ASCORBATE FREE RADICAL REDUCTASE, A KEY ENZYME OF THE ASCORBIC ACID SYSTEM

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Received 30 January 1981

1. Introduction

Following the finding that lycorine (an alkaloid from Amarillidaceae) is a powerful inhibitor of ascorbic acid (AA) biosynthesis, it was possible to establish that large amounts of AA are utilized in plant cell metabolism [1,2], similarly to what happens in animals [3,4]. Cell metabolism utilizes ascorbic acid essentially as an electron donor, so that AA undergoes continuous oxidation and reduction. Oxidation products are ascorbic free radical (AFR) and dehydroascorbic acid (DHA). They can be reconverted to AA by two enzymes: AFR-reductase (NADH:AFR oxidoreductase = NADH:monodehydroascorbate oxidoreductase, EC 1.6.5.4) [5–8] and DHA-reductase (GSH:dehydroascorbate oxidoreductase, EC 1.8.5.1) [9].

Here we report data concerning the relative contribution of these two enzymes in maintaining the ascorbic acid system in the reduced state.

2. Materials and methods

Onion and broad bean roots and etiolated pea seedlings were dark-grown in our laboratory on moist vermiculite. Other plant materials were obtained from commercial sources. The chopped tissue was placed in a medium containing 50 mM Tris–HCl, 0.3 M mannitol, 1 mM EDTA, 10 mM $MgCl_2$, 0.1% bovine serum albumin and 0.05% cysteine at pH 7.8. A Polytron was used for homogenization and the resulting homogenate was strained through nylon cloth.

Algae were harvested on the South Adriatic coast, washed and powdered in liquid nitrogen. The powder was placed in the above medium and grinded using a Potter glass homogenizer.

Mouse fibroblasts cultured according to [10] were provided by the General Pathology Institute of our University. The pelletted cells were resuspended in the above medium and ruptured by sonication with a Branson sonifier.

The homogenates from all assayed tissues were centrifuged at $600 \times g_{av.}$ to remove cell debris and nuclei. Mitochondria were precipitated at $7000 \times g_{av.}$ for 20 min, washed twice and resuspended in the homogenization medium minus EDTA and cysteine. The microsomal fraction was precipitated by centrifugation at $100\,000 \times g$ for 1 h and resuspended in the same medium as the mitochondria [11].

AFR-reductase activity was assayed by measuring the rate of NADH oxidation at 340 nm in the presence of AFR generating systems (see fig.1). DHA-reductase activity was assayed by measuring the rate of AA formation at 265 nm in a reaction mixture containing enzyme, 1 mM DHA, 1 mM GSH, and 100 mM phosphate buffer (pH 6.3) in 3 ml final vol. AA-oxidase activity was assayed by following AA oxidation at 265 nm. Protein was assayed by the method in [12].

3. Results and discussion

AFR- and DHA-reductase activities of various higher plants, algae and animal cells are listed in table 1. AFR-reductase appears to be an ubiquitous enzyme, while DHA-reductase does not. DHA-reductase, whenever present, is confined to the cytosol (table 2). AFR-reductase is present in mitochondria, microsomes, soluble fraction (table 2) and chloroplasts [13] of plant tissues. Animal tissues do not show DHA-reductase activity and AFR-reductase seems to be associated with mitochondria and microsomes in animal cells [8].

The presence of AFR-reductase in all these cell compartments makes it likely that AFR, a substrate

Table 1
AFR-reductase, AA-oxidase, and DHA-reductase activities of various plant tissues and animal cells

	AFR-[a] reductase	AA-[b] oxidase	DHA-[c] reductase
Potato tuber	0.58	0	0.16
Jerusalem artichoke tuber	0.47	0	0
Onion root	0.61	0	0.06
Broad bean root	0.30	0.16	0.20
Cauliflower bud	1.04	0	0.38
Cucumber squash	2.59	1.88	0.25
Etiolated pea hook	0.96	0	0.02
Pterocladia frond	0.23	0	0
Gigartina frond	0.33	0	0
Hypnaea frond	0.42	0	0
Gracilaria frond	0.62	0	0
Mouse fibroblasts (BHK)	0.12	0	0
Virus-transformed mouse fibroblasts (LS)	0.22	0	0

[a]μmol NADH oxidized . min^{-1} . mg $protein^{-1}$; [b]μmol AA oxidized . min^{-1} . mg $protein^{-1}$; [c]μmol DHA reduced . min^{-1} . mg $protein^{-1}$

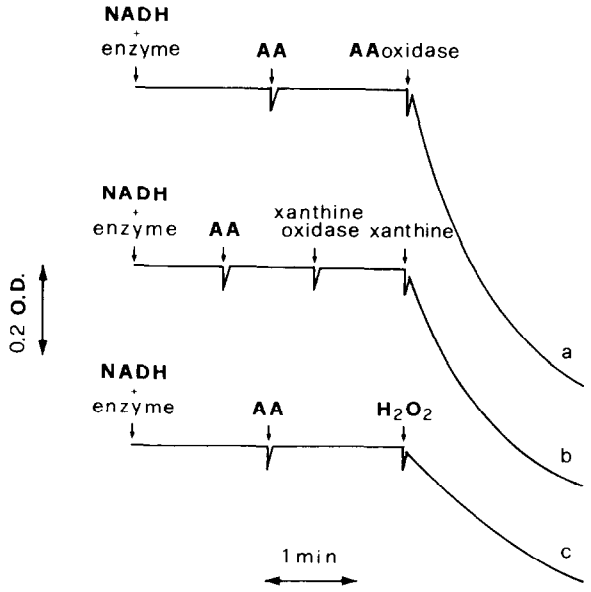

Fig.1. AFR-reductase activity assayed with different AFR generating systems. Reaction mixture contained 0.2 mM NADH, enzyme from potato tuber (~400 μg protein), 1 mM AA, and: (a) 1U AA-oxidase; (b) 0.15 mM xanthine and 0.25U xanthine oxidase; (c) 2 mM H_2O_2; in 3 ml final vol. Adding hydrogen peroxide prior to ascorbate did not affect NADH oxidation in curve (c). The change in absorbance was monitored at 340 nm.

of the enzyme, is generated in each of them. Different AA oxidizing systems can generate AFR:

(i) The aerobic oxidation of AA by ascorbate oxidase (L-ascorbate:oxygen oxidoreductase, EC 1.10.3.3) generates AFR (fig.1a). However, there is no correlation between the occurrence of AFR-reductase and AA-oxidase: the first is present in all assayed plant and animal tissues and the latter is present only in a few plant materials (table 1). Thus, AFR-reductase activity does not seem to be dependent 'in vivo' on the AFR generated by AA-oxidase.

(ii) The anion radical of oxygen, superoxide, oxidizes ascorbate and also yields AFR (fig.1b) [14]. The affinity of superoxide for AA in generating AFR must be very high, as the rate of AFR reduction by NADH was high. The reaction between superoxide and ascorbate to yield AFR may be a mechanism to eliminate the toxic superoxide anions formed during the cell metabolism in different cell compartments [15,16]. AFR-reductase is present in all these compartments and may reconvert AFR to AA. This system would then act as a superoxide 'scavenger' in addition to superoxide dismutase (SOD), particularly if SOD activity is low. Actually, we found increased AFR-reductase activity in virus-transformed mouse fibroblasts which showed reduced SOD activity compared to normal fibroblasts [17].

(iii) Fig.1c shows that AFR is generated during the oxidation of AA by hydrogen peroxide as well. However, the effectiveness of this system may be low. The rate of this reaction was lower than that observed with superoxide, though the concentration of peroxide used was as high as 2 mM. AFR generation by hydrogen peroxide may, however, become important if a peroxidase is operating in the system. The rate of ascorbate peroxidation in

Table 2
AFR-reductase and DHA-reductase activities in different cell compartments of potato tuber

	AFR-[a] reductase	DHA-[b] reductase
Mitochondria	0.29	0
Microsomes	0.10	0
Soluble fraction	0.58	0.16

[a]μmol NADH oxidized . min^{-1} . mg $protein^{-1}$; [b]μmol DHA reduced . min^{-1} . mg $protein^{-1}$

chloroplasts is very high [18]. Therefore, a more powerful system is necessary for the generation of AA than the non-enzymic reduction of dehydroascorbate by reduced glutathione [19]. The chloroplasts exhibit AFR-reductase activity [13]. Therefore, we suggest that the reduction of AFR by AFR-reductase is the main mechanism to regenerate AA in chloroplasts.

Ascorbic acid is known to be involved in biological defence mechanisms [20,21]. High levels of AA in tissues help to counteract the action of various pathogens [22]. Thus, high levels of both AFR production and AFR-reductase activity may be expected in the host cells engaged in the inactivation of the pathogen. We found increased AFR-reductase activity in resistant varieties of tomato plants subjected to nematode attack, as compared to susceptible varieties.

In conclusion, many ascorbate-dependent metabolic reactions generate AFR as the first oxidation product and DHA as the final product. AFR is efficiently reconverted to AA by AFR-reductase which appears to be an enzyme essential in maintaining the ascorbic acid system in the reduced state. In this respect, DHA-reductase seems to play a secondary role.

Acknowledgement

This work was supported by a grant from Consiglio Nazionale delle Ricerche (Italy).

References

[1] Arrigoni, O., Arrigoni-Liso, R. and Calabrese, G. (1975) Nature 256, 513–514.
[2] Arrigoni, O., Arrigoni-Liso, R. and Calabrese, G. (1976) Science 194, 332–333.
[3] Lewin, S. (1976) Vitamin C: Its Molecular Biology and Medical Potential, pp. 75–130, Academic Press London, New York.
[4] Atkins, G. L., Dean, B. M., Griffin, W. G. and Watts, R. W. E. (1964) J. Biol. Chem. 239, 2975–2980.
[5] Beevers, H. (1954) Plant Physiol. 29, 265–269.
[6] Nason, A., Wosilait, W. D. and Terrell, A. J. (1954) Arch. Biochem. Biophys. 48, 233–235.
[7] Kern, M. and Racker, E. (1954) Arch. Biochem. Biophys. 48, 235–236.
[8] Green, R. C. and O'Brien, P. J. (1973) Biochim. Biophys. Acta 293, 334–342.
[9] Yamaguchi, M. and Joslin, M. A. (1951) Plant Physiol. 26, 757–772.
[10] Matalon, R. and Dorfman, A. (1966) Proc. Natl. Acad. Sci. USA 56, 1310–1316.
[11] Rich, P. R. and Bendall, D. S. (1975) Eur. J. Biochem. 55, 333–341.
[12] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[13] Marré, E. and Arrigoni, O. (1958) Biochim. Biophys. Acta 30, 453–457.
[14] Nishikimi, M. (1975) Biochem. Biophys. Res. Commun. 63, 463–468.
[15] Fridovich, I. (1974) Adv. Enzymol. 41, 35–97.
[16] Boveris, A., Cadenas, E. and Stoppani, A. O. M. (1976) Biochem. J. 156, 435–444.
[17] Arrigoni, O., Arrigoni-Liso, R., Dipierro S., Borraccino, G., Zacheo, G. and Fumarulo, R. (1981) 20th Congr. Ital. Soc. Plant Physiol. 1980, in press.
[18] Groden, D. and Beck, E. (1979) Biochim. Biophys. Acta 546, 426–435.
[19] Foyer, C. H. and Halliwell, B. (1976) Planta 133, 21–25.
[20] Farkas, G. L., Király, Z. and Solymosy, F. (1960) Virology 12, 408–421.
[21] Arrigoni, O. (1979) in: Root-Knot Nematodes, Systematics, Biology and Control (Lamberti, F. and Taylor, C. E. eds) pp. 457–467, Academic Press London, New York.
[22] Arrigoni, O., Zacheo, G., Arrigoni-Liso, R., Bleve-Zacheo, T. and Lamberti, F. (1979) Phytopathology 69, 579–581.