Lett. 220, 162–166.
[31] Oquist, G. (1983) Plant Cell Environ. 6, 281–300
[32] Wise, R.R. and Naylor, A.W. (1987) Plant Physiol. 83, 272–277.
[33] Vass, I., Styring, S., Hundal, T., Koivuniemi, A., Aro, E.-M. and Andersson, B. (1992) Proc. Natl. Acad. Sci., USA 89, 1408–1412.
[34] De Las Rivas, J., Shipton, C.A., Ponticos, M. and Barber, J. (1993) Biochemistry 32, 6944–6950.
[35] Garrison, W.M. (1987) Chem. Rev. 87, 381–398.
[36] Thomas, E.L. (1979) Infect. Immun. 23, 522–531.
[37] Timmins, G.S. and Davies, M.J. (1993) J. Photochem. Photobiol. B: Biol. 21, 167–173.
[38] Gutteridge, J.M.C. and Wilkins, S.J. (1983) Biochim. Biophys. Acta 759, 38–41.
[39] Tang, C.-Q., Kuang, T.-Y., Yu, Z.-B., Lu, Y.-H., Peng, D.-C. and Tang, P.-S. (1992) in Research in Photosynthesis (Murata, N., ed.), Vol. IV, pp. 439–442, Kluwer, Dordrecht.