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Studies on the Energy-coupling Sites of Photophosphorylation

V. PHOSPHORYLATION EFFICIENCIES (P/e_2) ASSOCIATED WITH AEROBIC PHOTOOXIDATION OF ARTIFICIAL ELECTRON DONORS[1]

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ABSTRACT

The rate of Hill reaction can be measured accurately as O_2 uptake (the Mehler reaction) if a rapidly autoxidizable electron acceptor (e.g., methylviologen) is used. However, when an artificial electron donor-ascorbate couple (or ascorbate alone) replaces the natural donor, water, the rate of O_2 consumption is no longer a reliable measure of the electron flux, because superoxide radical reactions contribute to O_2 uptake. Such radical reactions, however, can be suppressed by adding enough superoxide dismutase to the reaction mixture. Indeed in all of the photosystem I- and photosystem II-donor reactions tested (except with benzidine which was tested without ascorbate added), the O_2 uptake was inhibited by 30 to 50% by the addition of superoxide dismutase. The rate of phosphorylation was totally unaffected by the enzyme. The reassessment of the phosphorylation efficiencies thus made by the use of superoxide dismutase led us to the following conclusions. The phosphorylation efficiency associated with the transfer of electrons from a donor to methlylviologen (than to O_2) through both photosystems II and I is practically independent of the donor used—catechol, benzidine, p-aminophenol, dicyanohydroquinone, or water. The P/e_2 ratio is 1.0 ± 0.1. Only ascorbate gives a slightly lower value ($P/e_2 = 0.9$). (NH_2OH-treated, non-water-splitting chloroplasts were used for reactions with these artificial donors.) The phosphorylation efficiency associated with DCMU-insensitive, photosystem I-mediated transfer of electrons from a donor to methylviologen (then to O_2) is again largely independent of the donor used, such as diaminodurene, diaminotoluene, and reduced 2,6-dichlorphenolindophenol. The P/e_2 ratio is 0.6 ± 0.08.

Artificial, low potential electron acceptors, represented by viologens and anthraquinone, have been used extensively for the study of chloroplast electron transport and photophosphorylation involving PS I[2]. These compounds have advantage over the reconstituted "natural" electron acceptor system ferredoxin-NADP in several respects. They are much less likely to contribute an undesirable rate-limiting step even in very fast PS I reactions and therefore are less likely to permit simultaneous cyclic electron flow around PS I. Also, they are much less susceptible to inactivation by various reagents and conditions and are much more economical to use. The reduction of these acceptors can be followed most easily as the O_2 uptake which results from the autoxidation of the reduced acceptors (12). The widely used formulae (e.g., 29) for the transfer of electrons from a donor (AH_2) to an acceptor (e.g., MV) and the subsequent aerobic reoxidation of the reduced form of the acceptor leading to the formation of H_2O_2 may be rewritten as follows, including a new term for the intermediate of H_2O_2 formation, the superoxide radical O_2^-(21).

$$AH_2 + 2MV^{2+} \xrightarrow[\text{light}]{\text{chloroplasts}} A + 2H^+ + 2\cdot MV^+ \quad (1)$$

$$2\cdot MV^+ + 2O_2 \xrightarrow{\text{spontaneous}} 2MV^{2+} + 2\cdot O_2^- \quad (2)$$

$$2\cdot O_2^- + 2H^+ \xrightarrow{\text{spontaneous}} O_2 + H_2O_2 \quad (3)$$

$$AH_2 + O_2 \xrightarrow[\text{MV, light}]{2e^- \text{ transport}} A + H_2O_2 \ (O_2 \equiv 2e^-) \quad (4)$$

In the normal Hill reaction where $AH_2 = H_2O$ (*i.e.*, $A = \frac{1}{2}O_2$), the over-all reaction becomes what is known as the Mehler reaction.

$$H_2O + \tfrac{1}{2}O_2 \xrightarrow[\text{MV, light}]{2e^- \text{ transport}} H_2O_2 \ (O \equiv 2e^-) \quad (5)$$

To date, all the computations of electron fluxes from O_2 uptake data have been made on the basis of the above two overall formulae (equations 4, 5).

The basic validity of the mechanism for the Mehler reaction was established by mass spectroscopic studies of the O_2 exchange reactions involved (8). The validity of the formulations for artificial electron donor systems, however, has never been rigorously proven. In fact, the now rapidly increasing knowledge of the role of $\cdot O_2^-$ in various oxidation reactions makes it more and more difficult to believe that the events involved in the aerobic photooxidation of artificial reductants are always as straightforward as represented by equations 1 to 4 (11). For instance, if any significant portion of the $\cdot O_2^-$ produced by aerobic reoxidation of $\cdot MV^+$ (equation 2) directly reacts with the artificial donor AH_2, then the same rate of electron transport through the photosynthetic chain would induce a faster rate of O_2 uptake than predicted by equation 4, and the relation $O_2 \equiv 2e^-$ would no longer be valid. This is because the $\cdot O_2^-$, which normally dismutates and regenerates half of the consumed O_2 (equation 3) is now simply reduced to H_2O_2:

$$AH_2 + 2\cdot O_2^- \longrightarrow A + 2HOO^- \quad (6)$$

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[2] Abbreviations: PS I and PS II: photosystems I and II; DAD: diaminodurene; $DCIPH_2$: reduced 2,6-dichlorophenolindophenol; DAT: diaminotoluene; MV: methylviologen; P/e_2: the ratio of the number of ATP molecules formed to number of pairs of electrons transported; SOD: superoxide dismutase.

However, such reactions and the resulting enhancement of O_2 uptake should be abolished when the dismutation of $\cdot O_2^-$ is greatly accelerated by added SOD (20). Thus, with sufficient SOD, the aerobic photooxidation of donors should follow exactly equations 1 to 4, and the relation $O_2 \equiv 2e^-$ should hold. This method of detecting or suppressing superoxide radical reactions has already been in wide use. For instance, Asada and Kiso (2, 3) have successfully adopted this method to detect the involvement of $\cdot O_2^-$ in the photooxidation of epinephrine and sulfite by isolated chloroplasts.

These considerations prompted us to re-examine a number of chloroplast reactions involving the light-dependent transfer of electrons from artificial donors to MV and then to O_2. The immediate objective was to assess, for each reaction system, exactly what portion of the O_2 uptake observed should be attributed to the true photosynthetic electron transport described by equations 1 to 4, and what portion to the chemical oxidation of donors by $\cdot O_2^-$. As indicated above, this can be achieved by observing the effect of excess SOD added to the reaction mixture which should abolish only the latter portion of O_2 consumption. While the importance of such fundamental data seemed obvious in view of the extensive use of these aerobic reaction systems by various workers, our primary concern was to establish the true efficiencies (P/e_2 ratios) of the phosphorylation reactions associated with the donor reactions, particularly those involving PS II. The experiments have not only provided clear answers to the question of the true electron fluxes and true P/e_2 ratios but have also disclosed an important quantitative relationship, in terms of the phosphorylation efficiency, between the reactions involving only PS I and those involving both PS I and PS II.

MATERIALS AND METHODS

Chloroplast Isolation and NH_2OH Treatment. Chloroplasts were isolated from commercial spinach (*Spinacia oleracea* L.) as described in an earlier paper (26). The hydroxylamine treatment was done in the dark at room temperature (21 C) as outlined below (for original procedure, see Ref. 26). Chloroplasts, at a final Chl concentration of approximately 100 μg/ml, were suspended in the following medium: 0.2 M sucrose, 5 mM HEPES-NaOH buffer (pH 7.5), 2 mM $MgCl_2$, 1 mM EDTA, and 3 mM NH_2OH. A stock solution of NH_2OH (0.1 M) was made by dissolving the hydrochloride salt in 0.1 N HCl and stored at 0 C (fresh solutions were made up every few days). The hydroxylamine was added to the treatment medium immediately before the chloroplast treatment, and the pH was adjusted to 7.5. Unless otherwise noted, the chloroplasts were treated in the above medium for 12 min at 21 C and then washed twice in the same medium (minus NH_2OH and EDTA) at 0 C to remove the amine.

Reagents. Lyophilized bovine blood superoxide dismutase (specific activity, 3000 units/mg) was purchased from Truett Laboratories (Dallas, Texas). The enzyme was dissolved in 10 mM HEPES-NaOH buffer at pH 7.8 at 2 mg protein/ml and dialyzed against 2 liters of the same buffer solution for 12 hr at 0 C, and then stored at −20 C. DAD, *p*-phenylenediamine dihydrochloride, and DAT were recrystallized from charcoal-treated aqueous alcohol solution by adding excess HCl at 0 C. Benzidine dihydrochloride, diphenylhydrazine hydrochloride, and *p*-aminophenol hydrochloride were recrystallized in an identical manner except the recrystallization was from charcoal-treated aqueous solutions. Dicyanohydroquinone(2,3-dicyano-*p*-benzohydroquinone) and *o*-tolidine were recrystallized from charcoal-treated aqueous solution by simply lowering the temperature to −25 C. Fresh solutions of these compounds were made up daily in 0.01 N HCl.

Measurements. The MV Hill reaction was assayed as the O_2 uptake resulting from aerobic reoxidation of reduced MV (equation 5). Electron transport from artificial donors to MV was assayed similarly, as O_2 uptake. A membrane-covered Clark-type oxygen electrode was used for O_2 assays. When artificial electron donors were used, the observed rate of O_2 uptake in the light was corrected for the slow rate of dark autoxidation of donors which accompanied some of the reactions and ranged from 5 to 20% of the rate observed in the light. The intensity of orange actinic light (600–700 nm) was approximately 600 $Kergs \cdot sec^{-1} \cdot cm^{-2}$. The reaction temperature was 19 C. ATP formation was measured as the residual radioactivity after the extraction of the ^{32}P-labeled orthophosphate as phosphomolybdic acid in butanol-toluene. Radioactivity was determined from the Čerenkov radiation as described by Gould *et al.* (13).

RESULTS

Photooxidation of Ascorbate by Normal, O_2-producing Chloroplasts. Aerobic photoxidation of ascorbate by isolated chloroplasts was a subject of rather intensive studies in 1950's, but no clearly defined mechanism has emerged (for a review, see Ref. 18). The original observation of Mehler (22) that the ascorbate photooxidation required O_2 and was stimulated by catalytic concentrations of quinone, may now be viewed in retrospect as already suggesting the involvement of the superoxide radical anion in the reaction mechanism.

Figure 1 shows the effect of ascorbate addition on the uptake of O_2 and phosphorylation in normal chloroplasts which are capable of actively transporting electrons from water to MV. As can be seen, the addition of ascorbate doubles the rate of the O_2 uptake mediated by MV. The phosphorylation rate is

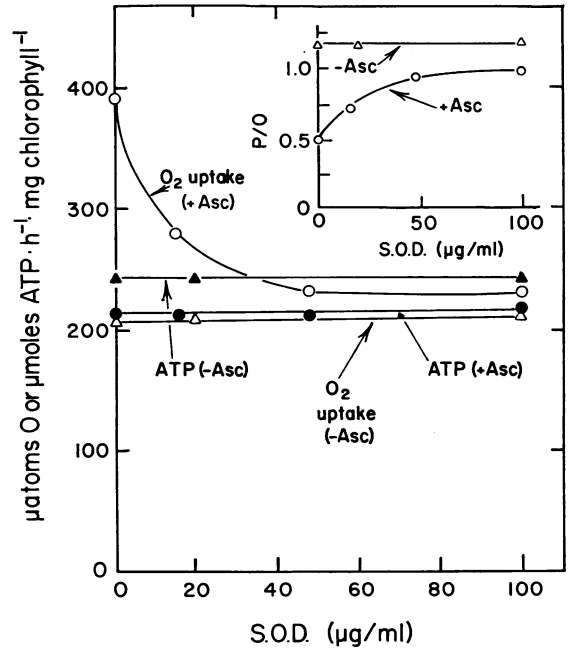

FIG. 1. Effect of SOD on ascorbate-stimulated O_2 uptake in normal (untreated) chloroplasts. The 2-ml reaction mixture consisted of: 0.1 M sucrose, 50 mM Tricine-NaOH buffer (pH 8.0), 2 mM $MgCl_2$, 0.75 mM ADP, 5 mM $Na_2H^{32}PO_4$ 0.5 mM methylviologen, and chloroplasts containing 40 μg of chlorophyll. When added (+Asc), D-ascorbate was 5 mM. All other pertinent reaction conditions are as described in "Materials and Methods." Note that O ($\frac{1}{2}O_2$) units are used in this particular figure (not O_2).

scarcely affected (10% inhibition), but, because of the stimulated O_2 uptake, the apparent phosphorylation efficiency P/O (= P/e_2 in the absence of ascorbate) drops sharply from 1.2, a value typical of the MV Hill reaction, to 0.5. However, the subsequent addition of SOD abolishes the ascorbate-stimulated portion of O_2 uptake without influencing the phosphorylation rate at all. Consequently the diminished P/O ratio is restored nearly to the original level of standard noncyclic photophosphorylation. We may deduce from these observations that, in the normal, O_2-producing chloroplasts employed here, ascorbate photooxidation is supported predominantly by the superoxide radical which is generated by the aerobic reoxidation of reduced MV, and, therefore, ascorbate does not significantly replace water as the electron donor. Böhme and Trebst (6) and Epel and Neumann (10) have also noticed the essentially unchanged rate of phosphorylation during the ascorbate-enhanced O_2 uptake. The above radical reaction mechanism has already been predicted by the latter authors (10).

Photosystem II-mediated Oxidation of Ascorbate in NH_2OH-treated Chloroplasts. Ascorbate does donate electrons to the photosynthetic electron transport chain if the water-splitting mechanism is inoperative or destroyed (5, 6, 9, 26, 32), suggesting that the inability of water to serve as reductant creates favorable conditions for the strong oxidant produced by PS II to oxidize exogenous reductants. In a preceding paper of this series (26) we have documented a new method of inactivating the water-oxidizing mechanism of chloroplasts (NH_2OH-treatment) without impairing the coupling efficiency of the chloroplast membrane. Our preliminary data on ascorbate photooxidation in these NH_2OH-treated chloroplasts indicated a P/O_2 value of 0.5 to 0.6, confirming the observation of Böhme and Trebst for their heat-treated chloroplasts (6). These P/O_2 values would have represented the P/e_2 values *if* the ascorbate photooxidation simply followed the mechanism expressed by equations 1 to 4 ($O_2 \equiv 2e^-$). However, it is already clear that these formulae do not apply even in these treated chloroplasts, since the superoxide radical, produced via the univalent reduction of O_2 by reduced MV, must still react with ascorbate.

As Figure 2 shows, the aerobic photooxidation of ascorbate in NH_2OH-treated chloroplasts indeed contains a large SOD-sensitive component indicative of the involvement of $\cdot O_2^-$. However, a larger, SOD-insensitive component remains as a well defined plateau. Clearly, it is the latter portion which must be considered as the "true" electron transport expressed by equations 1 to 4. As is clearly seen in Figure 2 (inset) in this plateau region the P/O_2 (now equivalent to P/e_2) reaches 0.9, a level which is no longer greatly different from that of the normal Hill reaction ($P/e_2 = 1.1$ to 1.2).

The pH dependence of O_2 uptake and phosphorylation in this ascorbate → MV → O_2 system is shown in Figure 3. As one would expect, the presence (Fig. 3b) or absence (3a) of SOD only affects the height of the O_2 uptake *versus* pH curves with little effect on their shapes. Phosphorylation remains totally unaffected by SOD at all pH levels. The "true" relationship between electron transport and phosphorylation is represented by Figure 3b where the radical-ascorbate interaction is prevented by SOD. The shapes of these activity-pH curves and the marked stimulation of electron transport by Pi (*i.e.*, by concomitant phosphorylation) are remarkably similar to those observed for the standard Hill reaction with MV as acceptor (14). Undoubtedly both reactions ascorbate → MV and H_2O → MV are governed by the same rate-limiting phosphorylation step.

Photosystem II-mediated Oxidation of Catechol and Other Electron Donors in NH_2OH-treated Chloroplasts. In this study we have found that catechol (*o*-hydroquinone; with 0.5 mM ascorbate as electron reservoir) serves as an excellent electron donor for PS II. The rates of electron transport and phosphorylation are comparable to those of the Hill reaction and are highly DCMU-sensitive (Fig. 4). The apparent P/O_2 ratio of 0.6 in the absence of SOD is close to that observed for ascorbate photooxidation. The addition of SOD again exerts a marked inhibitory effect (40%) on O_2 uptake, without affecting the phosphorylation rate at all. The maximum P/O_2 ratio obtained in the presence of SOD (where $P/O_2 = P/e_2$) now reaches 1.1, which is virtually identical with the ratio associated with the Hill reaction. Similar results were obtained with another new electron donor, 2,3-dicyanohydroquinone (with

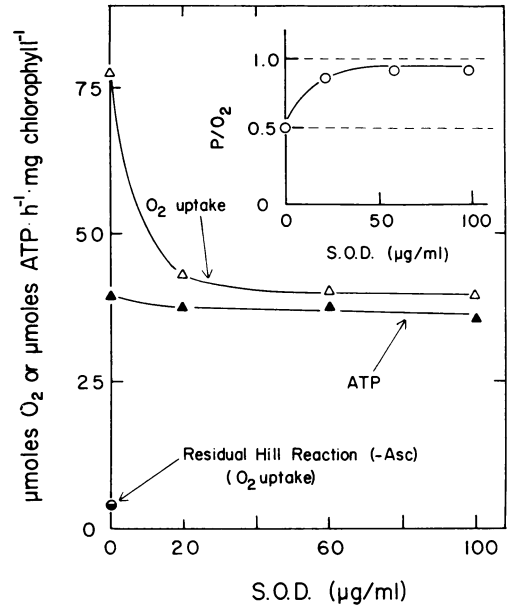

FIG. 2. Effect of SOD on O_2 uptake associated with photooxidation of ascorbate in hydroxylamine-washed chloroplasts. Chloroplasts were pretreated with NH_2OH as described in "Materials and Methods." Other conditions are as in Figure 1.

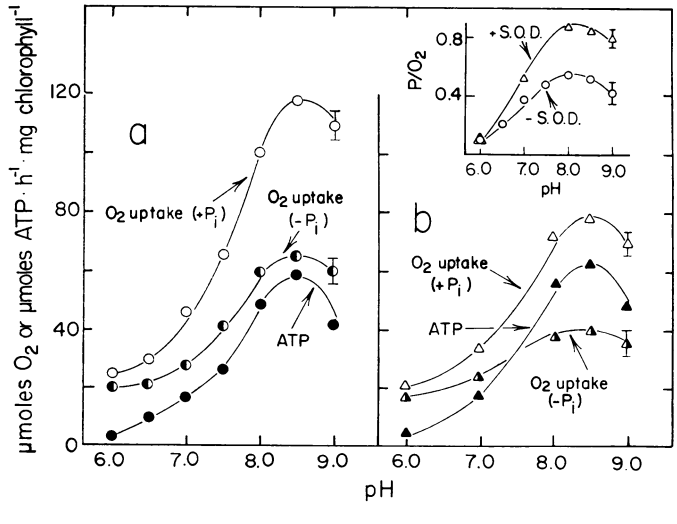

FIG. 3. Effect of SOD on O_2 uptake and phosphorylation associated with ascorbate photooxidation in NH_2OH-treated chloroplasts at various pH levels. The reaction conditions are identical to those in Figure 2 except for the buffers used: MES-NaOH (pH 6.0 to 6.5), HEPES-NaOH (pH 7.0 to 7.5), and Tricine-NaOH (pH 8.0 to 9.0). The dark oxidation of ascorbate at pH 9 became quite significant (20–30% of the rate in the light); as a consequence, exact determinations of rates became difficult. The concentration of SOD used (B) was 80 µg/ml; no SOD was used for obtaining data in part A.

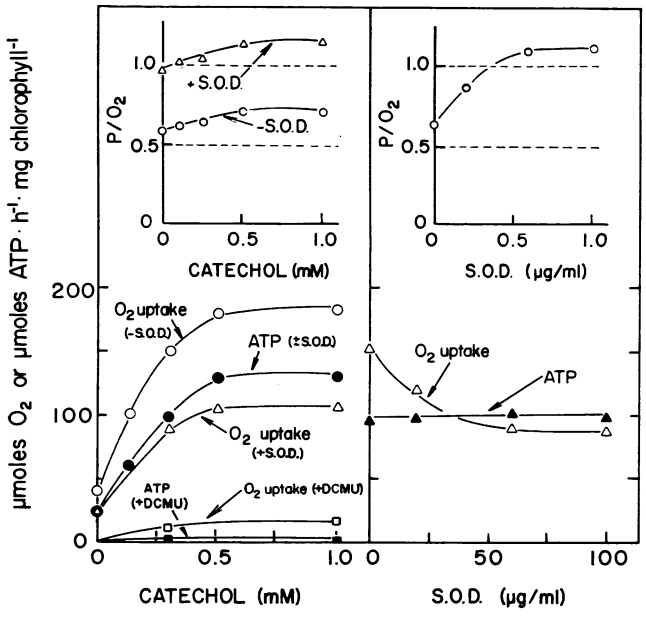

FIG. 4. Effect of SOD on O_2 uptake associated with the PS II-mediated oxidation of catechol in NH_2OH-treated chloroplasts. All the conditions were as in Figure 2 except for the reaction pH (7.8). When added, DCMU was 1.5 μM. Note that the P/O_2 ratio (=P/e_2) in the presence of SOD approaches 1.1, a value close to that of the Hill reaction. SOD, 80 μg/ml (left-hand figure).

0.5 mM ascorbate), although the reaction rates with this donor are not nearly as rapid as with catechol (Fig. 5).

Yamashita and Butler (32) showed that *p*-aminophenol acts as a good donor for PS II in their tris-washed chloroplasts. They reported a P/e_2 value of 0.97 measured under anaerobic conditions with NADP as acceptor. We have confirmed this value using the aerobic photoxidation system ($P/O_2 = 1.0$ with SOD). Benzidine is another compound reported by Yamashita and Butler to give a P/e_2 ratio approaching 1. As already indicated in a previous paper (26), the photooxidation of this compound can be followed for some time (1.5 min), without inducing a cycle, in the absence of added ascorbate. The P/O_2 ratio obtained was 1.05. As one would expect, the photooxidation of this compound (without ascorbate) is quite insensitive to SOD.

Photosystem I-mediated Oxidation of Artificial Electron Donors in DCMU-poisoned Chloroplasts. Aerobic reaction systems with autoxidizable electron acceptors are widely used for the studies of PS I-electron transport and phosphorylation, especially those involving very fast rates (*e.g.*, 15, 16, 25). In the following experiments all the reactions were run in the presence of 2.5 mM ascorbate as the electron reservoir.

Figure 6 shows the effect of SOD on the photooxidation of DAD, perhaps the most widely used PS I donor today, and DAT, a new donor. Oxygen uptake and phosphorylation in these PS I reactions are faster, by almost an order of magnitude, than the PS II-mediated reactions described above. However, a marked suppression of O_2 uptake by SOD clearly indicates that considerable portions (30–40%) of the high rates observed are due to the oxidation of the electron donors and/or ascorbate by $\cdot O_2^-$. Once again SOD has no effect on phosphorylation, and, consequently, apparent P/O_2 ratios of 0.3 to 0.4 in the absence of SOD are elevated to the level of 0.6 to 0.65 as the suppression of O_2 uptake by added SOD reaches a maximum.

The following electron donors were also tested: $DCIPH_2$, *o*-tolidine, diphenylhydrazine, and *p*-phenylenediamine. Of these compounds only $DCIPH_2$ is well known as a PS I donor. *p*-Phenylenediamine is better known as a PS II donor (31), but in our hands the photooxidation of this compound contains a significant part (30%) which is DCMU-insensitive and therefore considered to involve only PS I. All of these compounds are oxidized by PS I at much slower rates than are DAD and

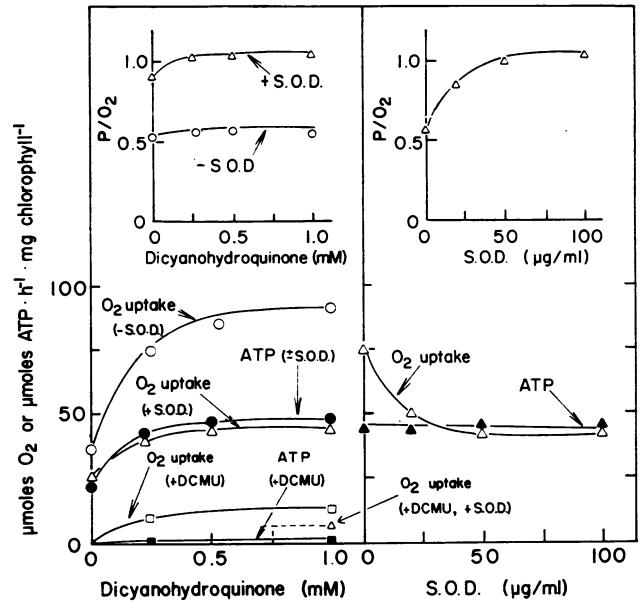

FIG. 5. Effect of SOD on O_2 uptake associated with the PS II-mediated oxidation of dicyanohydroquinone in NH_2OH-treated chloroplast. Conditions are as in Figure 4. In the left-hand figure, SOD was 80 μg/ml.

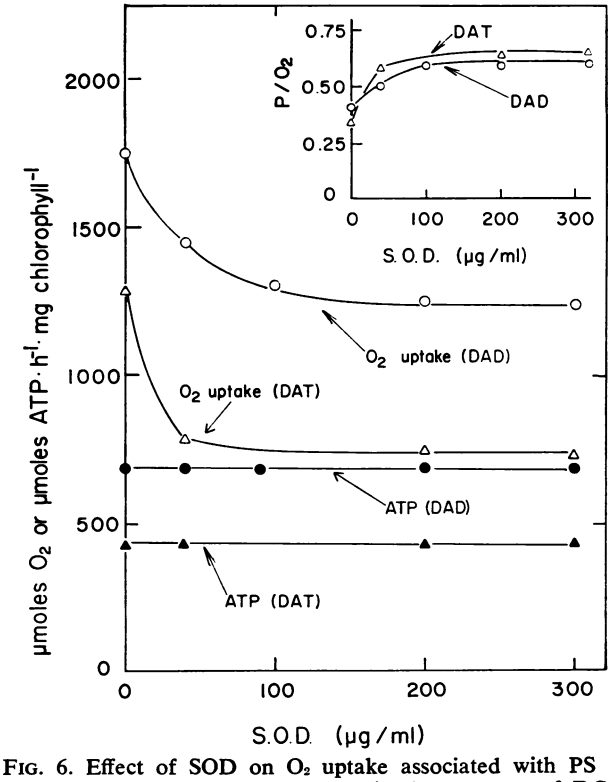

FIG. 6. Effect of SOD on O_2 uptake associated with PS I-dependent oxidation of DAD and DAT in the presence of DCMU. The 2-ml reaction mixture contained the following: 0.1 M sucrose, 50 mM Tricine-NaOH buffer (pH 8.0), 2 mM $MgCl_2$, 0.75 mM ADP, 5 mM Na_2-$H^{32}PO_4$, 50 μM methylviologen, 1.5 μM DCMU, 2.5 mM D-ascorbate, 0.5 mM diaminotoluene or diaminodurene, and chloroplasts containing 10 μg of chlorophyll.

DAT, their maximum activities as donors supporting only 100 to 200 μmoles O_2/hr·mg Chl. Nevertheless, all of these reactions support phosphorylation with similar efficiencies (P/O_2): 0.3 to 0.4 in the absence of SOD and 0.55 to 0.65 in its presence. Only $DCIPH_2$ gives significantly higher P/O_2 values—0.5 to 0.55 without SOD and 0.65 to 0.7 with SOD. These data are summarized in Table I together with data for all of the other PS I and PS II donors tested in our study.

The stoichiometric formation of H_2O_2 from the consumed O_2 was also confirmed for some typical donor reactions, in order to demonstrate that no significant peroxidatic or oxidase reactions were occurring which might further complicate the overall reaction mechanism and invalidate equation 4 (Table II).

DISCUSSION

In this work we have placed considerable weight on the characterization of the aerobic photooxidation of ascorbate. Our interest in this ascorbate photooxidation was aroused when Böhme and Trebst (6), using both normal chloroplasts and heated chloroplasts, showed that the P/e_2 ratio (assuming $P/e_2 = P/O_2$) associated with ascorbate photooxidation was 0.5, which was close to half of the value for normal noncyclic photophosphorylation. They interpreted this to suggest that one of two sites of noncyclic phosphorylation is associated with the water-splitting step of PS II, and the entry of electrons from ascorbate occurs after this phosphorylation site. We have confirmed the P/O_2 ratio of 0.5 to 0.6 using NH_2OH-treated chloroplasts (26, see also the "Results" of this paper). However, it is now quite clear that this apparently low phosphorylation efficiency was due to an overestimation of electron flux. Indeed, our data showed that 40% of the total O_2 consumption is due to the oxidation of ascorbate by the superoxide radical which is produced by the donor reaction itself: ascorbate → PS II → PS I → $O_2/\cdot O_2^-$. Thus, when this radical reaction is prevented by SOD added, the P/O_2 ratio is elevated to 0.9. This value, now equivalent to the P/e_2 ratio, is no longer low enough to make one suspect bypassing of a phosphorylation site. It is significantly lower, however, than the average P/e_2 ratio associated with the standard Hill reaction—1.0 to 1.2. We have no clear explanation for this at the present time. Ben-Hayyim and Avron (5) did obtain a P/e_2 ratio of 1 for the ascorbate → NADP reaction in tris-washed chloroplasts, but their results were not conclusive because of their conditions, which strongly favored a cyclic electron flow. In their original reports on tris-washed chloroplasts Yamashita and Butler (31, 32) listed rather poor P/e_2 ratios for the ascorbate → NADP system (0.1–0.4).

Table I. *Effect of Superoxide Dismutase on O_2 Uptake and Phosphorylation Associated with Various Photosystem II and Photosystem I Donor Reactions*

Conditions for reactions involving PS II are outlined in Figures 1 through 5. The concentration of ascorbate as a direct donor was 5 mM; the other PS II donors, 0.5 mM plus ascorbate 0.5 mM except benzidine which was added alone. Conditions for PS I-donor reactions are similar to those in Figure 6, except that the Chl concentration for reactions with $DCIPH_2$, *o*-tolidine, diphenylhydrazine (DPHZ), and *p*-phenylenediamine (PD) was 20 μg/ml. The concentration of donors, 0.5 mM plus 2.5 mM ascorbate. For the reaction involving H_2O as donor (Hill reaction), untreated chloroplasts were used. NH_2OH-treated chloroplasts were used for donor reactions involving PS II. PS I-donor reactions were run in the presence of DCMU added. DCHQ, 2,3-dicyano-*p*-hydroquinone.

Photosystem Involved	Donor	Rate of O_2 Uptake	Observed P/O_2 (or P/O for H_2O System)	O_2 Uptake in Presence of SOD	SOD-sensitive O_2 Uptake	"True" P/e_2
		μmoles/hr·mg Chl		μmoles/hr·mg Chl	%	
II + I	H_2O	225	1.22	225	0	1.22
	D-Ascorbate	100	0.55	65	35	0.85
	Catechol	170	0.70	100	41	1.15
	DCHQ	85	0.55	45	47	1.00
	Benzidine	90	1.05	90	0	1.05
	p-Aminophenol	145	0.64	85	41	1.00
I	$DCIPH_2$	160	0.50	115	27	0.68
	DAD	1750	0.39	1250	30	0.57
	DAT	1300	0.35	775	41	0.60
	o-Tolidine	100	0.27	50	50	0.53
	DPHZ	70	0.32	40	45	0.59
	PD	220	0.38	135	39	0.62

Table II. *Accumulation of H_2O_2 during the Photooxidation of Various Photosystem II- and Photosystem I Donors*

For the reaction conditions, see Table I. Immediately upon turning off the light, 10 μl of catalase (2×10^5 units/ml) were injected into the reaction vial, and O_2 evolution was monitored to determine H_2O_2 accumulation. Note that in all donor reactions the H_2O_2 accumulation to O_2 uptake ratio approaches the theoretical value of 1 (or 2 when H_2O serves as electron donor).

Donor	Presence (+) or Absence (−) of SOD	O_2 Taken Up	H_2O_2 Accumulated	H_2O_2/O_2
		μmoles		
H_2O	−	13.5	27	2.00
D-Ascorbate	−	8.6	8.0	0.93
	+	6.6	6.0	0.91
p-Aminophenol	−	10.0	10.2	1.02
	+	8.0	7.6	0.95
Catechol	−	11.0	9.4	0.86
	+	7.4	6.4	0.86
DAD	−	19.2	16.8	0.88
	+	13.4	12.0	0.90
DAT	−	15.9	16.4	1.03
	+	7.6	7.6	1.00

The involvement of superoxide radical ions in ascorbate photooxidation, which leads to an overestimation of electron flow, has already been predicted by Elstner *et al.* (11). However, their suggestion—that the monodehydroascorbate radical generated by the oxidation of ascorbate by $\cdot O_2^-$ may be the effective electron donor species rather than ascorbate *per se*—cannot readily explain our results, which showed that the SOD inhibition of O_2 uptake never exceeded 50% (in any donor reaction). In fact, this 50% inhibition is the theoretical maximal inhibition one could expect, if the whole reaction including O_2^--donor interactions is to be essentially described by equations 1 to 6.

Before the completion of this manuscript a paper by Allen and Hall (1) was published which deals with the effect of SOD addition on ascorbate photooxidation in normal, O_2-producing chloroplasts. Their observations include a marked enhancement of O_2 uptake by ascorbate addition (up to 3-fold increase), its complete reversal by SOD addition, and an unchanged rate of phosphorylation. They concluded that in normal chloroplasts ascorbate does not replace water as the electron donor, and the enhancement of O_2 uptake observed is almost entirely due to the oxidation of ascorbate by $\cdot O_2^-$. Our results agree with theirs rather well, and we agree with their conclusions, except that the slight inhibition of phosphorylation by ascorbate addition

we observed seems to indicate the existence of some interaction between the electron transport chain and ascorbate.

It is clear that the question of the superoxide radical involvement is shared by practically all of the reactions with artificial electron donors assayed as aerobic photooxidation since, in general, an electron donor is administered as a donor/ascorbate couple. Besides, many of the commonly used artificial electron donors themselves must be expected to be susceptible to $\cdot O_2^-$, as exemplified by *p*-hydroquinone (28) and catechols (23), although we have observed no sign of benzidine, given alone, consuming $\cdot O_2^-$. Nevertheless, our attempt to isolate true electron fluxes by abolishing donor-$\cdot O_2^-$ interactions by SOD addition was apparently successful with all of the donor reactions tested. This is indicated by the fact that the SOD-inhibition curves for O_2 uptake always reached a well defined plateau and that within the same group of donor reactions (PS I- or PS II-mediated) the P/O_2 ratios measured in these plateau regions were remarkably similar (Table I). As mentioned above, it is also important that the SOD inhibition of O_2 uptake never exceeded 50%.

It was surprising to find the P/e_2 ratios thus computed for all of the PS II-donor reactions tested fall near 1. Although the ascorbate system showed slightly lower values (0.85 to 0.9), they are still close to the range of normal P/e_2 values associated with the Hill reaction. Viewing these data in the light of the recent findings concerning the sites of energy coupling in noncyclic photophosphorylation (14, 17, 27, 30), there is no doubt that the transport of electrons from these donors to MV utilizes both coupling sites I and II. Here, coupling site I refers to the well known rate-limiting phosphorylation site between plastoquinone and cytochrome *f* (4, 7). Site II is considered to be located very close to PS II (17, 27, 30), possibly on its water-oxidizing side, as has been suggested by Böhme and Trebst (6), although the basis on which they made this suggestion is no longer valid. Since the true P/e_2 ratio associated with the transport of electrons through both photosystems is near 1 regardless of the donor used, it is evident that site II, if indeed located on the water-oxidizing side of PS II, does not require water as the obligatory electron donor. However, if one takes the view of chemiosmotic coupling (24), an interesting possibility arises. These artificial donors, with either amino or hydroxyl groups as the oxidizable groups, might mimic water as the hydrogen donor. That is, if the machinery of water oxidation operates in such a way that the protons released by water splitting are discharged to the inside of the thylakoid (which would create a trans-membrane hydrogen ion gradient), then the same directional discharge of protons might occur when the artificial "hydrogen" donors are oxidized by PS II. Alternatively, these donors may simply be oxidized inside of the thylakoid. Such a chemiosmotic view is now encouraged by the recent discovery of the involvement of a proton pump in the partial electron transport reaction $H_2O \rightarrow$ PS II $\rightarrow$ dibromothymoquinone (14).

Table I also displays unexpectedly high and uniform P/e_2 ratios (around 0.6) found for PS I-donor reactions, although the value for the $DCIPH_2$ system (0.65 to 0.7) seems appreciably higher than the rest. Significantly, all of these P/e_2 values are lower than those supported by the reactions involving both photosystems by about 0.4 to 0.5, a difference which could be explained if one assumes that the PS I reactions do not involve site II. In fact, the characteristics of the phosphorylation reaction associated with the $DCIPH_2 \rightarrow$ PS I $\rightarrow$ MV system have been well accounted for by the noninvolvement of site II (14).

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