Biochimica et Biophysica Acta 809 (1985) 167–172
Elsevier

BBA 41825

Uncouplers enhance photosynthetic electron transport from water to $NADP^+$ in the presence of plastoquinone inhibitors

Daniel I. Arnon and George M.-S. Tang

Division of Molecular Plant Biology, University of California, Berkeley, CA 94720 (U.S.A.)

(Received April 17th, 1985)

Key words: Photosystem II; Water splitting; Proton transport; Uncoupler; Plastoquinone; (Spinach chloroplast)

Uncouplers have been previously observed to relieve appreciably the inhibition of photosynthetic electron transport from water to $NADP^+$ by the plastoquinone analogues, dibromothymoquinone (DBMIB) and dinitrophenyl ether of iodonitrothymol (DNP-INT). These results were now extended by demonstrating that the reversal by uncouplers of DBMIB and DNP-INT inhibition occurred under conditions when the uncouplers did not stimulate or inhibit $NADP^+$ reduction in control treatments without the plastoquinone analogues. Since effects of uncouplers on photosynthetic electron transport depend on external pH, we determined for each of the four uncouplers, gramicidin, nigericin, FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) and SF 6847 (a ditertiary phenol derivative) its effect on oxygenic electron transport (H_2O to $NADP^+$) over a range of external pH from 6.7 to 8.7. The effect of each uncoupler on counteracting the inhibition of DBMIB and DNP-INT was then measured at its crossover external pH at which the uncoupler had little or no effect on electron transport in the uninhibited controls. Under these controlled conditions, uncouplers increased the rate of plastoquinone-inhibited electron transport, in some cases by almost 300%. To explain these results, a role for plastoquinone in processing protons released by the oxidation of water is postulated.

Introduction

One of the outstanding early successes of the chemiosmotic hypothesis [1] was its interpretation of uncoupler action, i.e., that uncouplers disrupt the coupling between ATP synthesis and electron transport because they facilitate the transport of protons across chloroplast and mitochondrial inner membranes [2]. Uncouplers are now recognized as lipophilic agents of diverse chemical nature which catalyze the equilibration of proton (and other ion) gradients across the hydrophobic core of energy-transducing membranes, noted for its low permeability to charged groups in general, and hydrogen and hydroxyl ions in particular [3,4]. By rendering the membranes permeable to protons, uncouplers dissipate the electrochemical proton gradient (formed as a consequence of electron transport and topological membrane asymmetry) that, according to the chemiosmotic theory, drives ATP formation [1,5].

We have recently used four chemically diverse uncouplers, two ionophores [6], the antibiotics gramicidin and nigericin, and two acidic aromatic compounds that act as proton carriers, carbonylcyanide fluorophenylhydrazone (FCCP) and 3,5-di-*tert*-butyl-4-hydroxybenzylidene malonitrile (SF

Abbreviations: PS, Photosystem; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DNP-INT, dinitrophenol ether of iodonitrothymol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; SF 6847, 3,5-di-*tert*-butyl-4-hydroxybenzylidene malonitrile; Mops, 4-morpholinepropane sulphonic acid; Tricine, *N*-[2-hydroxy-1,1-bishydroxymethyl)ethyl]glycine.

6847) [7], to probe the function of plastoquinone in photosynthetic electron and proton transport [8,9]. These experiments yielded novel evidence of considerable theoretical interest (see Discussion). When plastoquinone function was blocked by the use of the well-known plastoquinone inhibitors DBMIB and DNP-INT [10], the resulting blockage of photosynthetic electron transport from water to $NADP^+$ was appreciably relieved by each of the four uncouplers [8].

To assess the significance of these findings, it was necessary to establish that the observed stimulation by uncouplers of plastoquinone-inhibited photosynthetic electron transport [8] was not merely a reflection of a general stimulation by uncouplers of photosynthetic electron transport wheter plastoquinone inhibitors were present or not. Stimulation by uncouplers is known to depend on the pH of the external medium: uncouplers stimulate electron transport at acid pH's and inhibit at alkaline pH values [11].

These effects of uncouplers have led to a concept that the rate of oxygenic electron transport (i.e., of electrons originating from water) is governed not by the external pH whose optimum is near 8.0, but by the intrathylakoid pH whose optimum is near 5.0. A proton gradient of about 3 pH units established during electron transport generates the optimum acidic pH within the membranes [12–15]. The observed effects of uncouplers were consistent with this concept. By equalizing the internal and external pH, uncouplers would prevent the internal acidification. Thus, at an alkaline external pH, uncouplers would keep the internal pH in an alkaline range that was found to be damaging to the oxygen-evolving part of PS II [16,17]. At an acidic external pH, uncouplers would keep the internal pH in an acidic range favorable for electron transport.

In this study, we determined for each of the four uncouplers, its effect on oxygenic electron transport (H_2O to $NADP^+$) over a range of external pH. We then measured, at the predetermined crossover external pH, at which each uncoupler had little or no effect on the rate of electron transport in the uninhibited controls, the reversal by that uncoupler of the inhibition of electron transport by DBMIB or DNP-INT. Under these controlled conditions, uncouplers still appreciably increased the rate of the plastoquinone-inhibited electron transport, in some cases by almost 300%. The bearing of these results on a proposed role of plastoquinone in oxygenic proton transport is discussed.

Methods

Chloroplasts were isolated from spinach leaves (*Spinacea oleracea* var. Marathon) grown in a greenhouse in nutrient solution culture [18] and freshly harvested before each experiment. Whole chloroplasts were prepared essentially as described [19] except that leaves were blended in a solution containing 0.5 M sucrose/5 mM MgCl/50 mM Tricine-KOH buffer (pH 7.7). The preparation used consisted of osmotically disrupted chloroplasts obtained by suspending whole chloroplasts in a hypotonic solution of 5 mM $MgCl_2$ and 5 mM Tricine-KOH buffer (pH 7.7). After centrifugation, the pellet was washed, centrifuged and resuspended in the same solution to yield thylakoid membranes that retained the integrity of the thylakoid membrane structure needed for complete electron transport from water to $NADP^+$ and photosynthetic phosphorylation [20]. Chlorophyll was estimated [18], ferredoxin was isolated and purified [21] (by R.K. Chain) and the photoreduction of $NADP^+$ was measured [22] as described. DBMIB and DNP-INT were kindly supplied by Prof. A. Trebst, nigericin by Hoffman-La Roche Co., Nutley, NJ, and SF 6847 by Sumitomo Chemical Co., Ltd., Osaka, Japan. $NADP^+$, FCCP and gramicidin were purchased from Sigma chemical Co., St. Louis, MO.

Results

The effect of uncouplers on oxygenic electron transport (H_2O to $NADP^+$) over an external pH range from 6.7 to 8.7 is shown in Fig. 1. All four uncouplers stimulated electron transport below pH 7.5 and inhibited above pH 8. The rate of $NADP^+$ reduction in the presence and absence of an uncoupler when plotted against external pH, gave at the intersection of the curves the crossover pH for each uncoupler, i.e., the external pH at which the uncoupler neither stimulated nor inhibited electron transport. The crossover pH was found to be

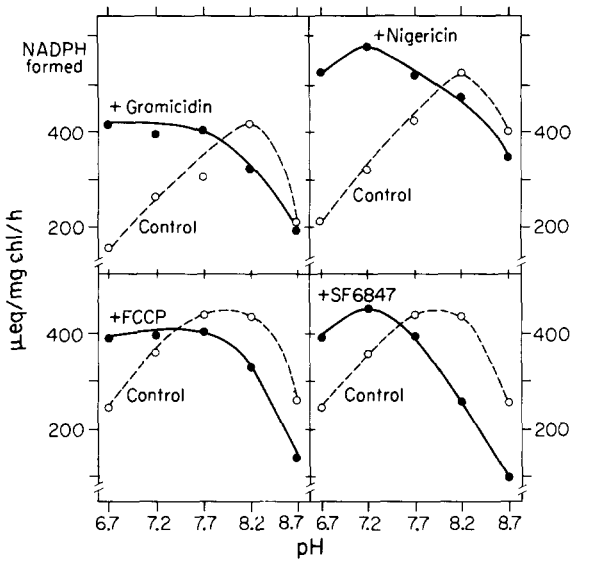

Fig. 1. Effect of uncouplers on oxygenic photorecution of $NADP^+$ (H_2O to $NADP^+$) at different external pH values. The reaction mixtures contained osmotically disrupted chloroplasts (equivalent to 50 µg chlorophyll per ml), 5 mM $MgCl_2$, 50 mM KCl, 2.5 mM ADP, 2.5 mM KH_2PO_4, 2 mM $NADP^+$, 10 µM ferredoxin and 50 mM of the appropriate buffer which was Mops for pH 6.7 and 7.2 and Tricine for pH 7.7–8.7. The concentration of gramicidin, nigericin, FCCP and SF 6847 was 0.5 µM each. Uncouplers were dissolved in methanol; equivalent concentrations of methanol were added to control treatments. The reaction mixtures were incubated for 3 min in the dark and then illuminated at room temperature in cuvettes (2 mm light path) open to air. Monochromatic illumination: 650 nm; 50 $J \cdot m^{-2} \cdot s^{-1}$.

about 8.0 for gramicidin and nigericin and about 7.5 for FCCP and SF 6847 (Fig. 1).

By measuring electron transport at the crossover pH appropriate for each uncoupler, it was possible to test whether uncouplers overcome the inhibition by the plastoquinone inhibitors DBMIB and DNP-INT under conditions when the uncouplers did not stimulate or inhibit electron transport in the controls. The concentrations of DBMIB used were well within its known inhibitory range (up to 1 µM) [10] and were predetermined for each experiment to insure appreciable inhibition of $NADP^+$ reduction. Table I shows that at the respective crossover pH values, gramicidin, nigericin, FCCP and SF 6847 gave a substantial enhancement of electron transport inhibited by DBMIB. With gramicidin and SF 6847 the increase in the rate of electron transport was almost 3-fold. At the same time, none of the uncouplers stimulated (or significantly inhibited) electron transport in the absence of DBMIB (Table I).

A similar enhancement by gramicidin, nigericin, FCCP and SF 6847 of electron transport inhibited by DNP-INT is shown in Table II.

In the experiments shown in Tables I and II relatively high concentrations of uncouplers were used similar to those found effective in previous experiments [8]. More recently, Theg and Junge [23] have reported that nanomolar concentrations of gramicidin and of other uncouplers caused the

TABLE I

REVERSAL BY UNCOUPLERS OF DBMIB INHIBITION OF OXYGENIC $NADP^+$ PHOTOREDUCTION

Except as noted in the footnotes experimental conditions were as in Fig. 1

Treatment	µequiv. NADPH formed ($mg \cdot Chl^{-1} \cdot h^{-1}$)			
	gramicidin (pH 8.0)	nigericin (pH 8.0)	FCCP (pH 7.5)	SF 6847 (pH 7.7)
Control	510	390	404	386
Control + uncoupler	510 [a]	390 [c]	382 [e]	376 [f]
DBMIB	138 [b]	52 [b]	162 [b]	54 [d]
DBMIB + uncoupler	346 [a,b]	102 [c,d]	276 [b,e]	148 [d,f]

[a] 11 µM gramicidin.
[b] 0.2 µM DBMIB.
[c] 0.5 µM nigericin.
[d] 0.3 µM DBMIB.
[e] 5 µM FCCP.
[f] 0.5 µM SF 6847.

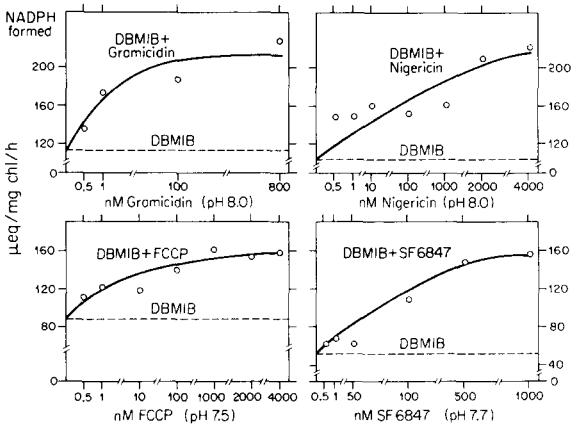

Fig. 2. Effect of uncoupler concentration on alleviating DBMIB inhibition of $NADP^+$ reduction. The rates of $NADP^+$ reduction in the presence of DBMIB are indicated by the dashed horizontal line. The DBMIB concentration was 0.2 µM for gramicidin, nigericin and FCCP and 0.3 µM for SF 6847. Other experimental conditions were as in Fig. 1.

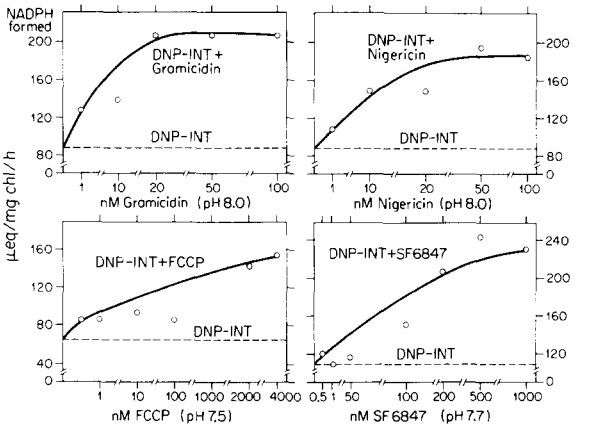

Fig. 3. Effect of uncoupler concentration on alleviating DNP-INT inhibition of $ANDP^+$ reduction. The rates of $NADP^+$ reduction in the presence of DNP-INT are indicated by the dashed horizontal line. The DNP-INT concentration was 3 µm for gramicidin, nigericin and SF 6847 and 2 µM for FCCP. Other experimental conditions were as in Fig. 1.

disappearance of the rapidly rising phase of the light-induced internal acidification in chloroplasts, as measured by the indicator neutral red. The rapidly rising phase of acidification, which they attribute to the deposition of protons derived from the photooxidation of water, was the only acidification phase they observed in the presence of DBMIB [23]. Since our interest was also in protons derived from the photooxidation of water and particularly in the possibility that uncouplers stimulated oxygenic electron transport in the presence of DBMIB (or DNP-INT) by facilitating the translocation of water-derived protons, we tested whether nanomolar concentrations of gramicidin and other uncouplers would also be effective under our experimental conditions.

Figs. 2 and 3 show that this was indeed the case. Despite some scatter of points, nanomolar concentrations of gramicidin, nigericin, FCCP and SF 6847 significantly alleviated the inhibition by DBMIB (Fig. 2) and by DNP-INT (Fig. 3) of photosynthetic electron transport from water to

TABLE II

REVERSAL BY UNCOUPLERS OF DNP-INT INHIBITION OF OXYGENIC PHOTOREDUCTION OF $NADP^+$

Except as noted in the footnotes experimental conditions were as in Fig. 1.

	µequiv. NADPH formed (mg Chl·h^{-1})			
	gramicidin (pH 8.0)	nigericin (pH 8.0)	FCCP (pH 7.5)	SF 6847 (pH 7.7)
Control	446	446	404	386
Control + uncoupler	414 [a]	506	382 [c]	376 [d]
DNP-INT (3 µM)	60	60	96	110
DNP-INT + uncoupler	138 [a]	142 [b]	192 [c]	244 [d]

[a] 10 µM.
[b] 0.2 µM.
[c] 5 µM.
[d] 0.5 µM.

$NADP^+$. We conclude, therefore, that one reason why blockage of plastoquinone turnover by DBMIB or DNP-INT inhibits oxygenic electron transport is because it impedes the translocation of protons derived from the photooxidation of water. When proton removal is facilitated by uncouplers, electron transport in the presence of DBMIB or DNP-INT is significantly enhanced.

Discussion

The results of this investigation extend and substantiate earlier observations [8] by demonstrating that uncouplers enhance to an appreciable degree the inhibition of photosynthetic electron transport (H_2O to $NADP^+$) by plastoquinone analogues under conditions when the uncouplers neither stimulate nor depress the rate of electron transport in the uninhibited controls. These effects of uncouplers require special explanation – they are not encompassed by the currently popular concepts of photosynthetic electron transport [24]. The ensuing discussion will focus on DBMIB, the best known and most widely investigated plastoquinone analogue, but similar results and conclusions were drawn from experiments with DNP-INT.

Plastoquinone is an essential link between PS II and PS I [25]. It transports to PS I electrons liberated in the photooxidation of water by PS II and concomitantly acts as a transmembrane shuttle of protons from stroma into the thylakoid lumen. When plastoquinone function is blocked by DBMIB, electron flow from water cannot reach PS I; it is restricted to PS II [10]. In fact, oxygenic electron transport by PS II is often defined as electron transport that proceeds in the presence of DBMIB [26–29].

There are no common features in the diverse chemical structures of the four uncouplers we used that would justify a speculation that they chemically counteracted the inhibition of plastoquinone function by DBMIB. The only common property of these uncouplers, a property that we hold relevant to our results, is that they all facilitate proton transport across the thylakoid membranes. We are therefore led to the hypothesis that plastoquinone not only serves as a link between PS II and PS I but is also required by PS II alone, when its connection to PS I is blocked by DBMIB. Specifically, we propose that plastoquinone-plastoquinol transitions are required for translocation into the thylakoid lumen of protons released in the thylakoid membrane by the photooxidation of water. We envision that electron and proton transport are coupled. When DBMIB, by inhibiting plastoquinol oxidation [10] blocks proton translocation, uncouplers provide a nonphysiological substitute mechanism for proton transfer and thereby reactivate to a significant degree oxygenic electron transport.

Central to this hypothesis is the notion that photooxidation of water takes place in the hydrophobic core of the membrane that is poorly permeable to the released protons and therefore, the mediation of plastoquinone is required as a carrier for the conductance of protons into the lumen. This view differs from frequent representations (see, for example, Fig. 1 in Ref. 30) that water-derived protons are released directly into the lumen, without the assistance of a carrier. More recently, work in the laboratories of Dilley [31], Junge [23] and Homann [32] led to a concept that protons from water are initially deposited in hydrophobic membrane compartments ('domains') on the inner side of the membrane from which they are released by special mechanisms: none of these mechanisms, however, implicate plastoquinone.

It should be stressed that, aside from electron transport, we envision a dual role for plastoquinone in the transfer of protons into the thylakoid lumen: one in the conductance of protons, that originate from the intramembrane photooxidation of water by PS II, and another, linked to PS I, in the transmembrane shuttle of protons originating in the stroma [8,9]. Since the transfer of protons into the lumen depends on the oxidation of plastohydroquinone by the FeS-cytochrome f/b_6 complex (reviewed in Refs. 33–35), it follows that this complex may be an operational component of the oxygenic as well as the anoxygenic system – a matter that we are now investigating.

Of considerable conceptual interest are the present findings that PS II, disconnected by DBMIB from PS I, is competent of an appreciable oxygen photoreduction of $NADP^+$ in presence of uncouplers, under conditions when the uncouplers do

not stimulate $NADP^+$ reduction in control treatments without DBMIB. These findings are in agreement with earlier observations [8] and with recent evidence of significant oxygenic photoreduction of $NADP^+$ by inside-out vesicles, under experimental conditions that appear to exclude the participation of PS I [36]. They are not compatible with the currently popular Z-scheme, but are compatible with a briefly outlined alternative concept [8] that will be discussed in more detail elsewhere.

Acknowledgement

This investigation was aided by a grant from the National Science Foundation.

References

1 Mitchell, P. (1961) Nature 191, 144–148
2 Greville, G.D. (1969) in Current Topics Bioenergetics (Sanadi, D.R., ed.), Vol. 3, pp. 1–78, Academic Press, New York
3 Mitchell, P. and Moyle, J. (1967) Biochem. J. 104, 588–600
4 Cunarro, J. and Weiner, M.W. (1975) Biochim. Biophys. Acta 387, 234–240
5 Mitchell, P. (1979) Science 206, 1148–1159
6 Szabo, G. (1981) Fed. Proc. 40, 2196–2201
7 Terada, H. and Van Dam, K. (1975) Biochim. Biophys. Acta 387, 507–518
8 Arnon, D.I., Tsujimoto, H.Y. and Tang, G.M.-S. (1981) Proc. Natl. Acad. Sci. USA 78, 2942–2946
9 Arnon, D.I., Tsujimoto, H.Y. and Tang, G.M.-S. (1982) Biochem. Biophys. Res. Commun. 106, 450–457
10 Trebst, A. (1980) Methods Enzymol. 69C, 675–715
11 Avron, M. (1972) Proceedings of the 2nd International Congress on Photosynthesis Research, Vol. 2, pp. 861–871, Junk, The Hague
12 Rumberg, B. and Siggel, U. (1969) Naturwissenschaften 58, 130–132
13 Rottenberg, H., Grunwald, T. and Avron, M. (1971) FEBS Lett. 13, 41–45
14 Rottenberg, H., Grunwald, T. and Avron, M. (1972) Eur. J. Biochem. 25, 54–60
15 Bamberger, E.S., Rottenberg, H. and Avron, M. (1973) Eur. J. Biochem. 34, 557–563
16 Harth, E., Reimer, S. and Trebst, A. (1974) FEBS Lett. 42, 165–168
17 Cohn, D.E., Cohen, W.S. and Bertsch, W. (1975) Biochim. Biophys. Acta 376, 97–104
18 Arnon, D.I. (1949) Plant Physiol. 24, 1–15
19 Kalberer, P.P., Buchanan, B.B. and Arnon, D.I. (1967) Proc. Natl. Acad. Sci. USA 57, 1542–1549
20 Arnon, D.I. and Chain, R.K. (1975) Proc. Natl. Acad. Sci. USA 72, 4961–4965
21 Losada, M. and Arnon, D.I. (1965) in Modern Methods of Plant Analysis, (Linskens, H.W., Wanwal, B.D. and Tracy, M.V., eds.), Vol. 7, pp. 569–615, Springer-Verlag, Berlin
22 McSwain, B.D. and Arnon, D.I. (1968) Proc. Natl. Acad. Sci. USA 61, 989–996
23 Theg, S.M. and Junge, W. (1983) Biochim. Biophys. Acta 723, 294–307
24 Trebst, A. (1974) Annu. Rev. Plant Physiol. 25, 423–453
25 Amesz, J. (1973) Biochim. Biophys. Acta 301, 35–51
26 Trebst, A. and Reimer, S. (1973) Biochim. Biophys. Acta 305, 129–139
27 Trebst, A. and Reimer, S. (1973) Biochim. Biophys. Acta 325, 546–557
28 Selman, B.R. (1976) J. Bioenerg. Biomembranes 8, 143–156
29 Izawa, S. (1980) Methods Enzymol. 69, 413–434
30 Trebst, A. and Avron, M. (1977) in Photosynthesis I, Encyclopedia of Plant Physiology, New Series, Vol. 5, pp. 1–4, Springer, New York
31 Dilley, R.A., Baker, G.M., Bhatnagar, D., Millner, P. and Laszlo, J. (1981) in Energy Coupling in Photosynthesis (Selman, B.R. and Selman-Reiner, S., eds.), pp. 47–58, Elsevier, Amsterdam
32 Johnson, J.D., Pfister, V.R., and Homann, P.H. (1983) Biochim. Biophys. Acta 723, 256–265
33 Malkin, R., Chain, R.K. and Lam, E. (1983) Chem. Scr. 21, 75–80
34 Hauska, G., Hurt, E., Gabellini, N. and Lockau, W. (1983) Biochim. Biophys. Acta 726, 97–133
35 Croffs, A.R. and Wraight, D.A. (1983) Biochim. Biophys. Acta 726, 149–185
36 Albertsson, P.-Å., Hsu, B.-D., Tang, G.M.-S., and Arnon, D.I. (1983) Proc. Natl. Acad. Sci. USA 80, 3971–3975