FEBS 14922

FEBS Letters 357 (1995) 55–57

Photoreduction of silicomolybdate in chloroplasts by agents accelerating the deactivation reactions of the water-oxidizing system

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Received 11 November 1994

Abstract Uncouplers of photosynthetic phosphorylation, CCCP, TTFB and PCP, inhibited light-induced O_2 evolution in the Hill reaction with SiMo (I_{50} ~20, 3 and 45 μM, respectively), but only insignificantly diminished SiMo photoreduction by pea chloroplasts. The same properties were exhibited by the ADRY agent ANT2p. CCCP, TTFB and PCP are oxidizable compounds with redox potentials of +1.17, +1.18 and +1.09 V (pH 6.0), as determined by cyclic voltammetry. Similarly to NH_2OH, the tested uncouplers can apparently serve as electron donors for photosystem II.

Key words: Photosystem II; Silicomolybdate; ADRY agent; Uncoupler; Cyclic voltammetry

1. Introduction

The water-oxidizing complex of chloroplasts and cyanobacterial membranes contains a tetranuclear Mn cluster which cycles through five oxidation states designated S_0–S_4 (for review, see [1]). S_2 and S_3 states are unstable and relax in the dark down to S_1 in tens of seconds [2]. Deactivation of the S_2 and S_3 states that is followed by the inhibition of O_2 evolution is accelerated by compounds known as ADRY agents [3]. These agents also induce a light-dependent oxidation of carotenoids [4–7] and chlorophyll *a* [4], and transform cytochrome *b*-559 from the reduced high-potential form to the oxidized low-potential form [8,9]. The most powerful ADRY agents are ANT2p and uncouplers of photosynthetic and oxidative phosphorylation, FCCP and CCCP [10].

ADRY agents were thought to act as mobile catalysts [11,12], reducing the Mn cluster in the S_2 and S_3 states [12,13]. In Tris-washed chloroplasts that are deprived of O_2-evolving activity, ADRY agents accelerate the re-reduction of component Y_Z, Tyr-161 of the photosystem II D1 polypeptide [14,15]. The data on the ADRY effect were explained by the assumption that the ADRY agents induce a cyclic electron flow around PSII [3,13]. However, as shown by cyclic voltammetry [16], ANT2p becomes irreversibly oxidized at a redox potential of +0.9 to 1.0 (pH 7.5).

On the other hand, uncouplers FCCP and 2,6-di(*t*-butyl)-4-(2′,2′-dicyanovinyl)phenol (SF 6847) induced the oxidation of plastoquinol and the oxidation of cytochrome *b*-559 that appeared to be part of the cyclic pathway for the oxidation of plastoquinol in the PSII [17,18]. As was noted by Arnon and Tang [18], the uncouplers "must have acted not as electron acceptors but as promoters of the oxidation of cytochrome *b*-559 by virtue of their special capacity for reversible binding of protons".

In chloroplasts incubated with gramicidin D, CCCP was recently shown to inhibit the DCMU-sensitive FeCy photoreduction and coupled O_2 evolution, but was practically without effect on the DCMU-resistant O_2 evolution with SiMo as an electron acceptor within the 0.2–10 μM concentration range [19]. The same effects were caused by the uncouplers, PCP and TTFB [20]. In conjunction with inhibitor study data, these results were interpreted as an indication that the tested uncouplers, redox active compounds, shunt the non-cyclic electron transfer to FeCy at the level of the membrane plastoquinone pool and thereby establish cyclic electron transfer around PSII [19,20].

This paper reports data on the electron-donor activity of uncouplers in PSII. Their redox potentials were measured using cyclic voltammetry.

2. Materials and methods

Pea chloroplasts were isolated as described earlier [21]. O_2 evolution by chloroplasts was recorded using a closed Clark-type Pt electrode [21] at the white light of saturating intensity of ~0.1 $W \cdot cm^{-2}$. FeCy photoreduction was measured at 420 nm using a Uvidec-4 Jasco spectrophotometer (Japan). Red light of saturating intensity with λ >600 nm was used for illumination of chloroplasts. The photodiode of the spectrophotometer was protected from stray light by a blue filter transmitting the light with λ < 550 nm. The incubation medium contained 0.4 M sucrose, 35 mM NaCl, 50 mM Tricine-KOH (pH 7.8) and chloroplasts at a chlorophyll concentration of 5–6 μg/ml in the spectrophotometric measurements and 12–15 μg/ml in the oxymetric measurements.

The cyclic voltammetry was performed as in [22], with an electrochemical measuring system ECM-700. The scan rate was 250 mV/s. A Pt electrode was used with a saturated calomel reference electrode. The potentials are given relative to a normal hydrogen electrode.

3. Results and discussion

CCCP inhibited the electron transfer from H_2O to FeCy in chloroplasts with an I_{50} of ~2 μM (Fig. 1). Oxygen evolution insensitive to DCMU in the presence of SiMo + FeCy was

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Abbreviations: ADRY, acceleration of the deactivation reactions of the water-splitting enzyme system Y; ANT2p, 2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene; CCCP, carbonyl cyanide chlorophenylhydrazone; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; FeCy, potassium ferricyanide; P680, the reaction center of photosystem II; PCP, 2,3,4,5,6-pentachlorophenol; PSII, photosystem II; SiMo, silicomolybdate; TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole.

SSDI 0014-5793(94)01309-8

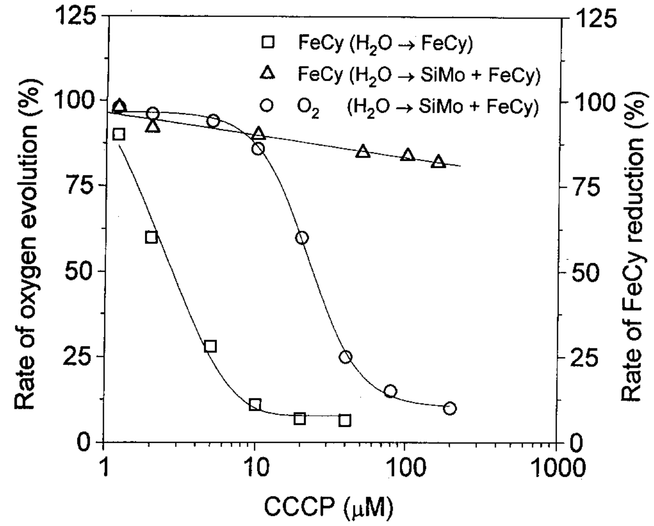

Fig. 1. Effect of CCCP on light-induced reduction of FeCy, SiMo and O_2 evolution by pea chloroplasts incubated with gramicidin D. Additions: 0.5 mM FeCy at measurement of FeCy photoreduction or 2.0 mM FeCy at measurement of light-induced O_2 evolution, 50 μM SiMo + 10 μM DCMU, 4 μM gramicidin D. Chloroplasts were preincubated with SiMo and FeCy in the dark for 1–2 min before switching on the light. Photoreduction of SiMo was measured as ferrocyanide formation in the reaction: $SiMo_{red} + FeCy_{ox} \rightarrow SiMo_{ox} + FeCy_{red}$. The 100% rates of FeCy reduction through the electron transfer $H_2O \rightarrow FeCy$ and $H_2O \rightarrow SiMo + FeCy$ and the 100% rate of O_2 evolution were 430–450, 380–420 and 125 μmol·mg^{-1} Chl per h, respectively.

unaffected by CCCP at concentrations < 10 μM. With a rise in the concentration, CCCP inhibited O_2 evolution with SiMo + FeCy as the electron acceptor (I_{50} ~20 μM). At the same time, the SiMo photoreduction rate measured by ferrocyanide formation was almost unaltered by CCCP. 2,5-Dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (2 μM) inhibiting plastoquinol Q_ZH_2 oxidation in the cytochrome *b*/*f* complex [23] decreased the CCCP-dependent SiMo photoreduction rate by no more than 5% (data not shown). PSII acceptor-side non-heme iron apparently donates the electron to SiMo [24].

Sayre and Homann [25] suggested that the interaction of ADRY agents with the oxidizing side of PSII can lead to H_2O_2 formation. Wydrzynsky et al. [26] reported on H_2O_2 production by intact PSII preparations without the addition of ADRY agents. This H_2O_2 production was visualized by an exogenous catalase-induced enhancement of O_2 evolution. H_2O_2 produced by PSII was responsible for photoinhibition of PSII reaction centers [27]. The addition of catalase to thylakoids decreased the rate of photoinhibition [27]. The experiment indicated that the CCCP-induced inhibition of O_2 evolution was unaltered upon the addition of catalase (data not shown). Therefore, the suggestion of H_2O oxidation to H_2O_2 seems an unconvincing explanation of the inhibition of O_2 evolution by CCCP. The above results imply that CCCP serves as the electron donor for SiMo photoreduction occurring in the absence of O_2 evolution.

Data similar to those for CCCP were obtained in experiments with other uncouplers (PCP and TTFB) and with ANT2p. I_{50} values of PCP, TTFB and ANT2p as inhibitors of O_2 evolution with SiMo were approximately 45, 3 and 1 μM, respectively. The effect of these compounds was compared with the effect of NH_2OH, a known inhibitor of photosynthetic water oxidation and an efficient electron donor to PSII (for review, see [1]). Table 1 shows that CCCP, PCP and TTFB produced similar results to NH_2OH: they inhibited light-induced O_2 evolution and displayed a high electron donor activity (assuming the electron-donor activity of H_2O to be 100%) in the reaction of SiMo photoreduction. The same properties were manifested by the ADRY agent ANT2p.

A residual O_2 evolution (~10%) was retained, even though the ADRY agent concentration was greatly in excess of its I_{50} value (100-fold for ANT2p). This is apparently due to the competition between H_2O and ADRY agents as electron donors. The diffusion mobility and binding/release kinetics of the ADRY agent molecules seem to determine the occurrence of the residual O_2 evolution when 'the enzyme' is saturated with substrate (with the ADRY agent molecules). Thus, ADRY agents at low concentrations are catalysts of the cyclic electron transfer around PSII [3,13,19,20], and at high concentrations they appear to serve as electron donors for non-cyclic electron transfer involving PSII.

The measurement of redox potentials was carried out with the goal of characterizing the electron donor properties of the tested compounds. Using cyclic voltammetry in aqueous buffer solution, Hanssum et al. [16] revealed that ANT2p becomes irreversibly oxidized with a redox potential of the order of 1.0 V at pH 7.5. Cyclic voltammetry data with CCCP in aqueous buffer demonstrated a well-resolved oxidation peak with a corresponding redox potential of +1.17 V (Table 2). The subsequent reductive sweep of the voltammogram reveals a hump with a redox potential of +0.34 V. Very similar data were obtained for TTFB and PCP. Redox potential differences for oxidative and reductive titrations were 0.80–0.86 V, indicating that the tested reagents become irreversibly oxidized.

The irreversibility can be due to dimerization of radical forms

Table 1
Rates of O_2 evolution in the Hill reaction with SiMo and of SiMo photoreduction in pea chloroplasts incubated with different agents

Agent	Concentration (mM)	Rate of electron transport (%)	
		O_2 evolution	SiMo reduction
NH_2OH	1	5	75
CCCP	0.1	9	86
PCP	0.1	12	78
TTFB	0.02	10	70
ANT2p	0.1	10	80

Experimental conditions were as in Fig. 1. The 100% rates of O_2 evolution and SiMo reduction in the absence of added agents were 100–110 and 380–400 μmol·mg^{-1} Chl per h, respectively.

Table 2
Redox potentials of CCCP, TTFB and PCP determined by means of cyclic voltammetry between −0.2 and +1.2 V

Agent	Redox potentials (V) in the	
	Oxidative sweep	Reductive sweep
CCCP	+1.17	+0.34
TTFB	+1.18	+0.32
PCP	+1.09	+0.29

The tested agents at concentrations of 100 μM were dissolved in aqueous 10 mM sodium phosphate buffer (pH 6.0) plus 0.1 M $LiClO_4$.

generated upon one-electron oxidation of the tested uncouplers (U) on the electrode surface:

$$U \rightarrow U^{\bullet} + e^{-} + H^{+}$$
$$U^{\bullet} + U^{\bullet} \rightarrow U\text{–}U$$

Accumulation of radicals is required for dimerization. This condition seems to be unrealizable in chloroplasts when the ADRY agent-induced cyclic electron transfer occurs and the photooxidized ADRY agent is quickly reduced. However, it can be satisfied when electrons are transferred to SiMo through the non-cyclic pathway. Besides, the radicals generated upon oxidation of the uncouplers can exist in different resonance states. This can be another factor responsible for irreversible oxidation of the tested reagents.

It should be noted that CCCP, TTFB and PCP are oxidizable compounds with similar redox potentials, ranging from +1.09 to +1.18 V. A value of about +1.12 V was quoted for the redox potential of the $P680^{+}/P680$ pair [28].

Acknowledgements: We wish to thank Professor G. Renger for the gift of ANT2p. This work was supported by a grant from the International Science Foundation (N ML 7000) and by a grant from Russian Foundation of Fundamental Research (N 12227a).

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