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Characterization and physiological function of glutathione reductase in *Euglena gracilis* z

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The purified glutathione reductase was homogeneous on polyacrylamide-gel electrophoresis. It had an M_r of 79000 and consisted of two subunits with a M_r of 40000. The activity was maximum at pH 8.2 and 52 °C. It was specific for NADPH but not for NADH as the electron donor; the reverse reaction was not observed. The K_m values for NADPH and GSSG were 14 and 55 μM respectively. The enzyme activity was markedly inhibited by thiol inhibitors and metal ions such as Hg^{2+}, Cu^{2+} and Zn^{2+}. *Euglena* cells contained total glutathione at millimolar concentration. GSH constituted more than 80% of total glutathione in *Euglena* under various growth conditions. Glutathione reductase was located solely in cytosol, as were L-ascorbate peroxidase and dehydroascorbate reductase, which constitute the oxidation–reduction cycle of L-ascorbate [Shigeoka *et al.* (1980) Biochem. J. **186**, 377–380]. These results indicate that glutathione reductase functions to maintain glutathione in the reduced form and to accelerate the oxidation–reduction of L-ascorbate, which scavenges peroxides generated in *Euglena* cells.

INTRODUCTION

Since spinach chloroplasts produce H_2O_2 under illumination and yet contain no catalase or non-specific peroxidase, Foyer & Halliwell (1976) suggested that H_2O_2 is eliminated by the redox pairs of AsA–DAsA and GSH–GSSG as shown below:

$$AsA + H_2O_2 \rightarrow DAsA + 2H_2O \quad (1)$$

$$DAsA + 2GSH \rightarrow AsA + GSSG \quad (2)$$

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+ \quad (3)$$

The validity of this view has been supported by the discovery of AsA peroxidase (EC 1.11.1.11) (reaction 1).

We have reported previously that, in *Euglena gracilis* z, which lacks catalase, AsA peroxidase occurs in cytosol, but not in chloroplasts, and that the oxidation–reduction system of AsA, consisting of AsA peroxidase, MDAsA reductase and DAsA reductase, plays a role in scavenging H_2O_2 generated *in vivo* (Shigeoka *et al.*, 1980*a*). AsA peroxidase has been purified for the first time from *Euglena gracilis* (Shigeoka *et al.*, 1980*b*), and some of its properties have been determined. Subsequently, we have shown that MDAsA reductase (EC 1.6.5.4) and DAsA reductase (EC 1.8.5.1) (reaction 2) work for the regeneration of AsA from MDAsA and DAsA produced by AsA peroxidase, although the latter mainly performs the operation rather than the former, as judged by its enzymic properties (Shigeoka *et al.*, 1986*a*).

It has been known that GSH is present in almost all organisms and serves as a reductant in numerous biochemical reactions, including counteraction of oxidative events and protection of the thiol groups of intracellular proteins (Arias & Jakoby, 1976; Meister & Tate, 1976). Accordingly, the reduction of GSSG is of fundamental importance in the metabolic function of GSH. Glutathione reductase (EC 1.6.4.2) catalyses the reduction of GSSG by NADPH (reaction 3). This enzyme has been purified from some sources: yeast (Mavis & Stellwagen, 1968); spinach leaves (Halliwell & Foyer, 1978); rat liver (Carlberg & Mannervik, 1975); rabbit liver (Zanetti, 1979); and human erythrocyte (Worthington & Rosemeyer, 1974). Despite the importance of glutathione metabolism, there has been no report on occurrence of glutathione and glutathione reductase in *Euglena gracilis* z.

In the present study we purified glutathione reductase from *E. gracilis* and studied some properties of the enzyme. We also determined the concentration of GSH and GSSG and the subcellular location of glutathione reductase and we discuss the physiological function of the enzyme and its association with the oxidation–reduction cycle of AsA.

MATERIALS AND METHODS

Cell culture

Euglena gracilis z and its streptomycin-bleached mutant were cultured in the Koren–Hutner (1967) medium at 26 °C for 5 days under illumination at 3000 lx or in the dark; they were designated as the light-adapted green (GL) and bleached (BL) cells and the dark-adapted green (GD) and bleached (BD) cells.

Enzyme assay

Glutathione reductase was assayed at 35 °C in 2 ml of reaction mixture containing 50 mM-phosphate buffer, pH 8.2, 1 mM-EDTA, 0.2 mM-NADPH and 1 mM-GSSG. One unit of glutathione reductase was defined as the amount of enzyme catalysing oxidation of 1 μmol of NADPH/min. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Abbreviations used: AsA, L-ascorbate; MDAsA, monodehydroascorbate; DAsA, dehydroascorbate.
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Table 1. Purification of glutathione reductase from *Euglena gracilis*

Details of the purification are described in the text.

Purification step	Protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Purification (fold)	Yield (%)
Crude extract	12276	1596	0.13	1.0	100
Heat treatment	5141	1594	0.31	2.4	99.9
DEAE-Sephadex	605	968	1.60	12.3	60.7
Blue Sepharose	4.3	495	115.0	885.0	31.0
DEAE-Sephadex	1.8	380	211.0	1623.0	23.8
Isoelectric focusing	–	320	–	–	20.1
Sephadex G-50	1.4	312	223.0	1715.0	19.5

Purification of glutathione reductase

Purification procedures were conducted at 0–4 °C unless otherwise noted. GL cells (170.9 g, wet weight) were washed with 10 mM-potassium phosphate buffer, pH 6.8, containing 10% (w/v) sucrose, and disintegrated in the same buffer by sonication (10 kHz) for total 60 min with 11 intervals of 5 min each. The homogenate was centrifuged at 20000 *g* for 15 min. The cell extract was rapidly brought to 52 °C in a thermostatically controlled water bath. After 5 min, the solution was immediately cooled on ice and centrifuged at 10000 *g* for 15 min. The supernatant was applied to a DEAE-Sephadex column (2.5 cm × 40 cm) equilibrated with 10 mM-phosphate buffer, pH 6.8, containing 10% (w/v) sucrose. The column was eluted with 1 litre of linear gradient of KCl (0–0.4 M) at an elution rate of 0.6 ml/min. Active fractions were combined and dialysed for 24 h against the above buffer. The active solution was chromatographed on a column (1.4 cm × 15 cm) of Blue Sepharose equilibrated with 10 mM-phosphate buffer, pH 6.8. After washing with 2 bed vol. of the same buffer, elution was left to equilibrate with the nucleotide solution for 20 min before collection of the active fractions. They were immediately applied to a column (1.5 cm × 20 cm) of DEAE-Sephadex, and glutathione reductase was eluted with 300 ml of 0–0.4 M-KCl gradient. The active fractions were concentrated in dialysis tubing with poly(ethylene glycol) 20000 powder for 12 h and subjected to isoelectric-focusing on a column (110 ml) containing an Ampholine gradient (pH 3–10) at 2 °C for 60 h at 400 V. Fractions (2 ml each) were withdrawn from the bottom of the column. The determination of pH was performed immediately at 4 °C. Enzyme fractions with a pI of 5.2 were collected, applied to a column of Sephadex G-50 (2 cm × 15 cm) to remove Ampholine, and concentrated to 1 ml by the above method. The purified enzyme was stored at −20 °C without extensive inactivation for several weeks.

Estimation of M_r by gel filtration

Gel filtration was performed on a Sephadex G-150 column (0.9 cm × 100 cm) equilibrated with 10 mM-potassium phosphate buffer, pH 8.2, containing 10% (w/v) sucrose, 0.1 mM-2-mercaptoethanol and 0.1 mM-KCl. The column was calibrated with glucose oxidase from *Aspergillus niger* (M_r 186000), lactate dehydrogenase from pig heart (M_r 109000), ovalbumin (M_r 43000), trypsin inhibitor from soybean (M_r 20100) and cytochrome *c* from horse heart (M_r 12400).

Polyacrylamide-gel electrophoresis

Disc electrophoresis in 7.5% (w/v) polyacrylamide gels was performed as described previously (Shigeoka *et al.*, 1980*b*). Electrophoresis was carried out at a constant current (2 mA/gel) with Bromophenol Blue as a migration marker. Protein in the gel was stained with Coomassie Brilliant Blue R-250 and destained in 5% (v/v) acetic acid. Electrophoresis in SDS was done with 12.5% (w/v) polyacrylamide gel as described by Weber & Osborn (1969) in the presence of the following standard proteins: rabbit muscle phosphorylase *b* (M_r 94000), bovine serum albumin (M_r 67000), egg-white ovalbumin (M_r 43000), bovine erythrocyte carbonic anhydrase (M_r 30000) and soybean trypsin inhibitor (M_r 20100).

Subcellular localization of glutathione reductase

A cell homogenate was obtained by partial trypsin digestion of the pellicle followed by mild mechanical disruption, and subcellular fractionation by differential centrifugation was performed as described previously (Shigeoka *et al.*, 1986*b*). Ribulosebisphosphate carboxylase (EC 4.1.1.39) was used as a marker enzyme of chloroplasts (Rabinowitz *et al.*, 1975); succinate semialdehyde dehydrogenase (EC 1.2.1.16) as a mitochondrial marker enzyme (Tokunaga *et al.*, 1976); glucose-6-phosphatase (EC 3.1.3.9) as a microsomal marker enzyme (De Duve *et al.*, 1955) and glutamate dehydrogenase (EC 1.4.1.2) as a cytosolic marker enzyme (Tokunaga *et al.*, 1979).

Determination of glutathione

Euglena cells were washed with distilled water and suspended in 5 ml of 1% (w/v) picric acid. The cells were disrupted by sonication (10 kHz) for a total of 2 min with two intervals of 30 s each. The homogenate was centrifuged at 10000 *g* for 10 min, and to the supernatant was added an equal volume of 100 mM-potassium phosphate buffer, pH 7.4. The solution was used for the assay of GSH and GSSG. The total GSH was determined with an enzymic recycling assay based on glutathione reductase (Tietze, 1969). The reaction mixture contained 5 mM-phosphate buffer, pH 7.4, 1 mM-EDTA, 0.2 mM-NADPH, 0.6 mM-5,5′-dithiobis-(2-nitrobenzoic acid) and cell extract in a final volume of 1 ml. After 5 min preincubation, the reaction was started

by the addition of glutathione reductase (2 units) and A_{412} was monitored for 3 min with the spectrophotometer. GSSG was selectively determined by assaying samples in which glutathione was masked by pretreatment with 2-vinylpyrimidine (Griffith, 1980). To 500 μl of sample was added 10 μl of 1 M-2-vinylpyrimidine; after being vigorously mixed for 1 min the solution was left at 25 °C for 1 h, after which time it was assayed as described above. The relationship between the quantity of GSSG and ΔA_{412}/3 min was linear over the range of 0–0.4 nmol. The difference between total glutathione and GSSG contents is presented as the GSH content.

Chemicals

GSH and GSSG were purchased from Sigma and Blue Sepharose from Pharmacia. All other chemicals were of reagent grade and obtained from commercial sources.

RESULTS AND DISCUSSION

Table 1 summarizes the purification of glutathione reductase from *Euglena gracilis* z by a seven-step procedure. The reductase activities with NADPH and NADH were recovered in the same fractions in all column chromatographies, indicating that a single protein is certainly responsible for the two activities. The specific activity with NADPH and GSSG was 223.0 units/mg of protein; the completely pure yeast (Mavis & Stellwagen, 1968), spinach (Halliwell & Foyer, 1978) and animal (Carlberg & Mannervik, 1975; Zanetti, 1979) enzymes have specific activities close to this value. Polyacrylamide-gel electrophoresis of the purified enzyme showed only one detectable protein band (Fig. 1). The enzyme activity, determined by the activity in 1 mm slices of the gel column, was detected at the position corresponding to the single band.

The enzyme retained full activity up to 67 °C between pH 5.8 and 8.4 and lost activity completely at 78 °C. The optimum pH was 8.2 and optimum temperature 52 °C. NADPH was the best electron donor. The activity with NADH was 1.3% of that with NADPH, whereas the optimum pH with NADH was shifted to a much lower value (pH 5.7), as observed for the erythrocyte enzymes (Worthington & Rosemeyer, 1974), rat liver (Carlberg & Mannervik, 1975) and rabbit liver (Zanetti, 1979). The cause of this shift is not clear at present. These results show that glutathione reductase requires NADPH virtually as the sole physiological electron donor. Similarly the enzyme was highly specific for GSSG. NADPH oxidation was never observed when GSSG was replaced by lipoic acid or cystine. With 1 mM-NAD(P) and GSH the enzyme did not exert the reverse reaction, indicating that the physiological function of the enzyme is to reduce GSSG but not to oxidize GSH. In the yeast (Mavis & Stellwagen, 1968) and spinach (Halliwell & Foyer, 1978) enzymes the reverse reaction was detected in the presence of 10 mM-dithiothreitol, although the rate of the reverse reaction is very low compared with that of the forward reaction. By using double-reciprocal plots of GSSG concentration versus reaction velocity, the enzyme systems gave parallel lines (Fig. 2), suggesting that the reaction proceeds by a branching mechanism as shown in yeast (Mannervik, 1973) and rat liver (Carlberg & Mannervik, 1975). Secondary plots of intercepts allowed us to determine the kinetic constants; the K_m for NADPH was 14 μM and that for GSSG, 50 μM. The

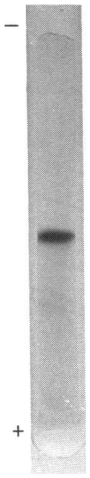

Fig. 1. Polyacrylamide-gel electrophoresis of glutathione reductase from the final purification step

For details, see the text.

values for NADPH and GSSG are comparable with the values reported for other reductases.

Gel filtration on a Sephadex G-150 column with several standard proteins showed that the *Euglena* glutathione reductase had an M_r of 79000. The enzyme also gave a single band on SDS/polyacrylamide-gel electrophoresis. From the position of this band the subunit M_r was estimated to be about 40000. Although this value M_r is lower than that reported for the yeast (124000), rat liver (125000) and spinach leaf (145000) enzymes, the *Euglena* reductase resembles the other ones in being a dimer with subunits of the same size. At 1 mM, Hg^{2+} inhibited activity completely, whereas Cu^{2+}, Zn^{2+} and Co^{2+} markedly decreased the activity. Ni^{2+}, Mn^{2+} and Fe^{3+} also inhibited activity to some extent; Mg^{2+}, Ca^{2+}, Na^+, K^+, NH_4^+, I^-, Br^-, Cl^-, NO_3^-, $CH_3CO_2^-$ (acetate), CO_3^{2-} and SO_4^{2-} had no significant effect on the enzyme activity. The activity was inhibited 38.9% by NADP and 32.1% by GSH (both at 1 mM). The addition of KCN, NaN_3 and EDTA inhibited the enzyme activity hardly at all. The enzyme was highly susceptible to thiol inhibitors such as *p*-chloromercuribenzoate and *N*-ethylmaleimide, supporting the notion that the thiol group of the enzyme protein is involved in the active site of the enzyme, as has been shown in yeast (Massey & Williams, 1965) and human erythrocytes (Worthington & Rosemeyer, 1976).

The subcellular location of glutathione reductase has been extensively investigated in animal tissues (Jocelyn, 1972); it is mainly located in cytosol. In plants, the enzyme is also distributed in chloroplasts (Foyer & Halliwell, 1976). Table 2 shows distribution of the activities of glutathione reductase together with those of marker enzymes in subcellular fractions obtained by differential centrifugation of an *E. gracilis* homogenate. The marker enzymes for chloroplasts, mitochondria and microsomes showed high activities in their respective fractions. *Euglena* glutathione reductase is distributed solely in cytosol, as judged by the distribution of glutamate dehydrogenase, a cytosolic enzyme. It has been shown that, in *E. gracilis*, AsA peroxidase and DAsA reductase are located only in cytosol (Shigeoka *et al.*, 1980*a*). The facts support the notion that *Euglena*

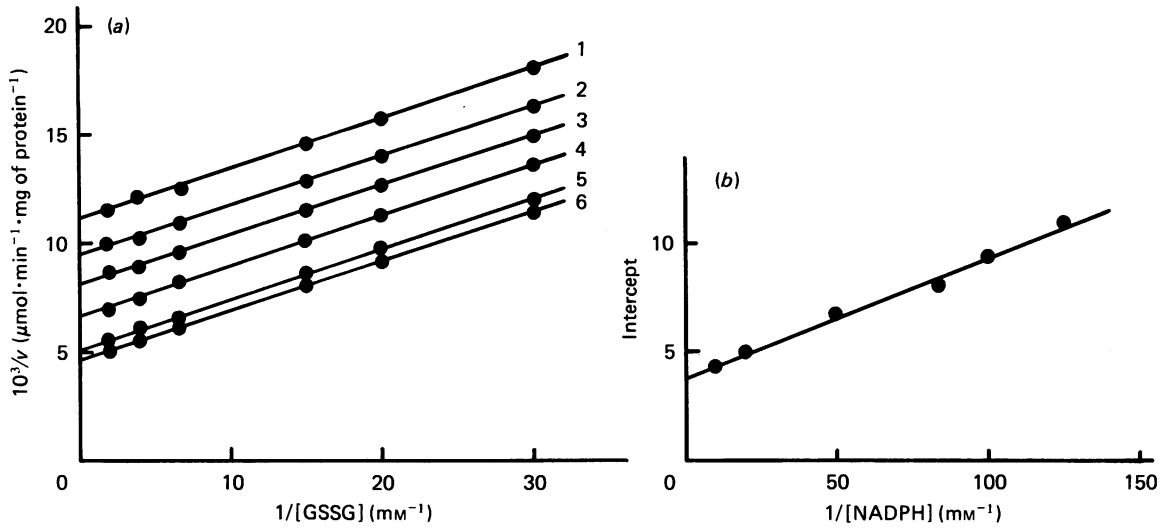

Fig. 2. Double-reciprocal plots of glutathione reductase activity and various concentrations of both GSSG and NADPH

(*a*) Double-reciprocal plots of initial velocity against variable GSSG concentrations at several fixed NADPH concentrations. NADPH concentrations were: 8 (line 1), 10 (line 2), 12 (line 3), 20 (line 4), 50 (line 5) and 100 (line 6) μM. (*b*) Replots of intercepts against the reciprocal of the NADPH concentration. Each experimental point presents the mean for four assays (coefficient of variation $\leqslant 5\%$).

Table 2. Distribution of glutathione reductase and marker enzymes in subcellular fractions of *Euglena gracilis*

GL cells were grown for 4 days under illumination (3000 lx). Partial trypsin digestion and subcellular fractionation by differential centrifugation were conducted as described by Shigeoka *et al.* (1986*b*). Glutathione reductase and marker enzymes were assayed as described in the Materials and methods section. Distribution of glutathione reductase and marker enzymes in each organelle is given as a percentage of the activity (μmol/min) in the crude homogenate given in parentheses. The crude homogenate contained 80.4 μg of protein.

Enzyme	Percentage of enzyme activity in crude homogenate				
	Crude homogenate	Chloroplasts	Mitochondria	Microsomes	Cytosol
Glutathione reductase	100 (10.85)	0	0	2.5	96.8
Ribulosebisphosphate carboxylase	100 (11.74)	87.3	3.6	0	11.5
Succinate semialdehyde dehydrogenase	100 (12.06)	4.2	89.7	0	8.1
Glucose-6-phosphatase	100 (1.87)	0	6.2	88.6	7.2
Glutamate dehydrogenase	100 (12.55)	0	0	3.1	97.2

Table 3. Contents of GSH, GSSG, AsA and DAsA in the different *E. gracilis* cell types

Euglena cells were grown under illumination or in the dark for 5 days, at which time they had reached the stationary phase of growth. GSH and GSSG were determined as described in the Materials and methods section. Each value is the mean $\pm$S.D. ($n = 4$).

Cell type	Content (nmol/10^9 cells)			
	GSH	GSSG	AsA*	DAsA*
GL†	958±31	140±11	884±37	156±10
GD	241±22	25±2	152±11	33±3
BL	349±28	79±4	308±18	42±3
BD	292±18	42±2	132±8	30±2

* Data from Shigeoka *et al.* (1979, 1980*c*).
† These abbreviations are defined in the text.

glutathione reductase is associated with the oxidation–reduction cycle of AsA in cytosol.

Table 3 shows GSH and GSSG contents in *Euglena* cells in the stationary phase of growth with and without illumination. GL cells, when grown heterotrophically under illumination, contained 1098 nmol of total glutathione/10^9 cells, enough to afford a glutathione concentration of 1 mM for an estimated cell volume of 1 ml/10^9 cells, which was determined with a haematocrit (Isegawa *et al.*, 1984); this concentration almost approaches that of total AsA (Shigeoka *et al.*, 1980*c*). The total glutathione contents of BL and GD cells were 39.0 and 24.2% respectively of that in GL cells. These results mean that the total glutathione content is affected by growth conditions and varies in parallel with the change in total AsA content (Shigeoka *et al.*, 1979, 1980*c*). In *Euglena* cells under illumination and in the dark, GSH constituted more than 80% of total glutathione, indicating that glutathione reductase participates in maintaining

glutathione in the reduced state. In addition, the maintenance of high [GSH/GSSG] and [AsA/DAsA] ratios in *Euglena* cells in both the light and the dark, which is in agreement with the experimental data for spinach chloroplasts (Law *et al.*, 1983), suggested that glutathione functions by coupling with the oxidation–reduction of AsA and removing H_2O_2 generated *in vivo*. It is well known that glutathione plays a role in stabilizing the enzymes requiring a thiol group for their activity (Meister & Tate, 1976; Halliwell, 1981). The large amounts of GSH together with the high activity of glutathione reductase in the *Euglena* cells may suggest an additional important function.

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