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FLUORESCENCE INDUCTION IN INTACT SPINACH CHLOROPLASTS

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SUMMARY

Under conditions in which the Photosystem II quencher is rapidly reduced upon illumination, either after a preillumination or following treatment with dithionite, the fluorescence-induction curve of intact spinach chloroplasts (class I type) displays a pronounced dip.This dip is probably identical with that observed after prolonged anaerobic incubation of whole algal cells ("I-D dip"). It is inhibited by 3(3,4-dichlorophenyl)-1,1-dimethylurea and occurs in the presence of dithionite, sufficient to reduce the plastoquinone pool. It is influenced by far red light, methylviologen, anaerobiosis and uncouplers in a manner consistent with the interpretation that it represents a photochemical quenching of fluorescence by an electron transport component situated between the Photosystem II quencher and plastoquinone. Glutaraldehyde inhibition may indicate that protein structural changes are involved.

INTRODUCTION

Fluorescence induction transients of chloroplasts in intact algal cells have long been known to display a dip immediately following the initial rapid rise. This dip, termed the "I-D dip" by Munday and Govindjee [1], and more recently dealt with by Schreiber et al. [2] and Schreiber and Vidaver [3] is usually observed after prolonged incubation of the algae under anaerobic conditions. The consensus is that it represents an oxidation of the Photosystem II quencher, Q, during an initial equilibration phase and this is driven by Photosytem I oxidation reactions. We have now observed a pronounced I-D dip in isolated intact spinach chloroplasts. This system is much more amenable to biochemical manipulation than the intact algal systems and we present data which indicate that the I-D dip represents the oxidation of Q by an electron acceptor which is not the plastoquinone pool.

METHODS

Class I (intact) spinach chloroplasts were prepared from freshly harvested spinach leaves, according to Heldt and Sauer [4]. All reactions were performed in a

Abbreviations: DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea; Q, photosystem II quencher.

medium containing 0.33 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM ethylenediaminotetraacetic acid, 0.05 M *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid, pH 7.6. Fluorescence induction was measured at room temperature in an instrument to which an oscilloscope equipped with memory storage was attached. The photomultiplier (EMI 9659Q/B) was situated at 90 ° to the exciting beam. Fluorescence was excited with a Baltzers 638-nm filter. Chlorophyll was measured according to Arnon [5], and was present at a concentration of 3–4 μg/ml for fluorescence measurements. Oxygen was removed by bubbling the reaction mixture with N_2 which had been passed through solutions of alkaline pyrogallol and saturated bicarbonate, before addition of the chloroplasts. Trace amounts of oxygen were added with the chloroplasts, but as the dilution factor was 150 this was ignored. Ferricyanide (0.8 mM), which does not penetrate intact chloroplasts, was added where indicated to eliminate the variable fluorescence of broken chloroplasts, which usually constituted about 30 % of the chloroplasts.

RESULTS

Fluorescence induction of intact spinach chloroplasts maintained for some time in the dark (Fig. 1a, curve 1), resembles that commonly observed with broken chloroplasts or chloroplast fragment preparations (e.g. Malkin and Kok [6]). The

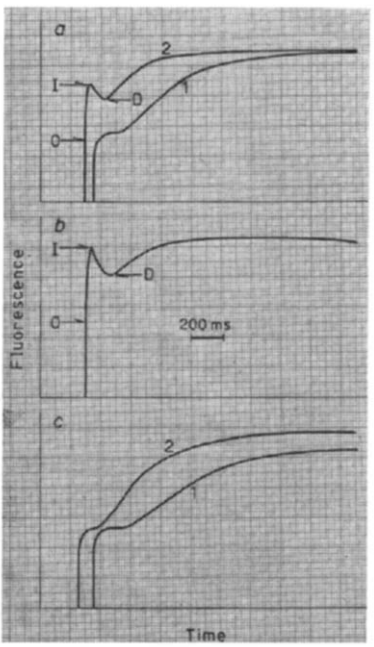

Fig. 1. Fluorescence induction of intact spinach chloroplasts after various treatments. (a) Curve 1, initial illumination of dark-adapted chloroplasts; curve 2, same experiments as curve 1, but after 4 s light and a subsequent 2 s dark. Ferricyanide (0.8 mM) was present to eliminate the variable fluorescence of broken chloroplasts. (b) Chloroplasts were incubated for 7 min with sodium dithionite (10 mM) at room temperature. (c) Curve 1, dark-adapted chloroplasts; curve 2, dark-adapted chloroplasts in the presence of dibromothymoquinone (1 μM), reduced by 5 mM sodium ascorbate.

induction time is mainly due to reduction of the electron acceptor pool up to and including the plastoquinone pool, as when reduced dibromothymoquinone was included in the reaction the area above the induction curve was not substantially reduced (Fig. 1c), as has also been observed in broken spinach chloroplasts [7]. Dibromothymoquinone is considered to inhibit electron transport at the level of plastoquinone re-oxidation [7, 8]. However, the induction pattern of these intact chloroplasts differs markedly from broken chloroplasts if a second induction is measured within a dark period of less than 20–30 s from the initial illumination (Fig. 1a, curve 2). The curve clearly resembles the O-I-D transition commonly observed in anaerobic intact algae [1–3], and we shall use the terminology usually employed for that phenomenon. From

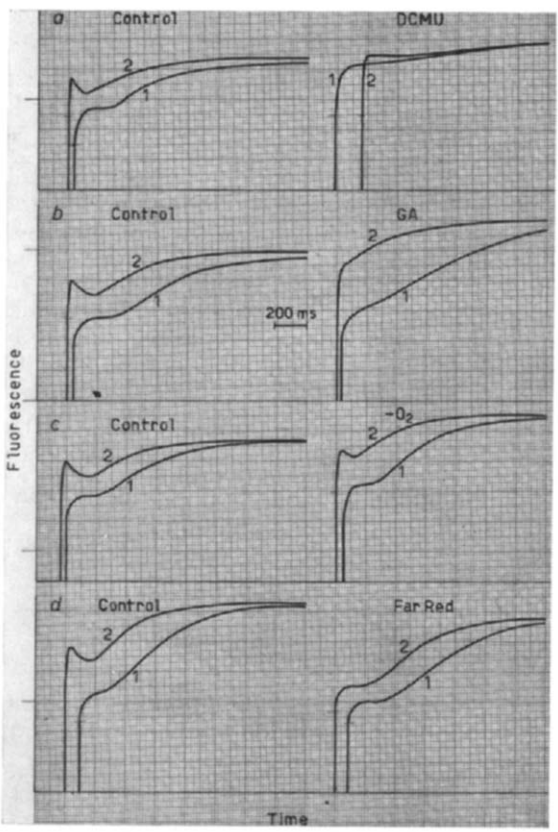

Fig. 2. Fluorescence induction of intact spinach chloroplasts after various treatments. In all cases ferricyanide (0.8 mM) was present to eliminate the variable fluorescence of broken chloroplasts. (a) Effect of DCMU ($3 \cdot 10^{-8}$ M). Curve 1, dark-adapted chloroplasts; curve 2, same experiment as curve 1 but after 4 s light and 2 s dark. (b) Effect of glutaraldehyde treatment (0.033 %, 1 min) at room temperature. Curve 1, dark-adapted chloroplasts; curve 2, same experiment as curve 1 but after 4 s light and 3 s dark. (c) Effect of oxygen removal. Chloroplasts were pre-incubated for 1 min in an oxygen-free buffer before commencement of the experiment. Curve 1, dark-adapted chloroplasts; curve 2, same experiment as curve 1 but after 4 s light and 3 s dark. (d) Effect of far red illumination (711 nm, 2000 ergs $\cdot$ cm^{-2} $\cdot$ s^{-1}). This was made for 0.5 s during the dark interval after the first illumination, i.e. after curve 1. Curve 1, dark-adapted chloroplasts; curve 2, same experiment as curve 1 after 4 s light and 5 s dark.

Fig. 1b it can be seen that the initial illumination is not necessary if the chloroplasts are previously incubated with dithionite for some time. As is observed with broken chloroplasts [9], dithionite reduced the pool of secondary acceptors more readily than Q itself, though the subsequent rate of Q reduction by light was greatly increased (O-I phase), and the I-D dip occurred. It therefore seems that to observe the I-D dip it is necessary for Q to be rapidly reduced. The effect of dithionite is clearly not related to lack of oxygen, as the anaerobic situation is much different (Fig. 2c).

Fig. 2 illustrates a number of treatments which inhibit the I-D dip. It is extremely sensitive to 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), which at the very low concentration of $3 \cdot 10^{-8}$ M very effectively inhibited the dip. Higher concentrations (10^{-6} M) completely eliminated it, and the fluorescence rise was extremely rapid as is usually observed in broken chloroplast preparations. A similar DCMU effect was seen with the dithionite-induced dip. These data are taken to indicate that the I-D dip represents a true photochemical quenching, whereby reduced Q is rapidly re-oxidised after the O-I transition. The data in Figs 1b and 1c suggest that this quenching agent is not identical with the plastoquinone pool as a very large I-D dip was observed when all or most of the plastoquinone pool was reduced by dithionite (see Discussion). Quenching was inhibited by low concentrations of glutaraldehyde (Fig. 2b), as is the dithionite-induced response. A brief illumination with 711 nm light during the dark period also eliminated the dip (Fig. 2d), and this can be correlated with the oxidation of the pool of Photosystem II acceptors, including Q, which therefore eliminates the O-I rise. Similar data has been obtained with anaerobic cells of *Chlorella* by Munday and Govindjee [10].

From Fig. 3 it is clear that the fast initial reduction of Q, manifest as the O-I rise, is not the only necessary prerequisite for observation of the I-D dip. In the aerobic sample two phases can be observed, depending on the time of dark incubation between illumination periods. During the first 5 s of dark, the extent of I-D quenching increased, despite the decreased levels of reduced Q, represented in Fig. 3b as the decline in O-I. Only after 5 s did the extent of the quenching decline. This may indicate that during the first 5 s dark, the I-D quencher was itself re-oxidising, and only after its oxidation was substantially complete was the inhibitory effect of lower initial levels of reduced Q observed, i.e. after 5 s.

This interpretation is supported by the observation that oxygen removal lengthened the dark phase required to attain the maximal quenching. Oxygen is known to act as a terminal electron acceptor in intact spinach chloroplasts [11], at least for the first few minutes of illumination (during the photosynthetic lag). Thus, oxygen removal is expected to lead to higher levels of reduction in the Photosystem II electron acceptor pool, and this is clearly evidenced in Fig. 3b where the O-I rise was greater under anaerobic conditions, which in turn would prevent the I-D quencher from becoming re-oxidised as rapidly as when oxygen is present.

The data presented in Fig. 3 may also indicate that the I-D quencher is a component of the electron transport chain operating between Photosystem II and Photosystem I. This conclusion arises from the faster attainment of maximal I-D quenching in the presence of oxygen, when Photosystem I is expected to be operating at a greater rate than in its absence, due to a Photosystem I-driven Mehler reaction [11]. However, it may be argued that the effect of oxygen is more direct and the I-D quencher is directly oxidised by oxygen during the dark period, or via such known slowly auto-

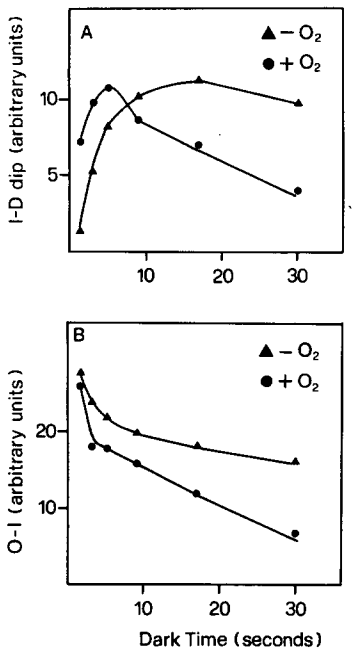

Fig. 3. Extent of (a) the I-D dip and (b) the O-I rise seen upon the second illumination after various dark incubation times in the presence and absence of oxygen. The initial illumination of dark-adapted chloroplasts was for 4 s. Ferricyanide (0.8 mM) was always present.

oxidisable intermediates as plastoquinone. This argument could suggest that the I-D quencher is not necessarily oxidised by Photosystem I at all. To test this we have examined the effect of low-intensity, far red light (711 nm) on the development of I-D quenching in the dark (Fig. 4). Here it can be seen that the extent of quenching was greatly reduced in the presence of the weak far red beam, for reasons concerned with Q re-oxidation as has already been mentioned with reference to Fig. 2d; but more significant for our purpose is the fact that the time required to attain maximal I-D quenching was less in the presence of far red light. Similar data has been obtained in other experiments with very low concentrations of methyl viologen, sufficiently low as to not maintain the electron carrier pool in a highly oxidised state. This hastening of the time required for maximal quenching by far red light and methyl viologen is consistent with the hypothesis that this period represents the re-oxidation of the quencher, and indicates that re-oxidation is achieved by photosystem I activity.

From Fig. 5 it can be seen that the uncoupler desaspidin greatly stimulated the I-D dip, and very similar effects have also been observed with carbonylcyanide *m*-chlorophenylhydrazone. This stimulation is seen for both light and dithionite-induced responses. The reduced I-D quenching seen in the presence of dithionite alone (Fig. 5b) upon the second illumination is probably due to the slow rate of quencher reoxidation. The apparent reversal of this by desaspidin seems to indicate that the uncoupler effect was to increase the rate of dark re-oxidation of the I-D quencher, and this hypothesis is supported by the data presented in Fig. 6 where desaspidin substantially decreased the dark time required to reach maximal quenching. The con-

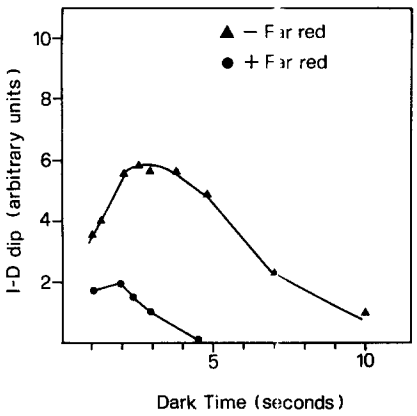

Fig. 4. Extent of the I-D dip seen upon the second illumination after various dark times in the presence or absence of a weak far red beam (711 nm, 200–300 ergs · cm^{-2} · s^{-1}). The far red light was switched on 10 s before the initial illumination with actinic light and remained on for the duration of the experiment. The initial actinic illumination was 4s. Ferricyanide (0.8 mM) was always present.

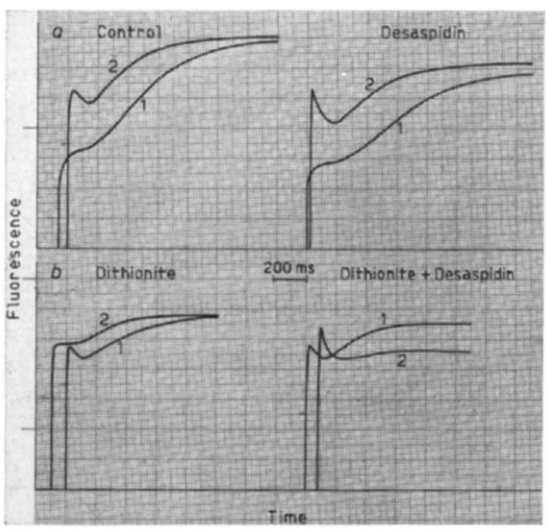

Fig. 5. Effect of desaspidin (3 μM) on fluorescence induction in intact spinach chloroplasts in the presence and absence of sodium dithionite. (a) Curve 1, dark-adapted chloroplasts; curve 2, same experiment as curve 1 but after 4 s light and 2 s dark. Ferricyanide was present. (b) Chloroplasts were pre-incubated with sodium dithionite (10 mM) for 5 min at room temperature in the dark. Curve 1, dark-adapted chloroplasts; curve 2, same experiment as curve 1 but after 4 s light and 1.5 s dark.

clusion that the uncoupler effect is exerted during the dark period is also supported by experiments in which the light intensity was varied. We have noticed that the ratio of the uncoupler stimulation of I-D quenching was very similar at exciting intensities of

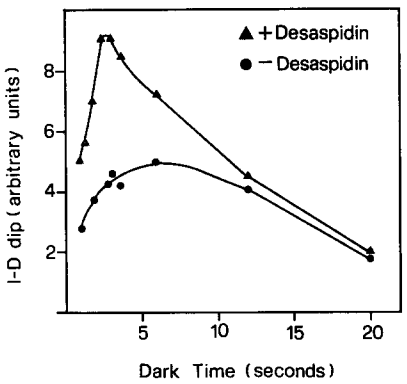

Fig. 6. Extent of the I-D dip seen upon the second illumination after various dark times in the presence and absence of desaspidin (3 μM). The initial illumination of dark-adapted chloroplasts was for 4 s. Ferricyanide was always present.

870 and 13 000 ergs · cm^{-2} · s^{-1} of 638 nm light and 160 000 ergs · cm^{-2} · s^{-1} of mainly blue light from a Corning 4-96 filter.

DISCUSSION

The data presented in this paper indicate that when the primary electron acceptor to Photosystem II is reduced rapidly in intact spinach chloroplasts there is a subsequent quenching of fluorescence (I-D dip). This observation is similar to those made by Munday and Govindjee [1], Schreiber et al. [2], and Schreiber and Vidaver [3] with intact algal cells exposed to lengthy periods of anaerobiosis, and by Mohanty et al. [12] with aerobic *Porphyridium* cells. This I-D quenching is certainly a photochemical quenching rather than a change in fluorescence yield, as it is inhibited by DCMU, and this observation moreover indicates that the phenomenon is brought about by the rapid re-oxidation of Q. Glutaraldehyde is very effective at low concentrations in inhibiting the I-D dip, and this may indicate that a protein structural change is involved. This could account for the fact that the quenching is not observed in the broken chloroplasts conventionally used for studies on fluorescence induction, after preillumination or dithionite treatment [6].

After preillumination, a period of between 3 and 6 s dark was required, in different experiments, for maximal I-D quenching. This dark time was reduced by treatments expected to stimulate Photosystem I activity, such as methyl viologen added before preillumination, and weak far red light shone during the dark period following pre-illumination. It is lengthened by treatments expected to slow Photosystem I activity, such as oxygen deprivation or treatment with relatively low concentrations of dithionite (data not shown here). These observations indicate that the dark period required for maximal I-D quenching is due to the requirement for re-oxidation of the I-D quencher. Thus, it would appear that the I-D quencher must be in an oxidised state upon the commencement of illumination for the phenomenon to be observed. This argues against the idea that I-D quenching is caused by a transient increase in Photosystem I activity during illumination, as has been previously suggested with algae [1, 3].

In the presence of sodium dithionite, shown to penetrate these chloroplasts by its reduction of most of the pool of Photosystem II electron acceptors (considered to be mainly the plastoquinone pool due to experiments with dibromothymoquinone), substantial I-D quenching was observed. This was often not less than that seen in a "light-dark-light" experiment. As Photosystem I activity would be extremely low or non-existent in these chloroplasts in the presence of dithionite due to oxygen removal [11], and due to the reduction by dithionite of ferredoxin-NADP reductase [13] and the soluble and probably also the bound ferredoxin [14], it is difficult to escape the conclusion that I-D quenching is due to a rapid re-oxidation of Q by a compound which is not part of the plastoquinone pool, and which is not readily reduced by dithionite. We cannot exclude the possibility that the I-D quencher is a special form of plastoquinone, i.e. plastoquinone in a different environment from the plastoquinone pool. The recent suggestion by Schreiber and Vidaver [3] that I-D quenching in *Scenedesmus* may be related to the oxygen gush, whereby the oxygen produced upon illumination is either directly reduced or operates as a positive controlling factor on Photosystem I activity, is not supported by these data, as dithionite is expected to reduce all oxygen evolved.

The problem therefore arises as to why the I-D quenching is not observed in broken chloroplasts which display high rates of elctron transport, coupled to phosphorylation. We have no definitive answer to this question though it may be concerned with relative rates of electron transfer processes during fluorescence induction being modified by breakage itself, or by the replacement of a stromal environment for the thylakoids with an artificial one. In this respect it is interesting to note that Velthuys and Amesz [9, 15] and Bouges-Bouquet [16] have recently proposed the existence of an electron carrier located between Q and plastoquinone in broken spinach chloroplasts. The electron carrier postulated by Velthuys and Amesz [9, 15], based largely on studies of the kinetics of dithionite reduction of the Photosystem II electron acceptor pool, is considered to be reduced only partly by dithionite. This is consistent with our observation on the I-D quencher. Furthermore, the pool size estimated by Velthuys and Amesz [9], where the postulated secondary electron acceptor was considered to be present at about 2 to 3 times the concentration of Q in terms of electron equivalents, is not inconsistent with some preliminary measurements we have made based on the area above the I-D dip, compared with the area attributed to Q reduction alone, in the presence of DCMU. These measurements suggest that the I-D quencher may be present at a concentration of roughly 2 equivalents per Q, though it should be emphasised that this figure is very approximate. Common to the models of both Velthuys and Amesz [9, 15] and Bouges-Bouquet [16] is the idea that electrons are transferred in pairs from this intermediate to the plastoquinone pool. We have no evidence on this point for I-D quenching at the moment.

The observation that uncouplers of electron transport stimulate the I-D quenching is also consistent with the idea that the quencher is on the normal electron transport chain. The specific mode of action of the uncouplers in this case is a little unclear, though they seem to stimulate quenching only after a previous illumination, as was demonstrated by experiments with dithionite. This observation, coupled with the fact that, as with far red light and methyl viologen treatment, the dark time required to attain maximal quenching was decreased, indicates that they accelerate the dark rate of I-D quencher re-oxidation. Experiments performed with 5 mM hydroxy-

lamine (not reported here, see also ref. 12) which rapidly reduces the positive charges accumulated on the water-oxidising side of Photosystem II [17], indicate that the I-D quencher is not oxidised by these positive charges. Thus, we may conclude that the uncoupler stimulation of quenching is via the electron transport chain and may be mediated at the well known coupling site located between plastoquinone and cytochrome *f*.

The question arises as to whether the necessity for a rapid Q reduction is only photochemical, i.e. Q needs to be reduced before its re-oxidation can be seen, or whether some other factor involved in the quenching, such as the putative glutaraldehyde-sensitive structural change, is dependent on reduced Q. The latter situation seems operative in the well known Mg^{2+} stimulation of fluorescence [18, 19], and may also apply here though we have no definite evidence. The reason that it is possible to observe I-D quenching during the early phase of fluorescence induction is probably related to the fact that the rise curve here is slow, the time of I-D quenching coinciding with the first plateau phase. We may assume, however, that electron transport to the I-D quencher is a continuous process, operative during normal electron transport in intact chloroplasts.

ACKNOWLEDGEMENT

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