LOSS OF SENSITIVITY TO DIURON AFTER TRYPSIN DIGESTION OF CHLOROPLAST PHOTOSYSTEM II PARTICLES

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1. Introduction

The membranes of the chloroplast contain numerous polypeptides, most of which have not yet been assigned to any photosynthetic function. Only the components of photosystem I [1], the chlorophyll *a/b* light harvesting protein [3,4] and of the coupling factor [2] have been clearly resolved. Most other photosynthetic functions have yet to be associated with any of the remaining polypeptides. One approach to relating individual polypeptides to function is to use selective trypsin digestion. When used on membrane systems, trypsin digestion has the added advantage of allowing exploration of the topographical organization of the membrane; only those functions that involve components exposed to the outer membrane surface would be expected to be affected by trypsin. When this approach is applied to chloroplast membranes, a variety of alterations in photosynthetic function result. These include a loss of Mg^{2+}-sensitive chlorophyll fluorescence [5–7], a loss of sensitivity to inhibition by diuron [8], a drop in the Em_7 of cytochrome *b*-559 [7,9] and inhibition of water oxidation [9]. Apart from cataloging these effects, it is very difficult to draw further conclusions because of the multitude of trypsin-induced changes. One approach to refining this technique has been to use a very brief exposure to low trypsin concentration such that a single function is lost, that of Mg^{2+}-regulated fluorescence and this was shown to coincide with digestion of a single polypeptide [5]. A second approach has been to study carefully the appearance of various trypsin-induced effects as a function of time [6]. In this paper a third approach is described, that of using a purified photosystem II fraction in which only a single effect of trypsin digestion is seen and which has a fairly simple polypeptide composition. This effect, the loss of sensitivity to inhibition by diuron, is accompanied by a marked alteration in the electrophoretic pattern of the photosystem II particle.

2. Materials and methods

Triton photosystem II particles were prepared by the method in [10]. Photochemical activity was assayed as described, using diphenyl carbazide as donor and dichlorophenolindophenol as acceptor [11]. Because these rates are often nonlinear with longer illumination times, sensitivity to diuron was tested by adding it in the dark piror to illumination, and measuring the initial rate.

Trypsin digestion of photosystem II particles was carried out at 25°C at 150 rev./min in a New Brunswick shaker. The chlorophyll (chl) concentration was 3.0–3.5 mg/ml and 10 μg/ml trypsin (Sigma T8253) were used in a medium containing 0.2 M sucrose and 10 mM Hepes (pH 7.5). After 20 min, 20 μg/ml trypsin inhibitor (Sigma I-5) was added, the sample stirred for 2 min and diluted to 1 mg chl./ml with a cold pH 6.8 medium. The photosystem II particles were

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collected by centrifugation for 1 h at 100 000 X *g* and assayed for activity.

Sodium dodecylsulphate (SDS)–polyacrylamide gel electrophoresis of photosystem II polypeptides was carried out essentially by the method in [12], but adapted to ensure complete solubilization and denaturation of the membrane components. Pigments were depleted from the particles by extraction in 90% methanol, the precipitated proteins were collected by centrifugation at 12 000 X *g* for 30 min and resuspended in 0.2 M sucrose, buffered at pH 7.5 with 10 mM Hepes. The sample was then mixed with a solution of 2% SDS, 1% mercaptoethanol and 8 M urea in phosphate buffer (pH 6.8) such that the protein concentration was ~0.80–0.85 mg/ml as assayed [13] using bovine serum albumin as a standard. The mixture was incubated at 100°C for 5 min and cooled. Between 50 μg and 90 μg protein together with 1 drop glycerol and 0.05% bromophenol blue were layered onto 10 cm gels of 10% polyacrylamide prepared as in [12]. Electrophoresis using phosphate buffer (pH 7) and 2.5 mA/tube was carried out until the bromophenol blue band had reached the bottom of the gel. The gels were fixed using methanol, water and acetic acid (5:5:1) and stained with 0.25% Coomassie blue. The destained gels were photographed and the Coomassie blue absorbance of the bands determined by scanning at 600 nm using a Gilford spectrophotometer equipped with a linear transport accessory. A molecular weight calibration curve was obtained by running cytochrome *c*, myoglobin, carbonic anhydrase, ovalbumin and bovine serum albumin as standards (all from Sigma).

3. Results

Figure 1 shows the inhibitory effect of the photosystem II inhibitor diuron on the rate of photoreduction of dichlorophenolindophenol by Triton photosystem II particles. In this experiment 10 μM diuron caused 60% inhibition of photosystem II activity, a range of 55–75% being observed with several different particle preparations. This level of inhibition is somewhat less than in whole chloroplasts. Incubation of the particles with trypsin markedly reduced the inhibitory effect of diuron, so that only 5–15% inhibition occurred. Trypsin treatment only slightly

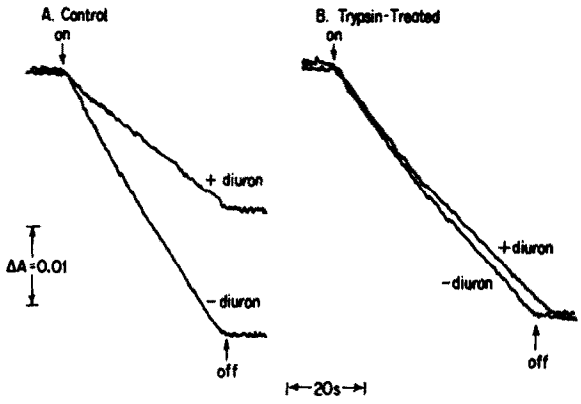

Fig.1. Inhibition of dichlorophenol indophenol reduction by diuron in trypsin-treated and control photosystem II particles. Particles were incubated for 20 min with and without trypsin as in section 2. Assays were performed at 15 μg chl/ml, with 0.4 mM diphenyl carbazide and 40 μM dichlorophenol indophenol. Reduction was measured at 550–500 nm using an Aminco DW-2 spectrophotometer using side-illumination filtered through a Corning 2-62 Filter. Diuron when added was 10 μM.

altered the rate of photosystem II activity observed in the absence of diuron, so that rates of 80–90% of controls were obtained. The concentration of diuron used was not an important contributing factor to these results; the trypsin-treated sample showed ⩽ 15% inhibition even with a 10-fold increase in diuron concentration (fig.2). At this concentration of

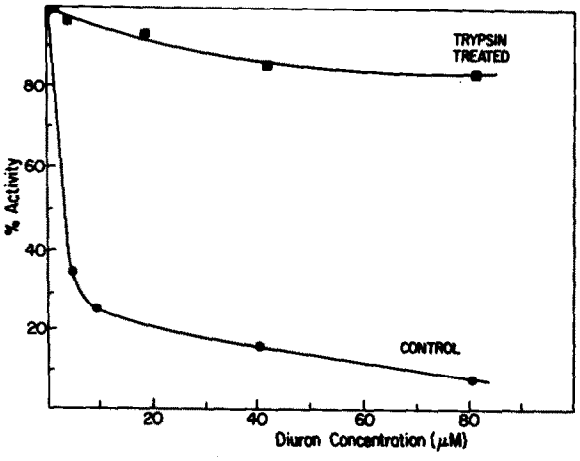

Fig.2. Inhibition of dichlorophenol indophenol reduction by different concentrations of diuron. Conditions as in fig.1.

diuron, the control sample was inhibited by 90%. This effect appears to be due to proteolytic action of trypsin, since incubation for $\geqslant$ 10 min is required before the effect is observed and since simultaneous addition of trypsin and trypsin-inhibitor prevented the loss of diuron inhibition. No other detectable alteration in photosynthetic function was seen, such as a change in the maximum yield of chlorophyll fluorescence or a loss of the effect of Mg^{2+} on the yield [11]. Thus trypsin digestion of photosystem II particles seems to have specifically blocked the action of the inhibitor diuron.

In an attempt to relate this effect to an alteration of individual polypeptides in the photosystem II particle, polyacrylamide gel electrophoresis of samples solubilised with SDS was carried out. Photographs of stained gels of particles incubated with and without trypsin are shown in fig.3. Two bands (marked X and Y) have been extensively degraded by trypsin and a new lower molecular weight band appears (Z). Figure 4 shows a densitometric scan of these gels, together with assigned molecular weights obtained from the calibration curve in fig.5. The two bands X and Y have mol. wt 32 000 and 27 000, respectively. The new band (Z) with mol. wt 17 000 appears to be different from one in the control at slightly higher molecular weight. Also it can be seen from the scan that there is a small decrease in amplitude of bands at mol. wt 58 000 and 44 000 and this could be related to a new band that appears at 35 000.

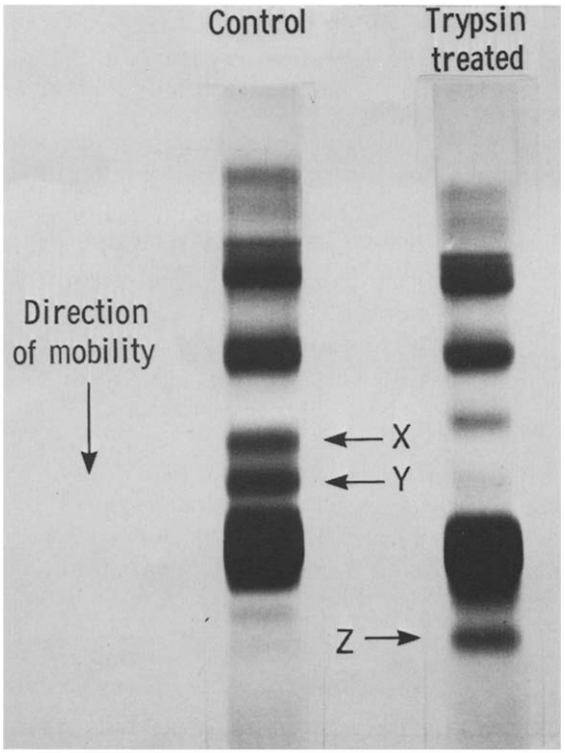

Fig.3. SDS–polyacrylamide gel separation of polypeptides from control and trypsin-treated photosystem II particles. For explanation, see text.

4. Discussion

Relief of electron transfer inhibition by diuron as a result of trypsin digestion was observed in whole

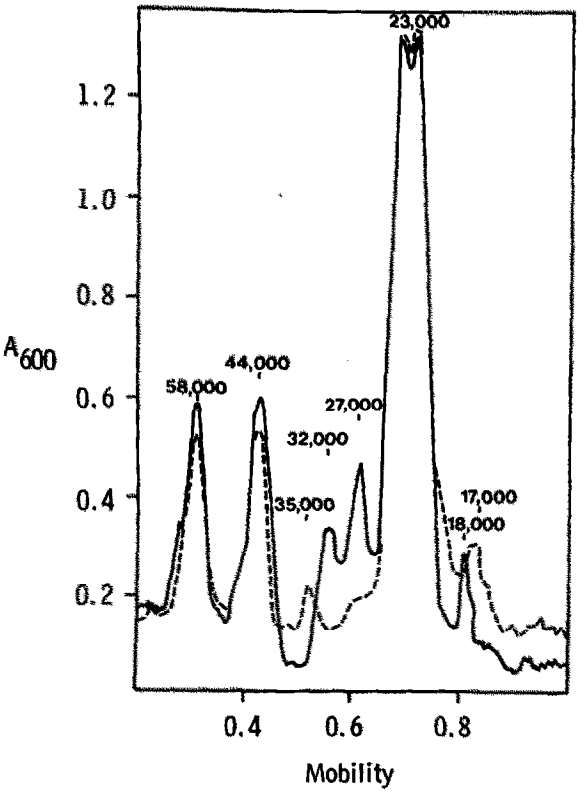

Fig.4. Densitometric scan of SDS–polyacrylamide gels of control (–) and trypsin-treated (– – – –) photosystem II particles. Molecular weights were assigned from the calibration curve in fig.5.

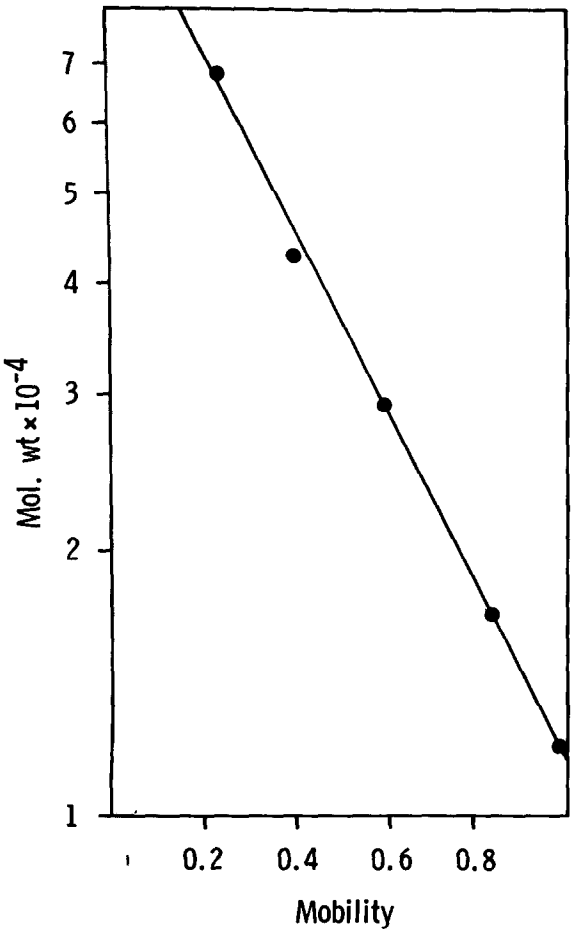

Fig.5. Calibration of molecular weights according to electrophoretic mobility. Data points are standards electrophoresed under identical conditions to the chloroplast proteins.

chloroplasts [8]. As an explanation, it was suggested that a surface proteinaceous shield prevents the primary acceptor of photosystem II (Q) from interacting with the electron acceptor ferricyanide, so that electrons have to normally flow through the diuron 'site'. Degradation of this shield by trypsin allows access of ferricyanide to Q and hence electron transfer becomes less sensitive to diuron. This explanation is supported by the observed inaccessibility of Q to added redox reagents [14], the ability of silicomolybdate, by interfering with membrane properties, to catalyse a diuron-insensitive photosystem II partial reaction [15] and the notion of a surface shield derived from the slow kinetics of proton binding by reduced plastoquinone [16]. Q is probably in a similarly shielded environment in photosystem II particles since electron flow in the control is almost completely inhibited by diuron, showing a lack of direct interaction between Q and dichlorophenolindophenol. In addition, in these particles, Q appears to still only slowly react with redox reagents (P. H., unpublished data). Thus, the effects of trypsin could be explained by a digestion of one or more polypeptides which shield Q from equilibration with the aqueous phase. An alternative explanation is that a membrane protein responsible for binding and mediating the effect of diuron has been digested by trypsin. These two alternatives can not be distinguished at present. The two most conspicuous changes in the gel pattern are the loss of two bands with mol. wt 32 000 and 27 000. Moreover, since the loss of diuron sensitivity is almost complete, one would predict that any polypeptide involved in the effect should be almost completely digested, as is seen for these two bands. Both these bands are enriched in the photosystem II particles; a photosystem I fraction isolated from the Triton supernatant [10], contained only trace amounts.

In comparison with published analyses of chloroplast polypeptide composition, it would seem that both the mol. wt 33 000 and 27 000 bands are included in the originally characterised group II polypeptides [17]. Photosystem II polypeptides with mol. wt 30 000–34 000, 25 000–26 000 and 21 000–23 000 have been characterised [4,18], in addition to a band of higher mol. wt ~50 000 which may be photosystem II reaction centre [18–20]. A larger band at mol. wt 60 000 and a smaller band at mol. wt 14 000 were also thought to be in photosystem II [18] although the larger may be photosystem I contamination [21]. Clearly, examination of the gel pattern in fig.4 shows good agreement with these analyses. The function of the mol. wt 33 000 and 27 000 bands have remained elusive; the latter was thought to be involved in granal stacking [17] but the former had no apparent role in this. Two similar polypeptides with mol. wt 30 000 and 25 000 were found in purified preparations of light harvesting complex [4]. Although a similar molecular weight does not prove that the same proteins are involved, it is of interest that a mol. wt 32 000 poly-

peptide has been proposed to be involved in proton translocation in the membrane CF_0 [22]. A relationship between proton-linked events and diuron action was indicated by an inhibition by diuron of an acid-induced decrease in redox potential of cytochrome *b*-559 [23]. The present study indicates that the mol. wt 33 000 and 27 000 polypeptides are surface polypeptides that form the 'proteinaceous shield' protecting the primary electron acceptor from interacting with the aqueous phase and/or the binding site for diuron.

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