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Rapid Report

The isolated D1/D2/cyt *b*-559 reaction centre complex of Photosystem II possesses a serine-type endopeptidase activity

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The isolated D1/D2/cyt *b*-559 reaction centre complex of Photosystem II has been shown to possess an endopeptidase activity which has been investigated using synthetic substrates and proteinase inhibitors. Substrate specificity and inhibition by serine type proteinase inhibitors suggest that the endopeptidase is of a serine type. Divalent cations, especially calcium, enhanced the activity of the intrinsic peptidase which also showed pH and temperature optima of 8.2 and 37 °C respectively. Treatment with strong light had little or no effect on the enzyme activity.

The reaction centre of Photosystem II (PS II) consists of a heterodimer of the D1 and D2 polypeptides [1] and can be isolated as a minimal functional unit [2,3]. In many ways this reaction centre is comparable with the reaction centre of purple bacteria [4,5]. There are, however, two main differences. The primary donor P680, in its oxidised state, has a potential of about 1.17 V, while the corresponding oxidising potential for the bacterial system is lower, being around 0.4 to 0.5 V [6,7]. The other significant difference is that, unlike its bacterial counterpart the L-subunit, the D1 polypeptide shows a light-dependent rapid turnover [8]. The reason for this turnover has been linked to the fact that PS II can be easily photodamaged, especially at high light intensities [9].

It is generally agreed that the degradation of the D1 protein is a secondary process resulting from deleterious and unavoidable photochemical processes occurring within the reaction centre [10]. Several studies indicate that the initial proteolytic cleavage occurs in a domain from Arg-238 to Ile-248 which is on the C-terminal side of a proposed α-helical destabilising region from Arg-225 to Arg-238 [11–13]. The fact that the cleavage of the D1 protein can occur in the dark following a preillumination at low temperatures and that its efficiency is increased by raising the temperature, indicates the enzymic nature of the process and dismisses the possibility of direct photocleavage reactions [10].

Several studies have indicated that the proteinase responsible for the initial cleavage of the D1 protein is bound to the thylakoid membrane [10,14] and is an intimate part of the PS II complex [15]. More recently, it has been found that the specific proteolytic breakdown can occur in isolated reaction centres of PS II consisting only of the D1 and D2 polypeptides, the α- and β-subunits of cytochrome *b*-559 and the product of the *psb*I gene [16]. This surprising result therefore suggested that the reaction centre contained an intrinsic peptidase activity. With this concept in mind we examined whether the isolated reaction centre showed any proteinase activity using artificial substrates and, indeed, we have shown that such activity exists.

The D1/D2/cyt *b*-559 reaction centre complex was isolated and characterised as previously reported [17]. Endopeptidase activity was assayed initially by azocasein hydrolase activity as described by Preston et al. [18]. The reaction mixture, consisting of 0.05 ml of enzyme solution or reaction centre preparation in McIlvain's citric acid/disodium phosphate buffer (pH 6.0), with 10 mM 2-decamercaptoethanol, was mixed with 0.35 ml azocasein substrate (1.4%) solution, also in McIlvaine's buffer (pH 6.0). The final concentration

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of enzymes and reaction centres was 8 μg ml^{-1} and 2 μg Chl ml^{-1}. After incubation at 37 °C for 3 h, the unreacted protein was precipitated with 0.5 ml 10% trichloroacetic acid (TCA) and the amount of azo-dye released by the hydrolysis of the substrate was measured at 440 nm after incubating for 10 min with 0.5 ml 0.5 M NaOH. One unit of the enzyme activity was expressed as one unit change in absorbance at 440 nm.

N-Benzoyl-L-isoleucyl-L-glutamyl-L-glycyl-L-arginine-4-nitroanilide (BIGGAN), a synthetic oligopeptide, was also used as a substrate to assay for D1/D2/cyt *b*-559 proteinase activity. The endopeptidase activity with this substrate was measured as described by Claeson et al. [19]. The reaction mixture containing 0.8 ml of 50 mM Tris (pH 8.2) and 20 mM Ca^{2+} was equilibrated to 37 °C and then 0.1 ml of enzyme or reaction centre added. After a further incubation of about 5 min at 37 °C, 0.1 ml of a 1 mM substrate solution was added. The level of enzyme and reaction centre in the 1 ml reaction cuvette was 0.8 μg and 5 μg Chl respectively. The change in absorbance at 405 nm was recorded using either a Shimadzu UV MPS2000 or a PW2000 spectrophotometer.

Trypsin and endopeptidase Lys-C were taken as checks for proteinase activities. Synthetic substrates were used for characterising the enzyme as described by Sarath et al. [20]. For controls the enzymes and the reaction centre were denatured by heating for 5 min in boiling water or given a mild treatment with acid. Inhibitors for endopeptidases, phenylmethanesulphonyl fluoride (PMSF), tosyl-lysine chloromethyl ketone (TLCK), antipain, leupeptin, soybean trypsin inhibitor, iodoacetate and 1,10-phenanthroline, were used at final concentrations of 1 mM, 10 μM, 10 μM, 100 μg/ml, 100 μM, 100 μg/ml and 10 mM respectively. All the chemicals used were AnalaR grade.

As Table I shows, using azocasein hydrolysis as an assay, an endopeptidase activity was detected with the isolated D1/D2/cyt *b*-559 reaction centre complex as well as with trypsin and with Lys-C. Acid and heat denaturation of the enzymes and the reaction centre gave no detectable amount of released azo-dye. This is the first report to show endopeptidase activity with the isolated PS II reaction centre complex. Therefore this activity was characterised using specific proteinase inhibitors. The inhibitor of metallic endopeptidase activity, 1,10-phenanthroline, had marginal effects on all the three proteinase activities. However, the inhibitors, TLCK, antipain and leupeptin, inhibited the azocasein proteolysis associated with isolated reaction centres by more than 50%. These inhibitors are known to act upon serine- and/or cysteine-type enzymes [21]. As the enzyme activity in the D1/D2/cyt *b*-559 complex, like that of trypsin and Lys-C, was inhibited less by iodoacetate, it is probable that the effect of these inhibitors could be through their action on a serine-type endopeptidase in the isolated reaction-centre complex. In support of this, it was found that the soybean trypsin inhibitor, specific for serine-type endopeptidases, strongly inhibited the enzyme activity of the reaction centre.

TABLE I

Endopeptidase activity of D1/D2/cyt b-559 complex, trypsin and endopeptidase Lys-C with azocasein as substrate, without or with proteinase inhibitors

The data are mean values of three separate samples with 3–5 replicates in each sample.

Treatment	D1/D2/cyt *b*-559 [a]	Trypsin [b]	Lys-C [c]
Control (– inhibitor)	100	100	100
+ 1,10-phenanthroline	98	97	100
+ TLCK	43	–	–
+ leupeptin	21	–	–
+ antipain	27	–	–
+ trypsin inhibitor (soybean)	15	6	4
+ iodoacetate	69	87	92

[a] 5.367 ± 0.903 A_{440} (mg Chl)$^{-1}$ h^{-1}.
[b] 14.52 ± 0.46 A_{440} (mg protein)$^{-1}$ h^{-1}.
[c] 56.53 ± 3.48 A_{440} (mg protein)$^{-1}$ h^{-1}.

Further studies were conducted with synthetic substrates to characterise the endopeptidase activity of the D1/D2/cyt *b*-559 complex. In general, all serine-type proteinase substrates tested were hydrolysed after incubation with the isolated reaction-centre complex. Substrates with 4-nitroanilide conjugates were found to give the greatest activity, with *N*-benzoyl-L-isoleucyl-L-glutamyl-L-glycyl-L-arginine-4-nitroanilide (BIGGAN) being better than others tested. The endopeptidase activity of the D1/D2/cyt *b*-559 complex using BIGGAN was estimated to be 0.2% of the trypsin activity (see Table II). This estimate was calculated on a mole

TABLE II

Effect of endopeptidase inhibitors on the proteolytic activity of D1/D2/cytochrome b-559 complex and trypsin on N-benzoyl-L-isoleucyl-L-glyamyl-L-glycyl-L-arginine-4-nitroanilide

Incubation with the inhibitors was at 4 °C for at least 1 h before conducting the assay.

Treatment	D1/D2/cyt *b*-559 [a]	Trypsin [b]
Control (– inhibitor)	100	100
+ 1,10-phenanthroline	99	97
+ leupeptin	10	0
+ antipain	26	0
+ trypsin inhibitor (soybean)	8	0
Heat-denatured enzyme	1	0

Control activities:
[a] $1 \cdot 10^9$ nkat mol^{-1} min^{-1}.
[b] $471 \cdot 10^9$ nkat mol^{-1} min^{-1}; where $(16.3 \times 10^3 \times \Delta A)/\Delta t$ = nkat/litre (see Ref. 22).

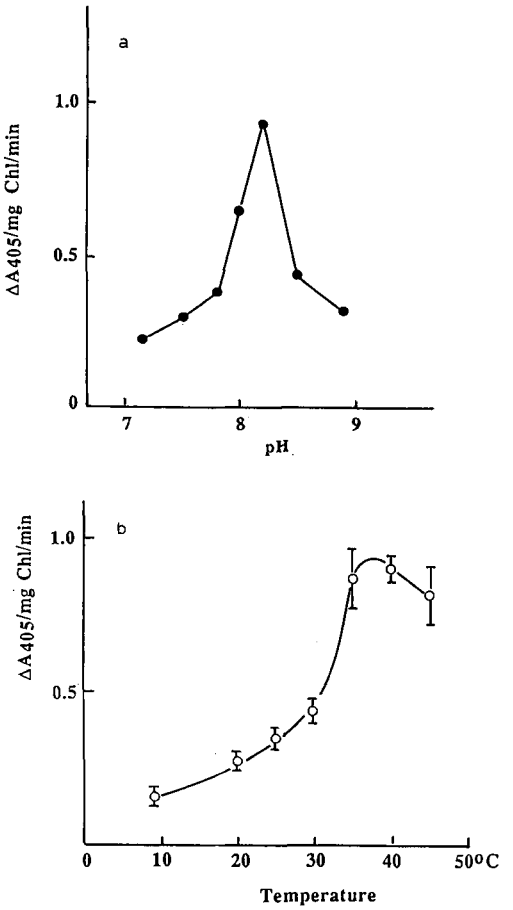

Fig. 1. pH (a) and temperature (b) sensitivity of the endopeptidase activity of the isolated PS II reaction centre using *N*-benzoyl-L-isoleucyl-L-glutamyl-L-glycyl-L-arginine-4-nitroanilide as the substrate.

basis assuming the PS II reaction centre binds six chlorophyll molecules.

The pH and temperature optima for the endopeptidase activity of PS II reaction-centre complex using BIGGAN as substrate were found to be 8.2 and 37 °C, respectively (Fig. 1). Similar pH and temperature optima were reported for trypsin activity on this substrate [22]. The Lineweaver-Burk plot for the enzyme activity of the D1/D2/cyt *b*-559 complex showed a K_m value of 0.05 mM and a V_{max} of 11 ΔA mg Chl^{-1} min^{-1}. The reaction, however, was stimulated by the presence of Ca^{2+}. Without Ca^{2+} the trypsin activity was 75% of the control value compared to only 36% with the D1/D2/cyt *b*-559 complex. Similarly, other divalent cations, especially Mg^{2+}, could also enhance the proteinase activity of the reaction centre.

The endopeptidase activities of the reaction centre complex on BIGGAN were compared with those of trypsin in the presence of several proteinase inhibitors (Table II). The insensitivity of the reactions to 1,10-phenanthroline was consistent with the data shown in Table I. Also, both the proteolytic activities were shown to be equally sensitive to PMSF and TLCK (data not shown). The comparable sensitivity of the reaction-centre complex and trypsin to soybean-trypsin inhibitor, leupeptin and antipain, as well as PMSF and TLCK, suggests that the enzymic activity associated with the D1/D2/cyt *b*-559 complex is a serine-proteinase. It is possible that this proteolytic activity is associated in some way with the degradation of the D1-polypeptide during its turnover. With this in mind we checked whether the peptidase activity could be enhanced by high light treatment known to cause D1 degradation [16], but we found no increase in activity.

In this paper we present, to our knowledge, the first report that the isolated PS II reaction centre, consisting of the D1 and D2 polypeptides, the apoproteins of cytochrome *b*-559 and the product of the *psb*I gene, possesses a serine-type peptidase activity. With the substrates tested the activity is very low as compared with well known serine-type proteinases such as trypsin. The possibility that the activity was due to contamination by exogenous proteinase was eliminated by carrying out further purifications involving sucrose density gradients and by subjecting the isolated reaction centre to resuspension after centrifugation. We therefore conclude that the endopeptidase activity is an intrinsic property of the PS II reaction-centre proteins. All the polypeptides of the isolated complex contain serine residues but none is totally conserved in the *psb*I protein or in the alpha and beta subunits of cytochrome *b*-559 between species. However, in the case of the D1/D2 heterodimer, 18 serine residues are highly conserved in the D1 polypeptide and 13 in the D2 polypeptide. Neither the unconserved nor conserved serines in the subunits are associated with amino-acid sequences typical of known serine-type peptidases, so it is difficult to identify at this stage on which subunit the active site is located.

It is tempting to speculate that the peptidase activity that we have identified plays some role in the degradation of the D1 polypeptide. Bearing in mind that the initial proteolytic cleavage occurs in the hydrophilic domain around Arg-238 near to the Q_B binding site [11–13] then conserved serines of particular interest are likely to be located on the outer surface of the reaction centre complex. Candidates are serine residues 23, 232 and 264 on the D1 polypeptide and 34, 246 and 263 on the D2 polypeptide. If our speculation is correct, the photoinduced damage that brings about proteolytic degradation of the D1 polypeptide does so by inducing a conformational change which allows the hydroxyl of the serine residue to act as a nucleophile to the carboxyl of the peptide bond. This conformation-dependent proteolytic event could be contained just within the D1 polypeptide itself or involve an active site on the D2 polypeptide. The observation that the peptidase activity using artificial substrate is not stimulated by preillumination with strong light suggests that

the proposed conformational change presents the cleavage site on the D1 to the active serine rather than activating the catalytic site itself. Clearly, these important new findings need further study to establish whether or not they have any relevance to the rapid turnover of the D1 polypeptide. Indeed, B. Andersson and colleagues (personal communication) have recently found that the photoinduced degradation of the D1 polypeptide in thylakoid membranes of spinach involves a serine-type proteinase.

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References

1 Trebst, A. (1986) Z. Naturforsch. 41c, 240–245.
2 Nanba, O. and Satoh, K. (1987) Proc. Natl. Acad. Sci. USA 84, 109–112.
3 Barber, J., Chapman, D.J. and Telfer, A. (1987) FEBS Lett. 220, 67–73.
4 Barber, J. (1987) Trends Biochem. Sci. 12, 321–326.
5 Michel, H. and Deisenhofer, J. (1988) Biochemistry 27, 1–7.
6 Klimov, V.V., Allakhverdiev, S.I., Demeter, S. and Krasnovskii, A.A. (1979) Dokl. Akad. Nauk. SSSR 249, 227–230.
7 Cogdell, R.J. (1982) in Topics in Photosynthesis, Vol.4, Electron Transport and Photophosphorylation (Barber, J., ed.), pp. 177–196, Elsevier, Amsterdam.
8 Edelman, M., Goloubinoff, P., Marder, J.B., Fromm, H., Devic, M., Fluhs, R. and Mattoo, A.K. (1985) in Molecular Form and Function of the Plant Genome (Van Vloten-Doting, L., Groot, G.S.P. and Hill, T.C., eds.), pp. 291–300, Plenum, New York.
9 Kyle, D.J., Ohad, I. and Arntzen, C.J. (1984) Proc. Natl. Acad. Sci. USA 84, 4070–4071.
10 Aro, E.-M., Hundal, T., Carlsberg, I. and Andersson, B. (1990) Biochim. Biophys. Acta 1019, 269–275.
11 Greenberg, B.M., Gaba, V., Mattoo, A.K. and Edelman, M. (1987) EMBO J. 6, 2865–2869.
12 Shipton, C.A., Marder, J.B. and Barber, J. (1990) Z. Naturforsch. 45c, 388–394.
13 Trebst, A. and Depka, B. (1990) Z. Naturforsch. 45c, 765–771.
14 Ohad, I., Kyle, D.J. and Hirshberg, J. (1985) EMBO J. 4, 1655–1659.
15 Virgin, I., Ghanotakis, D.F. and Andersson, B. (1990) FEBS Lett. 269, 45–48.
16 Shipton, C.A. and Barber, J. (1991) Proc. Natl. Acad. Sci. U.S.A. in press.
17 Chapman, D.J., Gounaris, K. and Barber, J. (1991) in Methods in Plant Biochemistry, Vol. 5 (Rogers, L., ed.), pp. 171–193, Academic Press, London.
18 Preston, K.R., Dexter, J.E. and Kruger, J.E. (1978) Cereal Chem. 55, 877–888.
19 Claeson, G., Aurell, L., Karlsson, G., Gustausson, S., Friberger, P., Arielly, S. and Simonssen, R. (1979) in Cromogenic Peptide Substrates (Scully, M.F. and Kakkar, V.V., eds.), pp. 20–31, Churchill Livingston, London.
20 Sarath, G., De la Motte, R.S. and Wagner, F.W. (1989) in Proteolytic Enzymes; A Practical Approach (Beynon, R.J. and Bond, J.S., eds.), pp. 25–56, IRL Press, Oxford.
21 Beynon, R.C. and Bond, J.S. (eds.) (1989) Proteolytic Enzymes: A Practical Approach, IRL Press, Oxford.
22 Geiger, R. and Fritz, H. (1984) in Methods of Enzymatic Analysis (Bergmeyer, J. and Grassl, M., ed.), Vol. 5, pp. 119–129, Verlag Chemie, Weinheim.