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New evidence suggests that the initial photoinduced cleavage of the D1-protein may not occur near the PEST sequence

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When isolated reaction centres of photosystem 2 from pea or wheat are exposed to photoinhibitory illumination in the presence of an electron acceptor, breakdown products of the D1-protein are observed having molecular masses ranging from about 24 to 10 kDa. By using antibodies raised to the C-terminal or N-terminal portions of D1 it was shown that the major breakdown fragment of 24 kDa was derived from the C-terminus. This conclusion was supported by phosphorylation studies and from the digestion pattern obtained by lysine specific endoprotease-induced proteolysis. The complementary N-terminal breakdown fragment was found to have an apparent molecular mass of 10 kDa. The implications of these data are discussed in terms of the possible relationship between the 24 kDa C-terminal fragment and the 23.5 kDa breakdown fragment detected in vivo by Greenberg et al. [1987, EMBO J. 6, 2865–2869] and it is suggested, based on limited proteolysis using papain, that the latter may not be derived from the N-terminus as previously thought but also originates from the C-terminus.

Photosynthesis; Photosystem 2; D1-polypeptide; Photoinhibition

1. INTRODUCTION

The D1-protein of the photosystem two (PSII) reaction centre rapidly turns over in vivo in the light [1]. This turnover has been linked with the phenomenon of photoinhibition where D1 is degraded and resynthesised as part of the repair system [2]. From in vivo studies, Greenberg et al. [3] were able to show that an initial breakdown product of the D1-protein had an apparent molecular mass of 23.5 kDa and proteolytic mapping of this major fragment suggested that it was derived from the N-terminus of the intact 32 kDa D1-protein. This conclusion was consistent with the proposal that the likely site for the initial cleavage was close to an α-helix destabilising stretch of amino acids, rich in glutamate, serine and threonine residues ('PEST'-like region) [3,4]. According to predicted models for the structure of the D1-protein in the membrane [5–7], this region is located between the IV and V transmembrane segments.

Until recently, it was thought that the protease responsible for the initial cleavage was not active in vitro [8]. However, a breakdown product in the region of 21–24 kDa has been observed when isolated thylakoids or PSII cores are exposed to photoinhibitory illumination [9–11]. Even more surprising is that a D1 degradation product of 24 kDa is observed when the isolated reaction centre of PSII, consisting of only the D1- and D2-proteins, the subunits of cytochrome b_{559} and the product of the *psb*I gene, is exposed to strong illumination in the presence of an electron acceptor [12]. This latter finding indicates that the initial cleavage of D1 involves an autoproteolytic activity of the reaction centre itself [12]. Recent evidence suggests that this intrinsic endopeptidase activity is of a serine type [13,14].

In this communication we present evidence which shows that the 24 kDa breakdown fragment obtained by illuminating isolated PSII reaction centres with photoinhibitory light is derived from the C-terminus, while the N-terminal fragment has a molecular mass of about 10 kDa. We therefore suggest that the initial cleavage site is located in the peptide region spanning the putative transmembrane segments I and II in the D1-protein. We further present some evidence indicating that the 24 kDa fragment generated by illuminating isolated PSII reaction centres could be the same as the 23.5 kDa fragment observed in vivo suggesting that it too is C-terminal in origin and not N-terminal as generally believed to date [3,15,16].

Abbreviations: Chl, chlorophyll; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; PSII, photosystem 2; RC, reaction centre; SDS, sodium dodecyl sulphate.

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2. MATERIALS AND METHODS

Isolation of PSII reaction centre (RC) complexes from pea and wheat was performed according to the method described in [17] which is a modification of the original procedure [18]. To obtain a preparation in which the D1- and D2-protein were labelled with ^{32}P at their N-termini, RC complexes were isolated from pea thylakoids that had

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been incubated under reducing conditions in the presence of [γ-^{32}P]ATP [19].

Photoinhibition of the isolated reaction centre was carried out according to [12] in a buffer containing 50 mM Tris-HCl, pH 8.0, and 2 mM dodecyl-β-maltoside. An electron acceptor, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) was added to a final concentration of 0.2 mM. Illumination was performed for fixed periods of time at 20°C using white light with an intensity of 4500 $\mu E \cdot m^{-2} \cdot s^{-1}$. Chlorophyll concentrations were 50 or 100 μg/ml.

SDS-PAGE in the presence of 6 M urea and immunoblotting of proteins was performed as described previously [10,20]. Following incubation with the primary antibody (see below), immunoreactions were detected by the alkaline phosphatase-conjugated secondary antibody method.

Cleveland digests were carried out according to [21]. After electrophoresis, SDS gels were stained for 1 h in 0.5% Coomassie R250 in 50% methanol and destained in 20% methanol. Bands containing the D1-protein and its photoinduced breakdown fragments were cut out of the gel and soaked in 135 mM Tris-HCl, pH 6.8, containing 0.1% SDS. The gel slices were loaded onto a second SDS gel and the polypeptides subjected to proteolysis. After electrophoresis, the gels were either stained with Coomassie or prepared for immunoblotting.

Three different anti-D1 polyclonal antisera have been utilized during this study and are referred to as anti-$D1_c$, anti-$D1_t$ and anti-$D1_n$. The anti-$D1_c$ antiserum recognises epitopes located on the C-terminal region of the D1-protein [10] and was raised in rabbit using the spinach D1-protein isolated from PSII core complexes [22] by preparative SDS-PAGE. The anti-$D1_t$ antiserum was raised against the *psbA* gene product expressed in *E. coli* [23]. To prepare an anti-D1 antiserum specific for the N-terminal region of the protein (anti-$D1_n$), we used the N-terminal fragment generated by lysine specific endoprotease (Lys-C) proteolysis of the wheat D1-protein as the antigen. The D1-protein of wheat was isolated as previously described [10]. Lys-C proteolysis was performed overnight at 37°C with 0.5 $mg \cdot ml^{-1}$ protein and 1.4 $units \cdot ml^{-1}$ protease. Fragments were resolved by preparative electrophoresis and then electroeluted. The N-terminal fragment was emulsified with complete Freund's adjuvant and injected into a rabbit. Immunization schedule and bleeding of the rabbit were according to standard procedures. Anti-$D1_c$ and anti-$D1_t$ were utilized at 1/400 dilution, whilst the anti-$D1_n$ was used at a dilution of 1/100.

3. RESULTS

As previouisly shown in [12] illumination of the isolated PSII RC complex in the presence of an electron acceptor results in degradation of D1 giving rise to several immunodetectable fragments. In Fig. 1A, we present the results of an experiment in which the PSII RC complex (50 μg Chl$\cdot ml^{-1}$) isolated from wheat thylakoids was exposed to 4500 $\mu E \cdot m^{-2} \cdot s^{-1}$ of white light for 45 min and the breakdown products of the D1 protein detected by Western blotting with the anti-$D1_c$ antiserum. This polyclonal antiserum was previously been shown to recognise only epitopes localised on the C-terminal region of D1 corresponding to the amino acids 239–343 [10]. Under these conditions three fragments are clearly detected (lane 1) at approximately 24, 22 and 14 kDa. An immunoblot of a dark control is shown in lane 2.

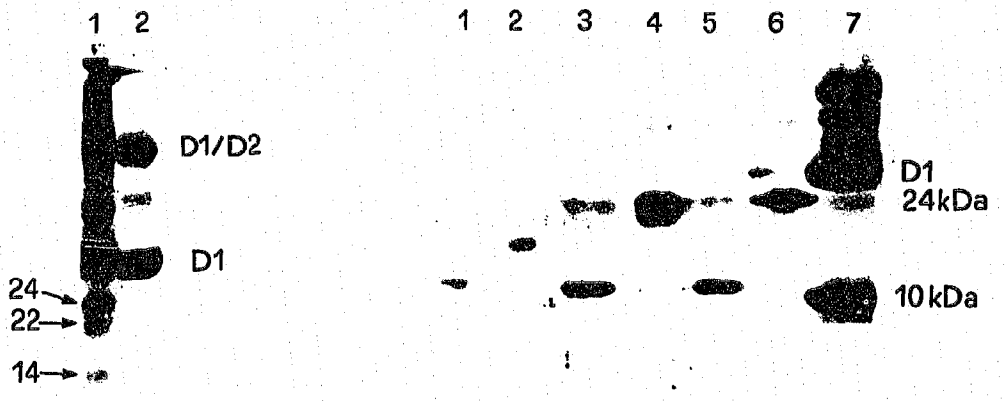

Fig. 1. (A) Immunodetection of the photoinduced D1 breakdown products. Wheat RC were illuminated for 45 min (100 μg Chl/ml) in the presence of 0.2 mM DBMIB and then analysed by immunoblotting using the anti-$D1_c$ antibody (lane 1). For comparison, an immunoblot of a dark control is shown in lane 2. (B) Characterisation of the D1 breakdown products. Photoinhibited wheat RC were subjected to urea/SDS-PAGE and polypeptides detected by Coomassie staining. Bands containing D1 fragments were cut out of the gel and loaded onto a second SDS gel. Lys-C protease was added (0.3 units/slot) and proteolysis carried out according to Cleveland et al. [21]. After electrophoresis, proteins were electroblotted to nitrocellulose filter and assayed with the anti-$D1_c$ antibody. Lanes 1, 3 and 5 contain respectively, the 14, 22 and 24 kDa fragment digested with Lys-C protease. Lanes 2, 4 and 6 contain the undigested fragments. Lane 7 shows the proteolysis with Lys-C of the D1 protein isolated from a dark control.

The choice of wheat as a source for our RC preparation has the advantage that a single lysine residue is contained in the D1-protein located at position 238 of the amino acid sequence [24]. By treating the D1 breakdown fragments with the endoprotease Lys-C it is possible to test whether they contain this lysine residue. The result of such an experiment is shown in Fig. 1B. All three fragments detected by anti-$D1_c$ are cut by the Lys-C protease and give rise to an immunodetectable fragment of about 10 kDa (lanes 1, 3 and 5). The same 10 kDa fragment was also obtained after proteolysis of the entire D1-protein (lane 7).

To further characterise D1 breakdown in isolated reaction centres, we prepared a polyclonal antibody raised against the N-terminal fragment of D1 (anti-$D1_n$) obtained by Lys-C digestion of the protein from wheat (see Materials and Methods). The monospecificity of the antibody for D1-protein is shown in Fig. 2A, where an immunoblot (lane 2) of the thylakoid polypeptides (lane 1) is shown. The reactivity of this antiserum to fragments generated by Lys-C proteolysis of the D1-protein is shown in Fig. 2B; it specifically recognises the N-terminal fragment (lane 2) with no cross-reaction with the C-terminal fragment (lane 1). Conversely, anti-$D1_c$ recognises the C-terminal fragment (lane 1, Fig. 2C) but not the N-terminal Lys-C induced fragment (lane 2, Fig. 2C).

The two polyclonals (anti-$D1_c$ and anti-$D1_n$) which have been proven in Fig. 2 to be specific towards different regions of D1 were tested against the light-induced

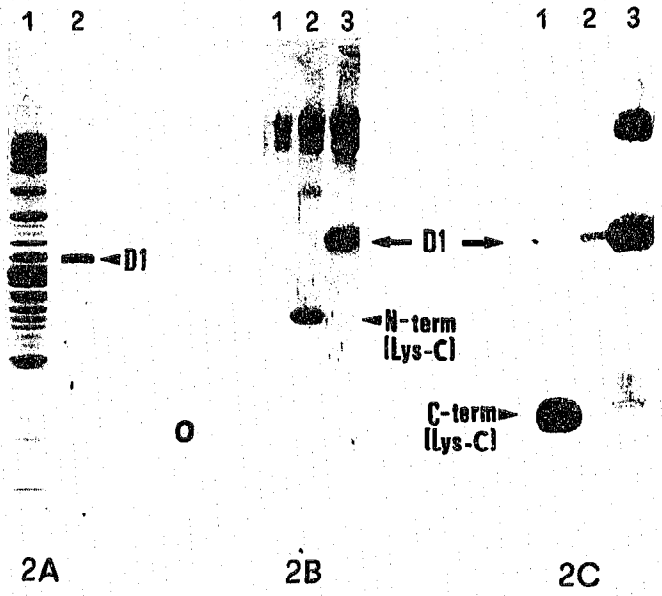

Fig. 2. Characterisation of the anti-$D1_c$ and anti-$D1_n$ antibodies. (A) Coomassie staining (lane 1) and immunoblot with the anti-$D1_n$ polyclonal antiserum (lane 2) of wheat thylakoids. (B and C) Specificity of anti-$D1_n$ (B) and anti-$D1_c$ (C) polyclonals towards different regions of the wheat D1 protein produced as described in the legend to Fig. 1. Lane 1, Lys-C generated C-terminal fragment (amino acids 239–343); lane 2, Lys-C generated N-terminal fragment (amino acids 1–238); lane 3, wheat PSII RC.

breakdown fragments of D1 generated from the isolated reaction centre complex. As shown in lane 1 of Fig. 3, the anti-D_c polyclonal antiserum, which is reactive towards the C-terminal region, gave a positive reaction with the 24 kDa fragment. The absence in this blot of the 22 and 14 kDa fragments shown in Fig. 1A is due to the shorter illumination time (15 min vs 45 min). However, the most significant result in this experiment is the absence of an immune reaction with the 24 kDa band when the anti-$D1_n$ (N-terminal specific) antiserum is used (lane 2). Instead, a 10 kDa fragment is recognised by this polyclonal, possibly representing the N-terminal fragment produced by the first photoinduced cleavage. Thus, the findings presented above indicate that the 24 kDa fragment observed as a photoinduced breakdown product of D1 in the isolated PSII RC is derived from the C-terminal portion of the D1-protein.

An alternative approach which we have employed, to give additional support to the proposed origin of the 24 kDa fragment, involved protein phosphorylation. It has been established that the phosphorylation of the D1-protein occurs near the N-terminus [25] at the first threonine residue [26]. Therefore, if the 24 kDa degradation fragment is derived from the C-terminus it will not carry the phosphate group if the label is retained by any of the fragments. Before isolating PSII reaction centres from pea thylakoids, we incubated the membranes with radioactive ATP under reducing conditions in order to phosphorylate the D1- and D2-proteins [19]. The PSII RCs were then isolated and subjected to photoinhibitory light. As the autoradiogram shows in Fig. 4A, the 24 kDa breakdown fragment was not phosphorylated. Instead three lower molecular weight bands

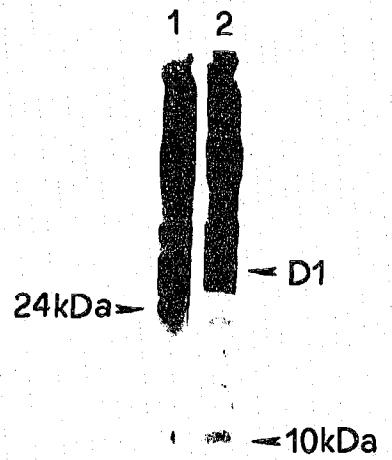

Fig. 3. Immunodetection of the photoinduced D1 breakdown products of the anti-$D1_c$ (C-terminal antibody) (lane 1) and the anti-$D1_n$ (N-terminal antibody) (lane 2). The isolated PSII RCs were pre-illuminated for 15 min in the presence of 0.2 mM DBMIB.

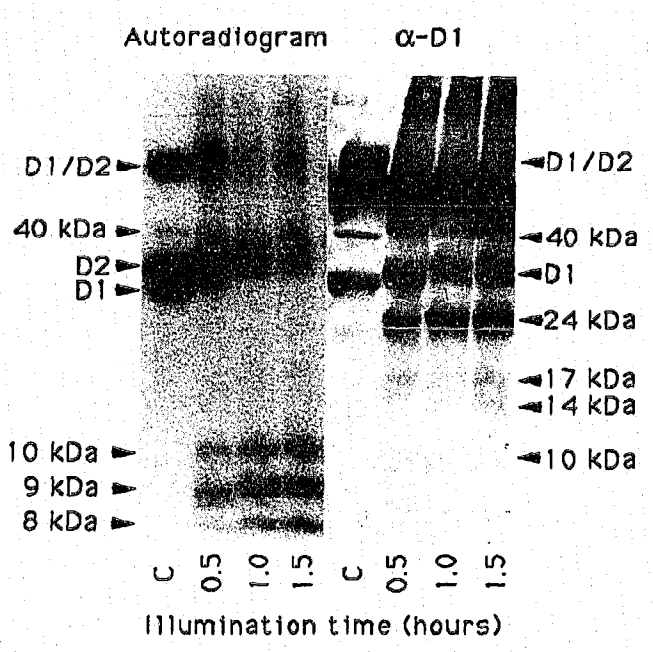

Fig. 4. (A) Detection of ^{32}P labelled polypeptides in reaction centres of peas which had been phosphorylated prior to isolation and subjected to photoinhibitory illumination in the presence of 0.2 mM DBMIB for the times indicated in hours. (B) Western blot used for the autoradiogram in (A) generated with anti-$D1_t$ antiserum. This polyclonal antiserum was raised to a D1-fusion protein [23] and like anti-$D1_c$ shows a specificity for the C-terminus but also weakly detects additional fragments, one of which is N-terminal (10 kDa).

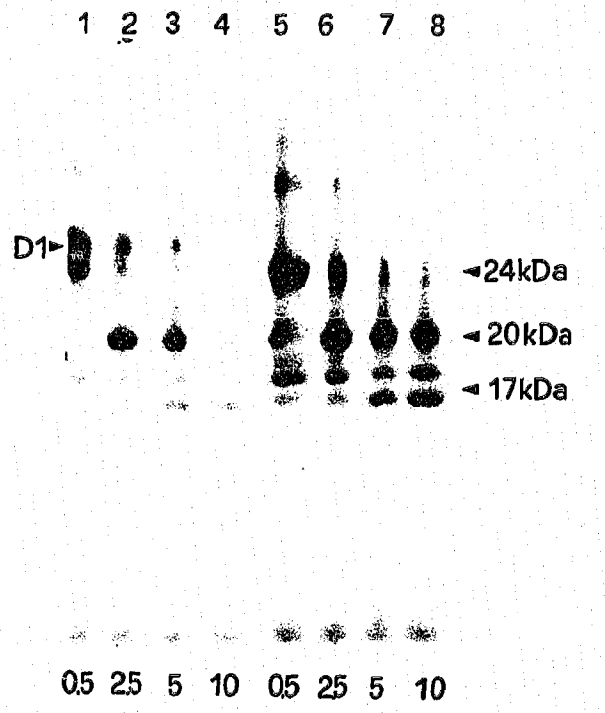

Fig. 5. Limited proteolysis with papain of D1 (lanes 1–4) and of its 24 kDa photoinduced fragment (lanes 5–8) as revealed by immunoblotting with the anti-$D1_c$ antiserum. Isolation, proteolysis and immunoblot of D1 and of its 24 kDa fragment are described in the legend of Fig. 1. The quantity of papain used in each slot is indicated at the bottom of the figure.

were observed. One having a molecular mass of 10 kDa was identified as a D1 fragment by immunoblotting with anti-$D1_t$ antiserum as shown in Fig. 4B. The origin of the other two phosphate labelled bands is unclear but they may derive from the breakdown of the D2-protein although our anti-D2 antiserum does not appear to react with any D2 fragment lower than 21 kDa (data not shown, see [12]). This experiment also records the finding that phosphorylated D1 in the isolated PSII RC is degraded in exactly the same way and with similar kinetics to non-phosphorylated D1, suggesting that phosphorylation/dephosphorylation is not a controlling factor in this process.

Data so far presented do not support the expectation that the 24 kDa fragment, observed when the isolated PSII RC is illuminated in the presence of DBMIB, is derived from the N-terminus of the D1 protein, but rather they unequivocally show that it represents the C-terminus. What is not clear, however, is the relationship between the 24 kDa fragment we observe and the 23.5 kDa fragment detected during light-induced D1-turnover in *Spirodella oligorrizha* [3] and also after in vivo photoinhibition of *Lemna minor* [15]. Greenberg et al. [3] argued that the 23.5 kDa polypeptide was an N-terminal breakdown product of the D1-protein, based on proteolytic mapping. In particular they found that the 23.5 breakdown product gave characteristic digestion fragments with papain and *Staph. aureus* V8 protease (P20 with papain and Sa22 and Sa21 with V8 protease) which had previously been suggested to be indicative of the N-terminus of the protein [27]. Thus we have checked whether the 24 kDa breakdown product of the D1-protein observed in our experiments gives a papain fragment similar to P20 described in [3]. The result of this experiment is shown in Fig. 5, where an immunoblot with anti-$D1_c$ (C-terminus specific) antiserum is shown of D1 and of the 24 kDa breakdown fragment digested with papain is shown. In both cases an immunoreactive fragment of about 20 kDa is clearly detected. Also slightly lower molecular mass papain fragments were obtained, weakly in the case of the whole D1-protein but more strongly with the 24 kDa fragment.

4. DISCUSSION

Our data indicate that the major breakdown fragment of the D1-protein (molecular mass approximately 24 kDa) observed after illumination of isolated PSII RCs in the presence of 0.2 mM DBMIB is derived from the C-terminal region of the protein. The other photoinduced breakdown products (i.e. the 22 and 14 kDa) detected by the anti-$D1_c$ antiserum must also contain the C-terminus of the protein since all of them are cleaved by Lys-C protease giving rise to the same 10 kDa immunoreactive product. The two latter fragments

are probably the result of a subsequent degradation of the 24 kDa fragment. A D1 fragment of approximately 14 kDa has been identified during photoinhibition of isolated thylakoids [9,10], PSII core complexes [11] and isolated RCs [12]. This fragment has been assigned to the C-terminal region of the protein [10] and is probably the 14 kDa product observed in the present work. An additional 10 kDa fragment has also been identified in this study and assigned to the N-terminal region of the D1 protein based on its detection with anti-$D1_n$ antiserum. Moreover, the same fragment seems to be heavily phosphorylated when photoinhibition is carried out on ^{32}P labelled reaction centres. Thus under our conditions, the primary cleavage of the D1 protein seems to give rise to two fragments, one at 24 kDa derived from the C-terminus and one at 10 kDa derived from the N-terminus. Although it is not possible to map the cleavage site accurately, a rough calculation based on the apparent molecular mass of the fragments, locates the site somewhere in the hydrophilic loop, connecting the putative transmembrane segments I and II on the inner side of the membrane. The same region must also contain a second cleavage site that brings about the 22 kDa fragment, whilst the 14 kDa fragment is possibly generated by cleavage at the hydrophilic loop between the putative transmembrane segments III and IV, again on the inner side of the membrane.

An important point raised by our results concerns the relationship between the 24 kDa DI fragment described here, and shown to be the C-terminus, and the 23.5 kDa fragment, observed during in vivo light-induced turnover of the D1-protein which is proposed to be N-terminal [3]. Actually in the absence of definitive N-terminal sequencing, the well-accepted model described in [3,4] has not been absolutely proven and relies partly on proteolytic mapping [27] and partly on indirect arguments. The apparent existence of a 'PEST'-like region in the stretch between transmembrane segments IV and V has been one important argument [3,4]. Further, the blocking of D1 protein degradation by herbicide binding to the Q_B site also focused attention on the same region [28]. There was also the idea that the cleavage site would be exposed to the outer surface so that after an appropriate photoinduced trigger it could be subjected to attack by an exogenous proteolytic enzyme [4]. The presence of an immuno-detectable 20 kDa fragment produced by papain digestion both in D1 and the 24 kDa fragment derived by photoinhibition of isolated PSII RC seems to indicate the similarity of the latter with the in vivo 23.5 kDa fragment. If this is proved to be correct the above arguments concerning the location of the primary cleavage site involved in the turnover of the D1-protein will need to be reconsidered.

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REFERENCES

[1] Edelman, M., Mattoo, A.-K. and Marder, J.B. (1984) in: Chloroplast Biogenesis (Ellis, J. ed.) pp. 283–302, Cambridge University Press.
[2] Kyle, D.J., Ohad, I. and Arntzen, C.J. (1984) Proc. Natl. Acad. Sci. USA 81, 4070–4074.
[3] Greenberg, B.M., Gaba, V., Mattoo, A.-K. and Edelman, M. (1987) EMBO J. 6, 2865–2869.
[4] Mattoo, A.-K., Marder, J.B. and Edelman, M. (1989) Cell 56, 241–245.
[5] Trebst, A. (1986) Z. Naturforsch. 41c, 240–245.
[6] Barber, J. (1987) Trends Biochem. Sci. 12, 321–326.
[7] Michel, H. and Deisenhofer, J. (1988) Biochemistry 27, 1–7.
[8] Schuster, G., Shochat, S., Adir, N. and Ohad, I. (1989) in: Techniques and New Developments in Photosynthesis Research (Barber, J. and Malkin, R. eds) pp. 499–510, Plenum Press, New York.
[9] Aro, E.-M., Hundal, T., Carlsberg, I. and Andersson, B. (1990) Biochim. Biophys. Acta 1019, 269–275.
[10] Barbato, R., Friso, G., Giardi, M.T., Rigoni, F. and Giacometti, G.M. (1991) Biochemistry (in press).
[11] Virgin, I., Ghanotakis, D.F. and Andersson, B. (1990) FEBS Lett. 269, 45–48.
[12] Shipton, C.A. and Barber, J. (1991) Proc. Natl. Acad. Sci. USA (in press).
[13] Misra, A.N., Hall, S.G. and Barber, J. (1991) Biochim. Biophys. Acta (in press).
[14] Virgin, I., Salter, A.H., Ghanotakis, D.F. and Andersson, B. (1991) FEBS Lett. (in press).
[15] Shipton, C.A., Marder, J.B. and Barber, J. (1990) Z. Naturforsch. 45c, 388–394.
[16] Trebst, A. and Depka, B. (1990) Z. Naturforsch. 45c, 765–771.
[17] Chapman, D.J., Gounaris, K. and Barber, J. (1990) in: Methods in Plant Biochemistry, vol. V (Rogers, L.J. ed.) pp. 171–193, Academic Press, London.
[18] Nanba, O. and Satoh, K. (1987) Proc. Natl. Acad. Sci. USA 84, 109–112.
[19] Telfer, A., Marder, J.B. and Barber, J. (1987) Biochim. Biophys. Acta 893, 557–563.
[20] Marder, J.B., Chapman, D.J., Telfer, A., Nixon, P.J. and Barber, J. (1987) Plant Mol. Biol. 9, 325–333.
[21] Cleveland, J.W., Fischer, S.G., Kirschen, M.W. and Laemmli, U.K. (1977) J. Biol. Chem. 252, 1102–1106.
[22] Barbato, R., Rigoni, F., Giardi, M.T. and Giacometti, G.M. (1989) FEBS Lett. 251, 147–154.
[23] Nixon, P.J., Dyer, T.A., Barber, J. and Hunter, C.N. (1987) FEBS Lett. 209, 83–86.
[24] Barros, M.D.C. (1986) Ph.D. Thesis, University of Cambridge, Cambridge.
[25] Marder, J.B., Telfer, A. and Barber, J. (1988) Biochim. Biophys. Acta 932, 362–365.
[26] Michel, H., Hunt, D.F., Shabanowitz, J. and Bennett, J. (1988) J. Biol. Chem. 263, 1123–1130.
[27] Marder, J.B., Goloubinoff, P. and Edelman, M. (1984) J. Biol. Chem. 259, 3900–3908.
[28] Mattoo, A.-K., Hoffmann-Falk, H., Marder, J.B. and Edelman, M. (1984) Proc. Natl. Acad. Sci. USA 81, 1380–1384.