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Review

Ascorbic acid: much more than just an antioxidant

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Abstract

Vitamin C (ascorbic acid (AA)) is very popular for its antioxidant properties. Consequently, many other important aspects of this multifaceted molecule are often underestimated or even ignored. In the present paper, we have tried to bring to the foreground some of these aspects, including the peculiarities of the AA biosynthetic pathway in different organisms, the remarkable function of AA as a co-substrate of many important dioxygenases, the role of AA-regenerating enzymes and the known pathways of AA catabolism, as well as the intriguing function of AA in gene expression.

Keywords: Ascorbic acid; Dehydroascorbic acid; Oxoglutarate-dependent dioxygenase

1. Introduction

Since its discovery in the late 1920s [1], probably no other chemical has ever been as celebrated as ascorbic acid (AA). The beneficial effect of vitamin C is almost universally recognised. The reason for this unprecedented popularity is probably linked on one hand to common sense (AA is present in relatively high amounts in fruit and vegetables, which are known to be healthy), and on the other hand – especially today – to expensive advertising campaigns for vitamin C-based products.

Until a few decades ago, the most common answer to the question 'what is the function of vitamin C?' would have been the same given by Albert Von Szent Györgyi in 1937, when he was awarded the Nobel Prize for the discovery of AA: it is the factor able to cure the variety of clinical symptoms known as scurvy, a syndrome occurring in humans whose diet is deficient in fresh fruit and vegetables. At that time, however, little molecular explanation for this effect was available. Nowadays, the same question would receive a different answer. Not many would mention scurvy, as this pathological state is no longer very common. Some specialists in the field would explain that the inability of humans to synthesise AA is due to the lack of L-gulono-lactone oxidase (GulL-ox), the last enzyme in its biosynthetic pathway. A large majority of people asked (irrespective of their scientific education) would promptly answer that AA is an antioxidant which efficiently scavenges toxic free radicals and other reactive oxygen species (ROS) formed in cell metabolism. Actually, ROS are associated with several forms of tissue damage and disease and also with the process of ageing [2]. Aerobic organisms have evolved intricate and interrelated processes for protection against the effects of free radicals and derived toxic species, including both enzymatic and non-enzymatic defences. Enzymatic mechanisms include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-px) and, in plants, ascorbate peroxidase (AA-px).

According to the generally assumed model of enzyme-mediated removal of ROS [3], SOD catalyses the disproportionation of superoxide anion to H_2O_2 and oxygen; in turn H_2O_2 is converted by CAT into water and molecular oxygen. CAT turnover number is very high, but its affinity for H_2O_2 is relatively low, and consequently a certain amount of H_2O_2 remains in the cell. This is potentially troublesome, since H_2O_2 can react with superoxide anion formed in oxidative metabolism and generate the highly reactive hydroxyl radical. GSH-px and AA-px are capable of removing low amounts of H_2O_2 due to their high affinity for H_2O_2. Thus, the co-operativity of SOD, CAT and peroxidases ensures low levels of superoxide anion and H_2O_2 and therefore limits the risk of hydroxyl radical formation (Fig. 1). In addition to enzymatic mechanisms, some compounds, known as antioxidants, are present in the cell that, on entering into redox reactions, contribute

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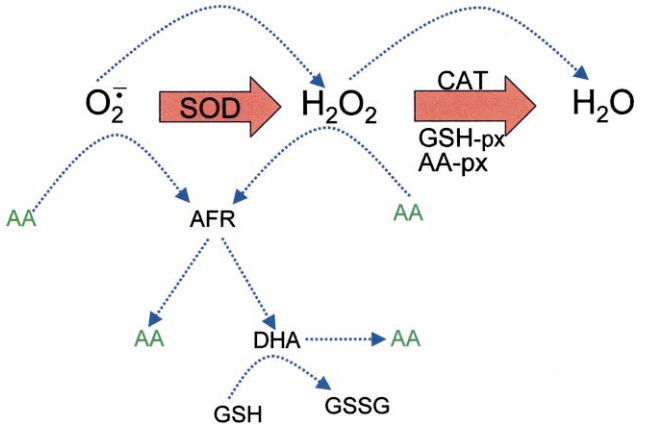

Fig. 1. The role of AA in the detoxification of ROS. Blue dotted lines indicate non-enzymatic reactions.

an electron to neutralise free radicals to non-reactive species. Many chemicals could serve this purpose because the high reactivity of free radicals results in extracting an electron from almost any available molecule. However, an efficient biological antioxidant is supposed to do more than simply react with free radicals: (a) it must be present in an adequate amount in the cell, (b) it must react with a variety of free radicals, and (c) it must be suitable for regeneration [4]. This combination of properties is typical of AA; therefore this compound is considered the perfect antioxidant for the cells of nearly all aerobic organisms. However, it should be considered that under several circumstances ROS and free radicals in general also play a pivotal role in signal transduction and the mechanism of enzyme action [5,6], thus indicating that the function of antioxidants is the fine regulation, rather than total extinction of free radicals. Moreover, in some cell compartments (i.e. plant cell walls) AA regeneration is impaired due to the absence of NAD(P)H and GSH. Eventually, if AA can be considered an efficient antioxidant and consequently an efficient free radical scavenger, it cannot be ignored that under some circumstances (such as the presence of metal ions and adequate pH conditions) AA has a pro-oxidant and even mutagenic effect [7–9].

All the above-mentioned observations lead us to conclude that deciphering the details of the antioxidant action which is attributed to vitamin C it is still very difficult for at least two reasons: (a) we lack reliable analytical methods to accurately measure AA distribution in different cell compartments, and (b) AA antioxidant action takes place by means of non-enzymatic (and therefore more difficult to predict) reactions, excepting AA-px. In addition, considering the key role of AA in cell metabolism to be limited to its antioxidant action could be misleading. In fact, this approach does not take into consideration many AA-dependent metabolic reactions that influence essential physiological processes, ranging from cell division to gene expression and the activation of biological defence mechanisms [10].

The aim of the present article is to summarise our present knowledge on the AA system in both animals and plants, in order to demonstrate that the pleiotropic action of vitamin C is, in all organisms, largely due to its function as a co-substrate of dioxygenases, a versatile family of enzymes. Moreover, we have also tried to suggest new directions for research, in order to give a three-dimensional portrait of this very well known, but still poorly understood compound.

2. Biosynthesis and evolution

Biosynthesis of AA was a relatively late event in evolution. Apparently neither AA nor AA-related enzymes are present in *Escherichia coli* or other bacteria [11]. The presence of D-araboascorbic acid was detected in *Penicillium* [12], whereas *Saccharomyces cerevisiae* and *Candida albicans* can synthesise the 5-carbon AA analogue D-erythroascorbic acid (EAA) [13] via D-arabinose and D-arabino-no-1,4-lactone [14]. All plants can synthesise AA at high rates following a four-step pathway (the Smirnoff–Wheeler pathway) which proceeds from GDP-D-mannose, via GDP-L-galactose, L-galactose and L-galactono-1,4-lactone (GalL), but possible minor biosynthetic pathways have not been excluded [15]. Almost all animals synthesise AA via UDP-D-glucuronic acid, glucuronic acid/glucuronolactone, L-gulonic acid/L-gulono-1,4-lactone [16,17], with the exception of humans, some primates, guinea pigs, some birds and teleost fishes [18]. The final steps of the three main pathways of AA synthesis are compared in Fig. 2.

It has been suggested that AA biosynthesis first appeared in amphibians, in connection with the passage of early tetrapods from an aquatic to a terrestrial environment, where oxygen could actually be a threat [18]. However, this hypothesis does not fit with more recent findings on the presence of AA biosynthetic enzymes in aquatic organisms, such as archeopterigian fishes and lamprey [19,20]. In support of the hypothesis that AA biosynthesis evolved side by side with the increase in atmospheric oxygen, it has been observed that animals which have lost the capability to synthesise AA (guinea pig, bat, monkey and man) show higher SOD activities in both liver and kidneys [21]. Following this line of reasoning, however, cyanobacteria, being the first organisms to produce O_2, should also have been the first ones to sense it and to find a way to avoid ROS accumulation; in this case, AA would have been a useful tool. Unfortunately, scarce and contradictory information is available concerning AA biosynthesis and metabolism in cyanobacteria [22].

It is now clear that man and other animals cannot synthesise AA because the gene encoding L-gulono lactone oxidase (GulL-ox), the enzyme catalysing the last step in AA biosynthesis, is not functional anymore [23]. Rat GulL-ox expression in guinea pig cells yielded AA [24].

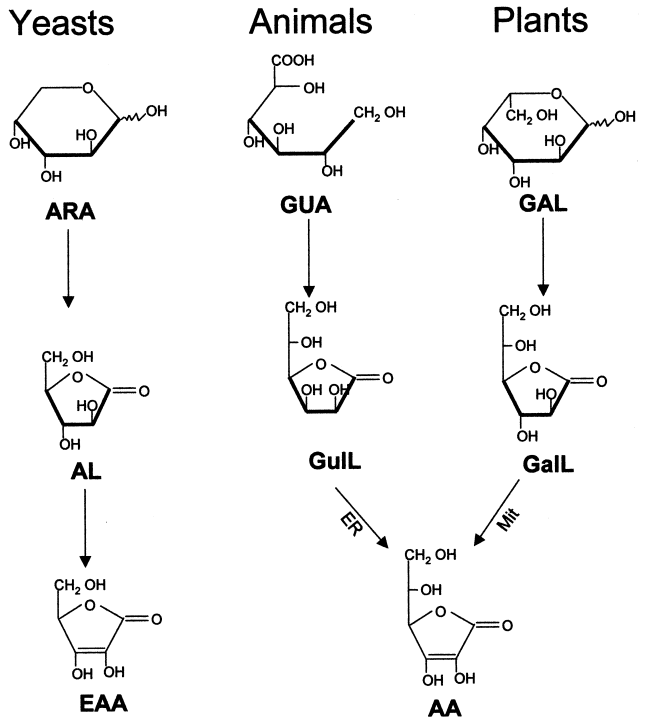

Fig. 2. A comparison of EAA and AA biosynthesis in different organisms. Only the final two steps occurring in yeasts, animals and plants are reported. ARA: D-arabinose; GUA: D-gulonic acid; GAL: L-galactose; AL: D-arabinono lactone; GulL: L-gulono lactone; GalL: L-galactono lactone. EAA: D-erythroascorbic acid; AA: L-ascorbic acid. GulL conversion to AA occurs in the ER, whereas GalL conversion occurs in mitochondria (Mit).

This confirms earlier reports showing that previous steps in the biosynthetic pathway are still operating in organisms which do not synthesise AA [17], although it is not clear whether the enzymes catalysing such activities are involved in other metabolic pathways. Surprisingly, this point has almost completely been neglected by scientists. It has been suggested that the loss of function of the GulL-ox gene, and the consequent impaired synthesis of the antioxidant AA, increased the mutation rate in the genome of our ancestors, inducing faster evolution of Anthropoidea and *Homo sapiens* [25,26]. On the other hand, the opposite could also be true: since GulL-ox activity produces hydrogen peroxide, it cannot be excluded that the loss of GulL-ox activity could have been a favourable trait in terms of evolution [9]. The fact that all other enzymes in the animal biosynthetic pathway are still present in guinea pig and man could be in line with this hypothesis. It should also be considered that even in AA-synthesising organisms AA is apparently not produced during early life stages, as in the case of newborn rats [27] and in developing seeds of plants [28].

The location of AA biosynthetic enzymes is another interesting issue. In primitive fishes, amphibians and reptiles, AA synthesis takes place in kidneys, whereas the liver is the site of synthesis in mammals [20,21]. A more complex situation is observed in birds, where all three conditions have been observed (kidney, liver, or no synthesis) [29]. In plants, virtually all cells synthesise AA, but the synthesis is much higher in juvenile (meristematic) cells [30].

Subcellular location of AA biosynthetic enzymes also shows peculiar features in different organisms. In plants, the first steps of AA synthesis are likely to be cytosolic, whereas GalL dehydrogenase, catalysing GalL conversion into AA, is located at the inner mitochondrial membrane [31]. Similarly, AA biosynthesis in animals is carried out by soluble enzymes, apart from the last step catalysed by GulL-ox, which is located in the endoplasmic reticulum (ER) [16,21].

It has been suggested that the transfer of the AA biosynthetic machinery in animals from the kidney of cold-blooded reptiles to the much larger and more active liver of mammals reflected the increased need for AA due to increasing atmospheric oxygen [21]. Whatever the reason for this change, it is surprising that a compound so very useful in high concentrations in all organs and cell compartments (the more, the better!) is only synthesised in a single organ. The comparison with hormones, synthesised in a specific site and then transferred to their different targets, is clearly not consistent, as hormones are effective at very low concentrations and, in general, hormone targets are represented by a few cells responding to the hormone stimulus. On the other hand, the reason for localised AA synthesis could reside in the necessity to keep it under tight control, suggesting that generalised and unleashed AA synthesis could be as dangerous as AA deficiency. The direct consequence of limiting to a single organ the synthesis of an essential compound, required for the metabolic activity of all cells, has been the development of short- and long-range efficient AA transport. The presence in AA-synthesising organisms of a specific delivery system capable to chaperone AA and avoid oxidative processes during its transport has been hypothesised [10], but as yet there is no direct evidence of such a mechanism.

The evolutionary reason for the presence in mitochondria of the final step of the AA biosynthetic pathway in plants is not clear. It is known that AA synthesis is linked with the mitochondrial electron transport chain and that GalL dehydrogenase seems to utilise cytochrome *c* as a specific electron acceptor [31], but this point requires further investigation. As discussed below, the location in the ER of the last step of AA biosynthesis in animals is a remarkable example of biological organisation.

More information is available concerning exogenous vitamin C uptake, which is very important in non-synthesising organisms. In animals, both AA and dehydroascorbic acid (DHA) are actively transported. However, the oxidised vitamin C form DHA is preferentially transported across plasma membranes by means of the GLUT1 glucose transporter [32].

In plant cells DHA is also actively transported across the plasma membrane [33]. This mechanism is tightly as-

sociated to DHA reduction to AA and seems to be less relevant in the framework of the AA system. In plants, all cells (both photosynthetic and non-photosynthetic) can synthesise AA de novo from sugar precursors, therefore the exchange of AA and DHA between cells is less important. It is possible to hypothesise an AA long-range transport mechanism in plants from young leaves and reproductive organs. This is consistent with the observation that the *Vicia faba* embryo has a high AA content despite not being able to synthesise AA until 30 days after anthesis, as GalL dehydrogenase is not functional at this stage [28]. Developing seeds acquire the capability to oxidise GalL to AA just 10 days before the onset of the desiccation period. A few days before entering the resting state, the seed is endowed with the entire AA biosynthetic pathway, so that the seed can promptly synthesise the amount of AA needed to re-start metabolic activity at the onset of germination. From this stage on, the AA biosynthetic machinery is always functional, and appears to be regulated by a feedback mechanism (Fig. 3). Freshly harvested potato tubers show high endogenous AA content and are not capable of synthesising AA when fed with glucose. AA content gradually decreases during storage, along with an increase in the capability to synthesise AA from glucose [34]. Similar data have recently been obtained by studies with ^{14}C-labelled glucose in pea seedlings pre-loaded with AA [35]. Furthermore, GalL administration strongly induces AA synthesis irrespective of endogenous AA content, clearly indicating that the feedback mechanism operates upstream of GalL dehydrogenase (Fig. 3).

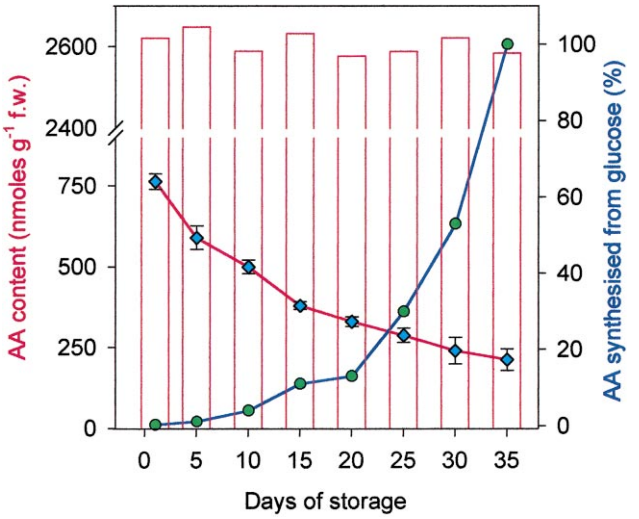

Fig. 3. The feedback mechanism of AA biosynthesis in plants. Freshly harvested potato tubers were stored and their endogenous AA content (red line) measured at different time points. Biosynthetic capability was measured in potato tuber slices fed for 24 h with 5 mM D-glucose (blue line). Administration of 5 mM GalL to slices for 24 h always markedly increased AA content (red bars). Modified from [34].

3. AA-dependent dioxygenases (AADs)

Dioxygenases catalyse the incorporation of O_2 into an organic substrate. This class of proteins includes a wide variety of enzymes, which differ in substrate requirement and mechanism of action. A subgroup of dioxygenases require oxoglutarate for their catalytic activity, and are therefore usually referred to as '2-oxoglutarate-dependent dioxygenases' [36]. In turn, some of these enzymes specifically require AA, and at least in one case (the plant enzyme 1-aminocyclopropane-1-carboxylate oxidase) AA is specifically required as a co-substrate, whereas 2-oxoglutarate is not [37]. Hence, from this point on, we will refer to these enzymes as AADs. In addition, extending to all AADs the nomenclature previously used by Kivirikko [38] to describe the mechanism of prolyl-hydroxylase (see below), we suggest the use of the term co-substrate for AA, rather than the commonly used cofactor.

The identification of AA as a co-substrate of AADs is a long story, taking its origins from the circumstantiated clinical observations of James Lind, who in 1747 observed that an unknown compound present in fruit and vegetables was able to cure scurvy [39]. It took two more centuries to identify AA as the antiscorbutic factor, and some more decades to understand its biochemical action. By the early 1960s, AA involvement in the processes of collagen synthesis, tyrosine oxidation, corticosteroid synthesis and aromatic hydroxylation had been more or less ascertained ([40] and references therein), but the biochemical basis of AA functions would only be fully understood some years later, starting with the discovery of the post-translational modification of peptidyl-prolyl residues by prolyl-4-hydroxylase (P4H) [41]. Data on the specificity of AA requirement for P4H activity were questioned by Udenfriend and co-workers on the basis of in vitro experiments, in which other agents were capable of taking over AA function in collagen hydroxylation [42]. Further experiments have demonstrated that inhibition of P4H activity in AA-synthesising organisms induces an increase in AA content in vivo [43,44], thus clearly indicating that P4H activity actually consumes AA.

Hydroxylation at carbon 4 of proline residues incorporated in polypeptide chains is a post-translational modification which is extremely important for protein folding and secretion. In vertebrates, P4H is a tetramer made up of two α- and two β-chains. The hydroxylation reaction is catalysed by α-subunits, whereas the role of β-subunits is not yet clear. Notably, it has been ascertained that β-subunits and protein disulfide isomerase (PDI) are the product of the same gene [38], but at present the role of the PDI protein as a subunit of P4H is not clear. The association between α- and β-subunits is much less constant in the plant enzyme [45]. P4H resides in the ER and recognises specific proline-containing sequences [38].

Hydroxyproline residues are typically present in proteins of the extracellular matrix: collagen in animals, and an array of different plant proteins (generically known

as hydroxyproline rich glycoproteins, HRGPs) ranging from structural elements to proteins involved in developmental processes [46]. Interestingly, in the absence of efficient hydroxylation of peptidyl-prolyl residues, all these proteins are non-functional and are rapidly degraded in the ER, whereas correctly hydroxylated proteins are exported and localise in the extracellular matrix [47]. In animals, collagen underhydroxylation is not only the main cause of scurvy, but also of other clinical disorders such as Ehlers–Danlos syndrome [48]. In plants, the inhibition of P4H activity induces dramatic alterations to cell wall regeneration and cell division in tobacco protoplasts [49] and markedly affects cell morphology and development of onion roots [44].

Collagen maturation is a complex multi-step process, in which, after protein synthesis and hydroxylation of peptidyl-prolyl residues, stable trimers are formed by a mechanism involving thiol/disulfide exchanges [50]. It is noteworthy that, in animals, AA biosynthesis, P4H activity and collagen maturation occur in the same cell compartment (the ER). A scheme of all these activities related to the AA system is reported in Fig. 4. As mentioned above, this intricate network of functions and activities is a fascinating example of biological organisation. We hypothesise the co-operative action of the β-subunits of P4H and the ascorbate system to ensure correct folding and the formation of the triple helix of collagen. The hydroxylation process catalysed by P4H α-subunits, utilising AA as an electron donor, generates ascorbate free radical (AFR, see below). This radical is in part recycled to AA by AFR reductase, which is present in the ER, but part of AFR is thought to escape reduction and spontaneously disproportionate yielding DHA. Such DHA could be the physiological electron acceptor necessary to form the disulfide bonds necessary for protein folding. Moreover, it has recently been reported that both PDI and the heat shock protein HSP47 bind to underhydroxylated procollagen synthesised in the absence of AA and display co-operative but distinct chaperone functions during procollagen biosynthesis [51].

Beside P4H, other AADs are affected by AA deficiency. Carnitine synthesis requires the sequential action of two AADs: ε-*N*-trimethyl-L-lysine hydroxylase and γ-butyrobetaine hydroxylase [52]. In addition, AA administration induces carnitine synthesis in guinea pigs [53]. Weakness, which is the first detectable symptom of scurvy, has been correlated with incorrect functioning of these enzymes. We have already mentioned that AA involvement in 'tyrosine oxidation' has been known for five decades [54]. This reaction is catalysed by tyrosine hydroxylase and represents the rate-limiting step in the biosynthesis of the catecholamines dopamine, norepinephrine, and epinephrine [55]. 4-Hydroxyphenylpyruvate dioxygenase catalyses the formation of homogenisate from 4-hydroxyphenylpyruvate and molecular oxygen [56]. Aspartyl (asparaginyl) β-hydroxylase activity is responsible for the post-translational modification of Asp residues present in EGF-like domains [57].

AADs operate in plants for the synthesis of flavonoid and important hormones: gibberellic acid, ethylene and abscisic acid (ABA) [58]. The pathway of ABA biosynthesis [59] has striking similarities to the synthesis of carotenoids involved in visual processes [60]. Deoxyhypusine hydroxylase, an enzyme catalysing the last step of the post-translational modification of a specific lysine residue forming the unusual amino acid hypusine, is present in the eukaryotic initiation factor 5A [61]. Interestingly, inhibi-

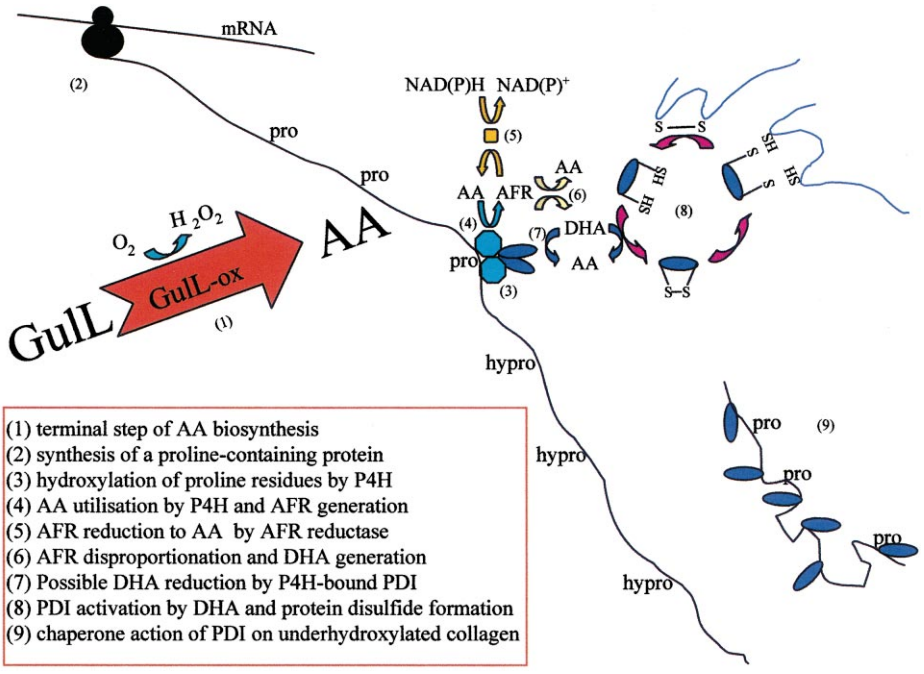

Fig. 4. The AA system in the ER. See text for details.

tion of deoxyhypusine hydroxylase activity blocks the G1–S transition of the cell cycle [62].

The list of AADs identified so far is quite long, and it is conceivable that some more will be identified in the near future. An exhaustive listing of known AADs is far beyond the scope of this article. However, we would like to stress that so many different enzymatic functions all requiring AA may well be the basis to understand the pleiotropic action observed for this compound.

4. AFR and DHA reduction to AA: simple regeneration or more complex role?

Dioxygenase activity and all other AA-requiring reactions (either enzymatic and non-enzymatic) generate AFR (also referred to as monodehydroascorbate radical). Several reports indicate a specific biological role for AFR, which is apparently involved in growth processes in both plant and animal cells [63,64]. It has been suggested that such AFR action could be mediated by a *trans*-plasma membrane electron transport system [65]. It is unclear whether other enzymatic AFR-requiring reactions actually occur, but this field has not been fully explored. On the other hand, it is well-established that AFR spontaneously disproportionates yielding DHA (and AA). At the present time, there is no evidence of reactions catalysing direct oxidation of AA to DHA, therefore intracellular DHA is apparently exclusively due to AFR disproportionation.

Besides disproportionation, there is another possible fate for AFR: it can be reduced back to AA by NADH-dependent AFR reductase (EC 1.8.5.3). This enzyme was identified in different organisms in the 1950s [66–70]. As for the location of AA biosynthetic enzymes, the subcellular distribution of AFR reductase in different organisms is an intriguing topic. In animals, AFR reductase activity is known to be located both in the ER, mitochondria [71] and plasma membrane, the latter depending on cytosolic NAD(P)H in an electron transport system mediated by CoQ inserted in the bilayer [72,73]. In plants, AFR reductase is located in the cytosol, chloroplasts and mitochondria [70,74,75]. As mentioned above, the AFR generated by AA utilisation that escapes AFR reductase disproportionates generating both AA and DHA. The latter can be reduced back to AA either non-enzymatically or by enzyme-mediated mechanisms. In both cases, glutathione (GSH) has a prominent role as an electron donor [27]. The concomitant presence in chloroplasts of AFR reductase, DHA reductase and glutathione (GSH) reductase, suggested for plants a fascinating AA regeneration pathway known as the AA–GSH cycle [75], in which AFR and DHA generated as a consequence of AA utilisation (i.e. oxidation) are continuously recycled to AA.

However efficiently AA regeneration from AFR and DHA may occur, each cell and obviously any growing organism requires AA biosynthesis (or dietary intake in non-synthesising organisms) because AA and DHA undergo continuous degradation. We believe that AFR reductase plays a key role in limiting DHA formation, rather than AA generation, as DHA is potentially toxic and a powerful inhibitor of several enzymes [76–78]. High AFR reductase activity limits the amount of AFR available for the disproportionation reaction. This hypothesis is supported by the observation that actively dividing cells, which use large amounts of AA for their metabolism and for division processes, also show higher AFR reductase activity, lower DHA content and highly efficient de novo AA biosynthesis [30].

DHA can also be efficiently reduced back to AA. However, the presence of a specific DHA regeneration system is another controversial point. In animal tissues, the presence of specific enzymatic mechanisms has been repeatedly questioned [79,80]. GSH-dependent DHA reductase activity has been detected in many plant species, and enzymes showing DHA reductase activity have been purified from different sources [81]. Since the observation that glutaredoxin and PDI can also catalyse DHA reduction to AA [82], the list of animal and plant proteins that share DHA reductase activity, besides catalysing different reactions, is getting longer and longer, and presently also includes thioredoxin and Kunitz-type trypsin inhibitor [83], thioredoxin reductase [84], 3α-hydroxysteroid dehydrogenase [85], GSH peroxidase [86], dioscorins [87] and even rat serum albumin [88]. This list does not include other reports on enzymes with DHA reductase activity, the sequences of which are not related to previously described proteins [89–91]. This multiplicity of DHA-reducing enzymes is apparently linked to the peculiar reactivity of the typical dicysteinyl motif (C-X-X-C), which is present in virtually all the above-mentioned proteins sharing DHA reductase activity.

The physiological role of DHA reductase is still a matter for debate. In animals lacking the AA biosynthetic machinery, AFR and DHA reduction could in principle contribute to keeping the size of the AA pool constant. However, the overall activity of these two enzymes is not much different in AA-synthesising and non-synthesising organisms, therefore it is conceivable to conclude that the bulk of AA in all organisms results from de novo biosynthesis or dietary intake, and not from AFR and DHA recycling. This has been observed, for instance, when germinating maize embryos were detached from their storage tissues, thus reducing the availability of sugar AA precursors [92]; in spite of high AFR and DHA reductase activities, AA content was progressively slowed down. According to computer simulations, there would be no need for AA recycling from DHA in chloroplasts in order to achieve the AA concentrations measured in this organelle [93]. Although DHA reduction is unlikely to significantly contribute to AA content, it could nevertheless have a role in DHA removal. We have recently observed that DHA administration to lupin and onion

roots induces a remarkable increase in intracellular AA content, in parallel with a transient decrease in the size of GSH pool and a general oxidation of thiol-containing proteins. However, AA formed by means of DHA reduction (either enzymatic or non-enzymatic) is apparently not equivalent to AA formed via biosynthesis [94]. This is consistent with the observations that DHA administration inhibits plant growth and only partially reverses scurvy symptoms in guinea pigs [95], although DHA is massively reduced to AA. Therefore, we believe that considering the AA pool as the sum of AA plus DHA content (as sometimes reported) is incorrect, since many available data indicate that AA and DHA are chemical species with different reactivity and metabolic functions, rather than just two sides of the same coin.

In animal cells, a significant contribution to DHA reduction by protein thiols has been observed in the ER [96], further substantiating the hypothesis of a role for DHA in the regulation of thiol–disulfide exchanges and in protein folding (Fig. 4). The peculiar reactivity of DHA with protein SH groups, beyond its specific cellular context in the ER, could explain its toxicity and inhibiting effect on plant growth [94]. This could also give a convincing explanation of DHA involvement in diabetes [97], possibly linked to DHA accumulation due to impaired reduction [98,99].

5. AA catabolism and the DHA mystery

Almost all biological research on the AA system in plants and animals has been directed to the investigation of its three most representative chemical species, namely AA, AFR and DHA. Not much attention has been given to diketogulonic acid (DKG) and to downstream steps of the degradation pathway. In the first step of DHA degradation, the lactone ring is broken and DKG is formed [100]. Attempts to identify an enzyme catalysing DHA delactonisation have not been successful. It is known that this reaction is promoted by bicarbonate [101]. Although a clear view of the pathway of AA catabolism has not been obtained yet, it is known that in animal cells DKG degrades to 5- and 4-carbon species, including lyxonic acid, xylonic acid, lyxose and xylose [102]. In plants, Loewus and co-workers identified oxalate, threonate and tartrate as products of AA catabolism [103]. However, available information on AA catabolism is far from being conclusive. In particular, the 'DHA mystery' largely remains unsolved. As previously mentioned, AA is massively utilised (dioxygenase activity, enzymatic and non-enzymatic ROS scavenging). All these reactions generate AFR, and then DHA as a product of disproportionation. However, we have observed that DHA content is usually quite low, as compared to AA [30] and usually it is not accumulated, even when it is exogenously administered [94]. The amount of AA degradation products observed is apparently not consistent with the amount of AA consumed in cell metabolism [104]. Where does DHA end up? A possible answer to this question could be obtained from interesting work in the field of chemistry.

The stability of DHA solutions may vary according to pH conditions [100]. Interestingly, analysis of DHA solutions at different time points has provided an array of many different molecules [105]. Experimental conditions used for such experiments on AA degradation in non-biological systems are often very far from physiological conditions, and involve high pH values, high temperatures, and high DHA concentrations. Nevertheless, these studies deserve our attention, since at least part of the degradation products isolated by using this approach might be produced by (yet unknown) catalysed reactions in vivo. Such hypothetical enzyme-mediated reactions could possibly shed new light on AA catabolism in vivo and help in solving the mystery of the fate of DHA.

Jung and Wells observed three high-performance liquid chromatography peaks deriving from DHA solutions kept at pH 7 and 30°C for 10 min, and two of the peaks were identified as AA and EAA [106]. As previously mentioned, EAA is a 5-carbon analogue of AA synthesised in yeast cells [13]. There is little information about a possible biological role of EAA. However, this compound presents the same enediol group typical of AA and it has been reported to have AA-like activity in the larval growth of tobacco hornworm (*Manduca sexta*) [107] and to protect yeast cells from hydrogen peroxide [13]. In addition, EAA may function as an electron donor yielding erythroascorbate free radical (EAFR), and this in turn forms dehydroerythroascorbate. EAFR reductase activity has recently been described in *S. cerevisiae* [108]. At present, there is no indication that DHA degradation could actually yield EAA in biological systems, although this hypothesis deserves further investigation.

6. AA-induced mRNA transcription

An increasing number of studies have investigated the involvement of AA in the regulation of transcription and/or stabilisation of specific mRNAs. Apparently specific ascorbate–RNA complexation was observed through both G–C and A–U base pairs, without changing RNA secondary structure [109]. This could be related to the observed selective AA-dependent stabilisation of collagen transcript and destabilisation of elastin transcript in smooth muscle cells and skin fibroblasts [110]. Alternatively, transcription of collagen genes could be induced by mature (i.e. properly hydroxylated and folded) collagen, therefore AA could have an indirect effect on transcription. As a third hypothesis, a redox-controlled mechanism has been invoked. The transcription of the 72-kDa type IV collagenase (matrix metalloproteinase-2) is downregulated by AA in cultured human amnion-derived cells [111]. Tyrosine hydroxylase transcription is enhanced by

AA treatment [112], and the mRNA encoding various forms of cytochrome P450 in liver microsomes from guinea pigs is induced as well [113]. Other mRNAs whose transcription appears to be regulated by AA are those for ubiquitins in guinea pigs [114]. AA alters the expression of collagen integrins in bone culture [115]. Avigliano and co-workers have recently demonstrated that AA administration induces transcription of the *fra-1* gene, which encodes a transcription factor of the Fos family, and down-regulates the activator protein-1 (AP-1) target genes [116]. Moreover, AA has been observed to affect vitamin D-induced differentiation of leukemic cells by redox regulation of AP-1, and also affects nuclear factor κB [117]. In plants, the maize *Hrgp* gene is induced by AA [118]. It is noteworthy that at least part of the genes which have their transcription induced (or stability of the transcript enhanced) by AA encode proteins which contain hydroxylated proline residues or which require AA for their catalytic activity.

7. Conclusions

In an interview published a few years before passing away, Albert Von Szent-Györgyi described AA and its reactivity as the basis itself of the continuing changes of life [119]. In the light of our present knowledge, this can be viewed as a beautiful and stimulating poetical image, rather than a scientifically based theory. As mentioned in the Section 1, AA has been much studied, yet its potential contribution to many different aspects of cell metabolism has been neglected. It is worth mentioning, for instance, that the regulation of the process of cell proliferation via inhibition of the dioxygenase deoxyhypusine hydroxylase has recently been viewed as a possible target for anti-cancer strategies [62,120,121]. It is very easy to predict that in the near future many more intriguing aspects of the AA system will give rewarding results to researchers interested in their investigation.

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