THE EFFECT OF CATIONS ON DCMU-INSENSITIVE ELECTRON TRANSPORT IN TRYPSIN-TREATED SPINACH CHLOROPLASTS

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1. Introduction

Mild treatment of higher-plant thylakoids with the proteolytic enzyme trypsin digests a proteinaceous component (B) which normally overlies Q, the primary acceptor of photosystem II (PSII) and prevents access of externally added oxidants, such as ferricyanide (FeCN), to the PSII reaction centre [1]. In untreated chloroplasts the protein B is thought to regulate electron transfer between Q and the plastoquinone pool, and also to contain a binding site for the herbicide DCMU [1]. Thus when B is removed by trypsin treatment, exposing Q, oxidants such as FeCN can be reduced by Q in a DCMU-insensitive reaction.

In [2] the effects of trypsin were compared in normal 'high-salt' chloroplasts, in which a large proportion of the thylakoids are stacked to form grana, and in salt-depleted or 'low-salt' chloroplasts in which the grana unstack [3,4]. No DCMU-insensitive FeCN reduction was observed in low-salt chloroplasts after trypsin treatment [2]. Since trypsin was still very effective in inhibiting DCMU-sensitive FeCN reduction in low-salt chloroplasts, it was concluded that in low-salt chloroplasts a conformational change of membrane components had increased the accessibility of the water-oxidising enzyme itself to trypsin, so that inhibition of water oxidation by trypsin proceeded faster than digestion of the DCMU-binding protein B.

Here, the effects of trypsin on low- and high-salt spinach chloroplasts have been re-examined, in particular with regard to the effects of cations on electron transfer in trypsin-treated chloroplasts.

Abbreviations: PSII, photosystem II; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Mes, 4-morpholine ethane sulphonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine; FeCN, potassium ferricyanide; LHCP, the light-harvesting chlorophyll *a*/*b*–protein complex; chl, chlorophyll

2. Materials and methods

Fresh spinach leaves (*Spinacia oleracea*) were homogenised for 5 s in low-salt buffer (0.4 M sorbitol, 20 mM Tricine/KOH, pH 7.2) or high-salt buffer (0.4 M sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, 20 mM Tricine, pH 7.2) plus 5 mM isoascorbate. The brei was filtered through Miracloth (Calbiochem) and chloroplasts pelleted by centrifuging for 5 min at 2000 × *g*. The chloroplasts were washed once in low or high-salt buffer without sorbitol, then finally resuspended and assayed in low or high-salt buffer containing 0.4 M sorbitol. Chlorophyll was assayed as in [5].

Electron transport was measured at 20°C in saturating red light (Corning 2-62 filter) using a Rank O_2 electrode. Reaction mixtures (3 ml) contained 2 mM FeCN and chloroplasts equivalent to 30 μg chl. DCMU (5 μM) and NH_4Cl (3 mM) were added as required.

Trypsin treatment was carried out in the O_2 electrode chamber, in 1 ml of reaction buffer containing 30 μg chl and 50 μg trypsin (Sigma, type XI). The reaction was stopped by addition of a 3-fold excess of soybean trypsin inhibitor (Sigma type 1-S), and O_2 evolution assayed immediately after addition of 2 ml reaction buffer plus the appropriate reagents.

3. Results

High-salt chloroplasts assayed in high-salt buffer were capable of DCMU-insensitive FeCN reduction after brief trypsin treatment, but in low-salt chloro-

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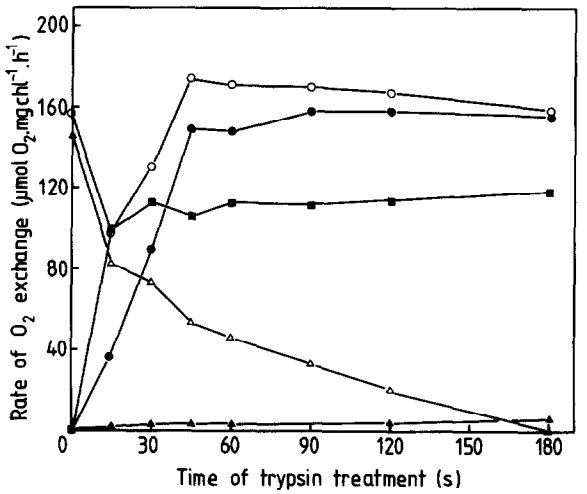

Fig.1. Effect of trypsin on electron transport in low-salt and high-salt spinach chloroplasts. Trypsin treatment was done as in section 2. High-salt chloroplasts treated and assayed in high-salt buffer (pH 7.2): $H_2O \rightarrow$ FeCN + NH_4Cl (o); $H_2O \rightarrow$ FeCN + DCMU (•). Low-salt chloroplasts treated and assayed in low-salt buffer: $H_2O \rightarrow$ FeCN + NH_4Cl (△); $H_2O \rightarrow$ FeCN + DCMU (▲); $H_2O \rightarrow$ FeCN + DCMU + 5 mM $MgCl_2$ (■).

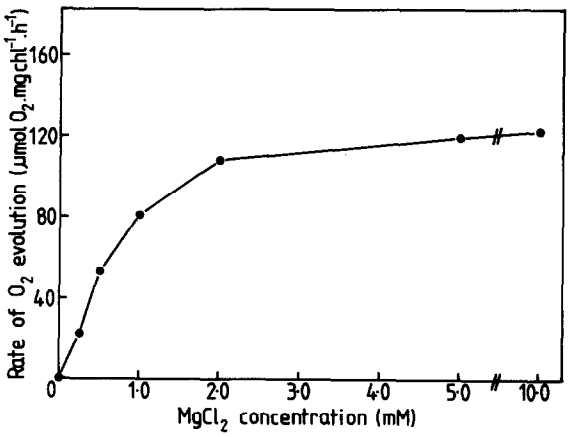

Fig.2. Effect of [$MgCl_2$] on DCMU-insensitive electron transport in trypsin-treated low-salt chloroplasts. Chloroplasts were treated with trypsin for 2 min in low-salt buffer (pH 7.2). O_2 evolution was assayed in the same buffer with 2 mM FeCN, 5 μM DCMU and [$MgCl_2$] as shown.

plasts assayed in low-salt buffer trypsin gradually inhibited FeCN reduction over ~3 min and any residual FeCN reduction was almost completely abolished by DCMU (fig.1). These results confirmed those in [1,2]. However if, instead of being assayed in low-salt medium as in [2], the trypsin-treated low-salt chloroplasts were assayed in a medium containing 5 mM $MgCl_2$, DCMU-insensitive FeCN reduction was restored, although the maximum rates were somewhat lower than for high-salt chloroplasts (fig.1). It was then found that trypsin-treated high-salt chloroplasts also required $MgCl_2$ in order to perform DCMU-insensitive FeCN reduction (not shown). $MgCl_2$ was not required during incubation with trypsin, but $MgCl_2$ in the final assay medium was essential.

In trypsin-treated chloroplasts, 2–5 mM $MgCl_2$ gave maximal rates of DCMU-insensitive FeCN reduction, both in low-salt and high-salt chloroplasts, with half-maximal rates at ~0.6 mM $MgCl_2$ (fig.2). When other salts were tried, $CaCl_2$ was found to be as effective as $MgCl_2$. 10 mM NaCl was relatively ineffective, so the observed requirement could not be explained by a chloride requirement for O_2 evolution. However at 50–100 mM both NaCl and KCl were partially effective in restoring DCMU-insensitive O_2 evolution (table 1).

The cation requirement for DCMU-insensitive FeCN reduction depended on the pH of the reaction medium. The results reported so far were obtained at pH 7.2, and there was also a strong cation requirement at pH 8.0 (fig.3). However, at pH 6.4 some DCMU-insensitive electron transport could be observed even in the absence of cations, although the rate could be increased further by 5 mM $MgCl_2$. The activity of trypsin itself was not markedly affected by pH, since electron transport through the complete chain from water to the photosystem I acceptor methylviologen was inhibited at all 3 pH-values. In the presence of 5 mM $MgCl_2$, DCMU-insensitive FeCN reduction was relatively unaffected by pH.

Table 1
Cation requirement for DCMU-insensitive ferricyanide reduction in trypsin-treated low-salt spinach chloroplasts

Salt added to assay medium[a]	Rate of DCMU-insensitive O_2 evolution (μmol O_2 . mg chl^{-1} . h^{-1})
None	0
5 mM $MgCl_2$	99
5 mM $CaCl_2$	113
10 mM NaCl	19
50 mM NaCl	59
100 mM NaCl	73
100 mM KCl	79

[a] Chloroplasts were treated with trypsin in low-salt buffer for 15 s as in section 2. O_2 evolution was assayed in low-salt buffer (pH 7.2) plus 2 mM FeCN, 5 μM DCMU and the additions of salts as shown

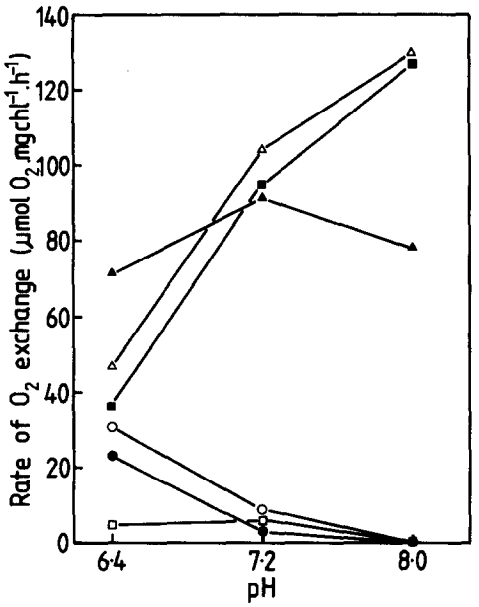

Fig.3. Effect of pH on the cation requirement for DCMU-insensitive electron transport in trypsin-treated low-salt chloroplasts. Chloroplasts were treated with trypsin for 3 min in low-salt buffer at the appropriate pH, then electron transport was assayed in the same buffer. Control chloroplasts: $H_2O \rightarrow$ FeCN + NH_4Cl (△); $H_2O \rightarrow$ 0.1 mM methylviologen + 0.5 mM NaN_3 + NH_4Cl (■). Trypsin-treated chloroplasts: $H_2O \rightarrow$ FeCN + NH_4Cl (○); $H_2O \rightarrow$ FeCN + DCMU (●); $H_2O \rightarrow$ FeCN + DCMU + 5 mM $MgCl_2$ (▲); $H_2O \rightarrow$ 0.1 mM methylviologen + 0.5 mM NaN_3 + NH_4Cl (□).

4. Discussion

It was concluded in [2], from the absence of DCMU-insensitive FeCN reduction in low-salt chloroplasts assayed in low-salt buffer, that in unstacked thylakoids an intra-membrane conformational change increased the susceptibility of the water-oxidising system to trypsin. This interpretation now requires re-examination, since I show here that both low- and high-salt chloroplasts can support DCMU-insensitive FeCN reduction in the presence of cations.

There have been reports that, apart from the requirement for cations for membrane stacking in higher-plant chloroplasts, there is also a cation requirement for activation of the PSII units themselves [6–8]. For example, in [6] at low light intensity where the number of active PSII units was the limiting factor for electron transport, $MgCl_2$ caused an increase in the rate of electron transport in low-salt chloroplasts. Since the increase was blocked by prior glutaraldehyde fixation of the membranes, it was suggested that micro-conformational changes of membrane components were involved. The cation activation effect was shown to be a separate effect from the cation-regulated distribution of light energy via the light-harvesting chlorophyll *a*/*b*–protein complex (LHCP), since it also occurred in plastids from intermittent-light-grown plants, which lack LHCP [9].

The cation requirement for DCMU-insensitive electron transport may involve a similar effect of cations on some part of the PSII unit. The [$MgCl_2$]-dependence of the reaction, and the fact that 50–100 mM monovalent cations showed the same effectiveness as 1–5 mM bivalent cations, suggest that electrostatic screening of membrane surface charges may be involved. The membrane surface in the region of PSII carries a net negative charge which is increased by trypsin treatment [10]. Screening of the negative charges by cations might prevent electrostatic repulsion between some intra-membrane components and thereby permit a conformational change which exposes Q to the membrane surface where it can be oxidised by FeCN. In the absence of cations, electrostatic repulsion would prevent the required conformational change and electron transport would be inhibited. At lower pH, the net negative charge on the membrane would be lower, and the need for electrostatic screening correspondingly less, thus explaining why some DCMU-insensitive ferricyanide reduction could be observed at pH 6.4 but not at pH 7.2 or 8.0. The molecular nature of conformational changes occurring in PSII during active electron transport remains unclear. However, ultrastructural studies of freeze-fractured thylakoids have revealed cation-induced changes in submembrane particles that were independent of the membrane stacking changes mediated by LHCP [11].

These results may also explain apparent discrepancies amongst reports on the effects of trypsin on electron transport. For example, in [12] the water-oxidising system was claimed to be more susceptible to trypsin digestion than the DCMU-binding protein, in contrast to [1]. However, the assay buffer used in [12] contained no $MgCl_2$ and only 10 mM NaCl, which as table 1 shows would be insufficient for significant rates of DCMU-insensitive electron transport.

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