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PHOTOOXIDATION OF THE CYTOCHROME *b*-559 IN THE PRESENCE OF VARIOUS SUBSTITUTED 2-ANILINOTHIOPHENES AND OF SOME OTHER COMPOUNDS, IN *CHLAMYDOMONAS REINHARDTII*

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Summary

Five substituted 2-anilinothiophenes and two substituted carbonylcyanide-phenylhydrazones were comparatively studied with respect to their capacities for inducing photooxidation of the cytochrome *b*-559 in chloroplast fragments and in whole cells of *Chlamydomonas reinhardtii* (wild type and *P*-700-lacking mutant *Fl* 5). In addition, some other compounds: antimycin A, picric acid, tetraphenylboron and NH_4Cl were also tested.

Cytochrome *b*-559 photooxidations were clearly observed in the presence of 2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene (ANT 2p), 2-(3,4,5-trichloro)anilino-3,5-dinitrothiophene (ANT 2s), 2-(4-chloro)anilino-3,5-dinitrothiophene and, with greater amplitudes, in the presence of carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone and carbonylcyanide-*m*-chlorophenylhydrazone, both in whole cells and in chloroplast fragments. Picric acid, antimycin A and tetraphenylboron were also able to induce cytochrome *b*-559 photooxidation in chloroplast fragments, but not in whole cells. In the wild type, the highest photoinduced redox changes were 1.1 (carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone, carbonylcyanide-*m*-chlorophenylhydrazone) and 0.6 (ANT 2p, ANT 2s) μmol of oxidized cytochrome *b*-559/

Abbreviations: ADRY, acceleration of the deactivation reactions of the water-splitting enzyme system Y of photosynthesis; ANT 2a, 2-(4-chloro)anilino-3,5-dinitrothiophene; ANT 2f, 2-(4-dimethylamine)-anilino-3,5-dinitrothiophene; ANT 2p, 2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene; ANT 2s, 2-(3,4,5-trichloro)anilino-3,5-dinitrothiophene; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; CCP, carbonylcyanidephenylhydrazone; CMU, 3-*p*-chlorophenyl-1,1-dimethylurea; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; NMANT 2p, 2-*N*-methyl(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene; *P*-700, chlorophyll *a* holochrome, active pigment in Photosystem I; S-13, 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide; 1799, α,α'-bis(hexafluoroacetonyl)acetone.

1 mmol of chlorophyll, corresponding to 40% and 23% of the redox changes which could be induced chemically. All these cytochrome *b*-559 photooxidations, the greater part of which was inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea and occurred in the mutant *Fl* 5, appeared to be mainly Photosystem II-dependent reactions. But 3-(3,4-dichlorophenyl)-1,1-dimethylurea-insensitive Photosystem I-dependent photooxidations of cytochrome *b*-559 occurred also in the wild type. On the other hand, 2-(4-dimethylamine)-anilino-3,5-dinitrothiophene, 2-*N*-methyl-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene and NH_4Cl did not induce any cytochrome *b*-559 photooxidation.

These results were discussed taking in consideration the nature of the molecular substitutions of the various tested substances and their respective acceleration of the deactivation reactions of the water-splitting enzyme system Y of photosynthesis capacities which had been defined elsewhere by Renger (Renger, G. (1972) Biochim. Biophys. Acta 256, 428—439) for spinach chloroplasts. Like the acceleration of the deactivation reactions of the water-splitting enzyme system Y effect, the capacity for inducing the cytochrome *b*-559 photooxidation appeared dependent on the acidity of the NH group and on the number of halogenous substituents in the aromatic ring of the molecule. The greatest action towards cytochrome *b*-559 photooxidation was obtained with the most active acceleration of the deactivation reactions of the water-splitting enzyme system Y agents: carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone, ANT 2p and ANT 2s.

Introduction

Most of the cytochrome *b*-559 which is present in chloroplasts in amounts corresponding to 2—2.8 molecules/reaction center and per cytochrome *f* is structurally bound close to Photosystem II. But generally no discernible light-induced absorbance change of cytochrome *b*-559 was observed, in higher plant chloroplasts or in whole cells of green algae, under physiological conditions. And it was necessary to perturb the photosynthetic membrane in a manner usually non-physiological, by addition of chemicals or by freezing, in order to observe ample light-induced absorbance changes which corresponded to oxidation reactions of this cytochrome *b*-559 (see review [1]). Among the various exogenous substances which were able to induce photooxidation of the cytochrome *b*-559 in higher plant chloroplasts, like desaspidin, antimycin A, CCCP [2], FCCP [3], salicylaldoxime, NaN_3, NH_2OH, KF [4], CCCP and FCCP appeared clearly the most active [3,4]. These substituted CCP compounds had been described as oxygen evolution inhibitors in chloroplasts, as uncouplers of the photosynthetic phosphorylations [5], as inhibitors of the reoxidation of the Photosystem II primary acceptor Q in DCMU-poisoned chloroplasts [6], as accelerators of the deactivation reactions of the water-splitting enzyme system Y of photosynthesis (ADRY agents) [7,8]. More recently other uncouplers: S-13, 1799 and tetrachloro-2-trifluoromethylbenzimidazole had been reported to induce photooxidation of the cytochrome *b*-559 [9]; two of these compounds, S-13 and 1799, had been tested and shown to act as ADRY agents [10].

Previously we have studied the photooxidation of the cytochrome *b*-559 induced by antimycin A or FCCP in chloroplast fragments and in whole cells of the wild type and of three non-photosynthetic mutants of *Chlamydomonas reinhardtii*. We have pointed out the occurrence of a Photosystem II-dependent photooxidation of cytochrome *b*-559 which was inhibited by CMU and which was observed in the *P*-700-lacking mutant *Fl* 5. This photooxidation was faster and greater in the presence of FCCP than in the presence of antimycin A. Weaker CMU-insensitive Photosystem I-dependent photooxidations of cytochrome *b*-559 occurred also in the wild type but not in the mutant *Fl* 5. An hydroquinone-reducible high-potential form of the cytochrome *b*-559 was, at least in part, involved in these photooxidations [11,12].

In the present work, we tested several compounds of another class of ADRY agents, the 2-anilinothiophenes, for photooxidation of cytochrome *b*-559 in the wild type and in the *P*-700-lacking mutant *Fl* 5 of *C. reinhardtii*, and we compared the properties of these agents to those of FCCP and CCCP. These 2-anilinothiophenes had been synthesized by Büchel and Schäfer [13] and had been initially characterized by these authors as potent uncouplers of oxidative phosphorylation in mitochondria. The ADRY capacity of these compounds had been pointed out then by Renger [14]. In addition, we tested picric acid (2,4,6-trinitrophenol) which is another ADRY agent [15] and also tetraphenylboron, an inhibitor of the oxygen evolution and of the reoxidation in the dark of the reduced primary acceptor Q in DCMU-poisoned chloroplasts [16,17]. The chemical formulae of the various compounds used are indicated in Fig. 1. The results showed a parallelism between the capacities of the different substances for inducing cytochrome *b*-559 photooxidation and their respective ADRY capacities.

Materials and Methods

The wild type of *C. reinhardtii*, the mutant *Fl* 5 which lacks *P*-700 and does not perform any Photosystem I reaction, and the mutant *Fl* 15 which lacks both cytochromes *b*-563 and *c*-553 have been described previously [18,19].

Algae were grown in the light, Tris/acetate/phosphate medium [20] as previously indicated [18,21]. For the preparation of chloroplast fragments, the cells were suspended in the buffer: 0.01 M potassium phosphate, 0.02 M KCl and $2.5 \cdot 10^{-3}$ M $MgCl_2$ (pH 7.5) and were disrupted for 15 s in an ultrasonic oscillator; then the chloroplast fragments were separated by means of two successive centrifugations at $480 \times g$ for 6 min and at $20\,000 \times g$ for 15 min, and they were finally suspended in fresh buffer. The chlorophyll contents were measured according to MacKinney [22] and Arnon [23].

The photooxidation of the cytochrome *b*-559 was measured at room temperature as previously described [12], using the dual-wavelength mode of an Aminco-Chance spectrophotometer. For the measurements, the chloroplast fragments or the cells were suspended in 0.01 M phosphate buffer (pH 7.5) at final concentrations corresponding to 100 μg of chlorophyll *a* + *b*/ml ($=1.1 \cdot 10^{-4}$ M). In the case of chloroplast fragments preparations, 0.04 M ascorbate was added to the suspensions in order to maintain the cytochrome *b*-559 reduced in the dark [12]. The different reactives were put directly in the

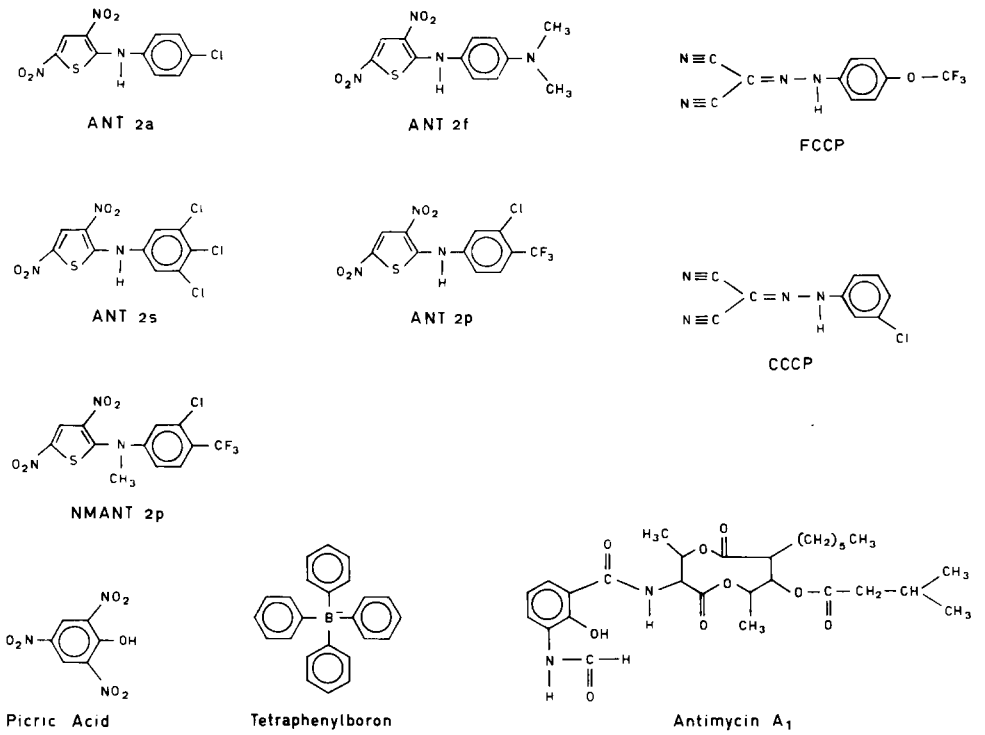

Fig. 1. Formulae of the different compounds tested for their capacities for inducing cytochrome *b*-559 photooxidation in *C. reinhardtii*. From Refs. 14, 29, 35 and 36.

cuvettes; in this way the incubation times before starting of the measurements were 1 min or less.

The various 2-anilinothiophenes had been kindly supplied by Dr. G. Renger (Max-Volmer-Institut für Physikalische Chemie, Technischen Universität, Berlin).

Results

The results concerning the occurrence of cytochrome *b*-559 photooxidation in whole cells and in chloroplast fragments of *C. reinhardtii* in the presence of various compounds are summarized in Table I. Fig. 2 shows some spectra of the photoinduced absorbance changes observed with the wild type or the mutants *Fl* 5 and *Fl* 15, in the presence of FCCP, ANT 2p, ANT 2s, picric acid or tetraphenylboron. Similar spectra were obtained in the presence of the other compounds CCCP, ANT 2a and antimycin. All showed maxima at 560 nm and the control spectra concerning the mutant *Fl* 15, which is devoid of the cytochromes *b*-563 and *c*-553 [18,19], indicated clearly that these absorbance changes were due to the cytochrome *b*-559. On the other hand, the curves of Fig. 3 illustrate how the effect of each compound varied with the concentration, both in whole cells and in chloroplast fragments.

The greatest cytochrome *b*-559 photooxidations were observed in the

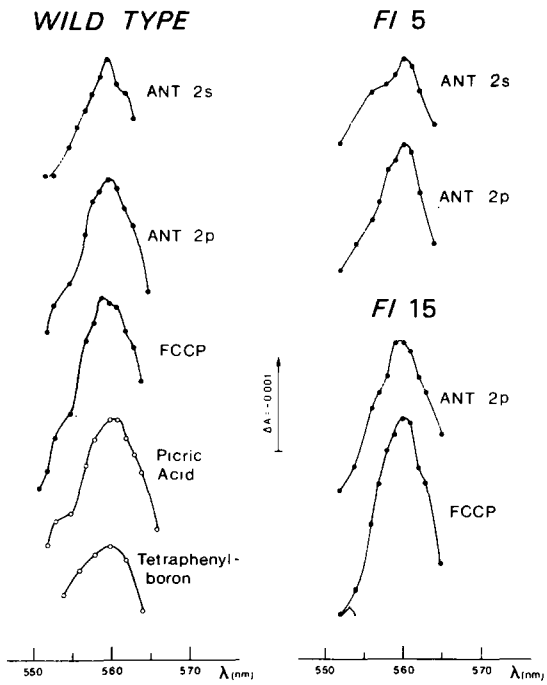

Fig. 2. Photooxidation of the cytochrome *b*-559 in whole cells or in chloroplast fragments of *C. reinhardtii* (wild type and mutants *Fl* 5 and *Fl* 15) in the presence of various ADRY reagents: spectra of some light-induced absorbance changes. Reference λ: 570 nm. The cells (•) or the chloroplast fragments (○) were suspended in 0.01 M phosphate buffer (pH 7.5) at concentrations corresponding to 100 μg of chlorophyll *a* + *b*/ml. In the case of whole cells, 5% dextran T 80 was added; in the case of chloroplast fragments, 0.04 M sodium ascorbate was added. Actinic light: 665—720 nm at half-transmission band of the filter (Balzers 'K7'), 24 W · m^{-2}. Concentrations of the reactives: 2.4 · 10^{-6} M ANT 2p, 2.4 · 10^{-6} M ANT 2s, 6.0 · 10^{-5} M FCCP, 2.4 · 10^{-4} M picric acid, 2.4 · 10^{-5} M tetraphenylboron (sodium salt).

presence of FCCP or CCCP (Table I). These photooxidation values, corresponding to absorbance decreases of 0.002—0.003 unit/100 μg of chlorophyll *a* + *b*, were comparable to the values reported elsewhere for higher plant chloroplasts [3,9]. Nevertheless, as previously reported [12], the photoinduced cytochrome *b*-559 oxidations measured in the presence of FCCP did not correspond to the total cytochrome *b*-559 content of the chloroplasts. Fig. 4 shows absorbance changes measured at 559 nm with chloroplast fragments of the wild type and of the mutant *Fl* 5 which were oxidized in the dark by $K_3Fe(CN)_6$ then reduced by ascorbate. These chemically induced absorbance changes corresponded to 2.6 (wild type) and 3.4 (*Fl* 5) μmol of cytochrome/1 mmol of chlorophyll *a* + *b*. As compared to these latter contents, the cytochrome amounts photooxidized in the presence of FCCP represented about 36% (chloroplast fragments) and 42% (cells) in the wild-type case, but only 16% (chloroplast fragments) and 22% (cells) in the mutant *Fl* 5 case, of the cytochrome *b*-559 present in the chloroplasts (Table I, Fig. 4).

As previously observed [11,12], a weak cytochrome *b*-559 photooxidation occurred in the presence of FCCP and DCMU, both in whole cells and in chloroplast fragments of the wild type (Table I). This photooxidation, which

TABLE I

PHOTOOXIDATION OF THE CYTOCHROME *b*-559 IN CHLOROPLAST FRAGMENTS AND IN WHOLE CELLS OF *C. REINHARDTII*, WILD TYPE AND MUTANT *Fl* 5, IN THE PRESENCE OF VARIOUS COMPOUNDS

Cytochrome oxidation: μmol of oxidized cytochrome/mmol of chlorophyll *a* + *b*. The chloroplast fragments or the cells were suspended in 0.01 M phosphate buffer (pH 7.5) at concentrations corresponding to 100 μg of chlorophyll *a* + *b*/ml. In the case of chloroplast fragments, 0.04 M sodium ascorbate was added; in the case of whole cells, 5% dextran T 80 was added. Actinic light: 665—720 nm at half-transmission band of the filter (Balzers 'K7'), 24 $W \cdot m^{-2}$. Analytic light: 559 nm (reference λ: 570 nm). Used concn., used concentration (i.e. concentration required to obtain maximum effect, if any); n.m., not measured. Values in parentheses: measured after addition of $1.2 \cdot 10^{-5}$ M DCMU.

Compounds	Chloroplast fragments			Whole cells		
	Used concn. (M)	Cytochrome oxidation		Used concn. (M)	Cytochrome oxidation	
		Wild type	Mutant *Fl* 5		Wild type	Mutant *Fl* 5
FCCP	$1.2 \cdot 10^{-6}$	0.94 (0.13)	0.55 (0.00)	$6.0 \cdot 10^{-5}$	1.10 (0.25)	0.77 (0.00)
CCCP	$1.2 \cdot 10^{-5}$	0.97	n.m.	$2.4 \cdot 10^{-4}$	1.10	n.m.
ANT 2p	$4.8 \cdot 10^{-7}$	0.66	0.43	$2.4 \cdot 10^{-6}$	0.60 (0.13)	0.47 (0.00)
ANT 2s	$4.8 \cdot 10^{-7}$	0.63	0.43	$2.4 \cdot 10^{-6}$	0.55 (0.17)	0.43 (0.00)
ANT 2a	$4.8 \cdot 10^{-6}$	0.58	0.39	$6.0 \cdot 10^{-6}$	0.47 (0.13)	0.51 (0.00)
ANT 2f	$4.8 \cdot 10^{-6}$	0.00	0.00	$4.8 \cdot 10^{-6}$	0.00	n.m.
NMANT 2p	$4.8 \cdot 10^{-6}$	0.00	0.00	$4.8 \cdot 10^{-6}$	traces	n.m.
Picric acid	$2.4 \cdot 10^{-4}$	0.47 (0.11)	0.55 (0.00)	$1.2 \cdot 10^{-3}$	traces	n.m.
Antimycin A	$6.0 \cdot 10^{-5}$	0.54	n.m.	$6.0 \cdot 10^{-5}$	0.00	n.m.
Tetraphenyl-boron *	$2.4 \cdot 10^{-5}$	0.47 (0.17)	n.m.	$2.2 \cdot 10^{-4}$	0.00	n.m.
NH_4Cl	$4.0 \cdot 10^{-2}$	0.00	n.m.	—	n.m.	n.m.

* The sodium salt was used.

was less than 25% of that without DCMU and which did not occur in the case of the *P*-700-lacking mutant *Fl* 5, was due to the Photosystem I.

Cytochrome b-559 photooxidation in the presence of ANT 2p

In the presence of ANT 2p, a photooxidation of cytochrome *b*-559 occurred in whole cells of the wild type of *C. reinhardtii*: the cytochrome *b*-559 was rapidly oxidized in the light, then it got reduced again in the dark; these reactions were reproducible, with decreasing amplitude, during further illuminations (Fig. 5). This photooxidation was greatly but not entirely inhibited by DCMU: the amplitude of this remaining reaction was about 22% of that without DCMU. Our technical equipment did not allow us to measure very rapid kinetics. Nevertheless, on the original recordings, the half-times for cytochrome oxidation in the whole cells appeared clearly to be less than 1 s. Cytochrome *b*-559 photooxidation and subsequent dark reduction occurred also in chloroplast fragments but their kinetics were slower, in particular that of the dark reduction: an endogenous reductant pool was probably lost in chloroplast fragments and the cytochrome *b*-559 was more slowly reduced by the exogenous ascorbate of the medium.

The amplitude of the cytochrome *b*-559 photooxidation in the presence of ANT 2p was 2/3 of that in the presence of FCCP. In chloroplast fragments, ANT 2p and FCCP were active at almost similar concentrations but, in whole

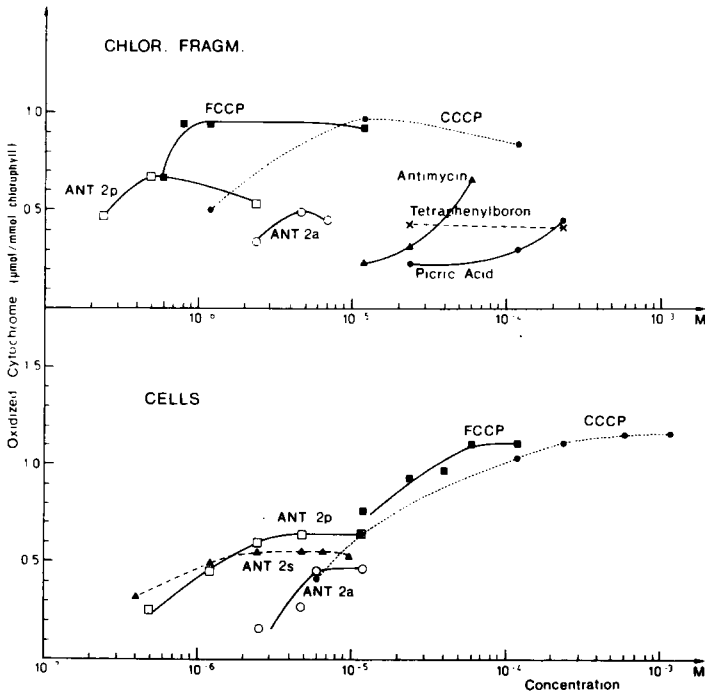

Fig. 3. Photooxidation of the cytochrome *b*-559 in chloroplast fragments and in whole cells of the wild type of *C. reinhardtii* in the presence of various ADRY reagents: variations of the amplitude with concentration of the different reagents. For the measurements, the chloroplast fragments or the cells were suspended in 0.01 M phosphate buffer (pH 7.5) at concentrations corresponding to 100 μg of chlorophyll *a* + *b*/ml. In the case of chloroplast fragments, 0.04 M sodium ascorbate was added; in the case of whole cells, 5% dextran T 80 was added. Analytic light: 559 nm (reference λ: 570 nm). Actinic light: 665—720 nm at half-transmission band of the filter (Balzers 'K7'), 24 W · m^{-2}. Chlor. fragm., chloroplast fragments.

cells, the efficient concentration was 25 times lower for ANT 2p than for FCCP.

A DCMU-sensitive photooxidation of cytochrome *b*-559 in the presence of ANT 2p, as in the presence of FCCP, occurred also in whole cells and in chloroplast fragments of the mutant *Fl* 5 which lacks *P*-700. Its amplitude, in whole cells, was similar to that of the DCMU-sensitive part of the photooxidation measured with the wild type. This confirms that a substantial part of the reaction we observed was Photosystem II-dependent (Table I). The photooxidation appeared relatively weaker in chloroplast fragments of the mutant *Fl* 5: in the case of chloroplast fragments preparations, the fragment size and the photosynthetic membrane integrity may vary somewhat from one strain to another.

Cytochrome b-559 photooxidation in the presence of other substituted 2-anilinothiophenes

We can see in Table I that the cytochrome *b*-559 was also photooxidized in the presence of ANT 2s and ANT 2a. On the contrary, no photooxidation occurred in the presence of ANT 2f and NMANT 2p.

In Table II (and in Fig. 1) are summarized some chemical characteristics of

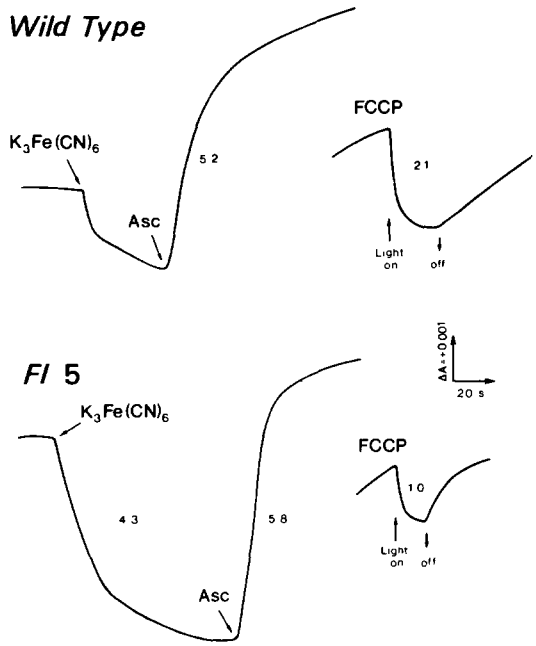

Fig. 4. Kinetics of chemically induced oxidation and reduction of the cytochrome *b*-559 in the dark and of its photoinduced oxidation in the presence of FCCP, in chloroplast fragments of the wild type and of the mutant *Fl* 5 of *C. reinhardtii*. The chloroplast fragments were suspended in 0.01 M phosphate buffer (pH 7.5) at concentrations corresponding to 100 μg of chlorophyll *a* + *b*/ml. Analytic light: 559 nm (reference λ: 570 nm). Concentrations of the reactives: for chemically induced reactions, $2.5 \cdot 10^{-4}$ M $K_3Fe(CN)_6$, $4.3 \cdot 10^{-3}$ M sodium ascorbate (ASC); for photoinduced oxidations, the chloroplast fragments were reduced by an excess of ascorbate (0.04 M) then $1.2 \cdot 10^{-6}$ M FCCP was added. Actinic light: 665—720 nm at half-transmission band of the filter (Balzers 'K7'), 24 W · m^{-2}. The numbers indicate the total absorbance changes ($X10^3$) for 100 μg of chlorophyll *a* + *b*/ml. Notice that, before $K_3Fe(CN)_6$ addition, the cytochrome was already almost fully oxidized in chloroplast fragments of the wild type but not in those of the *P*-700-lacking mutant *Fl* 5.

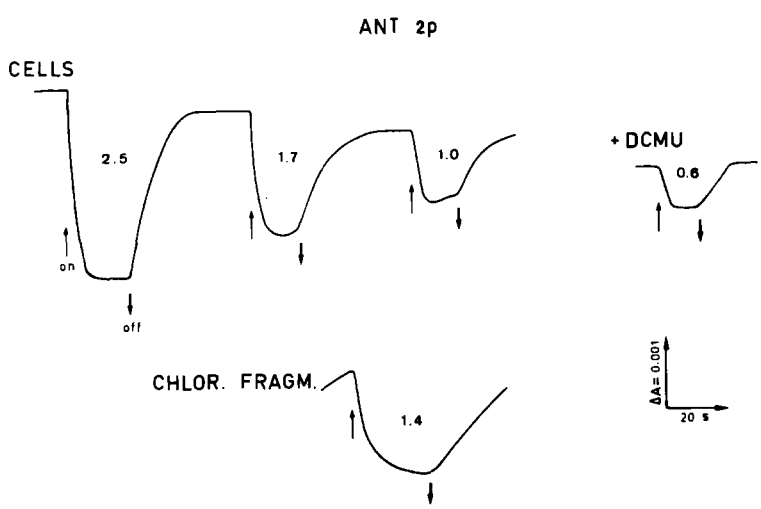

Fig. 5. Kinetics of the photooxidation of the cytochrome *b*-559 in whole cells and in chloroplast fragments of the wild type of *C. reinhardtii* in the presence of ANT 2p. The cells or the chloroplast fragments were suspended in 0.01 M phosphate buffer (pH 7.5) at concentrations corresponding to 100 μg of chlorophyll *a* + *b*/ml. In the case of whole cells, 5% dextran T 80 was added; in the case of chloroplast fragments, 0.04 M sodium ascorbate was added. Concentrations of the reactives: $2.4 \cdot 10^{-6}$ M ANT 2p, $1.2 \cdot 10^{-5}$ M DCMU. Analytic light: 559 nm (reference λ: 570 nm). Actinic light: 665—720 nm at half-transmission band of the filter (Balzers 'K7'), 24 W · m^{-2}; ↑, on; ↓, off. The numbers indicate the total absorbance changes ($X10^3$) for 100 μg of chlorophyll *a* + *b*/ml. Chlor. fragm., chloroplast fragments.

TABLE II

MOLECULAR SUBSTITUENTS OF THE CCP COMPOUNDS AND OF THE 2-ANILINO-3,5-DINITROTHIOPENE DERIVATIVES AND THEIR ADRY EFFECT IN SPINACH CHLOROPLASTS

ADRY effect in spinach chloroplast data are from Renger [8,14]: the ADRY effect is expressed by the reciprocal half-time of the apparent first-order decay of the average oxygen yield/flash. Chlorophyll concentration for the measurements corresponding to these data: $5 \cdot 10^{-5}$ M.

Compounds	Substituents on the aromatic ring	ADRY effect in spinach chloroplasts	Active concentration (M)
FCCP	OCF_3	4.6	$1.0 \cdot 10^{-6}$
CCCP	Cl	2.0	$1.0 \cdot 10^{-5}$
ANT 2p	Cl, CF_3	4.8	$4.0 \cdot 10^{-7}$
ANT 2s	Cl, Cl, Cl	4.9	$4.0 \cdot 10^{-7}$
ANT 2a	Cl	1.3	$2.0 \cdot 10^{-6}$
ANT 2f	$N(CH_3)_2$	<0.2	—
NMANT 2p	Cl, CF_3 *	0.0	—

* In addition: NCH_3 in place of the NH acidic group.

the various 2-anilino-3,5-dinitrothiophenes we tested and, in addition, their respective ADRY effects on higher plant chloroplasts as defined and published by Renger [14]. It appears clearly that a photooxidation of cytochrome *b*-559 occurred in the presence of compounds (ANT 2p, ANT 2s, ANT 2a) which are defined as ADRY agents. And the concentrations of each reagent needed for ADRY effect in spinach chloroplasts and for cytochrome *b*-559 photooxidation in chloroplast fragments of *C. reinhardtii*, at equivalent concentrations of chlorophyll (0.5 and $1.1 \cdot 10^{-4}$ M), were very close. On opposite, in the presence of ANT 2f and NMANT 2p which are not ADRY agents, there was no photooxidation of cytochrome *b*-559.

The substituted 2-anilinothiophenes appeared much more soluble in the cell walls than the substituted CCP compounds. Indeed, the respective concentrations of ANT 2p and ANT 2s required for maximum cytochrome *b*-559 photooxidations were five times greater for whole cells than for chloroplast fragments, whereas the corresponding concentrations of FCCP and of CCCP were, respectively, fifty and twenty times greater for whole cells than for chloroplast fragments (Table I, Fig. 3).

Cytochrome b-559 photooxidation in the presence of various other compounds

Some other compounds were also tested for inducing photooxidation of cytochrome *b*-559: picric acid, another type of ADRY agent [15]; tetraphenylboron which exhibited ADRY properties somewhat different from those of the typical ADRY agents, because it was also an electron donor to Photosystem II [16,17]; and antimycin A which was a less efficient ADRY agent (Renger, G., personnal communication). All these compounds induced photooxidation of cytochrome *b*-559 in chloroplast fragments, but not in whole cells: they are probably less soluble in the cell wall membranes than the substituted CCP compounds and the 2-anilinothiophenes derivatives (Table I, Fig. 3).

Finally, NH_4Cl which is uncoupler without ADRY action did not induce any photooxidation of cytochrome *b*-559.

Discussion

Depending on their concentrations all the compounds able to induce a photooxidation of cytochrome *b*-559 by Photosystem II are also inhibitors of oxygen evolution, phosphorylation uncouplers and ADRY agents. However, the occurrence of cytochrome *b*-559 oxidation is not directly linked to the inhibition of oxygen evolution since in a new mutant of *C. reinhardtii*, *Fl* 50, which we recently isolated and which does not evolve oxygen at all, no photooxidation of cytochrome *b*-559 occurred without any addition but this mutant performed clearly a cytochrome *b*-559 photooxidation in the presence of FCCP *. Besides, as previously pointed out in higher plant chloroplasts [24] and in cells of the wild type of *C. reinhardtii* [12], photooxidation of cytochrome *b*-559 occurred at concentrations of CCCP or FCCP at which oxygen evolution was only partially inhibited. On the other hand, uncoupling is not a sufficient condition for the occurrence of cytochrome *b*-559 photooxidation. Many uncouplers were shown to have no action towards the cytochrome *b*-559: NH_4Cl [25,26], methylamine [25,26], nigericin [26], gramicidin [10,26] and atebrin [25]. And it appears that the sole uncouplers which showed ADRY properties, like FCCP and ANT 2p, or S-13 and 1799 [9,10], were acting on the cytochrome *b*-559.

Cramer and coworkers [3,9] have shown that, in the case of FCCP-treated higher plant chloroplasts, far-red light induced a Photosystem I-driven photooxidation of cytochrome *b*-559 probably linked to a conformational change and to a lowering of the cytochrome midpoint potential. This photooxidation was achieved a few seconds after the addition of FCCP, period for which electron flow from water to methylviologen was not inhibited [28]. We observed also a Photosystem I-dependent photooxidation of cytochrome *b*-559 in whole cells or in chloroplast fragments of the wild type of *C. reinhardtii* in the presence of DCMU and FCCP (or ANT 2p or another active compound). But this reaction was only 1/3, or less, of that without DCMU. And the results concerning the *P*-700-lacking mutant *Fl* 5 indicated clearly that the cytochrome *b*-559 photooxidations were largely (at least 70% in the case of whole cells) Photosystem II-dependent reactions.

The concentration of each substituted CCP or 2-anilinothiophene needed for a maximum photooxidation of cytochrome *b*-559 in chloroplast fragments of *C. reinhardtii* is very close to the concentration indicated by Renger [8,14] for the ADRY effect in spinach chloroplasts (Tables I and II). Two important common characteristics of various ADRY agents are: (1) the presence of an acidic group NH or OH in their molecules, and (2) their ability to form an anion [29]. As showed by Renger [27], it is this negatively charged anion form which is important for the ADRY effect. Our present results indicate that the acidic group NH of the 2-anilinothiophenes was also necessary for the occurrence of a cytochrome *b*-559 photooxidation. Indeed, no cytochrome photooxidation occurred in the presence of NMANT 2p, the molecule of which has a NCH_3 group in place of an acidic NH group. Likewise, the halogenous sub-

* This mutant *Fl* 50 is partially devoid of cytochrome *b*-559, but the remaining pool of this cytochrome can be photooxidized in the presence of FCCP (Maroc, J. and Garnier, J., unpublished results).

stituents of the aromatic ring, which act on the NH group acidity [14] and probably on the solubility in membranes, also influence the cytochrome *b*-559 photooxidation: no cytochrome photooxidation occurred in the presence of ANT 2f, the aromatic ring of which has no halogenous substitution, and the active concentrations were higher for CCCP or ANT 2a, the aromatic ring of which has only one chloride substitution, than, respectively, for FCCP or for ANT 2p and ANT 2s which have more substituted aromatic rings. Concerning the other compounds we tested as also able to induce photooxidation of cytochrome *b*-559, picric acid has an OH acidic group ($pK = 0.38$) [15], tetraphenylboron is an anion, and the antimycin A molecule has OH and NH acidic groups ($pK_a = 5.1$) [30]. Therefore we observe parallelism between the capacities of the different substances for inducing photooxidation of cytochrome *b*-559 in *C. reinhardtii* and their ADRY activities which have been reported in the literature for higher plant chloroplasts. Both phenomena are induced by the same agents and influenced similarly by the chemical properties (capacity for forming an anion, number of halogenous substitutions) of these agents. The greatest action towards the cytochrome *b*-559 photooxidation was obtained with the most efficient ADRY agents: FCCP, ANT 2p and ANT 2s.

Renger and coworkers [29,31,32] showed that ADRY agents induced a cyclic electron flow which included Photosystem II but not necessarily Photosystem I, and which led to a dissipative recombination of the holes stored in the water-splitting enzyme Y with the electrons of an unknown donor. The cytochrome *b*-559 could participate in this deactivation pathway. However, the cytochrome *b*-559 photooxidation and the ADRY effect can also be two independent secondary consequences of structural modifications of the thylakoid membrane induced by the addition of the chemicals. Helgerson et al. [33] have shown that FCCP increases the microviscosity of the cell envelope in *Escherichia coli* and Cramer et al. [34] have suggested a similar mechanism for a possible action of FCCP on the thylakoid membrane structure. The fact that the ADRY agents were able to induce both Photosystem I and Photosystem II-dependent cytochrome *b*-559 oxidations seems in favour of this latter hypothesis of structural modification. Further work will be needed for a complete elucidation of these problems.

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