ISSN 0006-2979, Biochemistry (Moscow), 2014, Vol. 79, No. 3, pp. 282-289. © Pleiades Publishing, Ltd., 2014.
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REVIEW

Role of Ascorbic Acid in Photosynthesis

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Received November 22, 2013
Revision received December 5, 2013

Abstract—Experimental data concerning the role of ascorbic acid in both the maintenance of photosynthesis and in the protection of the photosynthetic apparatus against reactive oxygen species and photoinhibition are reviewed. The function of ascorbic acid as an electron donor in the "Krasnovsky reaction", as well as its physiological role as a donor to components of the photosynthetic electron transport chain, which was first studied by A. A. Krasnovsky in the 1980s, is discussed. Data on the content and transport of ascorbic acid in plant cells and chloroplasts are presented.

DOI: 10.1134/S0006297914030146

Key words: plants, photosynthesis, ascorbic acid, Krasnovsky reaction, chloroplasts, thylakoids, photosynthetic electron transport chain, cyclic electron transport, reactive oxygen species

Ascorbic acid, the simple organic compound commonly known as vitamin C, has played an important role not only in the development of seafaring by rescuing sailors from scurvy, but also in the development of science. Two scientists were awarded Nobel prizes in 1937 for studies of its properties: W. N. Haworth in Chemistry and A. Szent-Gyorgyi in Physiology or Medicine. Two-time Nobel Prize Laureate L. C. Pauling used ascorbic acid to heal the future Nobel Laureate P. Mitchell. This small molecule proved to be very useful also in studies of the mechanism of photosynthesis. It is this molecule that was used by A. A. Krasnovsky for experiments that resulted in the discovery of the reaction later named for him. The essence of this reaction, observed in pyridine solution, lies in the ability of chlorophyll and its analogs to engage in reactions of reversible photochemical reduction. Ascorbic acid acted as a reducing agent capable of transferring an electron to a light-excited chlorophyll molecule.

Abbreviations: APO, ascorbate peroxidase; Asc, ascorbic acid, ascorbate; CET PS1, cyclic electron transport around PS1; Chl, chlorophyll; DCMU, (the herbicide Diuron), 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DHA, dehydroascorbate; MDHA, monodehydroascorbate; $P700^+$, oxidized form of chlorophyll of the PS1 reaction center; PETC, photosynthetic electron transport chain; PS1 and PS2, photosystems 1 and 2; ROS, reactive oxygen species; VDE, violaxanthin de-epoxidase.

Although Krasnovsky used not only ascorbic acid, but also other electron donors, such as phenylhydrazine, hydrogen sulfide, and cysteine, most attention was given to ascorbic acid. When describing the mechanism of the newly discovered reaction, he wrote that "...participation of semi-oxidized forms of electron donor molecules of monodehydroascorbic acid-type in these reactions requires deeper study. The stationary concentration of monodehydroascorbic acid is rather high, as evidenced by measurements of electron paramagnetic resonance. These semi-oxidized compounds seem to be involved not only in reverse reactions with the reduced pigment forms, but can also be further oxidized (to dehydroascorbic acid), transferring their hydrogen to the acceptor – an oxygen molecule, methyl red, etc." [1].

The physicochemical properties of ascorbic acid are now quite well understood. It has two acidic groups with very different pK values: $pK_1 = 4.2$, $pK_2 = 11.8$. The term "ascorbate" is often used to describe this compound because it mainly takes the form of a monoanion at physiological pH values. Similar to most compounds capable of giving two electrons when being oxidized, the redox potential of ascorbate in aqueous solutions, +0.054 V, is comprised of redox potentials of two one-electron pairs: E'_0 of the pair $Asc^{\bullet -}$, $H^+/AscH^- = +0.282$ V, and E'_0 of the pair $DHA/Asc^{\bullet -} = -0.174$ V [2]. The dielectric constant of pyridine is lower than that of water (12.3 versus 80), and the pK and these potential values can vary somewhat, but it is significant that one-electron oxidation of the

ascorbic acid molecule is a very thermodynamically disadvantageous process, and Krasnovsky showed that it was light-excited chlorophyll that could participate in this process. The semi-oxidized form of ascorbate, which is usually referred to in literature as monodehydroascorbate (MDHA), is a radical; it has p$K = -0.86$ [3], and accordingly it is in the form of an anion radical at all pH values possible in living organisms.

Krasnovsky noted that "storage" of light energy, i.e. the increase in free energy of the system, takes place in systems where chlorophyll transfers the electron obtained from ascorbic acid to such acceptors as riboflavin, safranin, and pyridine nucleotides. He discovered similar effects also when studying heterogeneous systems [4, 5].

ASCORBATE AS AN ELECTRON DONOR FOR THE PHOTOSYNTHETIC ELECTRON TRANSPORT CHAIN

Having understood the general pathways of electron transfer in photosynthesis, Krasnovsky formulated the term "photosynthetic electron transport chain" (PETC) and then initiated studies that were intended to clarify the role of ascorbate as an electron donor in thylakoid membranes, a more complex system that chlorophyll solutions and colloid solutions of aggregated forms of this pigment. These works were published in Soviet journals in 1987 [6, 7]. Even though at the end of the 1960s certain data indicated that ascorbate could support DCMU-inhibited photoreduction of $NADP^+$ in thermally treated thylakoids of *Euglena gracilis* [8] and donate electrons in Tris-treated thylakoids of spinach [9], these facts were considered only as confirmation of the ability of the primary donors of photosystem 2 (PS2) to be re-reduced by easily oxidized electron donors. In the last paper data were presented showing that in the presence of DCMU ascorbate can donate electrons also to photosystem 1 (PS1).

Krasnovsky [6, 7] always considered the ability of ascorbate to donate electrons to the PETC to be of physiological importance. In these studies, it was shown that 50 mM ascorbate can act as an electron donor between the photosystems in the PETC, providing high rates of uncoupler-sensitive reduction of ferredoxin and $NADP^+$ in the presence of DCMU even without lipophilic mediators; the rate of reduction of ferredoxin reached 60 μmol/mg Chl per hour [6]. At this ascorbate concentration, the plastoquinone pool in pea thylakoid membranes was almost completely reduced after 15 min-long incubation; and rapid dark reduction of cytochrome *f* after its oxidation in the light could be observed [7]. It was suggested that under stress conditions, when PS2 is inactivated, electron transfer from ascorbate donating electrons to the PETC between photosystems can support all the necessary functions of the transport coupled to ATP synthesis. The same studies clearly stated the question of thylakoid membrane permeability for ascorbate; this question is still left without an answer (see below).

Within the next 10-20 years, other authors obtained numerous data on the ability of ascorbate to donate electrons, thus providing full PETC functionality. Japanese researchers, when working with isolated thylakoids [10, 11], showed that ascorbate could act as an effective electron donor for PS2 if the water-oxidizing complex was destroyed. Under these conditions, the affinity of ascorbate to PS2 was significantly higher than to PS1; the apparent K_m value for ascorbate as a PS2 donor was shown to be 2-4 mM [11]. It was shown that oxidation of ascorbate by such damaged PS2 resulted in the formation of MDHA, and electrons from ascorbate provided for reduction of $NADP^+$ at the rate of 50 μmol/mg Chl per hour in the presence of 20 mM ascorbate; the rate was only two times higher in intact thylakoids in the absence of an uncoupler [11].

A group of Hungarian scientists led by Prof. Garab [12-14] showed that high temperature treatment of *Arabidopsis* leaves of wild type and its *vtc2* mutant (the latter was ascorbate-deficient due to the reduced expression of GDP-L-galactose phosphorylase gene) resulted in ascorbate acting as an electron donor for PS2 *in vivo*. The half-time for electron donation by ascorbate was estimated to be in the range of 20-50 ms; it was also shown to depend on ascorbate concentration [13]. The data presented in this study also suggest that electron donation from ascorbate to PS1 *in vivo* is very low; according to the authors, the lumenal ascorbate concentration *in vivo* is not high enough to provide the same high donation rates as those observed in isolated thylakoids.

In experiments with bundle sheath cells isolated from maize leaves, it was shown that ascorbate from their chloroplasts could donate electrons to the PETC "between" the photosystems, i.e. to PS1 [15]. DCMU was added to these cells to stop electron flow from the very few PS2 complexes in their chloroplasts capable of oxidizing water; under these conditions, electron flow in these cells reached the rate of 50-100 μeq/mg Chl per hour with methyl viologen as the electron acceptor; ascorbate content in the incubation medium constituted 80 mM [15]. However, these rates were less than 10% of the electron transport rate under physiological conditions. Subsequent studies showed that ascorbate was a far more effective electron donor for PS2 than for PS1 in chloroplasts of intact bundle sheath cells from maize leaves that were not subjected to any treatment [16, 17].

PHYSIOLOGICAL EFFECTS OF ASCORBATE DONATING ELECTRONS TO THE PHOTOSYNTHETIC ELECTRON TRANSPORT CHAIN

Is there any physiological significance to the above-described ability of ascorbate to donate electrons to com-

ponents of the PETC? In their detailed review dedicated, in particular, to the analysis of possible functions of ascorbate in the lumens of thylakoids, Toth et al. [18] mainly focused on the fact that electron donation by ascorbate to PS2 was primarily important under stressful conditions, when electron donation from water is disrupted. It was shown that such a situation can be observed under the heat stress conditions [13], but it can also occur when PS2 is damaged by ultraviolet radiation [19]. The authors consider the absence of water-oxidizing complex or its malfunctioning to be one of the key conditions for ascorbate to be an effective electron donor, and then they suggest that such conditions may occur on the donor side of PS2 and under photoinhibition caused by strong light; they also think that ascorbate can protect this photosystem from photodegradation. In addition, in the case of synthesis and new assembly of PS2 components, when the reaction center is already able to separate charges but the water-oxidizing complex is not yet ready to transfer electrons, ascorbate (being an electron donor that does not require any special structure for its oxidation) can also protect the reaction centers, preventing the accumulation of $P680^+$.

Bundle sheath chloroplasts of leaves of grasses with C4-photosynthesis (the so-called malic enzyme species such as maize, sorghum, sugar cane, etc.) present a different situation. The amount of PS2 in mature parietal chloroplasts of these plant species is rather small, and it is either completely inactive in terms of oxidation of water, or this activity is minimal. Cyclic electron transport around PS1 (CET PS1) is the main pathway of photosynthetic electron transport in these chloroplasts; it is due to CET PS1 that the thylakoid membrane is energized, thus providing ATP synthesis necessary for CO_2 fixation in the Calvin cycle. Numerous studies [20-23] have shown that a certain reduction level of the plastoquinone pool ("redox poising") is required for CET PS1 functioning; this pool has to be reduced only partially so as to receive electrons from reduced ferredoxin. In the case of active functioning of PS2, low DCMU concentrations were added to stimulate CET PS1 in experiments on intact chloroplasts of C3-plants so as to prevent over-reduction of the plastoquinone pool under circumstances when the use of NADPH was limited and oxygen, being not so effective in this role, was the only final electron acceptor [22]. With a deficit of $NADP^+$, CET PS1 does not function under anaerobic conditions due to the complete reduction of the plastoquinone pool, and oxygen is required for the partial oxidation of the PETC [24]. However, under the usual aerobic conditions, oxygen oxidizes reduced ferredoxin and PS1 acceptors, taking electrons away from CET PS1, and this cycle cannot function without constant replenishment of electrons from the PETC to compensate their loss resulting from electron transfer to O_2 molecules. Nature seems to have created ideal conditions for CET PS1 in parietal chloroplasts of these C4-plants. PS2 in these chloroplasts have normal reaction centers capable of charge separation, but at the same time they are deficient in basic proteins of the water-oxidizing complex [25]. *In vitro* experiments have shown that disruption of the work of this complex is a prerequisite for ascorbate to act as a donor to PS2 (see above). According to the data of Ivanov et al. [16, 17], ascorbate is a very effective electron donor for PS2 in parietal chloroplasts *in vivo*, but the decreased number of reaction centers prevents over-reduction of the PETC section between the photosystems. Ascorbate provides electron flow sufficient for both initiation of CET PS1 and for the compensation of inevitable loss of electrons from the cycle due to their transfer to oxygen [17]. The strong wall of sheath cells is generally considered to prevent the leakage of CO_2 formed in these cells in the course of malate decarboxylation, but due to such a wall the supply of oxygen from the outside is also limited, and ascorbate oxidation produces no oxygen. Most of the electrons transferred to the PETC as a result of ascorbate oxidation reach PS1, and they are later transferred to O_2 molecules in the chloroplasts of sheath cells in the light (ferredoxin-NADP reductase is absent from these chloroplasts) [25]; as a result, O_2 concentration decreases in these chloroplasts. Replacement of water by ascorbate as a donor resulting in the absence of oxygen production has yet another advantage, namely it prevents the competition of O_2 molecules with CO_2 molecules for the catalytic sites of Rubisco, which is one of the reasons for increased growth rate of biomass of tropical grasses.

ASCORBATE CONTENT IN PLANT CELLS AND ITS BIOSYNTHESIS

Is there enough ascorbate in photosynthesizing cells to perform all the discussed physiological functions? Regarding the bundle sheath cells of maize leaves, direct measurements [26] including the average cell volume (0.5 ml/mg Chl [27]) give total ascorbate concentration in leaf cells of 114 mM, and the concentration of its reduced form is 74 mM. It was shown that in maize leaves ascorbate was primarily concentrated in the sheath cells; the reduced ascorbate pool was suggested to be maintained by transport of dehydroascorbate from parietal chloroplasts to mesophyll chloroplasts, which have the full PETC with actively functioning PS2, by dehydroascorbate reduction there and by the return of reduced ascorbate molecules to the sheath chloroplasts [26]. Thus, chloroplasts of bundle sheath cells of tropical grasses have all the necessary conditions for ascorbate to perform the role of a natural electron donor for PS2.

The ascorbate concentration in the chloroplasts of C3 plants was first reliably measured in 1983 [28, 29], and it was shown to be very high: 10-30 mM. Later studies showed ascorbate concentrations to be even higher, 25-50 mM [30-33]. In the highland plant *Soldanella alpine*,

ascorbate concentration reaches the value of 200 mM [34]. Interestingly, ascorbate content in algae and plant cells varies in accordance with the circadian rhythm [35].

Since humans and a number of other mammals cannot synthesize ascorbate, they need to get it from food, primarily from plants, for their normal existence. The main ascorbate biosynthesis pathway in plant cells (the Smirnoff–Wheeler pathway) has been fully described relatively recently [36, 37]. It starts with glucose, which after several reactions is transformed into L-galactose, and it (with catalysis by L-galactose dehydrogenase) turns into L-galactono-1,4-lactone; the latter is oxidized to ascorbate in mitochondria, L-galactono-1,4-lactone dehydrogenase being involved in this process, and this enzyme is absent from human cells. Even before the complete decoding of the ascorbate biosynthesis pathway, strong light was found to increase ascorbate content [30, 31]. Probably, the slow, several days-long changes in ascorbate content in leaves caused by light increase or decrease is due to the changes in its biosynthesis rate [31, 32, 37, 38]. According to the accumulated data, it is the *VTC2* gene that is responsible for these changes, because its expression and the activity of the enzyme that it encodes were shown to increase in response to increased light. Ascorbate biosynthesis is assumed to depend on the redox state of the PETC [39].

ROLE OF ASCORBATE IN PHOTOSYNTHESIS FOR PROTECTION AGAINST REACTIVE OXYGEN SPECIES AND PHOTOINHIBITION

In addition to the above-discussed role of ascorbate in the maintenance of photosynthetic electron transport under both normal and stress conditions, ascorbate is also required in many other processes taking place in plants. Its roles in synthesis of cell wall and in cell ontogenesis [40], in regulation of stomatal movement [41], in regulation of gene expression both by ascorbate molecules *per se* [42] and in combination with other signaling molecules [43] have been described (for details on the role of ascorbate in redox-signaling, see [44]). Its task is to reduce organic and inorganic substrates and important functional groups of enzymes. Currently, however, scholars and practitioners focus on the role of ascorbate in detoxification of reactive oxygen species (ROS). This detoxification can proceed both as a normal physicochemical reaction and as an enzymatic process. ROS are produced in all cellular compartments, and ascorbate is present in the cytoplasm, chloroplasts and mitochondria, vacuoles, cell wall, and apoplast [45, 46].

Ascorbate plays a special role in chloroplasts, where even under normal conditions the rate of ROS production in the light per volume unit is probably the highest in living organisms. Ascorbate is a component of the so-called ascorbate–glutathione cycle, a process ensuring protection of photosynthetic components located in chloroplast stroma. Hydrogen peroxide is the main danger for photosynthesis in healthy chloroplasts as it oxidizes primarily sulfhydryl groups of cell proteins. It is linked to its inhibitory effect on key enzymes of the Calvin cycle: fructose-1,6-bisphosphatase, glyceraldehyde-3-phosphate dehydrogenase, and ribulose-5-phosphate kinase [47]. Experiments with intact chloroplasts of higher plants have shown that 10 μM H_2O_2 inhibits CO_2 fixation by 50% [48]. Such H_2O_2 concentration in the stroma should be reached in less than 1 s at regular rates of electron transport along the PETC, even if only 1% of electrons are transferred from PETC carriers to O_2.

Catalase has not been found in chloroplasts, and detoxification of H_2O_2 is carried out by ascorbate peroxidase (APO) in them [49-52]. Two APO isoforms have been found in chloroplasts: stromal (sAPO) and thylakoid membrane-bound (tAPO) [53]. Two ascorbate molecules (Asc) take part in the ascorbate peroxidase reaction:

$$H_2O_2 + 2\ Asc \rightarrow 2\ MDHA + 2\ H_2O.$$

The K_m for catalase is 20-25 mM H_2O_2, while the K_m values of chloroplast APO are in the micromolar range: for sAPO and tAPO they are, respectively, 80 and 23 μM [50, 53]. It is this feature that allows APO to reduce H_2O_2 concentration to a value that is harmless for the enzymes of the Calvin cycle.

The APOs lose activity irreversibly within several seconds in medium without ascorbate and even at its low concentration [53, 54]. This seems to be a likely reason for high ascorbate concentration in chloroplast stroma. Ascorbate concentration in chloroplasts substantially exceeds the K_m (Asc) value in the ascorbate peroxidase reaction (0.5 mM) [53]. Studies of H_2O_2 accumulation in the light in chloroplasts of wild type and mutant *Arabidopsis* (mutants were characterized by different levels of ascorbate deficiency) have shown that in the case of chloroplasts of the *vtc2-2* mutant with ascorbate content being only 10% of that in the wild type plants [33], H_2O_2 accumulation was considerably higher, and in chloroplasts of the *vtc2-3* mutant with ascorbate content up to 30% of the wild type, there was hardly any difference in this parameter when compared to the one observed in wild-type chloroplasts [55, 56]. These data indicate that less than a third of the ascorbate amount present in wild-type plants is sufficient for the effective functioning of APO.

MDHA formed in the ascorbate peroxidase reaction can dismutate, producing Asc and dehydroascorbate (DHA); the rate constant of this reaction at pH 7.9 is $7 \cdot 10^4$ $M^{-1} \cdot s^{-1}$ [57]. In the stroma, DHA is reduced to ascorbate by reduced glutathione (dehydroascorbate reductase is involved in this reaction), and glutathione is maintained in a reduced state due to NADPH in the reaction catalyzed by glutathione reductase. Direct reduction of MDHA to ascorbate is also possible in chloroplasts –

enzymatically by NADPH and monodehydroascorbate reductase, or directly by PETC components. The latter process competes with electron transfer to $NADP^+$ and thus prevents NADPH formation. The rate of reduction of MDHA reduced by ferredoxin is 30 times higher than the rate of $NADP^+$ reduction [58], and MDHA reduction by membrane-bound carriers competes even with reduction of methyl viologen [59, 60]. Generally, available data suggest that PETC-mediated ascorbate regeneration in chloroplasts has priority over reduction of CO_2 in the Calvin cycle [61].

Water molecules oxidized in PS2 serve as electron donors both for O_2 reduction and H_2O_2 formation and for regeneration of ascorbate and glutathione; water is also the final product of detoxification of H_2O_2. This is the reason for naming the complex of reactions of H_2O_2 formation, its detoxification, and regeneration of ascorbate the water–water cycle [62].

Peroxiredoxins can also participate in H_2O_2 reduction in chloroplasts; it is assumed that their ability to reduce H_2O_2 can constitute up to half of such property of soluble APO, and moreover they can reduce peroxides of organic molecules [63, 64]. These proteins function as peroxidases with one difference: it is a sulfhydryl group of the peroxiredoxins that acts as electron donor:

$$\text{Per–SH} + \text{R}'\text{–OOH} \rightarrow \text{Per–S–S–R}'' + \text{R}'\text{–OH} + \text{H}_2\text{O},$$

where R′–OOH is organic peroxide or H_2O_2, and R″, depending on peroxiredoxin type, is a protein molecule, where an intramolecular disulfide bond is formed, or another molecule of a dimeric protein, where the oxidized molecule forms an intermolecular disulfide bond. Ascorbate along with thioredoxins, glutathione, glutaredoxins, and cyclophilins participates in regeneration of the sulfhydryl group from the disulfide bond.

In addition to participating in enzymatic detoxification of H_2O_2, ascorbate can participate in nonenzymatic reactions with other ROS ($OH^{\bullet}$, $O_2^{\bullet-}$, and $^1O_2$) protecting chloroplasts from their destructive action. The rate constant of the reaction of $O_2^{\bullet-}$ with ascorbate,

$$2\ O_2^{\bullet-} + 2\ H^+ + \text{Asc} \rightarrow 2\ H_2O_2 + \text{DHA},$$

is rather high ($3.3 \cdot 10^5\ M^{-1} \cdot s^{-1}$ at pH 7.8 [65]); it is much higher than the rate constant of the reaction of $O_2^{\bullet-}$ with glutathione, 10^2-$10^3\ M^{-1} \cdot s^{-1}$ [66]. The water-soluble ascorbate is also involved in the maintenance of the membrane pool of liposoluble antioxidant, tocopherol, in a reduced state [67].

Ascorbate plays an important role in protection of photosynthesis against photoinhibition, which develops at high light intensity, and this role is not connected to neutralization of ROS. Increasing thermal energy dissipation in the pigment matrix due to an increase in antheraxanthin and zeaxanthin content (violaxanthin de-epoxidase, VDE, is involved in their formation) in pigment–protein complexes is known to be one of the ways to protect photosynthetic apparatus from photoinhibition and photodestruction [68, 69]. VDE is located in the thylakoid lumen and is associated with the thylakoid membrane, and ascorbate serves as a highly specific donor for de-epoxidation of violaxanthin, the latter process being activated by decrease in pH inside the thylakoids. *Arabidopsis* mutants with reduced ascorbate content made it possible to evaluate its physiological significance in this process. The rate of zeaxanthin formation was lower, and its level was lower in these mutants when compared to the wild-type plants [70]. APO and VDE can compete for ascorbate, which was revealed in experiments with isolated spinach thylakoids [71] and intact maize chloroplasts [72]. The reason for this competition lies in the fact that APO catalyzes reactions in the stroma and stromal surface of the thylakoid membrane, and the reaction of de-epoxidation proceeds on its inner side. When using ascorbate in the stroma, its entry into the thylakoids can be significantly reduced, thus slowing the de-epoxidation reaction: because of the higher K_m value for ascorbate (3 mM), the reaction was completely stopped by the addition of hydrogen peroxide to thylakoids, and it resumed only after exhaustion of peroxide [71].

Although under normal growth conditions there is usually no damage observed in ascorbate-deficient *vtc* mutants, these mutants are more sensitive to such stressful conditions as ultraviolet radiation [73], drought [74], and salinization [75]. This clearly shows the role of ascorbate as a compound required by plants for normal metabolism under adverse conditions.

ASCORBATE TRANSPORT IN PLANT CELLS

Since ascorbate is formed in mitochondria, its presence in all parts of cells requires its diffusion across many cellular membranes. Specific ascorbate and dehydroascorbate carriers have been found in animal cells, but no similar proteins and genes encoding such proteins have been found so far in plant cells [18]. The mechanism of diffusion of ascorbate in plant cells is still a matter of debate, although many attempts have been made to disclose it. This issue was also addressed in the work of Krasnovsky [7], where he concluded that ascorbate was able to penetrate the thylakoid membrane, although at a low rate. It is logical to assume that only the protonated neutral form of ascorbic acid can diffuse across membranes, which have considerable negative charge on the surface at physiological pH values. However, the concentration of this form is rather low at such pH values: at ascorbate concentration 10 mM and pH 7.6, it is only 3.8 μM. Furthermore, the solubility of ascorbic acid in alcohol is 10 times less than in water, and it is practically insoluble in organic solvents.

Considerable rate of ascorbate transmembrane transport is required to ensure its participation in the reactions both in chloroplast stroma and in thylakoid lumen. Experimental data indicate that the ascorbate molecule can be transported across biological membranes as an anion. It was suggested that specific carriers ensure facilitated diffusion of ascorbate across both plasmalemma [76] and the chloroplast outer membrane [32, 77, 78]. The initial rate of ascorbate flow into an isolated intact spinach chloroplast at outer ascorbate concentration of 10 mM was shown to be 108 μmol/mg Chl per hour at 18°C [77]. Measurement of the rate of inflow of labeled ascorbate into intact pea chloroplasts at the same temperature and concentration gave a significantly smaller value, 2.2 μmol/mg Chl per hour [78]. These facts indicate the difficulties of studying the mechanism of ascorbate transmembrane transport into chloroplasts.

The situation with ascorbate transport from chloroplast stroma to thylakoid lumen is even less clear. Interpretation of data obtained on isolated thylakoids [78] should be taken with caution because of possible modifications of the membrane during the isolation. Since ascorbate quickly leaves the thylakoid lumen during extraction of whole cells from plants [13, 72], we can assume that it can easily cross the thylakoid membrane. It was suggested that a specific ascorbate carrier functions in the thylakoid membrane as well as in the outer chloroplast membrane [15, 32]. The initial rate of ascorbate flow from the medium to the thylakoid lumen of intact bundle sheath cells of maize leaves, estimated from the rate of $P700^+$ reduction, was found to be 128 μmol/mg Chl per hour at the outer ascorbate concentration of 10 mM [16], this value being even somewhat higher than the rate given above for ascorbate flow into isolated intact chloroplasts.

Toth et al. [18] considered the possibility of the ascorbate pool in the lumen being maintained in the reduced state not due to the inflow of its reduced molecules, but due to regeneration of the formed MDHA by external ascorbate molecules on the transmembrane reductase, as suggested for plasmalemma [79]. DHA molecules were suggested to be transferred to the lumen by a specific carrier, as in the case of plasmalemma [80].

Although currently we cannot exclude the involvement of carriers in ascorbate transfer across the plant cell membrane, in case of cells where ascorbate is synthesized and takes part in many processes we can assume the existence of special channels ensuring its unimpeded distribution to cells and tissues. The permeability of these channels for ascorbate is probably regulated by the redox state of the ascorbic acid pool. Very similar values were obtained for ascorbate flows (calculated per chlorophyll) into protoplasts, i.e. across the plasmalemma, and into intact chloroplasts, i.e. across its outer membrane [78]; it seems interesting that the areas of protoplast and chloroplast membranes (also calculated per chlorophyll) are very similar [81]. The thylakoid membrane area when expressed in the same units is about 10 times higher [81], and if the mechanism of transmembrane diffusion is universal, it ensures rapid ascorbate flow from the stroma into the thylakoid lumen, where it is also needed.

Krasnovsky et al. [6] also discussed the presence of high concentrations of ascorbic acid (tens of millimoles per liter) in cells and chloroplasts. Given the described functions of ascorbate as electron donor in many cellular processes, the apparent ascorbate excess in plant cells and primarily in chloroplasts is probably a necessary safety margin to ensure normal cell functioning in case of some problems with ascorbate biosynthesis and/or regeneration. Krasnovsky et al. [6] emphasized that due to their use of ascorbate concentrations close to physiological ones, they could observe its role as an electron donor to the PETC. Such concentrations are typically not used when working with isolated chloroplasts and thylakoids. Perhaps repeating some of the early experiments in media with high ascorbate content would enable a more adequate understanding of the processes taking place in the photosynthetic apparatus *in vivo*.

I express my appreciation to Dr. M. A. Kozuleva for her assistance in writing this review.

The work was supported by the Russian Foundation for Basic Research (grant No. 12-04-00320).

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