Metabolism of Hydrogen Peroxide in *Euglena gracilis* z by L-Ascorbic Acid Peroxidase

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Euglena gracilis was found to contain a peroxidase that specifically requires L-ascorbic acid as the natural electron donor in the cytosol. The presence of an oxidation–reduction system metabolizing L-ascorbic acid was demonstrated in *Euglena* cells. Oxidation of L-ascorbic acid by the peroxidase, and the absence of ascorbic acid oxidase activity, suggests that the system functions to remove H_2O_2 in *E. gracilis*, which lacks catalase.

O_2 -utilizing organisms possess enzymes capable of destroying toxic intermediates of O_2 metabolism. Catalase, peroxidases and superoxide dismutases are such enzymes ordinarily functioning to scavenge the toxic substances. *Euglena gracilis* lacks catalase (Graves *et al.*, 1971; Lord & Merrett, 1971; Asada *et al.*, 1977) and the method by which this organism disposes of H_2O_2 has not been elucidated. Cytochrome *c* was claimed to have a peroxidative activity (Brown *et al.*, 1975), but the view has apparently not gained wide acceptance as a convincing explanation.

In the course of studying the metabolism of L-ascorbic acid in *E. gracilis*, we have found that it is oxidized by the action of a peroxidase that specifically requires L-ascorbic acid as the natural electron donor. This peroxidase appears to be the sole agent destroying H_2O_2 in *Euglena* cells.

Materials and Methods

Heterotrophic cultures of *E. gracilis* were obtained by culturing the protozoon at 26°C in Koren & Hutner (1967) medium under illumination (2000lx) or in the dark, and photoautotrophic cultures were obtained in the pH 6.8 salt medium described by Cramer & Myers (1952).

Harvested cells (4×10^8) were washed with 25 mM-veronal buffer, pH 6.8, containing 30% (w/v) sucrose, and disrupted in 5 ml of the same buffer by sonication (10kc) for a total of 2 min with seven rest intervals of 15 s each. The supernatant obtained by centrifuging the sonicated cells at 20000g for 15 min was used as the enzyme source.

L-Ascorbic acid peroxidase was assayed at 32°C (optimum temperature) in a reaction mixture (2 ml) containing 25 mM-veronal buffer, pH 6.2 (optimum pH), 0.4 mM-sodium L-ascorbate, 0.11 mM- H_2O_2 and enzyme, by measuring the de-

crease in A_{285} . Peroxidase activity with pyrogallol (Nakarai Co., Kyoto, Japan) as an artificial electron donor was assayed by the method of Chance & Maehly (1955). Catalase (EC 1.11.1.6) was assayed by the method of Abei (1974). L-Ascorbic acid oxidase (EC 1.10.3.3) was assayed by measuring the decrease in A_{285} (Attridge, 1974) and also by measuring O_2 consumption with an oxygen electrode (Strothkamp & Dawson, 1977); the reaction mixture (2 ml) contained 50 mM-potassium phosphate buffer, pH 6.0, 1 mM-sodium L-ascorbate and enzyme. Dehydro-L-ascorbic acid reductase (EC 1.8.5.1) was assayed by a method similar to that for assaying glutathione peroxidase (EC 1.11.1.9) (Sies & Moss, 1978); the reaction mixture (2 ml) contained 50 mM-potassium phosphate, pH 6.5, 2.5 mM-dehydro-L-ascorbic acid, 2.5 mM-reduced glutathione, 0.2 mM-NADPH, glutathione reductase (1 unit; Sigma) and enzyme. This enzyme was also assayed by the method of Foyer & Halliwell (1977). Monodehydro-L-ascorbic acid reductase (EC 1.6.5.4) was assayed by the method of Marre & Arrigoni (1958). Total L-ascorbic acid was determined by the method described by Shigeoka *et al.* (1979a).

For studying the subcellular distribution of peroxidase activity, a cell homogenate was obtained by partial trypsin digestion of pellicle followed by mild mechanical destruction (Tokunaga *et al.*, 1976a; Shigeoka *et al.*, 1979b), and then submitted to linear sucrose-density-gradient centrifugation (Yokota *et al.*, 1978; Yokota & Kitaoka, 1979a). Succinic semialdehyde dehydrogenase as a marker for mitochondria (Tokunaga *et al.*, 1976b, 1979) and NADPH–cytochrome *c* reductase as a marker for microsomal fraction (Omura & Takesue, 1970), mitochondria (Yokota & Kitaoka, 1979b) and chloroplasts (Lazzarini & Pietro, 1962) were assayed by the reported methods. Chlorophyll and protein

were determined respectively by the methods of MacKinney (1941) and Lowry *et al.* (1951) (with bovine serum albumin as a standard).

Disc electrophoresis in polyacrylamide gel was performed as described by Davis (1964) with the use of 7.5% polyacrylamide gels. Electrophoresis was carried out in a cold-chamber by using a constant current (2mA/gel), with Bromophenol Blue as migration marker. The distribution of the peroxidase activity with L-ascorbic acid or pyrogallol as an electron donor was determined on 1 mm slices of the gel column.

Results and Discussion

Table 1 shows activities of the enzymes related to the oxidation-reduction system of L-ascorbic acid in the crude extract of *E. gracilis*. A very high activity was found for the peroxidase that utilized L-ascorbic acid only and not compounds such as NADH, NADPH, cytochrome *c*, glutathione and palmitic acid as the natural electron donor. The extract contained some monodehydro-L-ascorbic acid reductase and dehydro-L-ascorbic acid reductase activities, but no L-ascorbic acid oxidase. Scheme 1 illustrates the oxidation-reduction system of L-ascorbic acid in *E. gracilis* based on the results of the present experiments and those with plants (Mapson & Moustafa, 1956; Halliwell, 1978). Monodehydro-L-ascorbic acid, the primary oxida-

tion product of L-ascorbic acid, undergoes a non-enzymic dismutation, giving dehydro-L-ascorbic acid and L-ascorbic acid (Yamazaki *et al.*, 1960). The monodehydro compound is also reduced to form L-ascorbic acid by the action of monodehydro-L-ascorbic acid reductase, the presence of which in *E. gracilis* was demonstrated in the present experiments. Reduction of dehydro-L-ascorbic acid to L-ascorbic acid proceeds by the action of dehydro-L-ascorbic acid reductase, thus completing the oxidation-reduction cycle.

In *E. gracilis* this oxidation-reduction system starts by the oxidation of L-ascorbic acid catalysed by L-ascorbic acid peroxidase, with H_2O_2 as the oxidant; this is a characteristic difference from higher plants in which L-ascorbic acid is oxidized by L-ascorbic acid oxidase, with O_2 as the oxidant (Dawson *et al.*, 1975). Since only one peak of peroxidase activity with either L-ascorbic acid or pyrogallol as electron donor was detected in the gel column, a single protein appears to be responsible for the peroxidative activity. As reported in detail elsewhere this peroxidase has many unique properties and apparently belongs to a new type of peroxidase (Shigeoka *et al.*, 1979*d*). Groden & Berk (1977, 1979) have reported the occurrence of an L-ascorbic acid-specific peroxidase in spinach chloroplasts which decomposes H_2O_2 . The *Euglena* peroxidase with L-ascorbic acid and pyrogallol as electron donors was found to be located only in the

Table 1. Activities of the enzymes related to the oxidation-reduction system of L-ascorbic acid in *E. gracilis*

Enzyme	Enzyme activity	
	($\mu\text{mol/min per } 10^9 \text{ cells}$)	(nmol/min per mg of protein)
L-Ascorbic acid oxidase	Nil	—
L-Ascorbic acid peroxidase	78.4	768.4
Monodehydro-L-ascorbic acid reductase	2.1	20.5
Dehydro-L-ascorbic acid reductase	9.0	90.0
Catalase	Nil	—

Scheme 1. Oxidation-reduction system of L-ascorbic acid in *E. gracilis*

Key to reactions: (1) L-ascorbic acid peroxidase; (2) monodehydro-L-ascorbic acid reductase; (3) non-enzymic reaction; (4) dehydro-L-ascorbic acid reductase. GSSG and GSH, oxidized and reduced glutathione.

Fig. 1. Subcellular distribution of the peroxidase activities with L-ascorbic acid and pyrogallol as electron donors in *E. gracilis*

Activities of peroxidase with L-ascorbic acid and pyrogallol as electron donors, succinic semialdehyde dehydrogenase and NADPH-cytochrome *c* reductase are given in nmol of substrate transformed/min per 0.1 ml of fraction. Chlorophyll is expressed in mg/0.1 ml and sucrose density in g/cm³. ●, L-Ascorbic acid peroxidase; ▲, pyrogallol peroxidase; ○, succinic semialdehyde dehydrogenase; □, NADPH-cytochrome *c* reductase; △, chlorophyll; ■, sucrose density.

cytosol (Fig. 1); it was not present in mitochondria, chloroplasts and microsomal fractions. A similar centrifugation with 30–60% sucrose concentrations showed that the enzyme was not present in the microbody fraction either. Dehydro-L-ascorbic acid reductase was also detected in the cytosol, as in spinach leaves (Foyer & Halliwell, 1976).

Initiation of the oxidation–reduction system of L-ascorbic acid in *E. gracilis* by the peroxidase indicates that this system serves to destroy H₂O₂ generated in the cells. The L-ascorbic acid peroxidase activity is much higher than those of the L-ascorbic acid-regenerating enzymes in the recycling system. The high peroxidase activity together with the large amount of L-ascorbic acid in the cells (Shigeoka *et al.*, 1979a,c) suggests that L-ascorbic acid peroxidase and L-ascorbic acid are used for immediate and efficient detoxification of H₂O₂. Groden & Berk (1979) have found a similar situation in spinach chloroplasts in which the L-ascorbic acid-consuming activity is 5–25 times as high as the regenerating activity. The maximum rate of H₂O₂ generation was reported to be about 10 nmol/min per mg of protein in rat liver (Boveris *et al.*, 1972) and about 10 nmol/min per mg of protein in spinach chloroplasts (Asada *et al.*, 1974). If these values are applied to *Euglena* cells containing all organelles common to both samples, the maximum H₂O₂-generation rate is about 20 nmol/min per mg of protein. It is about 2.6% of the activity of L-ascorbic acid peroxidase and 18% of those of L-ascorbic acid-regenerating enzymes. Accordingly the H₂O₂-generating rate is the limiting factor of the oxidation–reduction cycle of L-ascorbic acid, controlling

the oxidation of L-ascorbic acid. This indicates that this cycle is sufficiently operative as the recycling system. The cell extract contained no catalase (Table 1), confirming results of previous papers (Graves *et al.*, 1971; Lord & Merrett, 1971; Asada *et al.*, 1977). The occurrence of the peroxidase only in the cytosol indicates the presence of a unique defence mechanism against O₂ toxicity in this protozoon.

A H₂O₂-generating system should exist in *E. gracilis*, since superoxide dismutase activity was detected in the cells (Asada *et al.*, 1977; Kanematsu & Asada, 1979). The activity of superoxide dismutase was reported to be 10.3 units/mg of protein in photoautotrophic cells, 6.0 units/mg in photoheterotrophic cells and 3.5 units/mg in heterotrophic cells (in the dark), indicating that the enzyme activity is affected by growth conditions. The contents of total L-ascorbic acid and activities of L-ascorbic acid peroxidase in *Euglena* cells grown under the corresponding conditions were 4010 nmol/10⁹ cells and 328.0 μmol/min per 10⁹ cells (photoautotrophic), 940 nmol/10⁹ cells and 78.4 μmol/min per 10⁹ cells (photoheterotrophic) and 185 nmol/10⁹ cells and 6.9 μmol/min per 10⁹ cells (heterotrophic) respectively. The results show that the cellular L-ascorbic acid content and L-ascorbic acid peroxidase activity vary in parallel with the change in superoxide dismutase activity and thus the generation of H₂O₂.

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