Determination of Superoxide Free Radical Ion and Hydrogen Peroxide as Products of Photosynthetic Oxygen Reduction

Erich F. Elstner, Claus Stoffer, and Adelheid Heupel

Ruhr-Universität Bochum, Lehrstuhl Biochemie der Pflanzen, Bochum

(Z. Naturforsch. **30 c**, 53–56 [1975]; received October 25, 1974)

Chloroplasts, Oxygen Reduction, Hydroxylamine Oxidation, Superoxide Free Radical Ion

Formation of Nitrite from Hydroxylamine in the presence of illuminated chloroplast lamellae is inhibited by superoxide dismutase but not by catalase, indicating that the superoxide free radical ion and not H_2O_2 is responsible for the oxidation of hydroxylamine. Decarboxylation of α-keto acids on the other hand is strongly inhibited by catalase but only slightly by superoxide dismutase. Light-dependent hydroxylamine oxidation and decarboxylation of α-keto acids can be used, therefor, as specific and sensitive probes for the determination of either the superoxide free radical ion or hydrogen peroxide, respectively.

Photosynthetic oxygen reduction in the presence of ferredoxin, (monitored by the above method) yields both H_2O_2 and $O_2^{\cdot-}$. The addition of an oxygen reducing factor (ORF, solubilized by heat — treatment of washed chloroplast lamellae) instead of ferredoxin, however, stimulates only the production of H_2O_2, while $O_2^{\cdot-}$ — formation is not observed. The cooperation of ferredoxin and ORF during photosynthetic oxygen reduction by chloroplast lamellae apparently produces H_2O_2 not only by dismutation of $O_2^{\cdot-}$, but also by a separate mechanism involving ORF.

Introduction

Photosynthetic oxygen reduction is thought to play an important role in photosynthetic energy conservation [1, 2] and photorespiration [3].

Recently it has been shown that oxygen is reduced monovalently by illuminated chloroplast lamellae, including either the primary electron acceptor of photosystem I [4–6] and/or ferredoxin as terminal reductants [5]. In the latter case, another component of chloroplast lamellae besides ferredoxin has been postulated to participate in photosynthetic electron flow from ferredoxin to oxygen [7]. This component has been called oxygen reducing factor (ORF). Several characteristics of ORF indicated, that an *o*-diphenol group might be responsible for the catalytic function of ORF during oxygen reduction [8].

The function of bound ORF depends on the presence of ferredoxin and the superoxide free radical ion as the product of autooxidation of reduced ferredoxin [8]; solubilized ORF is able to stimulate photosynthetic oxygen reduction in the absence of ferredoxin, but is still dependent on the activation by the superoxide free radical ion.

This paper tries to provide evidence that the products of photosynthetic oxygen reduction in the presence of Fd are both H_2O_2 and $O_2^{\cdot-}$, while ORF yields only in the formation of H_2O_2. The cooperation of Fd and ORF in photosynthetic oxygen reduction provides a system which is able to produce H_2O_2 and $O_2^{\cdot-}$ simultanuously. During photosynthetic oxygen reduction, H_2O_2 is not only the product of the dismutation of the superoxide free radical ion according to Eqn (1)

$$O_2^{\cdot-} + O_2^{\cdot-} + 2\,H^+ \rightarrow H_2O_2 + O_2 \qquad (1)$$

but is separately formed by a mechanism which includes the function of ORF.

Requests for reprints should be sent to Dr. E. F. Elstner, Ruhr-Universität Bochum, *D-4630 Bochum*, Lehrstuhl Biochemie der Pflanzen.

Material and Methods

Ferredoxin was isolated from spinach leaves [9] and SOD from dried green peas [10, 5]. Chloroplasts were isolated either from spinach [11] or from sugar beet leaves [12, 7]. ORF was prepared from isolated chloroplast lamellae [7] from either spinach or sugar beet leaves.

Photosynthetic decarboxylation of [1-^{14}C]glyoxylate [13], NADP-reduction [13] and nitrite-formation [14] from hydroxylamine were measured as described. Sugar beet leaves (greenhouse cultures) were a gift from the Kleinwanzlebener Saatzucht AG, Einbeck/Hann.

[1-^{14}C]sodium glyoxylate was obtained from the Radiochemical Center, Amersham. Catalase was purchased from Boehringer, Mannheim.

Abbreviations: MV, methylviologen; Fd, ferredoxin; ORF, oxygen reducing factor; SOD, superoxide dismutase.

Results

In order to elucidate the products of photosynthetic oxygen reduction in the presence of either ORF or Fd, two simple test systems were used which allow the identification of $O_2^{\cdot-}$ and H_2O_2. Superoxide dismutase[15] inhibits reactions which include the function of $O_2^{\cdot-}$; catalase on the other hand inhibits reactions driven by H_2O_2. Our test systems were based on the aid of these two enzymes.

Photosynthetic hydroxylamine oxidation

Hydroxylamine, at concentrations of lower than 5×10^{-4} M, is only a weak inhibitor of photosystem II[16]. Nitrite is the stoichiometric product of the oxidation of hydroxylamine in the presence of 10^{-3} M KCN, as shown in Fig. 1.

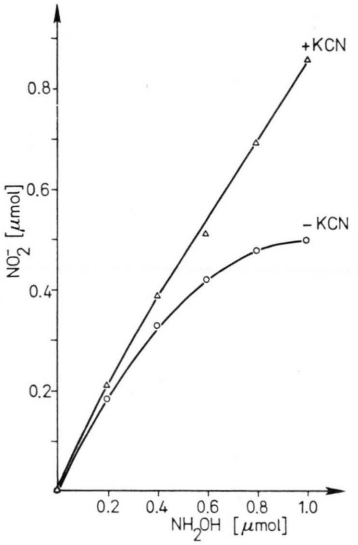

Fig. 1. Stoichiometry of photosynthetic nitrite formation from hydroxylamine. The reaction mixture contained in 3 ml: 80 μmol Tris buffer pH 8.0, 5 μmol NH_4Cl, 0.2 μmol MV, chloroplast lamellae from sugar beet leaves with 0.1 mg chlorophyll and hydroxylamine as indicated. The reaction was conducted in Warburg vessels in a photo-Warburg thermostate with illumination from the bottom (25 000 lx) at 15 °C for 15 min. △—△, NO_2^--formation in the presence of 10^{-3} M KCN; ○—○, NO_2^--formation in the absence of KCN.

The oxidation of hydroxylamine in the absence of KCN is inhibited to 50 per cent by 2 units[15] of SOD and to 90 per cent by 50 units of SOD. Addition of catalase (up to 140 units) has no influence on photosynthetic nitrite formation from hydroxylamine (Fig. 2). These results show that hydroxylamine is oxidized by the superoxide free

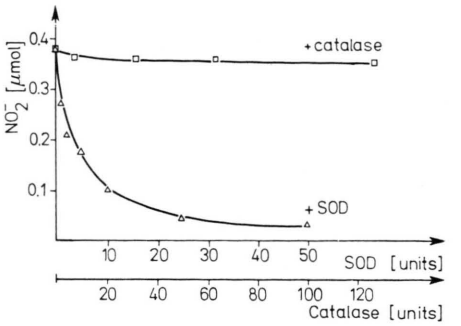

Fig. 2. Influence of SOD and catalase on photosynthetic hydroxylamine oxidation. Experimental conditions as described for Fig. 1 (with 1 μmol NH_2OH).

radical ion and not by H_2O_2 or by acting as an electron donor; it therefore provides a simple system for testing the production of $O_2^{\cdot-}$.

Decarboxylation of glyoxylate

Photosynthetic decarboxylation of glyoxylate or pyruvate in the presence of MV is inhibited to 50 per cent by 10 units of catalase and to more than 90 per cent by 140 units of catalase (Fig. 3).

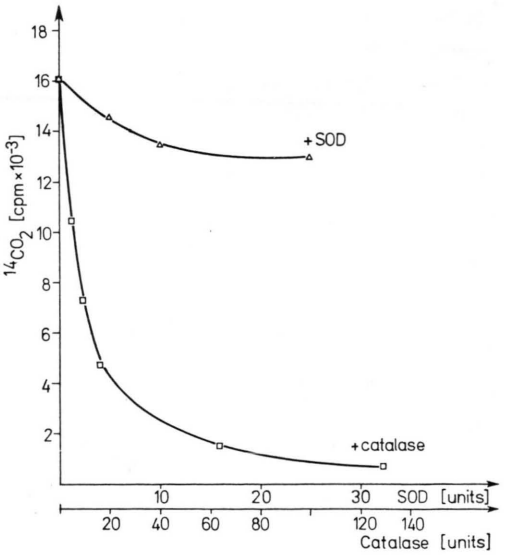

Fig. 3. Influence of SOD and catalase on photosynthetic decarboxylation of glyoxylate. The reaction mixture contained in 3 ml: 80 μmol Tris buffer pH 7.6, 5 μmol NH_4Cl, 0.2 μmol MV, chloroplast lamellae from sugar beet leaves with 0.1 mg chlorophyll and 3 μmol [1-^{14}C] sodium glyoxylate (0.2 Ci/mol)). The reaction was conducted as described for Fig. 1, $^{14}CO_2$ was determined as described in ref. 13.

Addition of 25 units of SOD, which inhibits hydroxylamine oxidation to at least 80 per cent show

less than 20 per cent inhibition of the decarboxylation. This is taken as proof that the decarboxylation of α-keto acids is mainly driven by H_2O_2 and not by $O_2^{\cdot-}$ (*cf.* ref 13).

Oxygen reduction in the presence of ferredoxin

As already described, by means of the decarboxylation of α-keto acids [13] photosynthetic oxygen reduction in the presence of Fd and NADP is observed as soon as all the available NADP is reduced. Fig. 4 shows that this oxygen reduction also yields

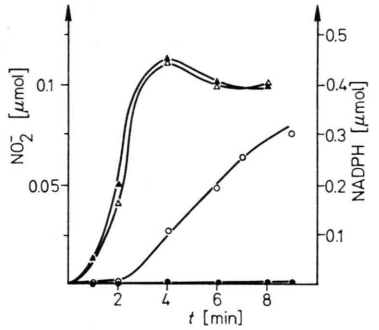

Fig. 4. Comparism of photosynthetic NADP-reduction with hydroxylamine oxidation in the presence and absence of SOD. The reaction mixture contained in 3 ml: 80 µmol Tris buffer pH 8.0, 5 µmol NH_4Cl, 10 nmol Fd, 0.7 µmol NH_2OH, 1 µmol NADP and chloroplast lamellae from sugar beet leaves with 0.1 mg chlorophyll; experimental conditions as described for Fig. 1, reaction time as indicated. △—△, NADP reduction; ▲—▲, NADP reduction in the presence of 25 units SOD; ○—○, hydroxylamine oxidation, ●—●, hydroxylamine oxidation in the presence of 25 units SOD.

the production of the superoxide free radical ion, measured as nitrite formation from hydroxylamine.

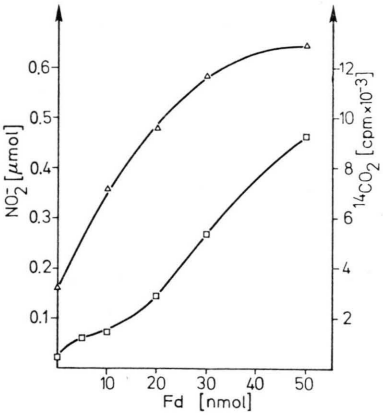

Fig. 5. Comparism of photosynthetic hydroxylamine oxidation and photosynthetic decarboxylation of glyoxylate in the presence of ferredoxin (Fd). Experimental conditions as described for Fig.s 1 and 3, with the indicated amounts of Fd instead of MV. △—△, hydroxylamine oxidation; □—□, decarboxylation of glyoxylate.

Fig. 5 shows that depending on the amount of Fd added, both decarboxylation of glyoxylate and hydroxylamine oxidation are stimulated by ferredoxin. This is taken as proof that both H_2O_2 and $O_2^{\cdot-}$ are products of oxygen reduction in the presence of Fd.

While the stimulation of hydroxylamine oxidation by Fd shows saturation-type kinetics, the decarboxylation of glyoxylate shows a shoulder at approx. 10 nmol of Fd, followed by a new increase of peroxide formation at higher concentrations of Fd.

Oxygen reduction in the presence of ORF

Addition of solubilized ORF to illuminated chloroplast lamellae shows an increase of both oxygen uptake and decarboxylation of glyoxylate upon increasing amounts of ORF [7, 8]. A comparism of glyoxylate decarboxylation with hydroxylamine oxidation shows, however, that ORF only stimulates the decarboxylation of glyoxylate and not the nitrite formation from hydroxylamine, indicating that H_2O_2 but not $O_2^{\cdot-}$ is the product of oxygen reduction in the presence of ORF (Fig. 6). ORF

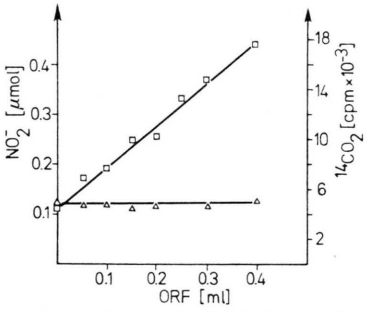

Fig. 6. Comparism of photosynthetic hydroxylamine oxidation and photosynthetic decarboxylation of glyoxylate in the presence of ORF. Experimental conditions as described for Figs 1 and 3, with the indicated amounts of ORF (isolated from spinach chloroplast lamellae) instead of MV. △—△, hydroxylamine oxidation; □—□, decarboxylation of glyoxylate.

therefor seems to reduce oxygen by a different mechanism compared to Fd or low potential dyes (like MV), both of which reduce oxygen monovalently (Fig. 7).

Identical effects are observed with chloroplasts or ORF from spinach and from sugar beet leaves, although there seems to be more than one compound in sugar beet leaves which exhibit ORF-activity and which are not bound to the chloroplast lamellae (unpublished results).

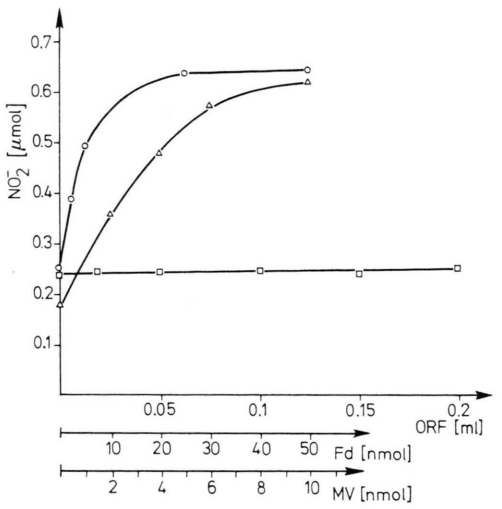

Fig. 7. Comparism of the influence of MV, Fd and ORF on photosynthetic hydroxylamine oxidation. Experimental conditions as described for Fig. 1. ○—○, hydroxylamine oxidation with MV; △—△, hydroxylamine oxidation with Fd; □—□, hydroxylamine oxidation with ORF.

Discussion

Stoichiometric formation of nitrite from hydroxylamine by illuminated chloroplast lamellae can be measured if the hydroxylamine concentration is kept below 5×10^{-4} M and if 10^{-3} M KCN is present. The fact that the formation of nitrite from hydroxylamine can be inhibited by SOD to more than 90 per cent suggests that hydroxylamine oxidation by illuminated chloroplast lamellae involves only the superoxide free radical ion and not H_2O_2 as oxidant and that hydroxylamine does not serve as an electron donor for photosystem II as postulated by several authors [17–19]. The stimulation of photosynthetic hydroxylamine oxidation by KCN is due to the inhibition by KCN of a cyanide-sensitive SOD which is tightly bound to the chloroplast lamellae [20].

The increase of oxygen uptake by addition of hydroxylamine to illuminated chloroplast lamellae which is observed in the presence of autooxidizable electron acceptors may be due to avoiding the dismutation of $O_2^{\cdot-}$ as outlined by Eqn (2) and as already postulated for ascorbate photooxidation [21–25, 5].

$$NH_2OH + 2\,O_2^{\cdot-} + H^+ \rightarrow NO_2^- + H_2O_2 + H_2O \quad (2)$$

Hydroxylamine, as the first product of oxidation of ammonia in the process of nitrification by *Nitrosomonas spec.*, is further oxidized by the copper-containing enzyme hydroxylamine oxidase. A possible role of peroxonitrite as a species of intermediates involved in hydroxylamine oxidation was discussed [26]. Whether the superoxide free radical ion is also involved in the enzymatic conversion of hydroxylamine to nitrite by the enzyme hydroxylamine oxidase from *Nitrosomonas spec.* during the process of nitrification is a matter for further investigations.

Nitrite formation from hydroxylamine and decarboxylation of α-keto acids presents a simple and specific system for testing the formation of either the $O_2^{\cdot-}$ or H_2O_2 during photosynthetic oxygen reduction. This method has been used for the determination of the products of photosynthetic oxygen reduction in the presence of either Fd (which includes the function of bound ORF [7, 8]), solubilized ORF or MV as electron acceptors. It has already been demonstrated that all three compounds can act as cofactors for the photosynthetic decarboxylation of glyoxylate [7]. This reaction depends on the presence of H_2O_2 as oxidant (*cf.* Fig. 3 and ref. 13). In the case of ORF it remained unclear, whether the production of H_2O_2 was due to the dismutation of $O_2^{\cdot-}$, as the first product of oxygen reduction or whether H_2O_2 itself was the first product of oxygen reduction.

The stimulation of decarboxylation of glyoxylate after addition of Fd may partly be due to the dismutation of $O_2^{\cdot-}$ yielding H_2O_2 (Eqn (1)), since $O_2^{\cdot-}$ is formed by Fd in the presence of illuminated chloroplast lamellae.

In an earlier paper we showed, however, that with Fd and chloroplast lamellae after a 15 min illumination about 70 per cent of the H_2O_2 formed was due to the function of ORF (ascorbate-sensitive rate) and only about 30 per cent of the H_2O_2 may have been formed by autooxidation of reduced Fd [27, 28] or the primary acceptor of photosystem I.

Solubilized ORF seems to function in the same way as in its bound form, except that the bound form seems to be dependent on the presence of ferredoxin [7, 8]. As shown in Figs 6 and 7, solubilized ORF does not stimulate the oxidation of hydroxylamine but does stimulate the decarboxylation of glyoxylate. This result is in agreement with the proposed chemical mechanism of ORF-function [8], which results in the formation of H_2O_2. $O_2^{\cdot-}$ is necessary for maintaining a chainreaction involving

both bound ORF and Fd or solubilized ORF and the reducing site of photosystem I.

The results presented may also explain why ethylene formation by illuminated chloroplast lamellae [29] from methional is strongly stimulated by the addition of Fd and to a lesser extent by low potential dyes [30]. Ethylene formation from methional is dependent on the presence of both H_2O_2 and $O_2^{\cdot -}$ [31]. Although the dismutation of $O_2^{\cdot -}$ provides H_2O_2, an addition of extra H_2O_2 has been shown to stimulate ethylene formation from methional with xanthin and xanthinoxidase as the oxygen reducing system [31]. By cooperation of Fd (producing $O_2^{\cdot -}$ [32]) and ORF (producing H_2O_2) in photosynthetic oxygen reduction, $O_2^{\cdot -}$ and H_2O_2 seem to be independently produced at a ratio which seems to be necessary for a rapid production of OH-radicals [33] as the active species in ethylene production.

We wish to thank the Kleinwanzlebener Saatzucht AG (Einbeck/Hann.) for financial support.

[1] U. Heber, Biochim. Biophys. Acta **180**, 302 [1969].
[2] U. Heber, Biochim. Biophys. Acta **305**, 140 [1973].
[3] W. A. Jackson and R. J. Volk, Ann. Rev. Plant Physiol. **21**, 385 [1970].
[4] K. Asada and K. Kiso, Agr. Biol. Chem. (Tokyo) **37**, 453 [1973].
[5] E. F. Elstner and R. Kramer, Biochim. Biophys. Acta **314**, 340 [1973].
[6] K. Asada and K. Kiso, J. Biol. Chem. **249**, 2175 [1974].
[7] E. F. Elstner and A. Heupel, Z. Naturforsch. **29 c**, 564 [1974].
[8] E. F. Elstner and A. Heupel, Z. Naturforsch. **29 c**, 559 [1974].
[9] K. Tagawa and D. I. Arnon, Nature **195**, 537 [1962].
[10] J. Sawada, T. Ohyama, and J. Yamazaki, Biochim. Biophys. Acta **268**, 305 [1972].
[11] N. Nelson, Z. Drechsler, and J. Neumann, J. Biol. Chem. **245**, 143 [1970].
[12] M. Burba and E. F. Elstner, Z. Zuckerind. **23**, 609 [1973].
[13] E. F. Elstner and A. Heupel, Biochim. Biophys. Acta **325**, 182 [1973].
[14] E. F, Elstner, A. Heupel, and S. Vaklinova, Z. Pflanzenphys. **62**, 184 [1970].
[15] J.. M. McCord and J. Fridovich, J. Biol. Chem. **244**, 6049 [1969].
[16] E. F. Elstner, A. Heupel, and S. Vaklinova, Z. Pflanzenphys. **62**, 173 [1970].
[17] S. Izawa, R. L. Heath, and G. Hind, Biochim. Biophys. Acta **180**, 338 [1969].
[18] R. L. Heath and G. Hind, Biochim. Biophys. Acta **189**, 222 [1969].
[19] S. Katoh, I. Ikegami, and A. Takamija, Arch. Biochem. Biophys. **141**, 207 [1970].
[20] E. F. Elstner and A. Heupel, manuscript in preparation.
[21] B. L. Epel and J. Neumann, VI. Intl. Congr. Photobiol. Bochum, Germany. Abstr. No. 237, 1972.
[22] B. L. Epel and J. Neumann, Biochim. Biophys. Acta **325**, 520 [1973].
[23] J. F. Allen and D. O. Hall, Biochem. Biophys. Res. Commun. **52**, 856 [1973].
[24] J. F. Allen and D. O. Hall, Biochem. Biophys. Res. Commun. **58**, 579 [1974].
[25] D. R. Ort and S. Izawa, Plant Physiol. **53**, 370 [1974].
[26] M. N. Hughes and H. G. Nicklin, Biochim. Biophys. Acta **222**, 660 [1970].
[27] H. Bothe, Z. Naturforsch. **24 b**, 1574 [1969].
[28] A. Telfer, R. Cammack, and H. C. W. Evans, FEBS Lett. **10**, 21 [1970].
[29] E. F. Elstner and J. R. Konze, FEBS Lett. **45**, 18 [1974].
[30] E. F. Elstner and J. R. Konze, Z. Naturforsch. **29 c**, 710 [1974].
[31] C. Beauchamp and I. Fridovich, J. Biol. Chem. **245**, 4641 [1970].
[32] H. P. Misra and I. Fridovich, J. Biol. Chem. **246**, 6886 [1971].
[33] F. Haber and I. Weiss, Proc. Roy. Soc., Ser. A, **147**, 322 [1934].