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Electron-transfer kinetics in cyanobacterial cells: Methyl viologen is a poor inhibitor of linear electron flow

Pierre Sétif *

iBiTec-S, CNRS UMR 8221, CEA Saclay, 91191 Gif-sur-Yvette, France

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ABSTRACT

The inhibitor methyl viologen (MV) has been widely used in photosynthesis to study oxidative stress. Its effects on electron transfer kinetics in *Synechocystis* sp. PCC6803 cells were studied to characterize its electron-accepting properties. For the first hundreds of flashes following MV addition at submillimolar concentrations, the kinetics of NADPH formation were hardly modified (less than 15% decrease in signal amplitude) with a significant signal decrease only observed after more flashes or continuous illumination. The dependence of the P700 photooxidation kinetics on the MV concentration exhibited a saturation effect at 0.3 mM MV, a concentration which inhibits the recombination reactions in photosystem I. The kinetics of NADPH formation and decay under continuous light with MV at 0.3 mM showed that MV induces the oxidation of the NADP pool in darkness and that the yield of linear electron transfer decreased by only 50% after 1.5–2 photosystem-I turnovers. The unexpectedly poor efficiency of MV in inhibiting NADPH formation was corroborated by *in vitro* flash-induced absorption experiments with purified photosystem-I, ferredoxin and ferredoxin-NADP$^+$-oxidoreductase. These experiments showed that the second-order rate constants of MV reduction are 20 to 40-fold smaller than the competing rate constants involved in reduction of ferredoxin and ferredoxin-NADP$^+$-oxidoreductase. The present study shows that MV, which accepts electrons *in vivo* both at the level of photosystem-I and ferredoxin, can be used at submillimolar concentrations to inhibit recombination reactions in photosystem-I with only a moderate decrease in the efficiency of fast reactions involved in linear electron transfer and possibly cyclic electron transfer.

1. Introduction

Inhibitors of electron flow have been widely used for the characterization of electron/proton transfer reactions in photosynthesis [1]. The herbicide methyl viologen (MV; also named paraquat) is thought to inhibit photosynthetic electron flow by accepting electrons from photosystem I (PSI) [2]. It is highly water-soluble and exhibits a low midpoint potential of −446 mV *vs* NHE [3]. After single reduction from the divalent to the monovalent cation state, MV is rapidly reoxidized by oxygen [4], a reaction that leads to superoxide production, with subsequent formation of other deleterious ROS species [5]. The ability of MV to induce oxidative stress has been extensively used to study the physiological adaptation of photosynthetic organisms and to study/select mutants resistant or sensitive to this stress (cyanobacteria: [6–15]; plants: [16–19]). Despite this, the MV reduction process is very poorly characterized *in vivo*, regarding both its exact site(s) of action and its kinetic properties.

During linear electron transfer (LET), NADP$^+$ is reduced into NADPH. This involves light-induced charge separation within PSI which, besides the oxidation of the primary donor P700, eventually leads to the fast (sub)microsecond reduction of the terminal electron acceptor (F_A, F_B) [20,21], a pair of closely spaced [4Fe–4S] clusters [22,23]. The different steps following PSI charge separation and leading to NADP$^+$ reduction have been studied *in vitro* [24–27] whereas only NADPH formation has been observed *in vivo* up to now [28,29] (see however [30]). These steps involve the fast microsecond reduction of the soluble acceptor ferredoxin (Fd) by PSI followed by NADPH formation by ferredoxin-NADP$^+$-oxidoreductase (FNR). This enzyme catalyzes the two electron reduction of NADP$^+$ using two reduced ferredoxins (Fd_{red}). In the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter named *Synechocystis*) as in many cyanobacteria, two FNR isoforms are present [31]. Under phototrophic conditions, the large isoform FNR_L, which is bound to the phycobilisome, constitutes the most part of FNR [32]. The short isoform FNR_S is similar to plastidial FNR and is expressed under growth conditions involving respiratory electron transfer [32]. Purified FNR_S and FNR_L (bound to a phycobilisome subcomplex) have been previously found to exhibit rather similar catalytic properties [33]. Apart from LET, PSI is

Abbreviations: CBc, Calvin–Benson cycle; CET, cyclic electron transfer; DCMU, the photosystem II inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea; ET, electron transfer; (F_A, F_B), terminal PSI acceptor; (F_A, F_B)$^-$, singly reduced terminal PSI acceptor; Fd, ferredoxin; Fd_{red}, reduced ferredoxin; FNR, ferredoxin-NADP$^+$-oxidoreductase; FNR_{ox}, oxidized FNR; FNR_{sq}, singly reduced FNR or semi-quinone form; FNR_L, large FNR isoform; FNR_S, small FNR isoform; LET, linear electron transfer; MV, methyl viologen; OPPc, oxidative pentose phosphate cycle; PSI, photosystem I

* Tel.: +33 169089867.
E-mail address: pierre.setif@cea.fr.

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also involved both in cyclic electron transfer (CET) and recombination reactions. The contribution of these two processes is expected to increase relatively to LET when the pool of stromal reductants (PSI acceptors, Fd, NADP) is highly reduced.

The main goal of the present work was to show that MV can be used as a relatively specific inhibitor of PSI recombination reactions in cyanobacterial cells of *Synechocystis*. For this purpose we studied *in vivo* both NADPH formation and decay and $P700^+$ kinetics. We also studied by flash-absorption spectroscopy the effect of MV addition in reconstituted systems comprising PSI, PS/Fd and PSI/Fd/FNR to tentatively correlate the *in vitro* electron transfer (ET) kinetics to the *in vivo* observations. This led us to identify the sites of MV reduction and to quantify the rates of this process both *in vivo* and *in vitro*.

2. Materials and methods

2.1. Biological materials

In vitro experiments were performed with PSI monomers from *Synechocystis* [34] and recombinant forms of Fd [35] and FNR_S [27] from the same organism. The PSI, Fd and FNR_S concentrations were estimated by assuming absorption coefficients of 7.7 mM^{-1} cm^{-1} for $P700^+$ at 800 nm [27], 9.7 mM^{-1} cm^{-1} at 422 nm [36] and 9.0 mM^{-1} cm^{-1} at 461 nm [33], respectively. Wild type cells of *Synechocystis* were grown photoautotrophically as described in [29]. In cell suspensions, the chlorophyll *a* concentrations were measured after methanol extraction using the absorption coefficient given in [37] and the PSI concentrations were calculated by assuming an *in vivo* chlorophyll to P700 ratio of 105, in accordance with an estimated PSI/PSII ratio larger than 4 [29].

2.2. In vitro flash-absorption spectroscopy

Measurements were made at 22 °C under aerobic conditions (open cuvettes) as described previously [25,27] in the presence or absence of MV with PSI alone, with PSI + Fd and with PSI + Fd + FNR_S. A short laser flash was used for saturating PSI photochemistry. The laser excitation (wavelength, 695 nm; duration, 6 ns; energy, 30 mJ; one flash every 10 s for data averaging) was provided by a dye laser (Continuum, Excel Technology France) pumped by a Nd:YAG laser that was frequency-doubled (Quantel, France). Measurements were made either in 1-cm square cuvettes or in 1-mm cuvettes (1.2 mm pathlength) in 20 mM Tricine, pH 8.0, in the presence of 30 mM NaCl, 5 mM $MgCl_2$, 0.03% β-dodecyl maltoside, 2 mM sodium ascorbate and 25 μM 2,6-dichlorophenolindophenol. Measurements were made at 3 different wavelengths, 480, 580 and 800 nm. The PSI concentrations were calculated from $P700^+$ measurements at 800 nm (data not shown). Fd reduction by PSI was observed at 480 and 580 nm, two wavelengths at which this process was already studied [25,26], and where chlorophyll absorption is not too large, thus making relatively easy to avoid the actinic effects of the measuring light. The reoxidation of $(F_A, F_B)^-$ by MV was measured at 480 nm and the signals were compared to those measured in the presence of Fd. Absorption changes in the presence of FNR_S were studied at 580 nm, a wavelength at which formation of the FNR semiquinone gives a strong contribution [24].

2.3. In vivo $P700^+$ photooxidation kinetics

$P700^+$ photooxidation kinetics were measured at 32 °C in the infra-red region with a PAM spectrophotometer (Walz, Effeltrich, Germany) [38] which was modified for synchronization of data acquisition and shutter-controlled actinic light. The infra-red measuring light was modulated at 100 kHz. Samples were illuminated for 5 s with actinic far-red light from a halogen lamp which was filtered by 2 RG-695 long-pass filters (Schott, Germany) and heat-absorbing filters eliminating infra-red light. The sample was continuously stirred in an opened 1-cm cuvette except during the measurements. Cells were incubated in darkness at 32 °C for 8 min before addition of 20 μM DCMU. For all samples, two measurements ($\Delta t = 2$ min) with DCMU were averaged after 3 min of dark incubation before the first addition of MV.

For the study of $P700^+$ kinetics as a function of MV concentration, MV was added sequentially and the sample was incubated for 15 min after each addition before the measurement. Two control experiments were made to check that the increase in the rate of $P700^+$ formation with [MV] (Fig. 4A/B) cannot be attributed to an increasing incubation time at 32 °C: firstly, a sample with a single addition of 15 μM MV was initially studied after 15 min of dark incubation and this measurement was repeated after 30, 45 and 60 min of incubation, giving identical kinetics for all times; secondly, a sample with a single addition of 0.5 mM MV was studied after 15 min of dark incubation and gave the same kinetics as those of Fig. 4 with the same MV concentration (cumulative time of incubation of 75 min after the initial addition of 7.5 μM MV). For the study of $P700^+$ kinetics as a function of incubation time following the addition of 0.25 mM MV (Fig. 4C), a control experiment was made to check that the increase in the rate of $P700^+$ formation with the incubation time was not dependent upon the number of illuminations given to the sample before the measurement: in the control sample, a single measurement was made after 12 min of incubation following the addition of 0.25 mM MV with kinetics identical to those observed for a sample which had been measured 3 more times (after 3, 6 and 9 min of incubation).

2.4. In vivo NADPH measurements

Light-induced measurements were performed on *Synechocystis* cell suspensions at 32 °C as described in [29] using the NADPH/9-AA module [39] of a DUAL-PAM spectrophotometer (Walz, Effeltrich, Germany) in square 1 × 1 cm opened cuvettes. As described in [29], the fluorescence levels could be converted into NADPH concentrations by measuring the fluorescence signal of a known concentration of exogenous NADPH that was added to the cell suspension at the end of the experiment. It was also previously found that the light-induced NADPH concentrations thus calculated are 2- to 4-fold overestimated due presumably to enhancement of *in vivo* fluorescence compared to that of exogenous NADPH. Therefore these light-induced concentrations are qualified as "apparent concentrations" whereas the real light-induced concentrations are 2- to 4-fold smaller than the apparent ones. All baselines before illumination were set arbitrarily to 0.

3. Results

3.1. In vitro MV reduction by the reduced PSI terminal acceptor and by reduced Fd

Fig. 1 compares the reduction of 50 μM MV by PSI (trace b) and PSI/Fd (trace d) at 480 nm measured by flash-absorption spectroscopy. Control measurements were made in the absence of MV with identical PSI concentrations either in the absence (trace a) or presence (trace c) of Fd. The fast absorbance increases (not time-resolved) observed at time 0 in traces a and b reflect both the photooxidation of the PSI primary donor P700 and the single reduction of the PSI terminal acceptor (F_A, F_B). At 480 nm, P700 oxidation leads to an absorbance increase whereas (F_A, F_B) reduction corresponds to an absorbance decrease. The resulting signal is positive as the contribution of P700 is larger than that of (F_A, F_B) [25]. In the sample containing only PSI (trace a), only a small decay is observable on a 10 ms timescale, which is attributed to the slow recombination reaction between $P700^+$ and $(F_A, F_B)^-$ ($t_{1/2} \approx 80$ ms in PSI from *Synechocystis*, [40]). In trace b, the absorbance increase is attributed to the oxidation of $(F_A, F_B)^-$ by MV [25,41].

When Fd is added to PSI (trace c), the initial rise is followed by an absorbance decrease due to reduction of Fd by $(F_A, F_B)^-$, as previously observed [25] and in line with the observation that reduction of the