Superoxide- and Ethane-Formation in Subchloroplast Particles: Catalysis by Pyridinium Derivatives

E. F. Elstner, H. P. Fischer*, W. Osswald, and G. Kwiatkowski

Institut für Botanik und Mikrobiologie, Techn. Universität München, Arcisstr. 21,
D-8000 München 2

Z. Naturforsch. **35 c,** 770–775 (1980); received May 23/June 6, 1980

Superoxide, Lipid Peroxidation, Ethane, Chloroplasts, Pyridinium Salts

Oxygen reduction by chloroplast lamellae is catalyzed by low potential redox dyes with E_0' values between -0.38 V and -0.6 V. Compounds of E_0' values of -0.67 V and lower are inactive. In subchloroplast particles with an active photosystem I but devoid of photosynthetic electron transport between the two photosystems, the active redox compounds enhance chlorophyll bleaching, superoxide formation and ethane production independent on exogenous substrates or electron donors. The activities of these compounds decrease with decreasing redox potential, with one exception: 1-methyl-4,4′-bipyridinium bromide with an E_0' value of lower -1 V (and thus no electron acceptor of photosystem I in chloroplast lamellae with intact electron transport) stimulates light dependent superoxide formation and unsaturated fatty acid peroxidation in subchloroplast particles, maximal rates appearing after almost complete chlorophyll bleaching. Since this activity is not visible with compounds with redox potentials below -0.6 V lacking the nitrogen atom at the 1-position of the pyridinium substituent, we assume that 1-methyl-4,4′-bipyridinium bromide is "activated" by a yet unknown light reaction.

Introduction

Low potential compounds of the bipyridinium family exhibit their light-dependent herbicidial activities due to their autoxidation after reduction by photosystem I, yielding superoxide radicals [1, 2] and eventually other toxic oxygen species [3, 4].

We recently found that photosystem I of isolated chloroplast lamellae or subchloroplast particles independently on electrons from photosystem II seems to catalyze monovalent oxygen reduction, stimulated by low potential compounds like paraquat. During this reaction, chlorophyll is bleached and unsaturated fatty acids are peroxidized forming ethane besides other oxidation products [5]. Surprisingly, maximal rates of ethane formation have been observed after bleaching of ca. 50 to 90% of the chlorophyll of subchloroplast particles. In order to further characterize this light dependent destructive reaction, we here report on the activities of five pyridinium compounds [6] with different redox potentials. Superoxide formation and ethane production by subchloroplast particles is compared with oxygen reduction by either isolated chloroplast lamellae in the light or by NADPH + H^+ in the dark, catalyzed by NADP-ferredoxin reductase.

Materials and Methods

Chloroplasts [7] and subchloroplast particles [5] were prepared from spinach. Chlorophyll was determined according to Arnon [8]. Superoxide formation [9], ethane production [10] and photosynthetic electron transport through both photosystems II and I [11] were determined as earlier described. Superoxide dismutase was prepared from dried green peas [12, 13]. Oxygen consumption was determined with a "Hansatech electrode" (Bachofer, Reutlingen) at 18 °C or 22 °C with side illumination (30 Klux).

Incubations of subchloroplast particles were done in ca. 14 ml Fernbach flasks in a photothermostate at 22 °C with illumination from the bottom (35 Klux). Ferredoxin was prepared according to Tagawa and Arnon [14], and NADP-ferredoxin reductase according to Shin *et al.* [15]. Glucose-6-

---

* Ciba-Geigy AG, Agrochemicals Division, CH-4002 Basel, Schweiz

Abbreviations: Chl, chlorophyll; SOD, superoxide dismutase; MV, methylviologen, paraquat, 1,1′-dimethyl,4,4′-bipyridinium dichloride; AQ, anthraquinone-2-sulfonic acid; TP_{420}, 1-methyl-4-(2-symtriazinyl)-pyridinium bromide ($E_0' = -0.42$ V); DTP_{470}, 1-methyl-4(4,6-dimethyl-2-symtriazinyl) pyridinium bromide ($E_0' = -0.47$ V); PP_{670}, 1-methyl-4(2-pyrimidyl)-pyridinium bromide ($E_0' = -0.67$ V); P_{1000}, 1-methyl-4-phenylpyridinium bromide (E_0' lower -1 V); B_{1000}, 1-methyl-4,4′-bipyridinium bromide (E_0' lower -1 V).

Reprint requests to Dr. E. F. Elstner.

0341-0382/80/0900-0770 $ 01.00/0

phosphate and glucose-6-phosphate dehydrogenase were from Boehringer, Mannheim. The pyridinium compounds were prepared and their half wave potentials determined as recently described [6].

Results

Oxygen uptake by chloroplast lamellae is stimulated by low potential compounds as AQ, diquat, paraquat or triquat with redox potentials between ca. −0.38 V and −0.55 V [16, 6]. Using hydroxylamine oxidation as a test system for monovalent oxygen reduction (and thus production of the superoxide free radical ion, $O_2^{\cdot -}$), we earlier demonstrated the AQ (E'_0 −0.38 V) as well as triquat ($E'_0 = -0.55$ V) catalyze a light dependent $O_2^{\cdot -}$ formation, whereas in the dark at the expense of NADPH and catalyzed by NADP-ferredoxin reductase only AQ was actively reducing O_2 [16].

If we test compounds with different redox potentials in a similar manner as mentioned above with either isolated chloroplast lamellae in the light or with isolated NADP-ferredoxin and NADPH in the dark (*cf.* ref. [17]), we observe a loss of activity in the light reaction with the compound PP_{670} exhibiting an E_0 value at pH 7.8 of ca. −0.72 V ($E'_0 = -0.67$ V), whereas the compound DTB_{470} with a redox potential of ca. −0.5 V at pH 7.8 ($E'_0 = -0.57$ V) is fully active, as compared to MV or AQ. In the dark reaction, however, the compound DTP_{470} shows very little activity as compared to MV or AQ. Thus, compounds with E'_0 values between −0.5 and −0.6 V should discriminate NADPH (or NADH) – driven reactions but are fully active in the light (Table I).

In this respect, the five compounds TP_{420}, DTP_{470}, PP_{670}, P_{1000} and B_{1000} behave as expected and catalyze oxygen reduction according to their respective redox potentials, compounds below −0.67 V being without activity both in the light and in the dark.

This result is confirmed by the *p*-nitrosodimethylaniline (pNDA)-test: as recently described [11] electron acceptors of photosystem I can easily and sensitively be detected by their inhibition of pNDA photobleaching by chloroplast lamellae with intact electron transport through both photosystems. Low potential electron acceptors as AQ or MV as well as NADP in the presence of ferredoxin by 100% inhibit pNDA photobleaching [11]; likewise, TP_{420} and DTP_{470} completely inhibit pNDA photobleaching whereas the compounds with E'_0 values below −0.6 V as PP_{670}, P_{1000} and B_{1000} have no significant influence (Fig. 1).

We also recently showed [5] that autoxidizable electron acceptors of photosystem I like MV catalyze Chl bleaching and unsaturated fatty acid peroxidation independently on photosynthetic electron transport and electrons from photosystem II. As shown in Table II both Chl bleaching and ethane formation are enhanced by compounds with redox potentials between −0.38 V (AQ) and −0.47 V (DTP_{470}) while the compounds PP_{670} ($E'_0 = -0.67$ V) and P_{1000} (E'_0 = lower −1 V) – are inactive. We observe one exception, however: Although the compound B_{1000} has no activity in oxygen reduction by illuminated chloroplast lamellae with intact electron transport it significantly enhances both Chl bleaching and ethane formation in subchloroplast particles (Table II).

In Table III we compare the influence of 5 low potential compounds (with E'_0 below −0.4 V) on

Table I. Effects of different low potential redox compounds on oxygen reduction by isolated chloroplast lamellae in the light or by NADPH + H⁺ in the dark.
Reaction conditions: Oxygen uptake was determined with a "HASATECH" oxygen electrode at 22 °C. The light reaction was conducted as described in ref. [23, 25], the dark reaction as described in ref. [25, 16].

Compound added [0.1 mM]	E'_0 [V]	E_0 (pH 7.8) [V]	Light reaction [nmol O_2 uptake per mg Chl × min^{-1}]	Dark reaction [nmol O_2 uptake per min]
none	–	–	430	0.0
P_{1000}	"−1"	"−1"	270	0.0
B_{1000}	"−1"	"−1"	380	0.0
PP_{670}	− 0.67	− 0.72	430	0.0
DTP_{470}	− 0.47	− 0.51	1250	4.8
TP_{420}	− 0.42	− 0.46	1300	40.7
MV	− 0.44	− 0.44	1100	79.4
AQ	− 0.38	n. i.	1400	566.3

n. i., information available, (see ref. [18]).

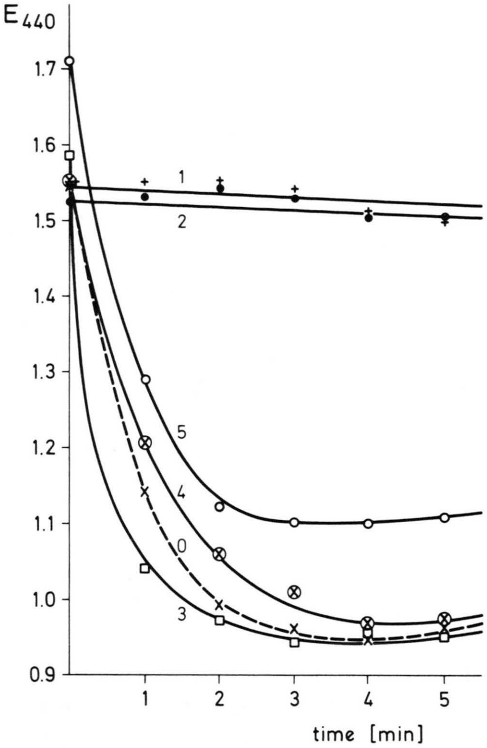

Fig. 1. Effects of five low potential pyridinium derivatives on photobleaching of *p*-nitrosodimethyl-aniline by chloroplast lamellae. For experimentel procedures see ref. [11]; pyridinium derivatives were added to yield concentrations of 0.1 mM. 0, control; 1, TP_{420} ($E_0' = -0.42$ V); 2, DTP_{470} ($E_0' = -0.47$ V); 3, PP_{670} ($E_0' = -0.67$ V); 4, P_{1000} (E_0' = lower − 1 V); 5, B_{1000} (E_0' = lower − 1 V).

ethane formation and hydroxylamine oxidation (forming nitrite; this reaction is indicative for the production of the superoxide free radical ion $O_2^{\cdot -}$, *cf.* ref. [9]) and the influence of SOD on these reactions: both ethane formation and hydroxylamine oxidation are enhanced by the compounds with E_0' values of −0.42 V (TP_{420}) and −0.47 V (DTP_{470}), whereas the compounds PP_{670} ($E_0' = -0.67$ V) and P_{1000} (E_0' = lower −1 V) show weak or no influence. Again, the compound B_{1000} (E_0' lower −1 V) represents an exception since it clearly stimulates ethane formation and hydroxylamine oxidation. The inhibition by SOD of both nitrite formation from hydroxylamine and ethane formation suggests that the peroxidation of unsaturated fatty acids like α-linolenic acid, yielding ethane [19–21], seems to involve the superoxide anion (Table III). The exceptional activity of the compound B_{1000} with is in contrast to our understanding of the function of low potential dyes in photosynthetic electron transport [1, 2] is further demonstrated in Fig. 2: comparing the time courses of Chl bleaching and ethane formation in the absence and in the presence of the compounds MV, DTP_{470}, PP_{670} and B_{1000} we observe that, in subchloroplast particles (as earlier reported, ref. [5]) Chl bleaching precedes ethane formation. After 60 min illumination when Chl bleaching is in the

Table II. Comparison of the effects of compounds with different redox potentials on chlorophyll bleaching and ethane formation by subchloroplast particles. For experimental conditions see Materials and Methods.

Compound added [10^{-4} M]	E_0' [V]	Chlorophyll [% bleached]	Ethane formed [pmol/mg Chl]
none	–	55	0.9
AQ	− 0.38	94	3.6
MV	− 0.44	87	4.0
TP_{420}	− 0.42	98	2.6
DTP_{470}	− 0.47	73	2.1
PP_{670}	− 0.67	65	0.8
P_{1000}	lower − 1	51	0.9
B_{1000}	lower − 1	**74**	**1.6**

Table III. Effects of low potential redox compounds on ethane formation and hydroxylamine oxidation by subchloroplast particles. Ethane formation: measured at pH 5.5, hydroxylamine oxidation at pH 7.5.

Compound added	E_0'	Ethane formed [a]			Nitrite formed [b]		
[10^{-4} M]	[V]	− SOD	+ SOD	% inhibition by SOD	− SOD	+ SOD	% inhibition by SOD
none	–	9	2.5	73	28	6	79
TP_{420}	− 0.42	14	10	30	74	4	95
DTP_{470}	− 0.47	15	7.6	50	33	6	82
PP_{670}	− 0.67	10	6.5	35	25	3	78
B_{1000}	lower − 1	14	5	65	40	8	80
P_{1000}	lower − 1	11.5	7	40	25	8	70

[a] pmol/mg Chl. [b] µmol/mg Chl.

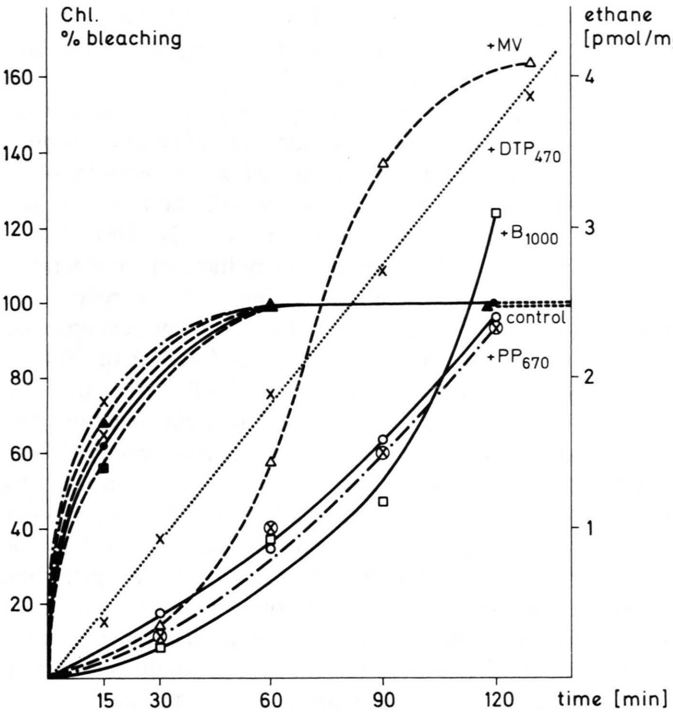

Fig. 2. Effects of MV, DTP_{470}, PP_{670} and B_{1000} on chlorophyll bleaching and ethane formation by chloroplast lamellae. For experimental procedures see Materials and Methods. Closed symbols, CHl. photobleaching; open symbols, ethane formation.

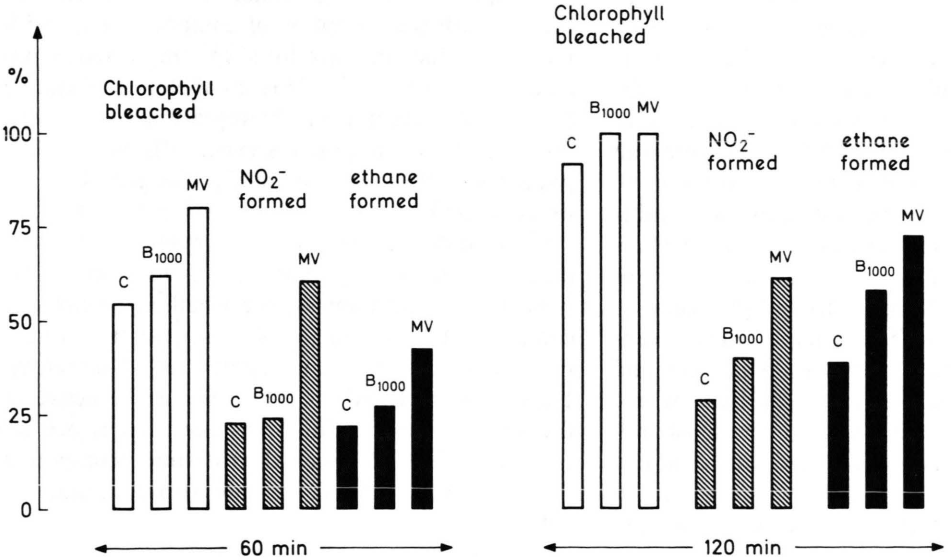

Fig. 3. Comparison of the effects of MV and B_{1000} on chlorophyll bleaching, superoxide formation (nitrite formation from hydroxylamine) and ethane production by subchloroplast particles. For experimental conditions see Materials and Methods; MV and B_{1000} were added to yield 0.1 mM solutions. 100% represent: 20 pmol ethane × mg Chl^{-1}; 220 μmol NO_2^- formed from hydroxylamine × mg Chl^{-1}; c, controls.

range of ca. 80%, only with the compound DTP_{470} a linear increase of ethane formation with time is observed. Different to the influence of compound DTP_{470} is the activity of MV which catalyzes a sigmoidally increasing ethane formation with maximal rates between 60 and 90 min illumination, when (with the applied method of Chl determination *cf.* ref. [8]) only ca. 2% Chl is left as compared to the start of the reaction. At 60 to 90 min illumination, neither compound PP_{670} nor B_{1000} show a significant influence on ethane formation; after 90 min illumination, however, compound B_{1000} catalyzes a strongly enhanced rate of ethane formation while in the presence of PP_{670} the rate of ethane formation is identical to the control (Fig. 2). In another experiment, we compare the effects of MV and B_{1000} on ethane formation, Chl bleaching and hydroxylamine oxidation by subchloroplast particles after 60 and 120 min illumination. As shown in Fig. 3, after 60 min illumination, only MV shows enhanced Chl bleaching nitrite formation and ethane production – while after 120 min all three parameters are clearly enhanced by both compound B_{1000} and MV.

Discussion

As recently reported [4, 5, 22, 23], ethane formation is a reliable indicator for unsaturated fatty acid peroxidation after treatment of green plants or unicellular algae with certain herbicides. Ethane formation correlates with Chl bleaching and is thus indicative for herbicide – induced photodestructions. In the case of low potential dyes as MV, the superoxide anion seems to play a role in the peroxidation of unsaturated fatty acids as indicated by the inhibition by SOD of ethane formation [4, 5]. Furthermore, we recently found that the catalysis by MV of the above photodestructions in strong light seems to merely be dependent on photosystem I-reaction center. Since MV enhances both Chl bleaching and ethane formation in subchloroplast particles where photosystem II is disconnected from photosystem I, apparently Chl itself and unsaturated fatty acid can function as electron donors for photosystem I after a certain degree of destruction of the tyhlakoids [5], maintaining MV reduction by the reaction center of photosystem I even after bleaching of more than 99% of the bulk chlorophyll.

In order to more closely study this photodestructions we compared the activities of several recently described [6] low potential redox dyes with those of MV. These different compounds catalyze oxygen reduction at the expense of either NADPH (catalyzed by NADP-ferredoxin reductase) or electrons from photosystem I according to their redox potentials. In the dark reaction at the expense of NADPH, compounds with E_0' of lower than -0.47 V are not active. The compound DTP_{470} with an E_0' of -0.47 V is fully active in the light reaction but shows very little activity in the dark reaction. This compound might well be a "safe" (as compared to MV, *cf.* ref. [24]) herbicide with little toxicity in animal systems. Compounds with E_0' values of higher than -0.47 V show almost identical activities in the light but are increasingly active with increasing redox potentials in the dark reaction indicating kinetically limited reactions in the light in contrast to thermodynamical limitations in the dark. Compounds with E_0' values lower than -0.67 V are inactive both in the light and in the dark.

In subchloroplast paticles, low potential redox compounds catalyze Chl bleaching, $O_2^{\cdot -}$ formation and ethane production as expected and according to the respective redox potentials (Tables II, III and Fig. 2), with the exception of compound B_{1000} with no clear reduction wave [6] suggesting a redox potential of lower -1 V. This compound is inactive as electron acceptor in chloroplast lamellae with intact electron transport (as expected), but exhibits photodestructive activities after prolonged illumination (2 h, 30 Klux) of subchloroplast particles.

Up to date we have no experimental proof for a certain mechanism operating during the above catalysis. We currently investigate whether the inactive compound B_{1000} = 1-methyl-4,4′-dipyridylium might photochemically be transalkylated into 1-dimethyl-1-alkyl-4,4′-dipyridylium which should be active in the above catalysis. This transalkylation is neither possible with the phenylpyridinium compound (P_{1000}) nor with the 4-(2-pyrimidyl)-pyridinium salt (PP_{670}).

[1] A. D. Dodge, Herbicides and Funicides. Factors Affecting Their Activity (N. M. McFarlane, ed.), pp. 7–21, The Chem. Soc., London 1977.

[2] J. F. Allen, Superoxide and Superoxide Dismutases (A. M. Michelson, J. M. McCord, and I. Fridovich, eds.), pp. 417–436, Academic Press London, New York, San Francisco 1977.

[3] E. F. Elstner, M. Saran, W. Bors, and E. Lengfelder, Eur. J. Biochem. **89,** 61–66 (1978).

[4] E. F. Elstner and R. Youngman, Ber. Deutsch. Bot. Ges. **91,** 569–577 (1978).

[5] E. F. Elstner, E. Lengfelder, and G. Kwiatkowski, Z. Naturforsch. **35 c,** 303–307 (1980).

[6] H. P. Fischer and L. A. Summers, J. Het. Chem. **17,** 333–336 (1980).

[7] R. G. Jensen and J. A. Bassham, Proc. Nat. Acad. Sci. USA **56,** 1095 (1966).

[8] D. I. Arnon, Plant Physiol. **24,** 1–4 (1949).

[9] E. F. Elstner, C. Stoffer, and A. Heupel, Z. Naturforsch. **30 c,** 53–56 (1975).

[10] E. F. Elstner and J. R. Konze, Nature **263,** 351–352 (1976).

[11] E. F. Elstner and H. Zeller, Plant Sci. Lett. **13,** 15–20 (1978).

[12] E. F. Elstner and R. Kramer, Biochim. Biophys. Acta **314,** 340–353 (1973).

[13] Y. Sawada, T. Ohyama, and I. Yamazaki, Biochim. Biophys. Acta **269,** 305 (1972).

[14] K. Tagawa and D. I. Arnon, Nature **195,** 537 (1962).

[15] M. Shin, K. Tagawa, and D. I. Arnon, Biochem. Zeitschr. **338,** 89 (1963).

[16] E. F. Elstner, A. Heupel, and S. Vaklinova, Z. Planzenphysiol. **62,** 184–200 (1970).

[17] P. Böger, Photosynthesis II, Encyclopedia of Plant Physiology, **Vol. 6,** p. 399–409 (M. Gibbs and E. Latzko, eds.), Springer Verlag Berlin-Heidelberg-New York 1979.

[18] R. L. Willson, Chemistry and Industry **5,** 183–193 (1977),

[19] J. R. Konze and E. F. Elstner, Biochim. Biophys. Acta **528,** 213–221 (1978).

[20] E. E. Dumelin and A. L. Tappel, Lipids **12,** 894–900 (1977).

[21] W. W. John and R. W. Curtis, Plant Physiol. **59,** 521–522 (1977).

[22] E. F. Elstner and I. Pils, Z. Naturforsch. **34 c,** 1040–1043 (1979).

[23] E. F. Elstner and W. Osswald, Z. Naturforsch. **35 c,** 129–135 (1980).

[24] A. P. Autor, Biochemical Mechanisms of Paraquat Toxicity. Academic Press New York, San Francisco, London 1977.

[25] E. F. Elstner and D. Frommeyer, Z. Naturforsch. **33 c,** 276–279 (1978).