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Flash-induced oxidation of cytochrome *b*-563 in algae under anaerobic conditions: effect of dinitrophenylether of iodonitrothymol

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Oxidation of cytochrome *b*-563 and reduction of oxidized plastocyanin were studied under reducing conditions in a mutant of *Chlorella sorokiniana* submitted to a single actinic flash. The rate of both processes is strongly decreased by addition of dinitrophenylether of iodonitrothymol. The slow increase of the membrane potential (phase *b*) is also strongly inhibited. Thus dinitrophenylether of iodonitrothymol is an efficient inhibitor of the site of oxidation of cytochrome *b* by Photosystem I (Q_z site) in living algae. These results are consistent with the view that the Q_z site can catalyze both cytochrome *b*-563 reduction and oxidation in a mechanism involving just one heme group of cytochrome *b*. The rate constant of the inhibitor release is higher than 100 s^{-1}.

Introduction

The electron flow from Photosystem II to Photosystem I involves a cytochrome *bf* complex which includes two hemes of cytochrome *b*-563, and one molecule of Rieske iron-sulfur protein and cytochrome *f*. The electron transfer from Photosystem II to the cytochrome *bf* complex occurs via a pool of plastoquinone. Photosystem II reduces plastoquinone to plastoquinol by transferring two electrons. Plastoquinol is then oxidized by the cytochrome *bf* complex at a quinol-binding site Q_z located on the internal side of the thylakoid membrane [1]. Plastoquinol transfers one electron to Photosystem I via the Rieske protein, cytochrome *f* and plastocyanin: this one-electron event results in an oxidation of plastoquinol to plastosemiquinone PQ^-. This plastosemiquinone transfers one electron to a cytochrome *b* heme close to the Q_z site. In the modified Q-cycle model [2,1] this electron is thought to be transferred across the membrane via a second cytochrome *b* heme to a molecule of plastoquinone at a site Q_c located on the external side of the membrane. In this hypothesis, the transfer of one electron across the membrane via two cytochrome *b* hemes accounts for the slow phase of the flash-induced membrane potential increase (phase *b*: cf Refs. 3–5).

However, the Q-cycle mechanism is not generally accepted as an obligatory pathway for the electron transfer [6,7]. Hartung and Trebst [8] pointed out that the inhibitors of the Q_c site (antimycin, halogenated hydroxypyridines) decrease the efficiency of ATP formation, but do not affect the rate of non-cyclic electron transfer. They suggested that a dismutation of plastosemiquinone could occur at the Q_z site alone, without any participation of the Q_c site. Such a mechanism

Abbreviations: DNP-INT, dinitrophenylether of iodonitrothymol; NQNO, 2-*n*-nonyl-4-hydroxyquinoline *N*-oxide; PQH_2, plastoquinol; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone.

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could account for the absence of slow electrogenic phase (phase b) under repetitive flash illumination [3].

Girvin and Cramer [7] have shown that phase b occurs with a normal amplitude in isolated chloroplasts at low redox potential (−100 mV), i.e., when cytochrome *b* is already fully reduced before the flash. Joliot and Joliot [9] observed a phase *b* of large amplitude in *Chlorella sorokiniana* incubated in anaerobic conditions so as to reduce both cytochrome *b* hemes prior to the illumination. Furthermore they observed in these conditions a large flash-induced oxidation of cytochrome *b*-563. The time-courses of both phenomena are identical. Following a single flash on dark-adapted algae, neither the oxidation of cytochrome *b* nor phase *b* is inhibited by NQNO, which is known to inhibit the Q_c site [10]. To account for these results Joliot and Joliot proposed a *b*-cycle type of mechanism (cf. Ref. 6) involving the Q_z site alone:

$$PQH_2 + (\text{FeS cytochrome } f)^+ \rightarrow PQ^- + (\text{FeS cytochrome } f) + 2H^+ \quad (1)$$

$$PQ^- + \text{cytochrome } b + 2H^+ \rightarrow PQH_2 + \text{cytochrome } b^+ \quad (2)$$

Reaction (1) leads to the reduction of PC^+ (oxidized plastocyanin) which is in equilibrium with the Rieske protein (FeS) and cytochrome *f*. The two protons involved in the reduction of semiquinone to quinol (reaction (2)) are picked up from the outer phase of the membrane via a proton channel connected to the Q_z site. An alternate hypothesis would be that the semiquinone formed by reaction (1) is able to cross the membrane and to be re-reduced at the Q_c site.

In both hypotheses the first step is the formation of PQ^- by reaction (1), which is known to occur at the Q_z site. In the present work we intend to test this hypothesis by studying the effect of DNP-INT, an inhibitor of the Q_z site [11], on the flash-induced oxidation of cytochrome *b* and reduction of plastocyanin. The inhibitory effect of DNP-INT on plastoquinol oxidation has been established by Trebst et al. in isolated chloroplasts. Hurt and Hauska [12]. O'Keefe [13] and Lam [14] studied the effect of this inhibitor on the redox reactions involving cytochrome *b*-563 in purified and reconstituted systems. Our results demonstrate that DNP-INT is an efficient inhibitor of the Q_z site in living algae. Contrary to DBMIB [4] the inhibitory effect of DNP-INT remains stable over periods of several hours.

Materials and Methods

Most of the absorption spectrophotometric measurements have been performed in a mutant strain of *Chlorella sorokiniana* (S52) isolated by Bennoun [15,16] and which lacks reaction centers of Photosystem II and a major part of the harvesting pigment-protein complex CP2. In this mutant the redox reactions of the electron-transfer chain give rise to large relative absorption changes, due to the high concentration of the electron carriers compared to that of the harvesting pigments [16]. However, in the experiment of Fig. 4 we used the mutant strain S8, which, unlike to S52, is normally pigmented [15,16].

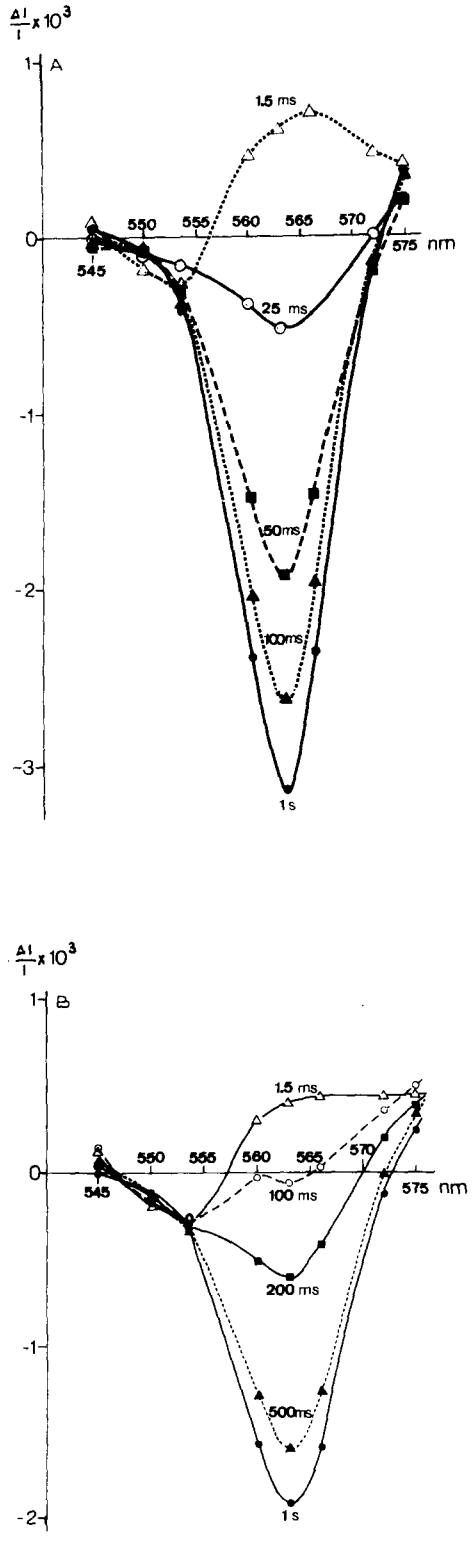
Algae were suspended in 0.05 M phosphate buffer (pH 6.5) with addition of 10% Ficoll to decrease both settling and light scattering. The anaerobic conditions were obtained as in Ref. 9: the oxygen concentration was lowered by bubbling nitrogen for a few minutes, then the suspension was incubated for more than 20 min in the measuring cuvette with addition of 20 mM glucose plus 3 mg/ml glucose oxidase.

The spectrophotometric measurements were performed using the absorption differential spectrophotometer described in Refs. 17 and 18. The source of actinic light was a xenon flash (duration, 3 μs at half-height) filtered through a red glass filter. The photodiodes were protected from the red actinic light by complementary blue filters.

Except in the experiment of Fig. 4, we added to the medium 1 mM dicyclohexyl-18-crown-6, which is known to induce a rapid decay of the membrane potential (Diner, B.A., unpublished results). Thus the absorption changes due to flash-induced electrochromic effect can be neglected at times longer than 20 ms following the actinic flash. The temperature was 20 °C.

Results

In the experiment of Fig. 1 we measured the absorption changes induced by a non-saturating



flash in the mutant strain S52 of *Chlorella sorokiniana* in anaerobic conditions. Each actinic flash was preceded by a dark incubation of 10 min. In these conditions most of the cytochrome *b* is in the reduced state prior to the flash. After a non-saturating flash the probability for a cytochrome *bf* complex to receive more than one positive charge from Photosystem I is negligible. In this case most of the positive charges are stored on the Rieske protein and plastocyanin, which explains why the absorption changes due to cytochrome *f* oxidation are very small (Ref. 19).

The flash-induced difference spectrum was plotted at different times following the actinic flash (a) without addition of inhibitor, and (b) with addition of 10 μM DNP-INT. As already reported by Joliot and Joliot [9] a large oxidation of cytochrome *b*-563 is observed with a half-oxidation time of about 40 ms in untreated algae. In the presence of 10 μM DNP-INT (Fig. 1b) the oxidation rate of cytochrome *b* is decreased by about a factor 10, while the maximum amount of cytochrome oxidized by the flash is lowered by about 30%. The difference spectrum of cytochrome *b* is not significantly modified.

In the experiment of Fig. 2 we measured the absorption changes at 572 nm induced by a saturating xenon flash following a 10 min dark anaerobic incubation in the presence of DNP-INT at various concentrations. Under these conditions there is an excess of positive charges produced by Photosystem I compared to the concentration of the cytochrome *bf* complex, so that a large positive signal due to oxidized plastocyanin is observed at 572 nm. At this wavelength, which is an isobestic point of the redox difference spectrum of P-700, the absorption changes due to cytochrome *f* can be neglected [19]. We subtracted from the signal at 572 nm the absorption change due to the oxidation of cytochrome *b* (10% of the signal plotted on Fig. 3). In the mutant strain S52 the flash-induced membrane potential causes a decrease of the absorption at 572 nm. Thus the

Fig. 1. Absorption difference spectrum induced by a non-saturating flash in mutant S52 under anaerobic conditions. Addition: 1 mM dicyclohexyl-18-crown-6. (a) no addition of inhibitor; (b) addition of 10 μM DNP-INT.

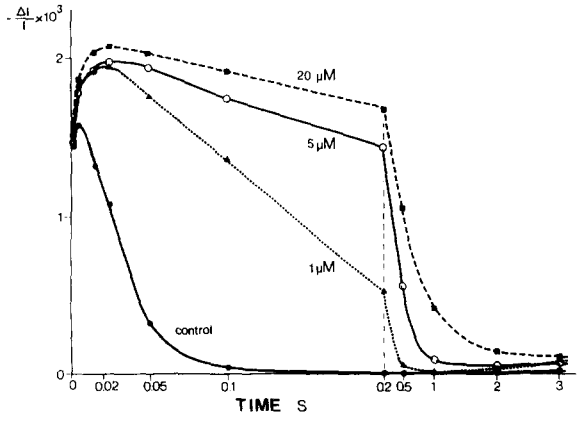


Fig. 2. Absorption changes at 572 nm induced by a saturating flash in mutant S52 under anaerobic conditions, at various concentrations of DNP-INT. The contribution of cytochrome *b* was subtracted from the measured signal. Addition: 1 mM dicyclohexyl-18-crown-6. The first detecting flash was given 1.5 ms after the actinic flash.

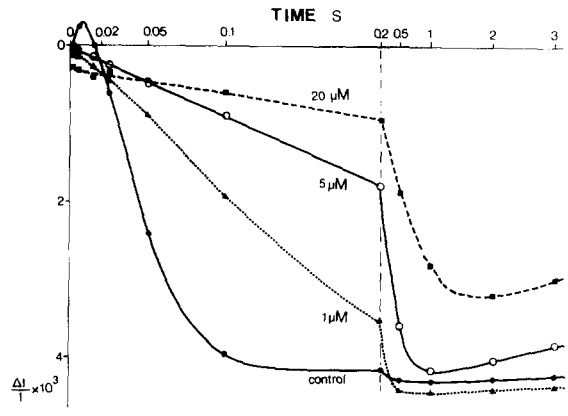


Fig. 3. Oxidation of cytochrome *b*-563 induced by a saturating flash in mutant S52 under anaerobic conditions, at various concentrations of DNP-INT. Addition: 1 mM dicyclohexyl-18-crown-6. Same conditions as Fig. 2.

absorption increase observed in the 1.5–25 ms range is due largely to the fast decay of the membrane potential in the presence of dicyclohexyl-18-crown-6. Beyond this range of time the signal is due mainly to oxidized plastocyanin.

The addition of DNP-INT considerably decreases the rate of reduction of the plastocyanin oxidized by the actinic flash (by a factor 5 at 1 µM DNP-INT). This result is consistent with the finding of Trebst [11] that DNP-INT inhibits the Q_z site. In isolated chloroplasts Trebst et al. observed that the rate of electron transfer from water to methyl viologen or ferricyanide is inhibited by a factor 9 in coupled chloroplasts and about 3 in uncoupled ones at 1 µM DNP-INT (Table III and Fig. 3 in Ref. 20). Thus the efficiency of this inhibitor appears similar in isolated chloroplasts and living algae.

Fig. 3 shows the flash-induced oxidation kinetics of cytochrome *b*-563 measured in the same experiment. The amount of oxidized cytochrome *b* was measured by the difference $(\Delta I/I_{563\,nm} - \Delta I/I_{572\,nm})$.

The same experiment was performed in the wild type of *C. sorokiniana*. Higher concentrations of DNP-INT (about 4 times) were required to observe the same inhibition as in the mutant S52.

Fig. 4 shows the effect of DNP-INT on the formation of the flash-induced membrane potential in the mutant strain S8 under anaerobic conditions. The membrane potential was measured by the difference $(\Delta I/I_{512\,nm} - \Delta I/I_{476\,nm})$. In the absence of inhibitor an increase of the membrane potential (phase *b*) is observed in the time range of 100 µs–25 ms, with a half-rise time of 5 ms. The amplitude of phase *b* is 1.2 times that of the fast increase (phase *a*). Addition of DNP-INT does not modify the amplitude of phase a, but considerably decrease the rate and amplitude of phase b. In this case phase b is scarcely visible because its rate is of the same order of magnitude as the decay of the membrane potential.

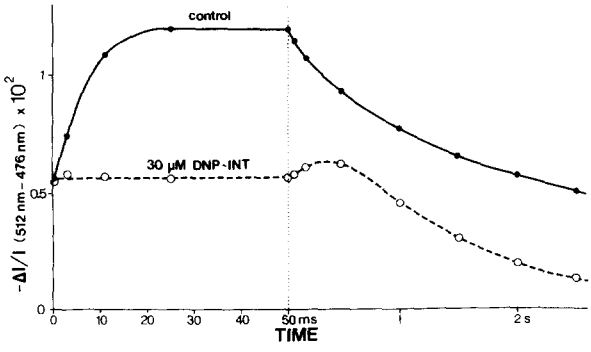


Fig. 4. Effect of DNP-INT on the flash-induced membrane potential in mutant S8 under anaerobic conditions. The actinic flash was preceded by a dark incubation of 10 min. The first detecting flash was given 100 µs after the actinic flash.

The effect of DNP-INT has been studied in the wild type cells submitted to a continuous illumination (weak light), under aerobic conditions and without addition of dicyclohexyl-18-crown-6. A short saturating flash superimposed on continuous light induced an electrochromic effect that was measured 100 μs after the flash. The amplitude of this electrochromic effect ($\Delta I/I_{515\,nm} - \Delta I/I_{474\,nm}$) is proportional to the transmembrane electric field induced by the flash, and thus to the fraction of reaction centers (Photosystem I + Photosystem II) which are in their active state. A 72% inhibition was observed in the presence of 20 μM DNP-INT ($\Delta I/I_{515\,nm} - \Delta I/I_{474\,nm} = 2.35 \cdot 10^{-3}$, compared with $8.35 \cdot 10^{-3}$ in the control).

The induction curves of fluorescence in continuous light were studied at various concentrations of DNP-INT in the wild type of *C. sorokiniana,* in the mutant strain S14 (lacking the cytochrome *bf* complex), and in isolated spinach chloroplasts. The amplitude of the fast phase of fluorescence increase was enlarged by DNP-INT while the slow phase was lowered. Concentrations of 50–100 μM were required to saturate this effect. This action of the inhibitor is consistent with the initial suggestion of Trebst et al. [20], further discussed in Ref. 21, that there is an inhibition site of DNP-INT involving the electron acceptors of Photosystem II.

Discussion

The inhibition of plastocyanin reduction by DNP-INT (Fig. 2) which has been observed in reducing conditions (plastoquinone pool totally reduced) clearly shows that this inhibitor blocks the Q_z site of the cytochrome *bf* complex where plastoquinol is oxidized, in agreement with the hypothesis proposed by Trebst [11]. The parallel inhibition of cytochrome *b* oxidation and plastocyanin reduction shows that the oxidant involved in the process of cytochrome *b* oxidation is very likely the semiquinone formed at the Q_z site. As already discussed in Ref. 9, two interpretations can be proposed: either the semiquinone formed at the Q_z site migrates towards the Q_c site where the oxidation of cytochrome *b* takes place, or the oxidation of cytochrome *b* occurs at the Q_z site (cf. Ref. 8). As expected in both models we observed that the slow increase of the membrane potential (phase *b*) is slowed down by DNP-INT similarly to the cytochrome *b* oxidation (Fig. 4).

When DNP-INT is added at concentrations higher than 5 μM the amount of cytochrome *b* oxidized after one flash is significantly decreased (Figs. 1b and 3). This result shows that plastocyanin can be slowly reduced by a pathway which does not involve the cytochrome *bf* complex. Lavergne et al. already showed that plastocyanin is reduced with a half-time of about 600 ms in a mutant which lacks the cytochrome *b/f* complex [16]. In the presence of DNP-INT this alternate pathway competes efficiently with the normal pathway including the cytochrome *bf* complex. In agreement with this interpretation several saturating flashes are required to fully oxidize cytochrome *b* (data not shown).

From the spectra of Fig. 1 it can be seen that the redox potential of cytochrome *f* is not modified by the fixation of DNP-INT on the Q_z site: the small extent of cytochrome *f* oxidation measured 1.5 ms after the flash is roughly the same in the presence or absence of DNP-INT, which shows that the equilibrium constant among the electron donors of Photosystem I is not altered by the inhibitor.

It is worth mentioning that whatever the concentration of inhibitor, the kinetics of cytochrome *b* oxidation or plastocyanin reduction are never biphasic (Figs. 2 and 3). This has implications regarding the turnover rate of DNP-INT. If the inhibitor release were slow compared to both redox reactions, we would expect biphasic kinetics, with a fast phase corresponding to the uninhibited cytochrome *bf* complexes, and a slow phase whose rate would be limited by the turnover of DNP-INT. Only the relative amplitudes of the fast and the slow phase would depend on the concentration of inhibitor. The total absence of biphasic character indicates that the turnover of DNP-INT is much faster than cytochrome *b* oxidation and plastocyanin reduction. The rate constant for inhibitor release is very likely higher than 100 s^{-1}.

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