Volume 248, number 1,2, 62–66 FEB 07097 May 1989

Two populations of the high-potential form of cytochrome *b*-559 in chloroplasts treated with 2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene (Ant 2p)

Klára Barabás and Győző Garab*

Institute of Biophysics and **Institute of Plant Physiology, Biological Research Center, Hungarian Academy of Sciences, PO Box 521, H-6701 Szeged, Hungary*

Received 2 March 1989

Two populations of the high-potential form of cytochrome *b*-559 (cyt *b*-559HP) can be distinguished in chloroplasts. In the presence of ≥ 1 μM Ant 2p [2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene] about one-half of the cyt *b*-559 is autooxidized in the dark. This fraction of cyt *b*-559HP undergoes rapid ($t_{1/2} \approx 2$ ms) photoreduction followed by reoxidation in the dark with $t_{1/2}$ 100–200 ms. Photoreduction can be inhibited by DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea] and UHDBT [5-(*n*-undecyl)-6-hydroxy-4,7-dioxobezothiazole] but not by DBMIB (2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinine), which suggests the involvement of Q_B or the plastoquinone pool. Another fraction of cyt *b*-559HP undergoes slow ($t_{1/2} \geq 1$ s), irreversible photooxidation in the presence of ≥ 0.1 μM Ant 2p. The presently available data suggest that the heterogeneity of cyt *b*-559HP originates from PS II_α centers.

Chloroplast; Chlorotrifluoromethylanilinodinitrothiphene; Cytochrome *b*-559; Photoreduction; Photooxidation

1. INTRODUCTION

The role of cyt *b*-559 in photosynthesis is unclear. It is associated with PS II and occurs in a high-potential form ($E_{m,7} \sim 400$ mV) and one or more lower potential forms [1,2].

Although cyt *b*-559 has been shown to be photooxidizable at 77 K or in the presence of different inhibitors at the donor side of PS II [3–5] and to be photoreducible via the Q_B or the plastoquinone pool [6], the question as to whether these hemes are distinct has not been resolved.

Here, we report that in chloroplasts treated with Ant 2p, two populations of cyt *b*-559HP can be distinguished. Approximately one-half of the molecules became capable of rapid reversible photoreduction, whereas the remaining molecules undergo virtually irreversible, slow photooxidation.

2. MATERIALS AND METHODS

Chloroplasts (approx. 70% intact) were isolated according to [7] from leaves of 2-week-old pea seedlings grown in a greenhouse. Thylakoid membranes for measurements involving ferricyanide were prepared from chloroplasts by osmotic shock in 5 mM $MgCl_2$. The standard reaction medium contained 0.35 M sorbitol, 20 mM Tricine (pH 7.7), 5 mM $MgCl_2$ and chloroplasts or thylakoid membranes at a chlorophyll concentration of 50 μg/ml. Other additions are indicated in the figure legends. Menodiol was prepared from menadion as in [8].

Flash-induced absorption transients were measured on a single-beam kinetic spectrophotometer [9] and averaged in a CAMAC data-acquisition system. The half-bandwidth of the

Correspondence address: K. Barabás, Institute of Biophysics, Biological Research Center, Hungarian Academy of Sciences, PO Box 521, 6701 Szeged, Hungary

Abbreviations: ADRY, acceleration of the deactivation reactions of the water-splitting enzyme system Y of photosynthesis; Ant 2p, 2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene; cyt *b*-559, cytochrome *b*-559; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; HP, high-potential form; PS, photosystem; UHDBT, 5-(*n*-undecyl)-6-hydroxy-4,7-dioxobezothiazole

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

measuring beam was 2 nm. The time resolution of the set-up was limited to about 0.4 ms. Unless stated otherwise 100 flash-induced transients were averaged at a flash frequency of 0.37 s^{-1}. The transients, $\Delta A_{559-572}$, in chloroplasts were corrected for the contribution of the electrochromic absorbance change. Correction factors at different wavelengths with respect to the amplitude at 515 nm were determined by using the gramicidin-sensitive transient spectrum of thylakoid membranes. No correction was necessary for samples containing Ant 2p at concentrations $\geq 1\ \mu$M, as we found that Ant 2p accelerates the decay of the electrochromic absorbance change (not shown).

Chemically induced absorbance changes associated with cyt *b*-559 were measured in the dual-wavelength mode in an Aminco DW-2 spectrophotometer. Inhibitors and redox compounds were added to magnetically stirred samples without interruption of measurements. Absorbance changes induced by continuous light were obtained by side-illumination of the cuvette with red light (680 nm Oriel interference filter) of intensity 2.5 $W \cdot m^{-2}$. The photomultiplier was protected from stray light by a complementary blue filter (Corning 4-96).

All measurements were carried out at room temperature (18–22°C).

3. RESULTS

In freshly isolated chloroplasts cyt *b*-559 (hydroquinone-reducible form) is mostly in the reduced state, whereas the lower potential forms reducible by ascorbate [10] and menadiol [11] are in the oxidized state. Fig.1a shows that in our preparation, the HP form constitutes about 2/3 of the cyt *b*-559 content.

In the presence of Ant 2p, an ADRY reagent [12] which also inhibits electron transport between the secondary electron donor Z and primary electron donor P680 of PS II [13], cyt *b*-559HP becomes photooxidizable [14]. As shown in fig.1b, photooxidation is virtually irreversible in the dark and is accompanied by an increase in the relative amount of lower potential forms at the expense of the HP form. Addition of 1 mM FeCy in the dark following photooxidation did not induce further absorbance decrease at 559–572 nm (not shown). Thus, photooxidation appears to involve all oxidizable cyt *b*-559 molecules. However, the number of molecules that undergo photooxidation is much less than that of oxidizable molecules in untreated chloroplasts (cf. fig.1a and b). This discrepancy can be explained by the observation that upon addition of Ant 2p the number of oxidized cyt *b*-559HP molecules increases relative to the untreated sample (cf. fig.1a and c). It can thus be concluded that Ant 2p promotes autooxidation of about half of the cyt *b*-559HP molecules. Autooxidation of cyt *b*-559 has been previously observed in the presence of different protonophores [15,16].

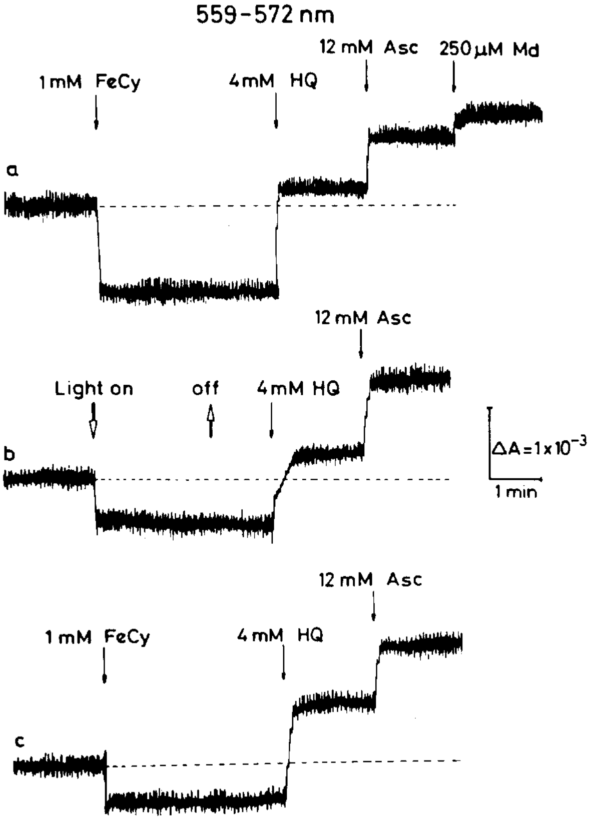

Fig.1. Redox changes of cyt *b*-559 in thylakoid membranes in the absence (a) and presence (b,c) of 1 μM Ant 2p. Successive additions of ferricyanide (FeCy), hydroquinone (HQ), Na-ascorbate (Asc) and menadiol (Md) were made or side-illumination applied as indicated.

Upon flash excitation of chloroplasts treated with Ant 2p, three kinetic components could be distinguished (fig.2a): a rapid photoreduction signal (biphasic rise; apparent half-rise time, $t_{1/2} \approx$ 2 ms); a decay ($t_{1/2} \sim$ 100–200 ms) corresponding to reoxidation of photoreduced molecules; and a slow ($t_{1/2} \sim$ 2.5 s) absorbance decrease originating from photooxidation of cyt *b*-559. The rate of photooxidation in these experiments was similar to that determined in [14].

Photoreduction and photooxidation of cyt *b*-559 could be separated experimentally. Photooxidation occurred only when the sample was changed between repeated excitations with a train of short

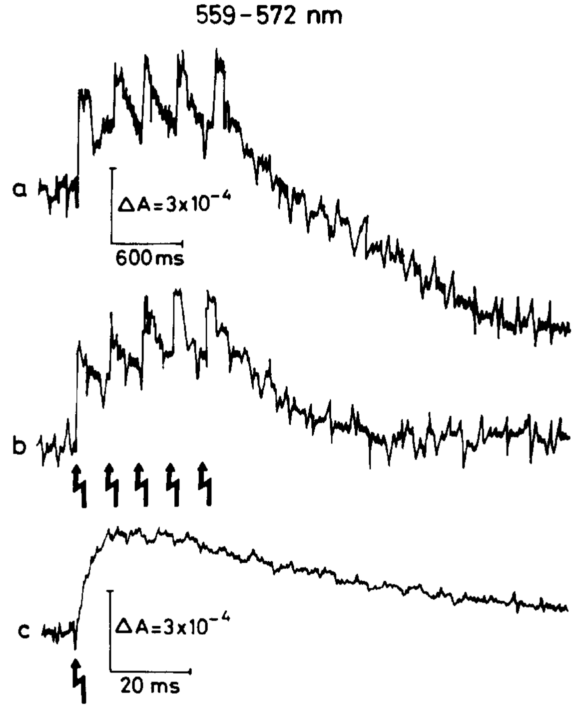

Fig.2. Absorbance transients, $\Delta A_{559-572}$, induced by a train of flashes (a,b) or by a single-turnover flash (c) in chloroplasts treated with 1 μM Ant 2p. Trace a, sample changed between repetitions; b, averaging on the same sample with 10-s dark intervals between excitations; a,b, average of 10 traces; c, average of 100 traces recorded at a repetition rate of 0.37 s^{-1}.

flashes (fig.2a) or a single flash (not shown). Due to its apparent irreversibility in the dark (fig.1b), photooxidation could not be observed under repetitive excitation. In contrast, photoreduction and reoxidation of molecules were independent of the number of repetitions (fig.2b,c). Photoreduction thus could be observed in the absence of a detectable amplitude of photooxidation (fig.2b). On the other hand, in low-intensity continuous light (fig.1b), or upon excitation with a train of low-intensity flashes (not shown), photooxidation occurred in the absence of a detectable amplitude of photoreduction signal.

The slow ($t_{1/2} \geq 1$ s) absorbance decrease has been identified as registering photooxidation of cyt *b*-559HP [5,14]. The rapid photoreduction signal, as shown by the flash-induced transient spectrum (fig.3), is also ascribable mainly to cyt *b*-559. In untreated chloroplasts the absorbance transient spectrum between 545 and 572 nm is dominated by

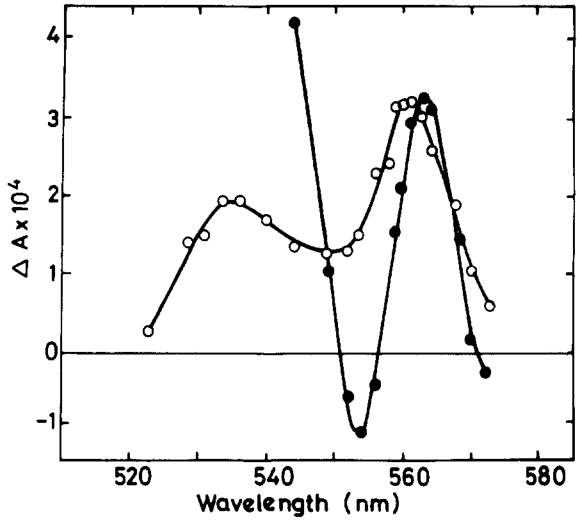

Fig.3. Transient absorbance spectrum at 10 ms following flash excitation of chloroplasts in the absence (●——●) and presence (○——○) of 1 μM Ant 2p. NB: In the absence of Ant 2p the absorbance transients of cytochrome b_6 and *f* are superimposed on the electrochromic absorbance changes.

the changes due to cytochrome *f* and b_6 with no detectable contribution from cyt *b*-559 (fig.3). Upon addition of 1 μM Ant 2p, the transmembrane electric field collapses, and owing to inhibition of the linear electron-transport chain [13,14], the amplitudes of the absorbance transients of cytochrome b_6 and *f* are diminished considerably. These changes are accompanied by a substantial increase in amplitude of the absorbance changes due to cyt *b*-559. (For the reduced minus oxidized spectrum see [17].)

The maximum amplitude of photooxidation, in agreement with [14], was found to occur in the presence of ≥ 0.1 μM Ant 2p, whereas photoreduction and autooxidation required much higher concentrations of Ant 2p (fig.4). This suggests that autooxidation and photoreduction of cyt *b*-559 cannot be correlated with the ADRY effect of Ant 2p which is observed at much lower concentrations (cf. [14]).

A similar concentration dependence of the photoreduction and autooxidation of cyt *b*-559 (fig.4) suggests that photoreduction originates from the same population of molecules as that which undergoes autooxidation upon addition of Ant 2p. Indeed, photoreduction was entirely sensitive to hydroquinone (fig.5), its extent being

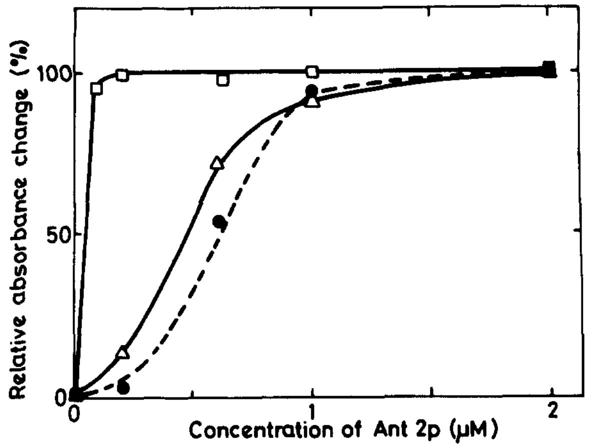

Fig.4. Dependence of absorbance changes, $\Delta A_{559-572}$, on concentration of Ant 2p. Amplitudes of photooxidation (□—□), autooxidation (△—△) and flash-induced photoreduction (●---●) of cyt *b*-559 were determined from absorbance changes measured as in figs 1b,c and 2c, respectively.

generally between 70 and 90% of the amplitude of autooxidation of cyt *b*-559. Furthermore, photoreduction cannot originate from the photooxidized fraction of cyt *b*-559, because photooxidation lags behind photoreduction (fig.2a). Thus, it can be concluded that photoreduction of cyt *b*-559 originates from the autooxidizable fraction of cyt *b*-559HP, and that photooxidation arises from another population of cyt *b*-559HP.

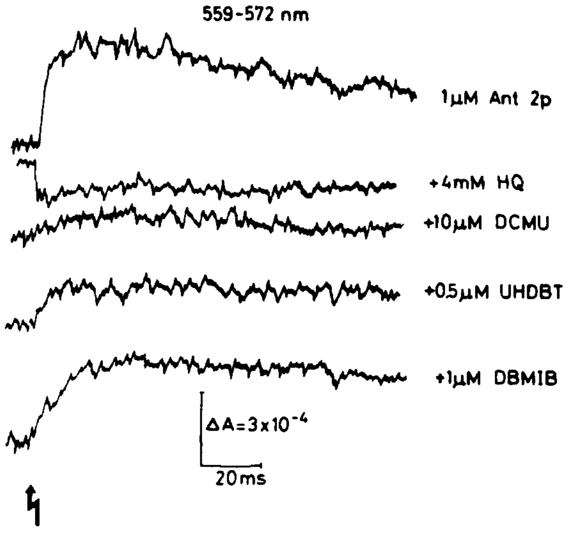

Fig.5. Effect of different compounds on the amplitude and kinetics of flash-induced photoreduction of cytochrome *b*-559 in chloroplasts treated with 1 µM Ant 2p.

The rapid ($t_{1/2} \approx 2$ ms) flash-induced photoreduction of cyt *b*-559HP, similarly to the slow photoreduction induced by continuous light [4], is fully inhibited by ≥1 µM DCMU; it is also inhibited by UHDBT but not by DBMIB (fig.5). (DBMIB decelerates the rate of reduction but does not affect its extent.) This suggests the involvement of Q_B or the plastoquinone pool.

4. DISCUSSION

Our results strongly suggest the existence of two distinct populations of cyt *b*-559HP in chloroplasts. One population of molecules undergoes autooxidation promoted by ≥1 µM Ant 2p (figs 1c,4). A rapid ($t_{1/2} \approx 2$ ms) reversible photoreduction of the autooxidizable population occurs probably via Q_B or the plastoquinone pool (fig.5). The other population of cyt *b*-559HP can undergo irreversible photooxidation ($t_{1/2} \geq 1$ s) in the presence of ≥0.1 µM Ant 2p (figs 2a,4).

Heterogeneity of cyt *b*-559HP is unlikely to arise from lateral heterogeneity of the PS II centers (i.e. from α and β centers [18]). PS II_β is known to be present in the stroma lamellae [18] and no HP form of cyt *b*-559 is detectable in this region [19]. Furthermore, both rapid reversible photoreduction and slow irreversible photooxidation of cyt *b*-559 occur in Ant 2p-treated PS II-enriched membrane fragments (BBY particles) [20] devoid of PS II_β centers (Barabás and Vass, unpublished). Thus, heterogeneity of cyt *b*-559HP appears to originate from within the PS II_α centers.

The source of this heterogeneity is unclear. One of the main difficulties in resolving this problem is that the stoichiometry of cyt *b*-559HP per PS II is ambiguous. A number of reports suggest that there are two hemes per reaction center [21–23], while others argue that there is only one heme within the PS II complex [24,25].

If cyt *b*-559 is present at a stoichiometry of one heme per PS II_α center, the heterogeneity may stem from an unrecognized microenvironmental factor, such as the protonation state of surrounding proteins or the redox state of the donor and acceptor molecules. This factor in Ant 2p-treated chloroplasts may selectively render one fraction of molecules capable of undergoing autooxidation followed by photoreduction while the other becomes photooxidizable.

If there are two hemes per PS II_α center, the heterogeneity suggests that the photoreducible and photooxidizable cyt *b*-559HP molecules are in different environments. Concerning the topography of the two hemes and the coordinating α- and β-subunits of the cyt *b*-559 molecule, several possible arrangements have been considered [26]. Recent data describing the orientation of the α-subunit with respect to the thylakoid membrane, assuming a heterodimer and/or a similar orientation of the β-subunit, would appear to favour a non-transmembrane arrangement of the two hemes [27]. Hence, the two hemes probably reside on the same side of the membrane. A heterogeneous behaviour of cyt *b*-559HP molecules may thus reflect association of the two populations of cyt *b*-559 molecule with different complexes. One of the forms of the cyt *b*-559 molecule is closely associated with the D_1-D_2 polypeptides of the PS II reaction center and is capable of undergoing DCMU-sensitive photoreduction [28]. On the other hand, cyt *b*-559 has also been shown to donate electrons to $P680^+$ when electron transport from the oxygen-evolving complex is blocked with inhibitors such as Ant 2p. This suggests that the second population of molecules may be associated with the water-splitting enzyme.

Acknowledgements: We are grateful to Professor W.A. Cramer for helpful discussions, and to Professor Barber for providing preprints of their work. The authors are indebted to Dr J. Davenport for critical reading of the manuscript. We would like to thank Professor K.H. Büchel, Bayer Forschungszentrum Wuppertal-Elberfeld, for the gift of Ant 2p, Dr I. Vass for discussions, Mrs Erika Rajtik and Etelka Mitrov for technical assistance and Mrs Zsuzsa Gonda for typing the manuscript. This work was supported by grants OKKFT (Tt) 222/1988 and 310/86 from the National Foundation of Technical Development (Hungary).

REFERENCES

[1] Cramer, W.A. and Whitmarsh, J. (1977) Annu. Rev. Plant Physiol. 28, 133–172.

[2] Anderson, B. and Akerlund, H.-E. (1987) in: The Light Reactions (Barber, J. ed.) pp.379–420, Elsevier, Amsterdam.

[3] De Paula, J.C., Innes, J.B. and Brudvig, G.W. (1985) Biochemistry 24, 8114–8120.

[4] Knaff, D.B. and Arnon, D.I. (1969) Proc. Natl. Acad. Sci. USA 63, 956–962.

[5] Maroc, J. and Garnier, J. (1979) Biochim. Biophys. Acta 548, 374–385.

[6] Tsujimoto, H.Y. and Arnon, D.I. (1985) FEBS Lett. 179, 51–54.

[7] Nakatani, H.Y. and Barber, J. (1977) Biochim. Biophys. Acta 461, 510–512.

[8] Rich, P.R. (1978) FEBS Lett. 96, 252–256.

[9] Barabás, K., Zimányi, L. and Garab, G. (1985) J. Bioenerg. Biomembranes 17, 349–363.

[10] Cramer, W.A. and Crofts, A.R. (1982) in: Photosynthesis (Govindjee ed.) vol.1, pp.387–467, Academic Press, New York.

[11] Rich, P.R. and Bendall, O.S. (1980) Biochim. Biophys. Acta 591, 153–161.

[12] Renger, G., Bouges-Bocquet, B. and Büchel, K.H. (1973) Bioenergetics 4, 491–505.

[13] Packham, N.K. and Ford, R.C. (1986) Biochim. Biophys. Acta 852, 183–190.

[14] Packham, N.K. and Barber, J. (1984) Biochem. J. 221, 513–520.

[15] Ortega, J.M., Hervas, M. and Losada, M. (1988) Eur. J. Biochem. 171, 449–455.

[16] Arnon, D.I. and Tang, G.M.-S. (1988) Proc. Natl. Acad. Sci. USA 85, 9524–9528.

[17] Wasserman, A.R. (1980) Methods Enzymol. 69, 181–202.

[18] Anderson, J. and Melis, A. (1983) Proc. Natl. Acad. Sci. USA 80, 745–749.

[19] Vallon, O., Hoyer-Harsen, G. and Simpson, D.J. (1987) Carlsberg Res. Commun. 52, 405–421.

[20] Bertold, D.A., Babcock, G.T. and Yocum, C.F. (1981) FEBS Lett. 134, 231–234.

[21] Murata, M., Miyao, M., Omata, T., Matsunami, H. and Kurabara, T. (1984) Biochim. Biophys. Acta 765, 363–369.

[22] Whitmarsh, J. and Ort, D.R. (1984) Arch. Biochem. Biophys. 231, 378–389.

[23] Yamada, Y., Tang, X.-S., Itoh, S. and Satoh, K. (1987) Biochim. Biophys. Acta 891, 129–137.

[24] Ghanotakis, D.F., Babcock, G.T. and Yocum, C.F. (1984) Biochim. Biophys. Acta 765, 388–398.

[25] Miyazaki, A., Shina, T., Toyoshima, Y., Gounaris, K. and Barber, J. (1989) Biochim. Biophys. Acta, in press.

[26] Cramer, W.A., Theg, S.M. and Widger, W.R. (1986) Photosynth. Res. 10, 393–403.

[27] Tae, G.K., Black, M.T., Cramer, W.A., Vallon, D. and Bogorad, L. (1988) Biochemistry 27, 9075–9080.

[28] Chapman, D.J., Gounaris, K. and Barber, J. (1988) Biochim. Biophys. Acta 933, 423–431.