Paraquat (Methylviologen): Its Interference with Primary Photochemical Reactions

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Paraquat (methylviologen), a widely used nonspecific herbicide, is photoreduced in the primary photochemical reaction of photosystem I. Using two types of the photosystem I reaction center preparations, *i.e.* one with FeS_A/FeS_B and the other without, the immediate electron donor to methylviologen was determined to be FeS_X (P_{430}) rather than FeS_A/FeS_B.

Introduction

Paraquat (methylviologen: 1,1′-dimethyl-4,4′-bipyridinium) has been used widely as a herbicide for some years. Methylviologen (MV) has also been known as an extremely electronegative artificial electron acceptor, and used in laboratories for the studies of biological electron transfer systems. Its midpoint redox potential, −446 mV, is as low as that of ferredoxin, the lowest redox potential among physiological electron carriers. In plant photosynthesis, electrons from photosystem I are highly negative enough to reduce MV. In its herbicidal action, paraquat (MV) that has been sprayed on leaves and penetrated into chloroplasts is photoreduced as above in place of ferredoxin, the natural electron acceptor. Reduced MV, a free radical, in turn reduces oxygen and generates a superoxide anion, O_2^-, a highly toxic radical that attacks numerous substances in the plant cells, thus fatally wounding the whole plant. This nonselective herbicide is useful and convenient in the field because it is quickly detoxicated by its strong adsorption to soil particles once it is in contact with the soil. This enables sowing and planting crops immediately after herbicide spraying. The immediate electron donor to MV is often regarded to be iron-sulfur clusters, FeS_A/FeS_B, mainly because the reoxidation of P_{430}, allegedly FeS_A/FeS_B (formerly Center A/Center B, respectively), is accelerated by MV [1, 2]. Another iron-sulfur cluster, FeS_X (formerly Component X) can also be a candidate, since this cluster was implicated as P_{430} [3]. In the present study, five types of photosystem I reaction center preparations were examined in terms of MV effects on the kinetics of flash-induced absorbance changes around 430 nm. These preparations fall into two categories: one with the PsaC subunit, the host protein for FeS_A/FeS_B, and another without PsaC.

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Materials and Methods

Reaction center preparations

All the preparations were performed in a cold room except when indicated otherwise. Broken chloroplasts were prepared from field grown spinach leaves suspended in 50 mM sodium phosphate, pH 7.0, 10 mM NaCl, grounded and washed by centrifugation. Several types of reaction center preparations were prepared from this broken chloroplast preparations as follows:

$ps1_a$ preparation: Broken chloroplasts were resuspended in 50 mM Tris-HCl, pH 8.8, 10 mM NaCl and 2% Triton X-100 at the chlorophyll concentration of 2 mg/ml, and kept stirring at 4 °C for 6 h, and then centrifuged at 12,000 × *g* for 30 min to remove debris. The supernatant was loaded on a DEAE-Toyopearl 650S column equilibrated with 10 mM Tris-HCl, pH 8.8 (at 20 °C) and 0.2% Triton X-100. The column was washed by the 20 column volumes of the above medium plus 10 mM NaCl, then with 4 column volumes of the above medium plus 50 mM NaCl, and eluted by the 4 column volumes of 50–200 mM linear gradient of NaCl. P700-active fractions were collected, diluted with an equal volume of 10 mM Tris-

HCl, pH 7.5 plus 0.6 mM $CaCl_2$ and loaded on a hydroxyapatite column (Pentax HP-40, Asahi Optical Co. Ltd, Tokyo) equilibrated with 10 mM Tris-HCl, pH 8.8 plus 0.3 mM $CaCl_2$ and 0.05% Triton X-100. The column was washed with five volumes of the above medium, and then eluted with a small volume of 50 mM sodium phosphate, pH 8.0 plus 0.05% Triton X-100. Green fractions were collected and dialyzed against 10 mM Tris-HCl, pH 8.8. Sucrose (0.2 M, final) was added before storage at −50 °C.

$ps1_b$ preparation: One volume of broken chloroplast suspension was mixed with two volumes of preheated (45 °C) medium (50 mM Tris-HCl, pH 8.8, 3% Triton X-100) and incubated at 45 °C for 30 min, then chilled in an ice bath, and centrifuged at 12,000 × *g* for 30 min to remove debris. The rest of the procedure was the same as described above for the $ps1_a$ preparation.

$cp1_a$ preparation: The $ps1_a$ preparation (protein concentration, 1 mg/ml) was incubated in 10 mM Tris-HCl, pH 8.0, 0.5% lithium dodecyl sulfate (LDS) and 0.05% Triton X-100 for 30 min at 4 °C. The aliquot was loaded on a Sephacryl S 300 HR column equilibrated with the above medium minus LDS. Fractions of the first-coming peak detected by the 280 nm absorbance were collected and concentrated by using ultrafiltration. This gel-permeation chromatography was repeated once more.

$cp1_b$ preparation: The $ps1_a$ preparation (protein concentration, 2 mg/ml) was incubated in 10 mM Tris-HCl, pH 8.0, 4 M sodium iodide (NaI) and 0.05% Triton X-100 for 10 min at 30 °C. The aliquot was chilled on ice and subjected to the gel-permeation chromatography as described above for $cp1_a$.

$cp1_c$ preparation: The $ps1_b$ preparation (final protein concentration, 0.1 mg/ml) was suspended in 2 M NaI containing 0.1% Triton X-100 and 10 mM Tris-HCl, pH 8.8, which had been exhaustively deoxygenated by flushing with purified nitrogen gas (99.9998%). The suspension was immediately ultrafiltrated through a UK-50 filter (Advantec, Tokyo) by using high pressure nitrogen (2.5 kg/cm^2). The filtrate that contained the small subunits was stored at −80 °C for the reconstitution experiment. The concentrate was diluted with the above medium minus NaI and filtrated again. This procedure was repeated three times to remove NaI and smaller subunits. The final concentrate was stored at −80 °C after supplementing 0.4 M sucrose.

Subunit compositions of the reaction center preparations

Subunit compositions were determined by using SDS-PAGE as described in ref. 4; $ps1_a$ (Type A in ref. 4) consisted of PsaA, PsaB, PsaC, PsaD, PsaE, and PsaL; $ps1_b$ (Type B in ref. 4), PsaA, PsaB, PsaC, psaD, psaE; $cp1_{a,b,c}$, PsaA and PsaB only. Identifications of those bands were done by immunostaining using corresponding antiserums as described [4].

Reconstitution of the photosystem I reaction center

The method of Parrett *et al.* [5] was used with some modifications as follows. The small subunit mixture prepared as described above was suspended in 10 mM Tris-HCl, pH 8.8 and 0.1% Triton X-100 (protein concentration, 0.2 mg/ml), and flushed thoroughly with nitrogen. Mercaptoethanol was added to the final concentration of 0.5%, flushed again with nitrogen and allowed to stand for 5 min. One two hundredth of 30 mM Na_2S was added to the final concentration of 0.15 mM, flushed with N_2 and incubated for 5 min. One two hundredth of 30 mM $FeCl_3$ was added to the final concentration of 0.15 mM, flushed with N_2 and incubated for 5 min. Reconstitution was completed by adding $cp1_c$ to the mixture at the final concentration of 0.1 mg protein/ml. The mixture was then flushed with N_2, incubated for 5 min, chilled on ice, and finally concentrated by an ultrafilter (Millipore Molcut MW 30,000). The ultrafiltration procedure was repeated once more.

Other methods

Flash kinetic spectroscopy was performed as described in [6]. Reaction mixtures contained, in addition to a reaction center preparation, 2 mM sodium ascorbate, 0.1 mM N,N,N′,N′-tetramethyl-*p*-phenylenediamine (TMPD), 50 mM Tris-HCl, pH 8.8 and 0.05% Triton X-100. P_{700} concentrations were determined as in [7].

Results and Discussion

Kinetics of flash-induced absorbance changes in the ps1 type reaction center preparations

Although the digitonin particles used originally in [2] were much cruder, kinetics of flash-induced

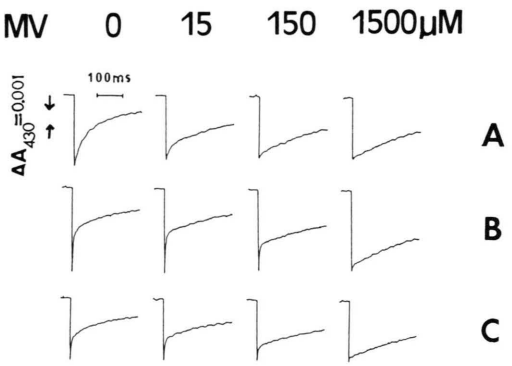

Fig. 1. Effect of methylviologen (MV) on flash-induced absorbance changes at 430 nm. A, psl_a; B, cpl_a; C, cpl_b. MV concentrations are indicated on top of the traces. Each trace was an average of 4 flash-induced kinetics.

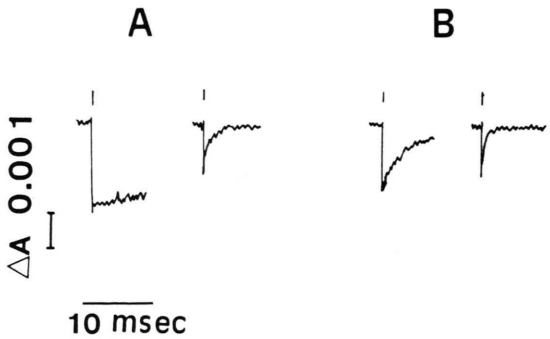

Fig. 2. Effect of methylviologen (MV) on flash-induced absorbance changes at 444 nm. A, psl_a (left, no MV; right, 0.128 mM); B, cpl_a (left, no MV; right, 0.182 mM). Each trace was an average of 16 flash-induced kinetics.

absorbance changes in Triton-treated psIs were basically the same; in the absence of artificial electron acceptor, the time course of an absorbance change at 430 nm was monophasic with $t_{1/2}$ of 100 msec (Fig. 1 A). Upon the addition of MV, decay kinetics became biphasic with faster and slower phases. By increasing the concentration, the faster phase became very fast and the slower one, quite slow; the whole kinetics looked monophasic, as the fast phase became invisible. This phenomenon had been interpreted [1, 2] as follows: at 430 nm, two components change absorbance. The one whose decay becomes very slow in the presence of a high concentration MV is P_{700}, because the difference spectrum is that of P_{700} including a typical 700 nm peak, and also because the decay is accelerated by increasing electron donor (TMPD/ascorbate) concentrations. The other one whose decay is accelerated by the addition of MV is apparently different from P_{700}, and named P_{430}. P_{430} is supposed to be an electron acceptor that is photoreduced concomitantly with the photooxidation of P_{700}. The decay in the dark after the flash represents reoxidation directly by oxidized P_{700}. When an artificial electron acceptor like MV was added, the decay can be accelerated depending on the acceptor concentration. This situation is more evident when the absorbance changes at 444 nm are monitored as in Fig. 2 A. At this wavelength, which is an isosbestic point of P_{700}, P_{430} alone is observed. With no artificial electron acceptor, the electron from P_{430} goes directly back to P_{700} with a half-decay time of about 50 msec in case of the spinach digitonin particles [2], and 100 msec in the present psl_a and psl_b particles. Upon the addition of MV, the decay became much faster (Fig. 2 A, left trace).

Kinetics of flash-induced absorbance changes in the cpl type reaction center preparations

In the cpl preparations that lacked the PsaC subunit as well as other small subunits, decay kinetics without MV were different: decays were much faster and often appeared biphasic (Fig. 1 B and C). Faster phase in case of cpl_a and cpl_b had a half decay time of about 8 msec. In case of cpl_c that had been more carefully prepared and kept anaerobic until use, the decay was more or less monophasic with a half decay of 7 msec (Fig. 3 B). At 444 nm, the decay was monophasic with a half time of 7–8 msec (Fig. 2 B, left trace). This decay was accelerated by the addition of MV (Fig. 2 B, right trace). At 430 nm, the same situation observed in psIs became evident as the MV concentration increased (Fig. 1 B and C; Fig. 3 B). At a very high concentration of 1.5 mM, there seemed to be no difference between psI and cpI with only one phase visible as seen in Fig. 1. The half decay times depended entirely on TMPD concentration in cpl_a, cpl_b and cpl_c as well as in psl_a and psl_b, indicating that these decay phases were those of P_{700} (data not shown). The rate constants of these pseudofirst order reactions were within the same range, indicating the property of P_{700} did not

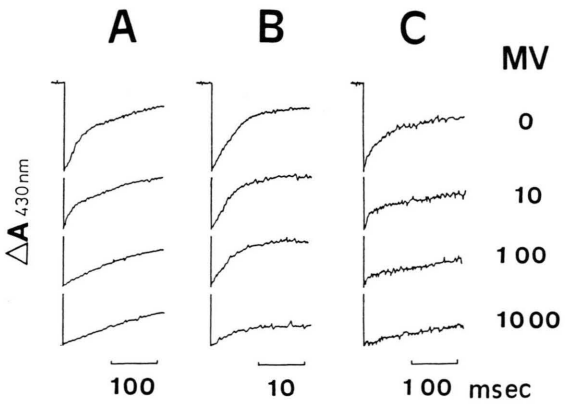

Fig. 3. Effect of methylviologen (MV) on flash-induced absorbance changes at 430 nm. A, psl_b; B, cpl_c; C, reconstituted psl. MV concentrations are indicated on the left column. Each trace was an average of 4 flash-induced kinetics.

change throughout the process of small subunit removal, either by a detergent (LDS) or a chaotropic agent (NaI).

Kinetics of flash-induced absorbance changes in a reconstituted psl preparation

As it was shown previously [5], the decay phase at 430 nm became as slow as that of the original psl in a reconstituted photosystem I reaction center preparation (Fig. 3C). The effect of MV was not much different from that in the original psl, either.

Difference spectra of P_{430}

Photoreduced-minus-oxidized difference spectra of P_{430} were obtained previously by subtracting photooxidized-minus-reduced difference spectra of P_{700} from those of total absorbance changes [1–3]. Fig. 4 shows those spectra (closed circles) obtained this time with psl_a (A), cpl_a (B) and cpl_b (C), together with those of P_{700} (open circles). Similar spectra were obtained with psl_b, cpl_c and reconstituted psl (data not shown). It is obvious that there is not much difference among these spectra. This result strongly suggests that P_{430} remains intact in cpls which do not have the PsaC subunit thus lacking FeS_A/FeS_B, and also supports a previous proposal that P_{430} is FeS_X [3].

Rate constants of P_{430} oxidation by MV

A reoxidation of P_{430} by MV was shown to be a pseudofirst order reaction [2]. Half decay times at 444 nm were plotted against MV concentrations in Fig. 5. There was no significant difference between psl_a (open circles) and cpl_b (closed circles), again indicating the presence of P_{430} in cpls.

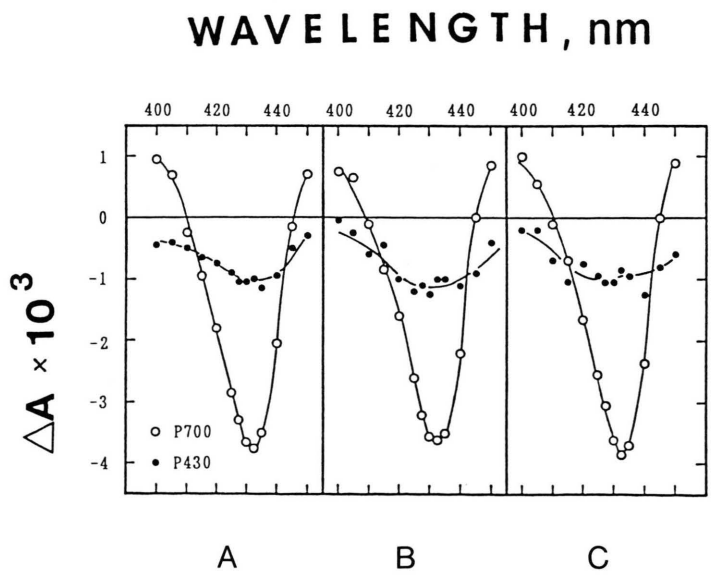

Fig. 4. Photoreduced-minus-oxidized difference spectra of P_{430} and photooxidized-minus-reduced difference spectra of P_{700}. A, psl_a; B, cpl_a; C, cpl_b.

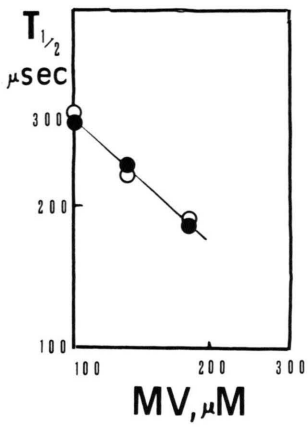

Fig. 5. Log-log plot of halv decay times at 444 nm against MV concentrations. Open circles, psl_a; closed circles, cpl_b.

Conclusion

The results shown in the present report indicates that MV (Paraquat) is reduced by P_{430}, which is present in reaction center preparations that lack the PsaC subunit (the FeS_A/FeS_B protein). P_{430} is most likely FeS_X rather than FeS_A/FeS_B, as a previous EPR experiment strongly suggested [3].

Acknowledgements

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