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The Function of Ascorbic Acid in Photosynthetic Phosphorylation[1]

Giorgio Forti* and Gianluca Elli

Centro di Studio Consiglio Nazionale delle Ricerche sulla Biologia Cellulare e Molecolare delle Piante, Dipartimento di Biologia, Università di Milano, Via Celoria 26, Milano 20133, Italy

Ascorbate is oxidized to the free radical monodehydroascorbate by $O_2^{\cdot-}$ and by H_2O_2 (through the action of ascorbate peroxidase) formed in the Mehler reaction by isolated spinach (*Spinacia oleracea*) thylakoids. Light-dependent electron transport from water to monodehydroascorbate is shown to be coupled to ATP formation with a ratio ATP:O_2 of 2. In the presence of ascorbate the net O_2 exchange balance of the Mehler reaction is close to zero, and the synthesis of ATP is increased 2 to 3 times due to the extra electron transport to the monodehydroascorbate free radical. A scheme of the electron transport in the presence of ascorbate is discussed.

Ascorbic acid is present at the concentration of about 10 to 25 mM in the stroma of chloroplasts (Foyer et al., 1983), and it has been shown to have important functions in photosynthesis, such as in the protection of the photosynthetic apparatus against the oxygen radicals and H_2O_2 that are formed during photosynthetic activity (Asada, 1994), and against photoinactivation, since it is a cofactor of carotenoid de-epoxidation (Siefermann and Yamamoto, 1974). Earlier work had demonstrated that ascorbate enhanced ATP synthesis coupled to electron transport in the Mehler reaction (Forti and Jagendorf, 1961). More recently, the enzyme APX, a peroxidase highly specific for ascorbate, has been shown to be present both bound to thylakoids and soluble in the stroma of chloroplasts, and the ascorbate free radical MDA was demonstrated to be a product of its activity (Miyake and Asada, 1992) and to serve as an electron acceptor in photosynthetic electron transport (Miyake and Asada, 1992; Forti and Ehrenheim, 1993). It was also shown that MDA is reduced at the reducing side of PSI in competition with the system Fd-$NADP^+$ (Forti and Ehrenheim, 1993). The rate of electron transport from H_2O to MDA is about 50% of that of $NADP^+$ reduction and generates ΔpH across the membrane (Forti and Ehrenheim, 1993). The same conclusion is indicated by the experiments of Schreiber and Neubauer (1990), who have shown that O_2 is required for photochemical quenching of PSII fluorescence in intact chloroplasts (in the presence of ascorbate), and that nonphotochemical (ΔpH-dependent) quenching, developed in the presence of O_2, was reversed by nigericin. On the basis of such observations they proposed that the combined Mehler-peroxidase reaction is responsible for ΔpH-dependent nonphotochemical quenching, an indirect indication that this reaction is coupled to membrane energization.

The generation of MDA in illuminated thylakoids, through the APX-catalyzed oxidation of ascorbate by H_2O_2 produced in the Mehler reaction, has been recently confirmed through electron paramagnetic resonance spectroscopy (Grace et al., 1995). These authors have also demonstrated, using the same technique, that MDA is reduced in the light and that a steady-state concentration of MDA is reached, resulting from the rates of the Mehler reaction, of APX activity and of electron transport (Grace et al., 1995).

The system ascorbate-MDA may therefore be of basic importance in providing the balance of ATP and NADPH in the stoichiometric amounts required by the CO_2 assimilation. It is well known that linear electron transport from H_2O to $NADP^+$ is coupled to ATP synthesis with a stoichiometry of 1 to 1.2 ATP/NADPH, whereas the operation of the carbon assimilation cycle requires 1.5 ATP/NADPH. In intact chloroplasts performing photosynthesis, light-dependent ATP production is thought to be balanced with its requirement for CO_2 assimilation either by cyclic electron transport around PSI or by electron transport from H_2O to O_2, the Mehler reaction (see a review by Forti, 1987). Although the Mehler reaction occurs at rather low rates in isolated thylakoids (Asada, 1994), it is shown here that in the presence of ascorbate it can trigger a faster electron transport from H_2O to MDA, which is coupled to ATP synthesis. On the other hand, we show that ascorbate has no influence on the photophosphorylation coupled to MV reduction nor on the reduction of $NADP^+$ in the presence of Fd, indicating that no MDA is produced when Fd and $NADP^+$ (or the alternative electron acceptor MV) are present. Furthermore, we report that the addition of ascorbate to stroma-free, Fd-free thylakoids suppresses the light-dependent O_2 uptake (defined as the Mehler reaction), bringing the O_2 exchange balance to zero while enhancing ATP synthesis 2 to 3 times. A scheme accounting for these observations is presented.

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* Corresponding author; fax 39-2-26604399.

Abbreviations: APX, ascorbate peroxidase; ΔpH, pH gradient; DHA, dehydroascorbate; MDA, monodehydroascorbate radical; MV, methyl viologen; SOD, superoxide dismutase.

MATERIALS AND METHODS

Stacked, stroma-free thylakoids were isolated from freshly collected spinach (*Spinacia oleracea*) leaves as previously described (Forti and Fusi, 1990). Ascorbate was present at the concentration of 1 mM in the isolation medium and in all subsequent manipulations to preserve the activity of APX (Miyake and Asada, 1992).

Oxygen uptake or evolution was measured at 22°C in a Clark-type oxygen electrode (Radiometer, Copenhagen, Denmark) under red light illumination at a wavelength from 630 to 720 nm as previously described (Forti and Ehrenheim, 1993). The reaction medium contained Tricine-NaOH buffer (30 mM at pH 8.0) or Hepes-NaOH (30 mM at pH 7.0), 100 mM Suc, 5 mM $MgCl_2$, 10 mM NaCl, 20 mM KCl, 0.5 mM ADP, 2.5 mM Pi, 0.01 mM P_1,P_5-di(adenosine-5')pentaphosphate (added as an inhibitor of the very low adenylate kinase activity present), and other additions as indicated. Samples were withdrawn before and after illumination for ATP estimation.

$NADP^+$ photoreduction was estimated as previously described (Forti and Fusi, 1990), measuring the increase of $A_{340-390}$ in a dual-wavelength spectrophotometer equipped with lateral illumination of the stirred sample. Actinic light was admitted at 90° with respect to the measuring beam, and filtered through a heat filter and a long-pass 630-nm filter. Its intensity was defined by neutral-density filters. The photomultiplier was protected from actinic light by the appropriate filters. In the presence of ADP and Pi, ATP synthesis was coupled to electron transport. ATP that formed was measured at the end of illumination, when *A* was constant, upon addition of Glc (4 mM), hexokinase (4 units), $NADP^+$ (0.5 mM), and Glc-6-P dehydrogenase (5 units), as the increase of *A* in the dark due to further $NADP^+$ reduction.

Light-dependent ATP formation, in the presence of any electron transport system different from $NADP^+$, was estimated enzymatically by measuring the increase of $A_{340-390}$ in the dual-wavelength spectrophotometer in the presence of the Glc-hexokinase-Glc-6-P dehydrogenase-$NADP^+$ system but in the absence of Fd (Fig. 1).

Catalase, SOD, hexokinase, and Glc-6-P dehydrogenase were obtained from Fluka, and their purity was tested for interfering activities. Chl was estimated according to Arnon (1949).

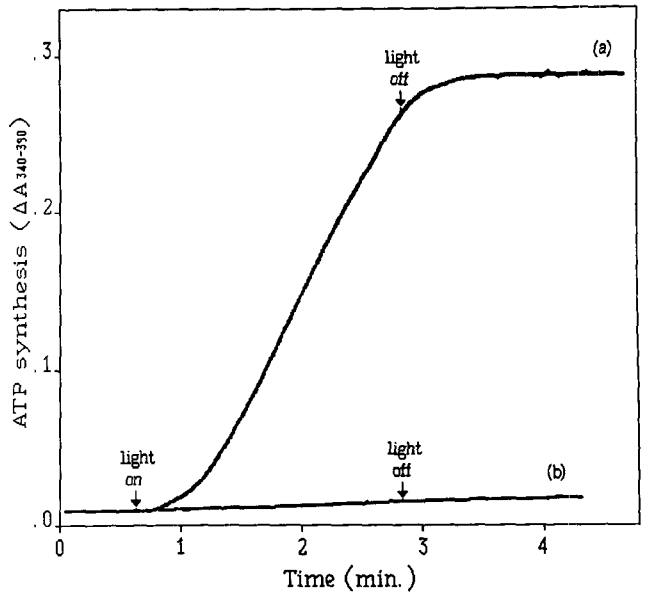

Figure 1. Light-dependent ATP formation determined enzymatically. For conditions, see "Materials and Methods." Chl concentration was 10 μg mL^{-1}. a, Ascorbate (5 mM). b, Same as a, plus nigericin at the concentration of 0.5 μM.

RESULTS

Electron transport from H_2O to MDA generated by APX in the presence of H_2O_2, measured as the ascorbate- and H_2O_2-dependent O_2 evolution at 22°C, was found to be coupled to ATP synthesis with the same ratio of ATP formed to O_2 evolved of about 2 both at pH 7 and pH 8. The rates were close to three times higher at pH 8 than at pH 7, when ascorbate concentration was saturating (Table I). The addition of H_2O_2 was an absolute requirement for O_2 evolution (Forti and Ehrenheim, 1993). The very low activity of catalase contaminating thylakoids was inhibited by 1 mM NaN_3. Table I also shows that the rates of electron transport and of ATP formation with MV as the electron acceptor were approximately 50 to 60% higher than the rates with ascorbate and H_2O_2, and the ratio ATP:2 electrons was the same. The same ratio of ATP:O_2 was observed, as usually reported in the literature, when $NADP^+$ was the electron

Table I. *Coupling of ATP synthesis to MDA photoreduction*

Conditions were as described in "Materials and Methods." H_2O_2 was added at the concentration of 0.5 mM at the onset of the 1-min illumination. Chl concentration was 25 μg mL^{-1} and NaN_3 was 1 mM. The bottom lines report the average of measurements done in duplicate on eight different thylakoid preparations, ± SD. All values are in μmol mg^{-1} Chl h^{-1}. Light intensity was 60 mW cm^{-2} (below saturation). In the case of MV electron transport, SOD was added in the amount needed to lower to a constant value the rate of O_2 uptake.

Condition	pH	O_2 Exchange	ATP Formed	ATP:O_2	ATP:$2e^-$
Ascorbate (1 mM)	7.0	15.8	32.0	2.02	1.01
	8.0	36.8	68.2	1.85	0.92
Ascorbate (3 mM)	7.0	22.6	41.8	1.85	0.92
	8.0	60.7	115.4	1.90	0.95
Ascorbate (3 mM)	8.0	60.7	115.4	1.90	0.95
Ascorbate (3 mM)	8.0	72.4 ± 10.6	140.1 ± 33.0	1.93	0.96
MV (100 μM)	8.0	−104.0 ± 38.0	222.0 ± 17.0	2.13	1.06

Table II. *Effects of cyanide, of ascorbate, and of H_2O_2 on photophosphorylation*

Conditions were as described in "Materials and Methods," pH 8.0. Chl was 10 μg mL^{-1}; ascorbate was 5 mM; H_2O_2 was 0.4 mM; KCN was 2 mM. The data are expressed in μmol ATP mg^{-1} Chl h^{-1}, and they are the average of duplicate measurements from four thylakoid preparations, ± SD. Light intensity was 86 mW cm^{-2}.

Condition	Additions		
	None	Ascorbate	Ascorbate + H_2O_2
Control	23.3 ± 1.8	49.3 ± 4.6	195.0 ± 14.5
KCN	20.4 ± 1.2	26.0 ± 3.0	31.1 ± 3.8

acceptor in the presence of Fd (not shown). These observations indicated that the reduction of MDA, MV, and $NADP^+$ share the same electron transport pathway from H_2O to the reducing side of PSI. This is also consistent with the previous report that the same sensitivity to the inhibitors DCMU and dibromothymoquinone and the same value of the Emerson enhancement were observed in the case of MDA and $NADP^+$ reduction (Forti and Ehrenheim, 1993). The involvement of APX in the ascorbate- and H_2O_2-dependent electron transport and ATP formation is clearly indicated by the observations reported in Table II. This table shows that ascorbate caused a more than 2-fold stimulation of the rate of ATP synthesis observed in the absence of added electron carriers, and the stimulation was more than 8 times when both ascorbate and H_2O_2 were added. The addition of KCN, a known inhibitor of APX (Miyake and Asada, 1992), almost completely suppressed the effect of ascorbate and H_2O_2. On the other hand, KCN did not affect photophosphorylation in the presence of MV or $NADP^+$ (data not shown), a well-known fact.

The observed inhibition by KCN is easily explained in terms of inhibition of the generation of MDA by APX. Some MDA is still produced by the spontaneous reaction of ascorbate with $O_2^{\cdot -}$ generated by the Mehler reaction even when APX is completely inhibited (see Grace et al., 1995), and this may account for the small stimulation of ATP synthesis by ascorbate in the presence of KCN (Table II).

The stimulation of ATP synthesis as a function of ascorbate concentration (in the presence of saturating concentration of H_2O_2) is shown in Figure 2. It can be observed that the stimulation was half-maximal at 500 μM ascorbate, the concentration corresponding to the K_m of spinach thylakoid APX (Miyake and Asada, 1992). This observation is in agreement with the central role of APX in the stimulation of coupled electron transport by ascorbate. Also consistent with the role of ascorbate-APX-MDA in electron transport is the lack of influence of ascorbate on ATP synthesis or electron transport when MV or $NADP^+$ were present as the electron acceptor (Tables III and IV), indicating that these acceptors prevent MDA from being formed and accepting electrons at the reducing end of PSI (in agreement with Grace et al., 1995).

It is known that most if not all of the light-dependent generation of H_2O_2 in the chloroplasts is due to the univalent reduction of O_2 at the reducing side of PSI, which produces $O_2^{\cdot -}$, followed by its disproportionation catalyzed by the thylakoid-bound SOD (Asada, 1994). We have

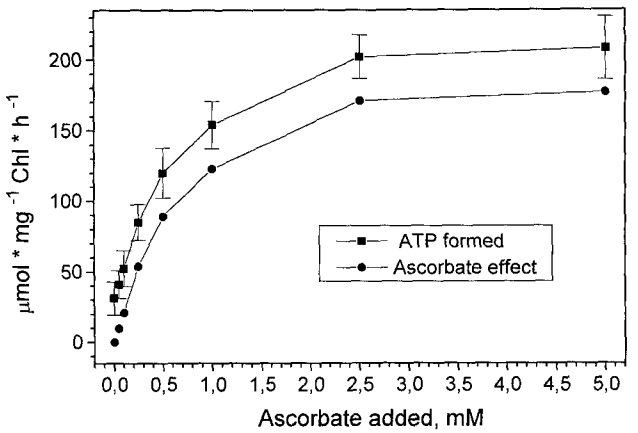

Figure 2. Stimulation of ATP synthesis as a function of ascorbate concentration in the presence of H_2O_2. Conditions were as described in Table II. Bars indicate SD.

therefore measured the rates of light-dependent O_2 uptake and the coupled ATP synthesis in isolated thylakoids in the absence of Fd or any other added cofactor or electron acceptor, and the effect of the addition of ascorbate under these conditions (Table V). The rates of O_2 uptake (the Mehler reaction) were about 10 to 25 μmol mg^{-1} Chl h^{-1} at pH 8, and the synthesis of ATP was coupled to such electron transport. Upon addition of ascorbate, O_2 uptake was drastically reduced, whereas the rate of ATP synthesis was enhanced 2 to 3 times. The addition of catalase or catalase plus SOD also suppressed O_2 uptake but had no effect on ATP synthesis, in agreement with a previous report (Ort and Izawa, 1974). When catalase plus SOD were added in the presence of ascorbate, the stimulation by ascorbate of ATP synthesis was drastically reduced (Table V).

DISCUSSION

Our observations on the influence of ascorbate on photophosphorylation and O_2 exchange in the absence of other electron acceptors can be understood by considering the following sequence of reactions.

$$2\,H_2O + 4\,O_2 \text{—(light)}\rightarrow 4\,O_2^{\cdot -} + O_2 + 4\,H^+ \quad (1)$$

$$4\,O_2^{\cdot -} \text{—(SOD)} \rightarrow 2\,O_2 + 2\,O_2^{2-} \text{—(+4 } H^+) \rightarrow 2\,H_2O_2 \quad (2a)$$

$$2\,H_2O_2 + 4\,\text{ASC—(APX)}\rightarrow 4\,\text{MDA} + 4\,H_2O \quad (3a)$$

$$4\,\textit{MDA} + 2\,H_2O \text{ — (light)}\rightarrow 4\,\text{ASC} + O_2 \quad (4a)$$

$$4\,O_2^{\cdot -} + 4\,\text{ASC} + 4\,H^+ \rightarrow 4\,\text{MDA} + 4\,H_2O_2 \quad (2b)$$

$$4\,H_2O_2 + 8\,\text{ASC— (APX)}\rightarrow 8\,\text{MDA} + 8\,H_2O \quad (3b)$$

$$12\,\text{MDA} + 6\,H_2O \text{— (light)}\rightarrow 12\,\text{ASC} + 3\,O_2 \quad (4b)$$

Scheme 1

The data in Table V are easily explained by this sequence of reactions. In the absence of any addition (column 1), the

Table III. *Lack of influence of ascorbate on ATP synthesis coupled to MV reduction*

Conditions were the same as in Table II, with the omission of H_2O_2. Other additions and light intensity were as indicated. MV was added at the concentration of 100 μM. The values are expressed in μmol mg^{-1} Chl h^{-1}, ± SD. The number of experiments are in parentheses.

Light Intensity	Additions		
	None	Ascorbate (5 mM)	Ascorbate (40 mM)
mW cm⁻²		*μmol mg⁻¹ Chl h⁻¹*	
0.20	20.4 ± 1.1 (2)	19.8 (1)	19.8 ± 1.0 (2)
0.81	88.5 ± 0.5 (2)	81.0 (1)	79.0 ± 1.0 (2)
43	266.0 ± 51.0 (5)	233.0 ± 3.0 (2)	277.0 ± 36.0 (5)

electron transport from H_2O to O_2 produced $O_2^{\cdot -}$, which is known to be disproportionated by the endogenous SOD to yield H_2O_2. Such sequence (reactions 1 + 2a) causes the net uptake of 1 O_2:4 electrons transported, and was coupled to ATP synthesis (Table V). The thylakoids contain a high activity of SOD (Asada, 1994); in our preparations it was estimated to be about 300 μmol O_2 mg^{-1} Chl h^{-1}, whereas catalase activity was absent or present as a contaminant at the level of 3 to 6 μmol O_2 produced mg^{-1} Chl h^{-1}. The addition of catalase or catalase + SOD disproportionates both the superoxide radical and H_2O_2. This is expected to lead to the evolution of O_2 and to a net balance of O_2 changes equal to zero. However, the ATP-generating electron transport (reaction 1) should not be affected, as was observed (Table V). The addition of ascorbate should bring the O_2 balance to zero, because the fast reaction of ascorbate with $O_2^{\cdot -}$ (reaction 2b) largely prevents $O_2^{\cdot -}$ disproportionation (reaction 2a; this was demonstrated by measuring MV-dependent O_2 uptake, which was increased to a constant value by successive additions of ascorbate, not shown). Reaction 2b will produce MDA and H_2O_2. The thylakoid-bound APX (Miyake and Asada, 1992) is then expected to produce two times more MDA (reaction 3b), and MDA would be rapidly reduced by the electron transport chain at the reducing end of PSI (Miyake and Asada, 1992; Forti and Ehrenheim, 1993; Grace et al., 1995). Therefore, O_2 evolution by PSII coupled to MDA reduction is expected to quantitatively balance O_2 uptake (see the sequences of reactions 1–2a-3a-4a or 1–2b-3b-4b in Scheme 1). However, the latter sequence increases by a factor of 4 the number of electrons transported along the chain, compared to the Mehler reaction (reaction 1), whereas the former increases it by a factor of 2. The rate of electron transport should also be enhanced by ascorbate, because the rates of APX and of MDA photoreduction are much higher than the rate of Mehler reaction. Therefore, it should be expected that photophosphorylation coupled to electron transport would also be enhanced, in agreement with our observations (Tables II and V; Fig. 2). The addition of a large excess of SOD + catalase drastically reduced the stimulation of photophosphorylation by ascorbate (Table V). This was expected according to the Scheme 1, because both $O_2^{\cdot -}$ and H_2O_2 are destroyed by these enzymes, thereby preventing their reaction with ascorbate, which produces MDA.

However, the stimulation of photophosphorylation by ascorbate was not completely suppressed by any amount of SOD + catalase (Table V). The residual effect of ascorbate on ATP synthesis may be explained by assuming that $O_2^{\cdot -}$ generated in the aprotic interior of the membrane (Takahashi and Asada, 1988), where it is not accessible to the water-soluble SOD and catalase added to the external medium, may oxidize ascorbate through some membrane-bound intermediate. In agreement with a previous report (Takahashi and Asada, 1988), we have observed that the photoreduction of added Cyt *c*, which has been proposed to be due to $O_2^{\cdot -}$ (Takahashi and Asada, 1988), cannot be inhibited completely by SOD (not shown). Of course, the possibility that Cyt *c* may be reduced by some thylakoid redox component other than $O_2^{\cdot -}$ cannot be ruled out.

The central role of APX in the stimulation by ascorbate of ATP synthesis was also demonstrated by the observation that inhibition by cyanide of this enzyme almost completely abolished the stimulation, both in the presence and absence of added H_2O_2, although it did not affect the basal rate of ATP synthesis observed in the absence of any added cofactor (Table II). Furthermore, we report here (Fig. 2) that the half-maximal stimulation by ascorbate was observed at the concentration of ascorbate coinciding with the K_m of APX (Miyake and Asada, 1992).

An important conclusion from the data reported here concerns the dual role of ascorbate in photosynthesis, namely as a scavenger of the toxic species of activated oxygen (Asada, 1994), and as a cofactor of photosynthetic electron transport coupled to photophosphorylation. The latter is performed through the activity of APX, which removes H_2O_2 while generating MDA, which has been shown to be an efficient electron acceptor at the reducing side of PSI (Table I) (Miyake and Asada, 1992; Forti and Ehrenheim, 1993; Grace et al., 1995). Ascorbate is therefore utilized as the redox couple ascorbate/MDA in photosynthetic electron transport and is not usually oxidized to dehydroascorbate in the chloroplast, because the thyla-

Table IV. *Lack of influence of ascorbate on $NADP^+$ reduction*

Conditions were the same as in Table II, with the omission of H_2O_2. Other additions and light intensity were as indicated. $NADP^+$ was added at the concentration of 0.5 mM in combination with 12 μM Fd. The values are expressed in μmol mg^{-1} Chl h^{-1}.

Light Intensity	Ascorbate Added	Control	Nigericin (0.5 mM)
mW cm⁻²	*mM*	*μmol mg⁻¹ Chl h⁻¹*	
0.7	None	31.0	38.2
0.7	25	30.0	38.2
74.0	None	97.0	349.0
74.0	25	77.0	372.0

Table V. *Effect of ascorbate on O_2 uptake and on ATP synthesis in isolated thylakoids*

Conditions were as described in "Materials and Methods," pH 8.0. Where added, ascorbate was 3 mM. Chl concentration was 25 $\mu g\ mL^{-1}$. The data are the average of duplicate measures from four thylakoid preparations. All figures are in $\mu mol\ mg^{-1}$ Chl h^{-1}, ± SD. Added catalase activity was 4.39 μmol of $O_2\ min^{-1}$; SOD was added in excess of the amount required to disproportionate $O_2^{\cdot -}$ produced at a rate of 375 $\mu mol\ mg^{-1}$ Chl h^{-1}.

Condition	Additions				
	None	Catalase	Catalase + SOD	Ascorbate	Catalase + SOD + ascorbate
O_2	−10.70 ± 0.69	0.70 ± 0.70	1.98 ± 0.80	−2.34 ± 1.10	1.81 ± 0.40
ATP	10.40 ± 1.13	10.20 ± 1.49	10.90 ± 1.49	28.20 ± 4.30	14.60 ± 2.60
Effect of ascorbate	—	—	—	+17.8	+4.4

koids keep it reduced photochemically. Furthermore, the stroma-located enzymes glutathione-DHA reductase and NADPH-glutathione reductase ensure the reduction of DHA (Asada, 1994), which might be formed by disproportionation of any small amount of MDA that may have escaped photoreduction. This accounts for the observation that the ratio ascorbate:DHA is very high in the chloroplasts in vivo, and does not change appreciably in the light or dark (Foyer et al., 1983).

In the presence of ascorbate, therefore, the low rate of O_2 uptake due to the Mehler reaction is suppressed and substituted by a Hill reaction in which MDA is the terminal acceptor functioning at a high electron flow rate (Table I) (Forti and Ehrenheim, 1993). Such an electron transport system, which has been shown here (Tables I and II) to be coupled to ATP formation, is the best candidate to be the source of ATP synthesis in the chloroplast that is capable of fulfilling the requirement for ATP in the assimilation of CO_2. It is well known that ATP formation coupled to $NADP^+$ reduction is stoichiometrically inadequate to support CO_2 assimilation by the Calvin cycle, which requires $(3ATP+2NADPH)/CO_2$. Photophosphorylation coupled to cyclic electron transport around PSI has been thought to fulfill the requirement for ATP (Arnon, 1977). However, cyclic photophosphorylation has been demonstrated in vivo only under nonphysiological conditions (Forti and Parisi, 1963; Tanner and Kandler, 1969), and no evidence for cyclic electron transport around PSI has been found in cyanobacteria (Myers, 1987). The MDA photoreduction (Miyake and Asada, 1992; Forti and Ehrenheim, 1993; Grace et al., 1995) has been shown here to generate ATP at rates adequate to the requirements for photosynthesis.

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