PHOTOCHEMICAL ACTIVITIES OF CHLOROPLASTS TREATED WITH DIAZONIUM-1,2,4-TRIAZOLE

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1. Introduction

Chloroplast membranes are composed of two major chlorophyll particles, one enriched in chlorophyll *a* and the other in chlorophyll *b*. Many works on the chlorophyll particles have shown that the particles enriched in chlorophyll *a* and *b* are correlated with photochemical system I and II, respectively [1–6]. We have previously shown that diazonium-1,2,4-triazole reacted preferentially with the particle enriched in chlorophyll *a* and inferred that the particles responsible for the photochemical system I may be located on the external surface of the grana membrane and more reactive with the diazonium compound [7–9]. The same conclusion was reached by Dilley et al. [10] who treated chloroplast with a non-penetrating ^{35}S-*p*-(diazonium)-benzene sulfonic acid and found that photochemical system I but not system II was readily labeled with this reagent.

Since diazonium compound reacts with a number of amino acid residues such as histidine, tyrosine and lysine in proteins, the coupling reaction may result in the inactivation of the modified protein molecule. Thus it is to be expected that the photochemical activities of system I are decreased by the treatment of chloroplasts with diazonium-1,2,4-triazole. The present study was undertaken to examine this possibility.

Abbreviations; DCIP, 2,6-dichlorophenolindophenol; CMU, 3-(4-chlorophenyl)-1,1-dimethylurea; PMS, phenazine methosulfate.

2. Materials and methods

Chloroplasts were isolated from spinach leaves as previously described [7] using 0.05 M phosphate buffer [pH 7.4). Ferredoxin and ferredoxin-NADP$^+$ reductase were extracted from spinach leaves in 0.25 M phosphate buffer (pH 7.8) and partly purified by acetone precipitations according to the method of San Pietro and Lang [11]. The acetone-precipitate was dissolved in 0.05 M Tris buffer (pH 7.8) and chromatographed twice on DEAE cellulose [12]. Ferredoxin-NADP$^+$ reductase was obtained from the first effluent of DEAE-cellulose column by ammonium sulfate precipitation (40–65%) [12]. The reaction of chloroplasts with diazonium-1,2,4-triazole was conducted by the same procedure as described earlier [7], except that the carbonate buffer of pH 8.9 was replaced by 0.05 M phosphate buffer of pH 7.7. The chloroplast suspension treated with diazonium-1,2,4-triazole for 15 min in the dark at room temperature (15 ± 5°C) was washed twice by centrifugation at 0°C and subjected to the activity measurement.

3. Results and discussion

Curve A in fig. 1 shows the effect of diazonium-1,2,4-triazole treatment of chloroplasts on the NADP$^+$ photoreduction with reduced DCIP as electron donor. The activity dropped steeply on the treatment with a low concentration of diazonium-1,2,4-triazole and became zero above 0.17 mM of the reagent. By contrast, the Hill activity with DCIP as electron acceptor was little affected by the treatment with diazonium-1,

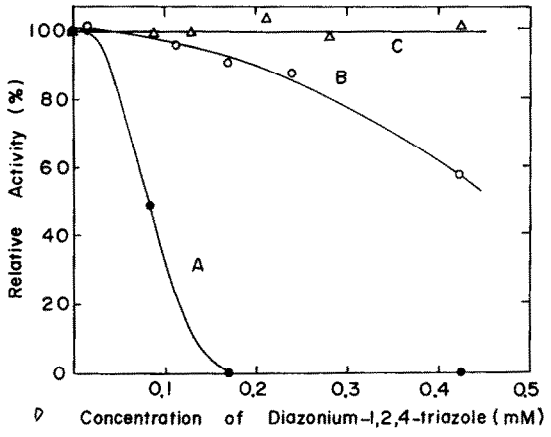

Fig. 1. Photochemical activities of chloroplasts treated with various concentrations of diazonium-1,2,4-triazole; curve A, $NADP^+$ photoreduction; curve B, Hill activity with DCIP as electron acceptor; curve C, oxygen uptake in the presence of methyl viologen and reduced DCIP. For $NADP^+$ photoreduction the reaction mixture contained diazonium-1,2,4-triazole-treated chloroplasts equivalent to 85 μg chlorophyll, Tris–HCl (pH 8.0) 60 μmoles, $MgCl_2$ 13 μmoles, NaCl 94 μmoles, $NADP^+$ 0.85 μmole, ascorbate 10 μmoles, DCIP 0.03 μmole, CMU 0.004 μmole and a saturating amount of crude preparations of ferredoxin and ferredoxin-$NADP^+$ reductase in a total volume of 4.6 ml. $NADP^+$ photoreduction of control chloroplasts treated similarly without diazonium-1,2,4-triazole was 90.2 μmoles $NADP^+$ reduced/mg chlorophyll per hr. The Hill reaction assay solution contained chloroplasts equivalent to 85 μg chlorophyll, Tris–HCl (pH 7.2) 130 μmoles and DCIP 0.16 μmole in 4 ml. The Hill activity of control chloroplasts was 158.0 μmoles DCIP reduced/mg chlorophyll per hr. The reaction mixture for the measurement of oxygen uptake obtained chloroplasts equivalent to 136 μg chlorophyll, tricine (pH 7.0) 200 μmoles, ascorbate 12 μmoles, $MgCl_2$ 8 μmoles, methyl viologen 0.4 μmole, CMU 12 μmoles and DCIP 0.4 μmole, CMU 12 μmoles and DCIP 0.4 μmole in 4 ml. The oxygen uptake of control chloroplasts was 102.5 μmoles O_2 absorbed/mg chlorophyll per hr. Light intensity at the position of reaction mixture was 40 000 lux. The reduction of $NADP^+$ and DCIP was followed at 340 and 610 nm, respectively, with a Shimadzu recording spectrophotometer model MPS-50 and the activity was determined from the initial decrease of the absorbance.

2,4-triazole as shown by curve B in the same figure and 92% activity of the control remained on the treatment with 0.17 mM of the reagent, where the activity of $NADP^+$ photoreduction was lost completely. These results indicate that the photosystem I-dependent electron flow was inhibited selectively or primarily by the treatment with diazonium-1,2,4-triazole. Although some preparations of chloroplasts did not lose the $NADP^+$ photoreduction activity completely on the treatment with diazonium-1,2,4-triazole above 0.4 mM, the decline of $NADP^+$ photoreduction activity was always much steeper than that of the Hill activity. When the Hill activity of chloroplasts treated with diazonium-1,2,4-triazole was measured with an ionic (class I) electron acceptor such as ferricyanide, the pattern of activity change was similar to that observed with DCIP as electron acceptor (results not shown).

In order to determine the site of inhibition by diazonium-1,2,4-triazole, photosystem I-dependent electron flow was measured by oxygen uptake in the presence of methyl viologen as electron acceptor and reduced DCIP as electron donor. As shown by curve C in fig. 1, the activity of oxygen uptake did not change at all on the treatment even with 0.42 mM of diazonium-1,2,4-triazole. A possible explanation for this is that electron transport from P_{430} to $NADP^+$ in the following scheme was blocked by the diazonium-1,2,4-triazole treatment. Since sufficient

$NADP^+ \leftarrow$ ferredoxin $\leftarrow P_{430} \leftarrow$ system I $\leftarrow$ plastocyanin $\leftarrow$ system II $\leftarrow H_2O$ methyl viologen

amounts of ferredoxin and ferredoxin-$NADP^+$ reductase were added to the reaction mixture for measuring the photosystem I-dependent activity after the treatment with diazonium-1,2,4-triazole, it is unlikely that the site of inhibition is ferredoxin or ferredoxin-$NADP^+$ reductase. We must, therefore, assume an intermediate carrier somewhere between P_{430} and $NADP^+$ which is inactivated by diazonium-1,2,4-triazole, although there is no direct evidence of the presence of the intermediate carrier.

Cyclic phosphorylation reaction catalyzed by PMS and non-cyclic phosphorylation reaction associated with the electron flow from H_2O to ferricyanide were all strongly inhibited by the diazonium-1,2,4-triazole treatment as shown by solid(cyclic) and open circles (non-cyclic) on curve A in fig. 2. As is evident from these results, the decline of cyclic phosphorylation was practically the same as that of non-cyclic phosphorylation. Curves B and C in the same figure show $NADP^+$ photoreduction and the Hill activity, respectively,

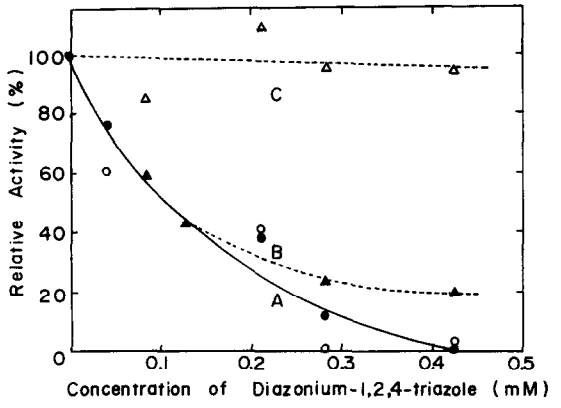

Fig. 2. Cyclic and non-cyclic phosphorylation reactions of chloroplasts treated with various concentrations of diazonium-1,2,4-triazole; curve A, cyclic phosphorylation(solid circles) and non-cyclic phosphorylation(open circles); curve B, $NADP^+$ photoreduction and curve C, the Hill activity measured using the same sample of chloroplasts treated with diazonium-1,2,4-triazole. The cyclic phosphorylation reaction mixture contained chloroplasts equivalent to 34 μg chlorophyll, tricine (pH 8.0) 50 μmoles, $MgCl_2$ 5 μmoles, NaCl 90 μmoles, ADP 2 μmoles, $Na_2H^{32}PO_4$ 5 μmoles and PMS 0.05 μmole in 3.2 ml. The non-cyclic phosphorylation reaction mixture was identical except that PMS was replaced by 1.5 μmoles of ferricyanide. Illumination was for 3 min at 40 000 lux. The rates of cyclic and non-cyclic phosphorylation reactions were 259.0 and 26.4 μmoles ATP/mg chlorophyll per hr, respectively. Reaction conditions of $NADP^+$ photoreduction and the Hill activity were as described in fig. 1.

measured using the same sample of chloroplasts. It is to be noted that cyclic and non-cyclic phosphorylation reactions were sharply decreased with increasing diazonium-1,2,4-triazole concentration to a greater degree than $NADP^+$ photoreduction. Since electron flow from reduced DCIP to methyl viologen as well as the flow from H_2O to DCIP were not inhibited by the diazonium-1,2,4-triazole treatment, it is inferred that diazonium-1,2,4-triazole may also inactivate a coupling factor which is known to be exposed on the outer surface of thylakoids [13]. As reported by Selman et al. [13], the uncoupling action of trypsin is due to attack on the coupling factor protein of thylakoids. Our result is also compatible with the observation of Bradeen and Winget [14] that $HgCl_2$ inhibits both cyclic and non-cyclic phosphorylation reactions but not electron transport from reduced DCIP to methyl viologen.

The inhibition of photosystem I-activity by the treatment with diazonium-1,2,4-triazole seems to be different from that caused by the inhibitors of photosystem I such as KCN [15], polycation [16] and $HgCl_2$ [17]. These inhibitors appear to inactivate primarily plastocyanin. Considering the finding of Kimimura and Katoh [17] that various electron acceptors such as DCIP and ferricyanide are predominantly reduced at the reducing side of photosystem I, it is unlikely that plastocyanin was inactivated by diazonium-1,2,4-triazole.

Diazonium-1,2,4-triazole seems have advantage in that the proteins which react with the reagent are marked. As reported previously, the products derived from the reaction of proteins and diazonium-1,2,4-triazole are stable [9]. If 3-amino-1,2,4-triazole labeled with $5\text{-}^{14}C$ is diazotized and used as a reagent, the modified proteins can be detected by following radioactivity. It is therefore hoped that the inhibition by diazonium-1,2,4-triazole treatment will be useful for the study of the location of thylakoid membrane proteins and their relation to the inhibition of photochemical activities.

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