Involvement of the Superoxide Free Radical Ion in Photosynthetic Oxygen Reduction

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Photosynthetic oxygen reduction by isolated chloroplast lamellar systems has been studied with the aid of superoxide dismutase. Two mechanisms of oxygen reduction by illuminated chloroplast lamellar systems can be differentiated:

1. In the presence of low potential electron acceptors like AQ or MV the superoxide free radical ion is the product of autooxidation of the reduced acceptor. Addition of superoxide dismutase has no influence on the initial rates of oxygen reduction.
2. Stimulation of photosynthetic oxygen reduction by *o*-diphenols is only observed in the absence of superoxide dismutase and ascorbate; apparently the superoxide free radical ions is involved in both initiation and propagation of a chain reaction.

If the ferredoxin-stimulated photosynthetic oxygen reduction is measured, both ascorbate and superoxide dismutase are active as inhibitors. By heating suspensions of chloroplast lamellar systems, a substance is released into the supernatant which exhibits the activity of an oxygen reducing factor (ORF) with properties similar to *o*-diphenols: The stimulation of photosynthetic oxygen reduction is reversed by addition of either SDM or ascorbate. A reaction sequence for photosynthetic oxygen reduction in the presence of ferredoxin is considered, which is initiated by the superoxide free radical ion produced by autooxidation of reduced ferredoxin; the superoxide free radical ion "activates" an endogenous oxygen reducing factor, which in this "active" state can reduce oxygen to $O_2^{\cdot -}$. The presence of either superoxide dismutase or ascorbate yields in chain termination by scavenging the superoxide free radical ion.

Introduction

Stimulation by light of oxygen uptake can be observed in intact leaves as well as with isolated chloroplasts [1, 2]. A possible role of photosynthetic oxygen reduction for energy conservation in the chloroplasts has been pointed out [3].

Photosynthetic oxygen reduction by isolated chloroplast lamellar systems primarily yields the superoxide free radical ion and finally H_2O_2 by dismutation of $O_2^{\cdot -}$ [4–7].

Several classes of compounds, including low potential dyes [8], certain quinones [9], ferredoxin [10] and other natural factors isolated from green plant material [11] have been shown to stimulate oxygen uptake in the presence of illuminated chloroplast lamellar systems.

We recently reported on the possible involvement of a bound factor in photosynthetic oxygen reduction, which is released from chloroplast lamellar systems upon heat-treatment [12]. Stimulation of photosynthetic oxygen reduction by addition of the solubilized oxygen reducing factor (ORF) to illuminated chloroplast lamellar systems is reversed in the presence of ascorbate. In contrast to this observation, oxygen uptake in the presence of low potential electron acceptors is stimulated by ascorbate [9, 5–7]. The different influence of ascorbate on photosynthetic oxygen reduction in the presence of either low potential acceptors (AQ, MV) or the "natural" cofactor ORF implies different chemical mechanisms.

In connection with the above findings earlier results of Trebst *et al.* [9] may be of special importance. They observed that oxygen uptake and H_2O_2-formation by isolated chloroplast lamellar systems in the presence of AQ are stimulated by ascorbate; if *o*-diphenols are used instead of AQ, however, ascorbate acts as an inhibitor of oxygen reduction. We reinvestigated these observations with the aid of superoxide dismutase and compared several natural cofactors of photosynthetic oxygen reduction with certain *o*-diphenols.

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Abbreviations: AQ, anthraquinone-2-sulfonic-acid; MV, methylviologen; SDM, superoxide dismutase; ORF, oxygen reducing factor.

Material and Methods

Ferredoxin was isolated from spinach leaves as described by Tagawa and Arnon [13]. Chloroplast lamellar systems were prepared from spinach leaves [14] and superoxide dismutase from dried green peas [15, 5]. Decarboxylation of [1-^{14}C]glyoxylate was measured as recently described [16].

Supernatants exhibiting an oxygen reducing activity in the presence of illuminated chloroplast lamellar systems [12] were prepared by heating suspensions of chloroplast lamellar systems (containing 4 mg chlorophyll/ml) in resuspending medium [14] and centrifugation (20 000 × g/15 min).

Oxygen uptake was measured in a Gilson oxygraph. The reaction mixture (maintained at 20 °C) was illuminated by a Leitz slide projector with a 400 W tungsten lamp (80 000 lx) through a heat filter and a "Schott" far red filter cutting off light below 658 nm.

Results

Isolated chloroplast lamellar systems (in the absence of ferredoxin and NADP) show a slow rate of oxygen uptake (Fig. 1, trace a). If an autooxidizable electron acceptor like AQ is present a fast initial rate of oxygen uptake is measured (Fig. 1, trace b). Addition of 100 units [17] SDM has no influence on the initial rate of oxygen uptake in the presence of AQ. Ascorbate accelerates the initial rate of oxygen uptake even in the presence of SDM.

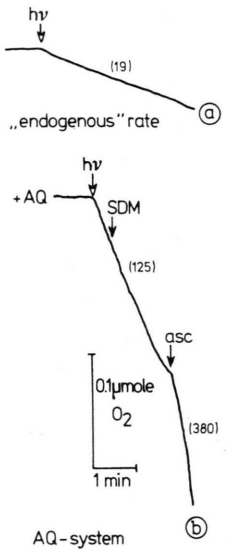

Fig. 1. Effect of SDM and ascorbate on photosynthetic oxygen reduction by isolated chloroplast lamellar systems in the presence of AQ. The reaction mixture contained in 1.4 ml: 40 μmol Tris buffer pH 8.0, 2.5 μmol NH_4Cl, chloroplast lamellar systems with 0.05 mg chlorophyll; 100 units of SDM, 0.1 μmol AQ and 5 μmol ascorbate were added as indicated. The traces represent O_2-uptake, measured in the oxygen electrode; the numbers in brackets represent rates of O_2-uptake in μmol/mg chlorophyll/hour.

If dopamin is added to illuminated chloroplast lamellar systems, oxygen uptake is stimulated after a short lag-phase of approximately 0.3 min (Fig. 2).

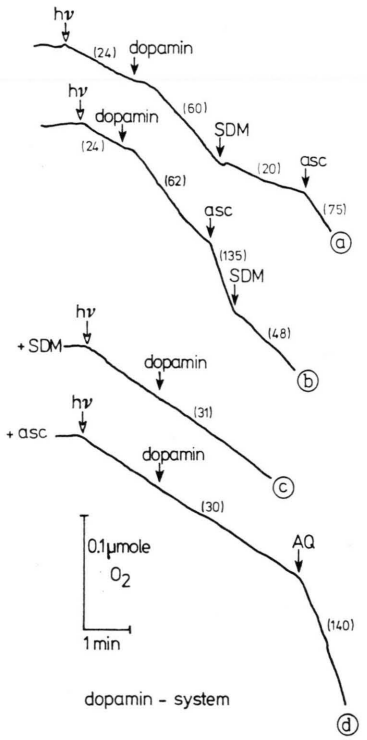

Fig. 2. Effect of dopamin, SDM and ascorbate on photosynthetic oxygen reduction by isolated chloroplast lamellar systems. For experimental conditions see Fig. 1; 0.15 μmol dopamin were added as indicated.

Addition of 100 units SDM reverses the stimulation by dopamin, ascorbate yields in a new stimulation (trace a). If ascorbate is added prior to SDM, first a stimulation and afterwards an inhibition of oxygen uptake is observed (trace b).

Dopamin has no effect on oxygen uptake by illuminated chloroplast lamellar systems if SDM is present before the light is turned on (Fig. 2, trace c). If ascorbate is present before illumination, neither dopamin nor SDM show an effect on the rate of oxygen uptake only addition of AQ yields in a stimulation (trace d).

Fig. 3 shows the rates of oxygen uptake in the presence of an "oxygen reducing factor" (ORF) isolated by heat treatment of chloroplast lamellar systems. As shown by trace a and b both addition of SDM or ascorbate inhibit the rate of oxygen uptake. If ascorbate is added in the presence of SDM the rate of oxygen uptake is trebled; addition of AQ causes a further stimulation (trace c).

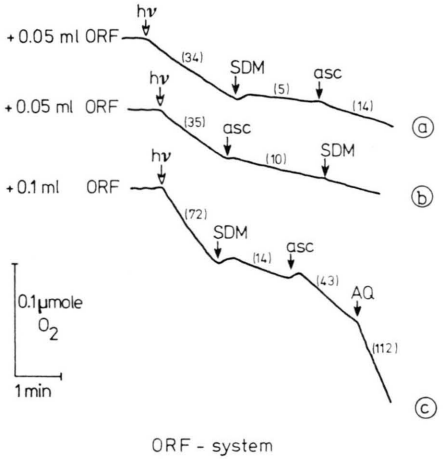

Fig. 3. Effect of ORF, SDM and ascorbate on photosynthetic oxygen reduction by isolated chloroplast lamellar systems. For experimental conditions see Fig. 1.

If ferredoxin is reduced by NADPH *via* NADP-feredoxin reductase in the dark, oxygen reduction by autooxidation of reduced ferredoxin is not inhibited by ascorbate [12, 7]. If however oxygen reduction or H_2O_2-production by illuminated chloroplast lamellar systems in the presence of ferredoxin [10, 18] is observed both SDM and ascorbate are inhibitory (Fig. 4). Since both ascorbate and SDM act as scavengers for $O_2^{\cdot -}$, one can draw the conclusion that oxygen reduction by illuminated chloroplasts

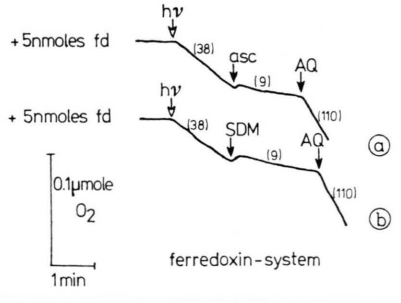

Fig. 4. Effect of ferredoxin (fd), SDM and ascorbate on photosynthetic oxygen reduction by isolated chloroplast lamellar systems. For experimental conditions see Fig. 1; 5 nmol fd were added to the reaction mixture.

lamellar systems in the presence of either ferredoxin, ORF or dopamin is dependent on the presence of the superoxide free radical ion in contrast to low potential electron acceptors like AQ.

In order to further investigate the characteristics of the oxygen reducing factor (ORF) isolated from either spinach or sugar beet leaves [12] several phenolic compounds and flavonoids have been tested for their activity to stimulate the decarboxylation of glyoxylate as a probe for peroxide formation [16]. As shown in the Table several natural factors like

Table I. Comparison of the effects of several "natural" factors, phenolic compounds and flavonoids on the decarboxylation of glyoxylate by isolated chloroplast lamellar systems. The reaction mixture contained in 3 ml: Chloroplast lamellar systems with 0.2 mg chlorophyll, 80 µmol Tris buffer pH 7.6; 3 µmol sodium [1-^{14}C] glyoxylate (0.07 Ci/mol), 5 µmol NH_4Cl, 1 µmol sodium azide and 10 µmol sodium ascorbate, where indicated.

Additions	[µmol glyoxylate decarboxylated per mg chlorophyll/h]	
	−Ascorbate	+Ascorbate
none	1.6	0.3
natural compounds:		
"CRS" (spinach), 0.5 ml	4.2	1.4
flavonoid (sugar beet leaves) 0.05 ml	3.0	0.7
ORF 0.1 ml (spinach chloraplasts)	3.5	0.2
phenolic compounds: (3×10^{-5} M)		
p-coumaric acid	1.7	0.1
benzoic acid	1.9	0.1
chlorogenic acid	4.3	0.0
dopamin	14.5	0.1
epinephrine	15.5	0.1
flavonoids: (3×10^{-5} M)		
apigenine	1.5	0.1
kaempferole	1.8	0.0
rutine	2.5	0.0
quercitrine	2.5	0.0

CRS [19], a flavonoid isolated from sugar beet leaves (Oettmeier, Elstner, and Heupel, unpublished results) and ORF show similar characteristics as certain phenolic compounds or flavonoids with an *o*-dihydroxy-configuration. Compounds which do not have an *o*-dihydroxy configuration (*p*-coumaric acid, benzoic acid, apigenine, kaempferole) show little or no effect on the decarboxylation of glyoxylate.

Stimulation of photosynthetic oxygen reduction by the above compounds is essentially abolished in the presence of ascorbate. It can be assumed that

ascorbate like SDM competes with these *o*-diphenols for the superoxide free radical ion. Since we also assume that bound ORF is the main oxygen reductant in chloroplast lamellar systems in the presence of ferredoxin [12] the influence of increasing amounts of ascorbate on this system was tested. As shown in Fig. 5, three ranges of inhibition can

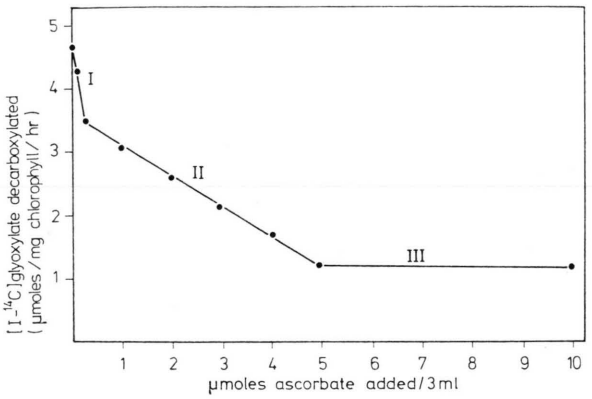

Fig. 5. Photosynthetic decarboxylation of [1-^{14}C] glyoxylate in the presence of ferredoxin, NADP and increasing amounts of ascorbate. For experimental conditions see "Methods". The reaction mixture contained in 3 ml: 80 μmol Tris buffer pH 7.6, 5 μmol NH_4Cl, 1 μmol sodium azide, 3 μmol [1-^{14}C] sodium glyoxylate (0.07 Ci/mol), 10 nmol ferredoxin, 1 μmol NADP and chloroplast lamellar systems with 0.1 mg chlorophyll.

be observed: Range I, -inhibition up to a concentration of about 1.5×10^{-4} M, followed by range II, up to about 2×10^{-3} M; higher concentrations of ascorbate than 2×10^{-3} M yield in no further increase of inhibition. This result can be interpreted as the competition of ascorbate for the superoxide free radical ion with another (perhaps other) compound(s), which is (are) responsible for oxygen reduction by chloroplast lamellar systems. While range I is not fully understood, range II seems to reflect the exclusion by ascorbate of ORF as oxygen reductant, range III seems to represent the slow rate of autooxidation of reduced ferredoxin, which is not influenced by ascorbate [12].

Discussion

Ferredoxin-dependent NADP- as well as cytochrome c-reduction cannot be inhibited by SDM [5, 20] but ferredoxin-stimulated oxygen reduction is inhibited by SDM as well as by ascorbate (Fig. 4). Neither the reducing side of photosystem I nor ferredoxin itself or NADP-ferredoxin reductase are identical with the site of inhibition by ascorbate [12]; at heat-stabile factor has been extracted from chloroplast lamellar systems however which shows the charatersitics of a SDM- and ascorbate-sensitive oxygen reductant in the presence of illuminated chloroplast lamellar systems (Fig. 3) [12]. In order to investigate the nature and the chemical mechanism of function of this oxygen reducing factor model compounds have been used to study the mechanism of photosynthetic oxygen reduction.

It is well established that oxygen reduction by reduced low potential electron acceptors is not inhibited by either ascorbate or SDM (Fig. 1) [5–7].

Stimulation of photosynthetic oxygen reduction by several *o*-diphenols however is reversed as well in the presence of ascorbate [9] as by SDM (Fig. 2, Table). If ascorbate or SDM are present in the reaction mixture before illumination no effect upon addition of dopamin on photosynthetic oxygen reduction is observed (Fig. 2). If dopamin is added to illuminated chloroplast lamellar systems in the absence of SDM or ascorbate however a stimulation of oxygen reduction is visible which can be inhibited by addition of SDM; subsequent addition of ascorbate accelerates the rate of oxygen uptake (Fig. 2, trace a+b). Apparently dopamin has to be converted into an "active form" by the superoxide free radical ion, which is formed by illumination of chloroplast lamellar systems only in the absence of SDM or ascorbate. As soon as enough of this "active compound" is present (*cf.* the lag phase in Fig. 2), the reaction seems to be self-propagating, and the influence of either SDM or ascorbate is reflected as a competition for the $O_2^{\cdot -}$, according to

$$\text{I,} \quad O_2^{\cdot -} + O_2^{\cdot -} + 2\,H^+ \xrightarrow{\text{SDM}} H_2O_2 + O_2$$

(decrease of O_2 uptake)

$$\text{II,} \quad 2\,O_2^{\cdot -} + 2\,Asc_{red} + 2\,H^+ \rightarrow 2\,H_2O_2 + 2\,Asc_{ox}$$

(increase of oxygen uptake by avoiding the dismutation).

According to Misra and Fridovich [21], and Heikkila and Cohen [22] the active form of dopamin is most likely the semiquinone radical, which is formed from the *o*-diphenole (QH_2) by reaction with $O_2^{\cdot -}$.

$$\text{III,} \quad QH_2 + O_2^{\cdot -} + H^+ \rightarrow QH^{\cdot} + H_2O_2\,.$$

The semiquinone radical in turn produces a new $O_2^{\cdot -}$ by autooxidation, maintaining a chainreaction. This mechanism is in agreement with recent findings of Augusto *et al.* [23], who reported that microsomal oxidation of NADPH is stimulated by epinephrine. This stimulation is suppressed by SDM, however,

if this enzyme is added before the reaction is started.

On the other hand, radiation induced oxidation of NADH in the presence of lactate dehydrogenase has been shown to proceed by a chain mechanism which is initiated by $O_2^{\cdot -}$ and propagated by oxygen [24]. The observation, that this chain reaction can be inhibited by ascorbate is also in good agreement with our results.

The results obtained with solubilized ORF (Fig. 3) are very similar to those obtained with dopamin. The fact, that the ferredoxin-stimulated photosynthetic oxygen reduction is also inhibited in the presence of either SDM or ascorbate is good evidence, that the same or a similar mechanism as in the case of solubilized ORF or dopamin is also operating in the ferredoxin-system. The function of both ferredoxin and bound ORF in photosynthetic oxygen reduction as previously postulated [12] may be expressed by the following equations:

Autooxidation of reduced ferredoxin (or of the primary electron acceptor) yields in the formation of the superoxide free radical ion which in turn is activating the bound oxygen reducing factor (ORF):

a. $Fd_{red} + O_2 \xrightarrow{\text{slow}^{12}} Fd_{ox} + O_2^{\cdot -}$,

b. $(ORF_{red}) + O_2^{\cdot -} + H^+ \rightarrow (ORF)^{\cdot} + H_2O_2$.

The activated ORF (radical?) is autooxidizable and reduces oxygen to the superoxide free radical ion:

c. $(ORF^{\cdot}) + O_2 \rightarrow O_2^{\cdot -} + (ORF_{ox}) + H^+$.

The oxidized bound ORF (ORF_{ox}) is finally reduced by reduced ferredoxin:

d. $2\,Fd_{red} + (ORF_{ox}) \rightarrow 2\,Fd_{ox} + (ORF_{red})$.

The reduced bound ORF (ORF_{red}) is oxidized again by the superoxide free radical ion, which is produced by reaction c, propagating a chain reaction. The stimulation by ferredoxin of photosynthetic oxygen reduction may be attributed to the fact, that bound ORF_{ox} (in contrast to solubilized ORF) cannot be directly reduced by photosystem I *e. g.* the primary acceptor. If solubilized ORF is added as the cofactor, oxygen reduction is no longer dependent on the presence of ferredoxin. A similar result was reported by Asada and Kiso [25], who showed that SDM-sensitive, photosynthetic oxidation of epinephrine was not stimulated by addition of ferredoxin, suggesting that another reduced cofactor of the electron transport chain is active in the formation of the superoxide radicals.

Several natural compounds, including a flavonoid isolated from sugar beet leaves and CRS (possibly an iron complex of *p*-coumaryl-mesotartaric acid) isolated from spinach chloroplast lamellar systems [26] show the activity to stimulate photosynthetic oxygen reduction. Although the structure of ORF is not yet known, the chemical mechanism would be in good agreement with an *o*-diphenol-derivative.

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