Purification and characterization of glutathione reductase from *Chlamydomonas reinhardtii*

TORU TAKEDA, TAKAHIRO ISHIKAWA, SHIGERU SHIGEOKA,* OSAMU HIRAYAMA and TOSHIO MITSUNAGA

Department of Food and Nutrition, Kinki University, Nara 631, Japan

(Received 5 January 1993; revised 2 April 1993; accepted 7 April 1993)

Glutathione reductase was purified to homogeneity from the unicellular alga *Chlamydomonas reinhardtii*. The enzyme was a monomer with a molecular mass of 54–56 kDa as judged by gel filtration and SDS-PAGE. The activity was maximal at pH 8.2 and 49 °C. The enzyme was specific for NADPH, but not for NADH. The reverse reaction with NAD(P)⁺ and GSH (glutathione, reduced form) was not observed in the pH range 4.8–8.2. K_m values for NADPH and GSSG (glutathione, oxidized form) were 10.6 µM and 54.1 µM, respectively. Thiol inhibitors and metal ions such as Hg²⁺ and Cu²⁺ markedly inhibited the enzyme activity. Activity was lost when the apoenzyme was prepared by dialysis, but was restored to 40% of the original activity by the addition of 50 µM-FAD. The enzyme reaction proceeded via a branching mechanism. Upon immunoprecipitation, glutathione reductase activity of *C. reinhardtii* was inhibited 50% and 90% by antibodies generated against spinach and *Euglena* glutathione reductases, respectively. Both antibodies cross-reacted with *C. reinhardtii* glutathione reductase in an immunoblot analysis.

Introduction

The thiol-containing tripeptide glutathione is widespread in plant cells in high concentrations (Alscher, 1989). Glutathione functions as an antioxidant and has a role in the detoxification of xenobiotics and air pollutants and the removal of toxic free-radical and hydroperoxides in the ascorbate–glutathione cycle (Alscher, 1989; Halliwell & Gutteridge, 1985).

We have previously demonstrated that in cultures of the photosynthetic alga *Chlamydomonas reinhardtii*, grown in medium containing sodium selenite, there is a *de novo* synthesis of glutathione peroxidase and the disappearance of ascorbate peroxidase (Yokota *et al.*, 1988). Selenium-dependent glutathione peroxidase in *C. reinhardtii* has recently been purified (Shigeoka *et al.*, 1990). *C. reinhardtii* glutathione peroxidase resembles other well-characterized enzymes from animal sources that contain selenium based on enzymic, physico-chemical and immunological properties (Shigeoka *et al.*, 1990, 1991). Subsequently, we have shown that enzymes involved in metabolism of oxygen and glutathione are

present in cells cultured in the presence or absence of selenite (Takeda *et al.*, 1992).

Glutathione reductase is ubiquitous in living organisms (Smith *et al.*, 1989). This enzyme is coupled directly with glutathione peroxidase in animals and indirectly with ascorbate peroxidase via dehydroascorbate reductase in higher plants and microorganisms including *Euglena* (Shigeoka *et al.*, 1980, 1987a), green algae (Kow *et al.*, 1982) and cyanobacteria (Tel-Or *et al.*, 1986). It seems likely that glutathione reductase participates in the maintenance of a large pool of glutathione in the reduced form and the acceleration of the H₂O₂ scavenging pathway *in vivo* (Smith *et al.*, 1989).

In the present work, we purified *C. reinhardtii* glutathione reductase from cells grown in medium containing selenite and characterized the enzyme. The immunological properties of the enzyme were studied using antibodies directed against the spinach and *Euglena* enzymes. We discuss the physiological function of glutathione reductase coupled with selenite-induced glutathione peroxidase in *C. reinhardtii*.

Methods

Organism and culture. *Chlamydomonas reinhardtii* Dangeard was grown in Allen's medium supplemented with 3 mg sodium selenite l⁻¹ at 26 °C for 5 d under constant illumination at 240 µE s⁻¹ m⁻² (Yokota

* Author for correspondence. Tel. 742 43 1511 ext. 3416; fax 742 43 1155.

Abbreviations: GSH, glutathione, reduced form; GSSG, glutathione, oxidized form.

et al., 1988). The batch cultures (8 l) were magnetically stirred and aerated with sterile air at a rate of 8 l min⁻¹.

Enzyme assay. Glutathione reductase activity was assayed at 35 °C in 2 ml of reaction mixture containing 50 mM-potassium phosphate buffer, pH 8.2, 1 mM-EDTA, 0.2 mM-NADPH, 1 mM-GSSG and the enzyme as described previously (Shigeoka *et al.*, 1987b). The reaction was initiated by the addition of enzyme. The progress of the reaction was monitored by measuring the decrease in absorbance of NADPH at 340 nm. One unit of enzyme activity was defined as the amount of enzyme catalysing oxidation of 1 µmol NADPH min⁻¹.

Purification of glutathione reductase. All steps were carried out at 4 °C. Potassium phosphate buffer (10 mM, pH 8.2) containing 10% (w/v) sucrose and 1 mM-EDTA was used unless otherwise noted. Cells (40 g wet wt) were harvested by centrifugation, resuspended in 100 ml buffer, and sonicated (10 kHz) for a total of 15 min with three intervals of 5 min each. This lysate was centrifuged at 15000 g for 20 min to remove cell debris. The supernatant, designated crude extract, was subjected to ultracentrifugation at 100000 g for 30 min. The supernatant was loaded onto a DEAE-cellulose column (2.5 × 30 cm) equilibrated with phosphate buffer. The column was eluted with 300 ml of a linear gradient of 0–300 mM-KCl (flow rate 0.6 ml min⁻¹, 5 ml fractions). Active fractions were combined and adjusted to 70% saturation with ammonium sulphate. The precipitate was collected by centrifugation at 15000 g for 30 min and dissolved in 3 ml phosphate buffer. The protein solution was loaded on a Sephadex G-150 column (2.2 × 90 cm), equilibrated with phosphate buffer and eluted with the same buffer at a flow rate of 20 ml h⁻¹. The combined active fractions were chromatographed on a column (1.7 × 11 cm) of Blue-Sepharose equilibrated with 10 mM-phosphate buffer. The column was washed with 2 bed vols of the same buffer and eluted with 200 ml of a linear gradient of 0–300 mM-KCl at an elution rate of 0.6 ml min⁻¹. The active solution was dialysed twice for 8 h against phosphate buffer to remove KCl and subsequently loaded onto an ADP-Sepharose column (0.9 × 6 cm) equilibrated with 5 mM-potassium phosphate buffer, pH 8.2, containing 1 mM-EDTA. The column was washed with 20 ml of the same buffer. Glutathione reductase was eluted with 2 ml 5 mM-NADP⁺. The purified enzyme was immediately dialysed for 7 h against the buffer, concentrated to a final volume of 1 ml by ultrafiltration (Amicon PM-30), and stored at -20 °C.

Determination of molecular mass. The molecular mass of native glutathione reductase was determined by gel filtration on a Sephadex G-150 column equilibrated with 10 mM-potassium phosphate buffer, pH 8.2. SDS-PAGE was performed on 12.5% (w/v) polyacrylamide slab gels as described previously (Shigeoka & Nakano, 1991). Phosphorylase *b* from rabbit muscle (94 kDa), BSA (67 kDa), ovalbumin from egg white (43 kDa), carbonic anhydrase from bovine erythrocytes (30 kDa), trypsin inhibitor from soybean (20.1 kDa) and α-lactalbumin from bovine milk (14.4 kDa) were used as standards. Proteins in the gel were stained with Coomassie Brilliant Blue R-250.

Preparation of antibodies from spinach and *Euglena*. *Euglena* glutathione reductase was purified to homogeneity by the method of Shigeoka *et al.* (1987b). The purified glutathione reductases (400 µg each) from *Euglena* and spinach were used to prepare antibodies by subcutaneous injection into male 6-month-old white rabbits as described previously (Shigeoka *et al.*, 1991). The rabbits were bled and the sera were prepared by standard methods (Hurn & Chantler, 1980) followed by purification and concentration of the IgG using Protein A-Sepharose.

Immunoblot analysis. To study immunological properties of glutathione reductases, a dot blot apparatus (Biodot SF, Bio Rad) was used. Glutathione reductases from cabbage and rat liver were partially

purified according to the procedure described above up to the (NH₄)₂SO₄ precipitation step. Each sample (50 and 100 µg protein) was applied to a nitrocellulose membrane, washed with 100 ml 0.9% (w/v) NaCl and filtered with suction for 1 h. BSA (100 µg) was used as a control. Skim milk [0.5% (w/v) dissolved in 20 mM-Tris/HCl, pH 8.3, containing 100 mM-NaCl] was used to block background. The subsequent blotting analysis procedure was done as described previously (Shigeoka & Nakano, 1991). Proteins were detected using antibodies against spinach and *Euglena* glutathione reductase and peroxidase-conjugated goat anti-rabbit IgG serum.

Other methods. To examine the cofactor requirements of glutathione reductase, the purified enzyme was suspended in 10 mM-potassium phosphate buffer, pH 8.2, and dialysed against 100 mM-citrate buffer pH 2.0 for 1 h at 4 °C, followed by incubation with FAD or FMN for 30 min at 30 °C as described previously (Carlberg & Mannervik, 1975). The optimum pH was determined in 10 mM-potassium phosphate buffer (pH 6.2–8.4) and 25 mM-Tris/HCl buffer (pH 7.0–9.5). The pH stability was determined by assaying activity after treatment of the purified enzyme at various pH values for 10 min at 48 °C. Thermal stability of the enzyme was determined after treatment at various temperatures for 10 min at pH 8.2. Protein determination was done by the method of Bradford (1976) using BSA as a standard.

Chemicals. Glutathione reductases from spinach and yeast, and Protein A-Sepharose were obtained from Sigma. All reagents for SDS-PAGE and goat anti-IgG serum-peroxidase conjugate were purchased from Pharmacia. Nitrocellulose for immunoblot analysis was obtained from Toyo Roshii Co., Osaka, Japan. All other chemicals were purchased from commercial sources and were of the highest purity available.

Results and Discussion

The purification scheme of glutathione reductase using a seven-step procedure is summarized in Table 1. Ultracentrifugation of the crude extract resulted in an increase in the total enzyme activity and yield, implying that there is a membrane-associated inhibitory material in this fraction. During purification of *C. reinhardtii* glutathione reductase, the enzyme activity was eluted from each column as a sharp and single peak. These results indicate that *C. reinhardtii* cells contain only one type of glutathione reductase. The enzyme was purified about 4000-fold over the crude extract with a yield of 28.7%. The purification was repeated several times with similar results. Polyacrylamide disc-gel electrophoresis of the native purified enzyme (data not shown) and SDS-PAGE (Fig. 1) showed only one detectable protein band (arrowed). The specific activity with NADPH and GSSG was 373.3 µmol min⁻¹ (mg protein)⁻¹; this value is comparable to those of enzymes from plants (Smith *et al.*, 1989) and *Euglena* (Shigeoka *et al.*, 1987b). The molecular mass of glutathione reductase was 54 kDa as indicated by SDS-PAGE (Fig. 1) and about 56 kDa by gel filtration on a calibrated Sephadex G-150 column. This result indicates that glutathione reductase of *C. reinhardtii* exists in a monomeric form in its native state. As shown in Table 2, glutathione reductase from several

Table 1. Purification of glutathione reductase from *C. reinhardtii*

Purification step	Total protein (mg)	Total activity (U)	Specific activity [U (mg protein) ⁻¹]	Yield (%)
Crude extract	710.0	78.1	0.11	100
Ultracentrifugation	321.1	112.4	0.35	143.9
DEAE-cellulose	52.5	67.7	1.29	86.7
(NH ₄) ₂ SO ₄ (70 %) precipitation	45.7	63.5	1.39	81.3
Sephadex G-150	10.6	41.9	3.95	53.6
Blue-Sepharose	1.35	30.9	22.9	39.6
ADP-Sepharose	0.06	22.4	373.3	28.7

Fig. 1. SDS-PAGE of purified glutathione reductase. Protein standards (lane 1) and 5 µg of purified enzyme (lane 2) were subjected to SDS-PAGE. Both lanes were stained with Coomassie Brilliant Blue R-250 and destained in 7% (v/v) acetic acid. The arrow indicates the glutathione reductase.

other sources is a dimer with subunits of the same size (Carlberg & Mannervik, 1985; Halliwell & Foyer, 1978; Shigeoka *et al.*, 1987b; Madamanchi *et al.*, 1992). Glutathione reductase belongs to the pyridine nucleotide-disulphide oxidoreductase family which includes lipoamide dehydrogenase and thioredoxin reductase, each of which is dimeric (Williams, 1976). The X-ray crystal structure of the human erythrocyte enzyme showed that elements from both polypeptides, including the redox-active disulphide and a histidine residue, contribute to each active site and to the GSSG binding site (Thieme *et al.*, 1981). Furthermore, Arscott *et al.* (1989) have demonstrated that the extent to which the inactivation of *Escherichia coli* glutathione reductase

proceeded was inversely related to the enzyme concentration, suggesting that dimer dissociation was involved. The novel existence of an active monomer of the *C. reinhardtii* glutathione reductase is the first case that we have observed. However, detailed information on the monomer in its native state is not yet available.

Glutathione reductase has an absorption spectrum which is typical of enzymes which contain FAD as coenzyme (Worthington & Rosemeyer, 1974). Since an absorption spectrum of *C. reinhardtii* glutathione reductase was not obtained owing to the low recovery of the purified enzyme, we examined the presence of the coenzyme according to the dialysis method of Carlberg & Mannervik (1975). Dialysis of the purified enzyme in citrate buffer caused the enzyme to lose activity, but this activity was recovered up to 22 and 38% of the original level following incubation with 20 and 50 µM-FAD, respectively. FMN (50 µM) had no effect on the enzyme activity. These data imply that FAD is necessary for catalytic activity of *C. reinhardtii* glutathione reductase and that the cofactor is non-covalently bound.

The optimal pH for glutathione reductase activity was 8.2, which is similar to those of the enzymes from spinach (Halliwell & Foyer, 1978) and *Euglena* (Shigeoka *et al.*, 1987b). The enzyme retained full activity between pH 6.8 and 8.4. The enzyme activity was stable up to 45 °C and was completely lost at 55 °C. The optimum temperature was 49 °C, while the activities at 31 °C and 54 °C were 49% and 68%, respectively. The activation energy was calculated to be 31.9 kJ mol⁻¹ from the Arrhenius plots. The enzyme utilized NADPH as an electron donor. Activity with NADH was not detected in the pH range 4.8–8.2, indicating that NADPH is the sole physiological electron donor. Glutathione reductases from many sources have a remarkably high preference for NADPH as a reductant (Smith *et al.*, 1989). The reverse reaction with 1 mM-NAD(P)⁺ and GSH was not measurable and this is similar to results for the *Euglena* enzyme (Shigeoka *et al.*, 1987b). The enzyme was specific for GSSG; lipoic acid and cystine did not substitute for GSSG. The enzyme reaction followed Michaelis–Menten kinetics

Table 2. Comparison of some properties of the purified glutathione reductase from several sources

Property	<i>C. reinhardtii</i>	<i>Euglena</i> *	Spinach leaf†	Pea chloroplast‡	Yeast§	Rat liver
Mol. mass (kDa)						
Native	56	79	145	114		125
Subunit	54	41	72	55	56	60
Optimum pH	8.2	8.2	8.5-9.0	7.6	7.1	7.0
K_m value (μM)						
NADPH	10.6	14.0	3.0	4.8	7.6	7.9
GSSG	54.1	55.0	200.0	56.0	61.0	56.7
Reaction mechanism	Branching	Branching			Branching	Branching

* Shigeoka, *et al.* (1987b). † Halliwell & Foyer (1978). ‡ Madamanchi *et al.* (1992). § Massey & Williams (1965). || Carlberg & Mannervik (1975).

toward GSSG over the range 0–100 μM and NADPH over the range 0–50 μM . In double-reciprocal plots of NADPH concentration versus reaction velocity with different GSSG concentrations, the enzyme systems gave parallel lines (Fig. 2), indicating that the reaction proceeds by a branching mechanism as has been reported for the yeast (Massey & Williams, 1965), rat liver (Carlberg & Mannervik, 1975) and *Euglena* (Shigeoka *et al.*, 1987b) enzymes. The secondary plots of slopes and intercepts allowed us to calculate the kinetic constants; the K_m values of the enzyme for GSSG and NADPH were 54.1 μM and 10.6 μM , respectively. These values were of the same order of magnitude as those reported for other sources; the K_m values for GSSG and NADPH are in the ranges 10–60 μM and 2–14 μM in plants (Smith *et al.*, 1989), respectively. *C. reinhardtii* cells grown in medium containing sodium selenite accumulated 100.5 ± 8.5 nmol total glutathione (mg chlorophyll)⁻¹, which corresponds to 1 mM. GSH represents more than 90% of the total glutathione, 10% of which is GSSG (Takeda *et al.*, 1992). The presence of 100 μM -GSSG in *C. reinhardtii* cells would account for the observation that glutathione reductase efficiently sequesters glutathione in the reduced form *in vivo*. The enzyme dialysed against 50 mM-Tris/HCl buffer, pH 8.2, for 6 h was used to examine the effect of some compounds on enzyme activity. Hg^{2+} , Cu^{2+} and Co^{2+} (each at 1 mM) completely inhibited enzyme activity. Similar concentrations of Ni^{2+} , Zn^{2+} and Mn^{2+} inhibited enzyme activity to 80%, 75% and 40%, respectively. Dithiothreitol, KCN, NaN_3 and EDTA (1 mM each) had no effect. Thiol inhibitors such as 1 mM-*N*-ethylmaleimide and 0.05 mM-*p*-chloromercuribenzoate completely inhibited enzyme activity, indicating that a thiol group is located at the active centre of the enzyme and is involved in catalysis, as has been shown in yeast (Massey & Williams, 1965), human erythrocytes (Worthington & Rosemeyer, 1976) and *Euglena* (Shigeoka *et al.*, 1987b).

Fig. 2. Kinetic analysis of glutathione reductase activity with GSSG and NADPH. (a) Double-reciprocal plots of initial velocity against variable NADPH concentrations at several fixed GSSG concentrations: 30 μM (1); 35 μM (2); 40 μM (3); 50 μM (4); and 100 μM (5). (b) Re-plots of intercepts against the reciprocal of the GSSG concentration. Each experimental point presents the mean for four assays (coefficient of variation < 5%).

To examine immunological relationships between *C. reinhardtii* glutathione reductase and those from other sources, immunoprecipitation experiments were carried

Fig. 3. Immunoprecipitation of glutathione reductases from several sources using antibodies directed against spinach (a) or *Euglena* (b) glutathione reductases. Each glutathione reductase was incubated with the indicated amounts of antibodies directed against spinach or *Euglena* glutathione reductases at 4 °C for 1 h, followed by centrifugation at 10000 g for 10 min. The supernatant was used to assay glutathione reductase activity. Normal serum had no effect on the enzyme activity. The data are means of values obtained from four experiments (coefficient of variation < 5%). Glutathione reductases: ▲, *C. reinhardtii*; ●, *Euglena*; ○, spinach; ■, cabbage; △, yeast; □, rat liver.

out using antibodies directed against the spinach and *Euglena* glutathione reductases (Fig. 3). After incubation with the indicated amounts of anti-glutathione reductase antibody, the insoluble complex was removed by centrifugation and the enzyme activity remaining in the supernatant was assayed. The antibody directed against spinach glutathione reductase removed the enzyme activity from the spinach supernatant almost completely, as described previously (Tanaka *et al.*, 1988). Glutathione reductases from cabbage and *C. reinhardtii* were also immunoprecipitated. Their enzyme activities were reduced by approximately 70% and 59% respectively, after immunoprecipitation with anti-spinach enzyme antibody, while those from *Euglena*, rat liver and yeast were unaffected. The antibody against *Euglena*

Fig. 4. Immunoblot analysis of glutathione reductases from several sources using antibodies raised against glutathione reductase from spinach (a) or *Euglena* (b). This was carried out using a dot-blot apparatus (Bio-Rad). Details of the procedure are described in Methods. Glutathione reductases: 1, spinach; 2, yeast; 3, *Euglena*; 4, rat liver; 5, cabbage; 6, *C. reinhardtii*.

glutathione reductase removed 100% of the enzyme activity from the *Euglena* supernatant, about 90% from the *C. reinhardtii* supernatant and about 20% from spinach supernatant, while the antibody showed no effect on the enzyme activities from yeast, rat liver and cabbage. On immunoblot analysis, the anti-spinach antibody reacted with glutathione reductase from spinach, cabbage and *C. reinhardtii*, while the anti-*Euglena* antibody reacted with those from *Euglena*, *C. reinhardtii* and spinach (Fig. 4). The results obtained from the immunoprecipitation experiments with spinach and *Euglena* antibodies were similar to the immunoblot analysis. It has been reported, that based on an Ouchterlony double immunodiffusion method, tobacco and petunia crude extracts reacted with the anti-spinach antibody, but these precipitin lines fused only partially with the precipitin band between spinach glutathione reductase and anti-spinach antibody (Tanaka *et al.*, 1988). Crude extracts from moss, fern, *Chlorella* and purified yeast enzyme did not produce any precipitin band with anti-spinach antibody (Tanaka *et al.*, 1988). These results indicate that, although the molecular structure of glutathione reductases among living organisms studied so far are antigenically fairly distinguishable, *C. reinhardtii* glutathione reductase has similar antigenic epitopes to the enzymes from *Euglena* and spinach.

Glutathione reductase appears to function to maintain glutathione in the reduced form *in vivo* (Alscher, 1989; Halliwell & Gutteridge, 1985; Meister & Anderson, 1983). Foyer *et al.* (1991) recently demonstrated that glutathione reductase in normal and transgenic tobacco leaves does not limit the glutathione content or the reduction state of the glutathione pool under optimal conditions or oxidative conditions induced by methylviologen. In *C. reinhardtii* cells cultured in the presence and absence of selenite, GSH constantly constituted more than 80% of total glutathione (Takeda *et al.*, 1992). Therefore, these results imply that glutathione reductase maintains the glutathione pool in a largely reduced form in *C. reinhardtii*.

We are grateful to the late Professor Y. Iizuka, Kinki University, for his stimulating advice and discussion and to Dr D. W. Copertino, University of Arizona, USA, for his critical reading of the manuscript.

References

- ALSCHER, R. G. (1989). Biosynthesis and antioxidant function of glutathione in plants. *Physiologia Plantarum* **77**, 457–464.
- ARSCOTT, L. D., DRAKE, D. M. & WILLIAMS, C. H., JR (1989). Inactivation–reactivation of two-electron reduced *Escherichia coli* glutathione reductase involving a dimer–monomer equilibrium. *Biochemistry* **28**, 3591–3598.
- BRADFORD, M. M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry* **717**, 1448–1454.
- CARLBERG, I. & MANNERVIK, B. (1975). Purification and characterization of the flavoenzyme glutathione reductase from rat liver. *Journal of Biological Chemistry* **250**, 5475–5480.
- CARLBERG, I. & MANNERVIK, B. (1985). Glutathione reductase. *Methods in Enzymology* **113**, 484–495.
- FOYER, C., LELANDIS, M., GALAP, C. & KUNERT, K. J. (1991). Effects of elevated cytosolic glutathione reductase activity on the cellular glutathione pool and photosynthesis in leaves under normal and stress conditions. *Plant Physiology* **97**, 863–872.
- HALLIWELL, B. & FOYER, C. H. (1978). Properties and physiological function of a glutathione reductase purified from spinach leaves by affinity chromatography. *Planta* **139**, 9–17.
- HALLIWELL, B. & GUTTERIDGE, J. M. C. (1985). *Free Radicals in Biology and Medicine*. Oxford: Oxford University Press.
- HURN, B. A. L. & CHANTLER, S. M. (1980). Production of reagent antibodies. *Methods in Enzymology* **70**, 104–142.
- KOW, Y. W., SMYTH, D. A. & GIBBS, M. (1982). Oxidation of reduced pyridine nucleotide by a system using ascorbate and hydrogen peroxide from plants and algae. *Plant Physiology* **69**, 72–76.
- MADAMANCHI, N. R., ANDERSON, J. V., ALSCHER, R. G., CRAMER, C. L. & HESS, J. L. (1992). Purification of multiple forms of glutathione reductase from pea (*Pisum sativum* L.) seedlings and enzyme levels in ozone-fumigated pea leaves. *Plant Physiology* **100**, 138–145.
- MASSEY, V. & WILLIAMS, C. H. (1965). On the reaction mechanism of yeast glutathione reductase. *Journal of Biological Chemistry* **240**, 4470–4480.
- MEISTER, A. & ANDERSON, M. E. (1983). Glutathione. *Annual Review of Biochemistry* **52**, 711–760.
- SHIGEOKA, S. & NAKANO, Y. (1991). Characterization and molecular properties of 2-oxoglutarate decarboxylase from *Euglena gracilis*. *Archives of Biochemistry and Biophysics* **288**, 22–28.
- SHIGEOKA, S., NAKANO, Y. & KITAOKA, S. (1980). Metabolism of hydrogen peroxide in *Euglena gracilis* Z by L-ascorbic acid peroxidase. *Biochemical Journal* **186**, 377–380.
- SHIGEOKA, S., YASUMOTO, R., ONISHI, T., NAKANO, Y. & KITAOKA, S. (1987a). Properties of monodehydroascorbate reductase and dehydroascorbate reductase and their participation in the regeneration of ascorbate in *Euglena gracilis*. *Journal of General Microbiology* **133**, 227–232.
- SHIGEOKA, S., ONISHI, T., NAKANO, Y. & KITAOKA, S. (1987b). Characterization and physiological function of glutathione reductase in *Euglena gracilis* Z. *Biochemical Journal* **242**, 511–515.
- SHIGEOKA, S., TAKEDA, T., HANAOKA, T., YOKOTA, A., KITAOKA, S. & IIZUKA, Y. (1990). Properties of selenium-induced glutathione peroxidase in low-CO₂-grown *Chlamydomonas reinhardtii*. *Current Research in Photosynthesis* **4**, 615–618.
- SHIGEOKA, S., TAKEDA, T. & HANAOKA, T. (1991). Characterization and immunological properties of selenium-containing glutathione peroxidase induced by selenite in *Chlamydomonas reinhardtii*. *Biochemical Journal* **275**, 623–627.
- SMITH, I. K., VIERHELLER, T. L. & THORNE, C. A. (1989). Properties and functions of glutathione reductase in plants. *Physiologia Plantarum* **77**, 449–456.
- TAKEDA, T., SHIGEOKA, S., HIRAYAMA, O. & MITSUNAGA, T. (1992). The presence of enzymes related to glutathione metabolism and oxygen metabolism in *Chlamydomonas reinhardtii*. *Bioscience, Biotechnology, and Biochemistry* **56**, 1662–1663.
- TANAKA, K., SAJI, H. & KONDO, N. (1988). Immunological properties of spinach glutathione reductase and inductive biosynthesis of the enzyme with ozone. *Plant and Cell Physiology* **29**, 637–642.
- TEL-OR, E., HUFLEIT, M. E. & PACKER, L. (1986). Hydroperoxide metabolism in cyanobacteria. *Archives of Biochemistry and Biophysics* **246**, 396–402.
- THIEME, R., PAI, E. F., SCHIRMER, R. H. & SCHULTZ, G. E. (1981). Three dimensional structure of glutathione reductase at two Å resolution. *Journal of Molecular Biology* **152**, 763–782.
- WILLIAMS, C. H., JR (1976). Glutathione reductase. *Enzymes* **13**, 89–173.
- WORTHINGTON, D. J. & ROSEMEYER, M. A. (1974). Human glutathione reductase: purification of the crystalline enzyme from erythrocytes. *European Journal of Biochemistry* **48**, 167–177.
- WORTHINGTON, D. J. & ROSEMEYER, M. A. (1976). Glutathione reductase from human erythrocytes. Catalytic properties and aggregation. *European Journal of Biochemistry* **67**, 231–238.
- YOKOTA, A., SHIGEOKA, S., ONISHI, T. & KITAOKA, S. (1988). Selenium as inducer of glutathione peroxidase in low-CO₂-grown *Chlamydomonas reinhardtii*. *Plant Physiology* **86**, 649–651.