Photoinactivation and Protection of Glycolate Oxidase *in vitro* and in Leaves

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Glycolate oxidase that was partially purified from pea leaves was inactivated *in vitro* by blue light in the presence of FMN. Inactivation was greatly retarded in the absence of O_2. Under aerobic conditions H_2O_2 was formed. The presence of catalase, GSH or dithiothreitol protected glycolate oxidase against photoinactivation. Less efficient protection was provided by ascorbate, histidine, tryptophan or EDTA. The presence of superoxide dismutase or of hydroxyl radical scavengers had no, or only minor, effects. Glutathione suppressed H_2O_2 accumulation and was oxidized in the presence of glycolate oxidase in blue light. Glycolate oxidase was also inactivated in the presence of a superoxide-generating system or by H_2O_2 in darkness. In intact leaves photoinactivation of glycolate oxidase was not observed. However, when catalase was inactivated by the application of 3-amino-1,2,4-triazole or depleted by prolonged exposure to cycloheximide a strong photoinactivation of glycolate oxidase was also seen in leaves. *In vivo* blue and red light were similarly effective. Furthermore, glycolate oxidase was photoinactivated in leaves when the endogenous GSH was depleted by the application of buthionine sulfoximine. Both catalase and antioxidants, in particular GSH, appear to be essential for the protection of glycolate oxidase in the peroxisomes *in vivo*.

Introduction

Visible light provides the energy source for autotrophic plant growth but may also exert detrimental effects on plant cells. In particular, photosynthetic pigments can act as photosensitizers mediating the formation of singlet oxygen, and the photosynthetic electron transport chain may contribute to the production of reactive oxygen species (ROS) that initiate photooxidative damage (Foyer *et al.*, 1994). A multitude of photoprotective systems exists that usually allow plants to either avoid or repair photooxidative damage under conditions to which they are adapted (Asada, 1994; Foyer, 1997). It has, however, become apparent that, in spite of efficient antioxidative mechanisms, certain compounds are generally not sufficiently protected against photodamage. Well investigated examples are the D1 reaction center protein of photosystem II (PSII) and the peroxisomal enzyme catalase. Whereas these proteins have most important functions for the photosyn-

thetic or photorespiratory plant metabolism in light, both the D1 protein (Aro *et al.*, 1993) and catalase (Feierabend and Engel, 1986; Hertwig *et al.*, 1992; Feierabend *et al.*, 1996; Shang and Feierabend, 1999) are inactivated in light. In order to maintain constant steady state levels of functional proteins, they need to be continuously replaced by *de novo* synthesis. Therefore, both the D1 protein of PSII and catalase exhibit a specific light-dependent turnover in leaves that increases with the photon flux.

Photoinactivation of catalase is mediated by blue light absorbed by its prosthetic heme (Cheng *et al.*, 1981; Grotjohann *et al.*, 1997; Shang and Feierabend, 1999). In leaves catalase inactivation may, in addition, be indirectly mediated by photooxidative events initiated in the chloroplasts (Feierabend and Engel, 1986; Shang and Feierabend, 1999). Photoinactivation of catalase in leaves can be detected when its repair is prevented either by translation inhibitors (Feierabend and Engel, 1986; Hertwig *et al.*, 1992) or by stress conditions that suppress protein synthesis, such as salt (Streb and Feierabend, 1996) or low temperature (Volk and Feierabend, 1989; Feierabend *et al.*, 1992). When protein synthesis is suppressed, cata-

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Abbreviations: Chl, chlorophyll; PAR, photosynthetically active radiation; PSII, photosystem II; ROS, reactive oxygen species.

lase activity of leaves can be largely depleted within one day of light exposure. The main function of peroxisomal catalase is to detoxify the H_2O_2 that is produced by the oxidation of glycolate during photorespiration. It has been reported that the enzyme glycolate oxidase is also light-sensitive. *In vitro* glycolate oxidase from tobacco leaves was inactivated by blue light absorbed by its FMN cofactor (Schmid, 1969). During our investigations of the photoinactivation of catalase we did, however, not observe a simultaneous inactivation of glycolate oxidase. During irradiation of isolated peroxisomes from rye leaves *in vitro* glycolate oxidase activity remained constant, while catalase activity was totally lost (Feierabend and Engel, 1986). In intact leaves the decline of catalase activity in light was not accompanied by any major loss of glycolate oxidase activity when repair was blocked by translation inhibitors or stress conditions (Feierabend and Engel, 1986; Volk and Feierabend, 1989; Feierabend *et al.*, 1992; Shang and Feierabend, 1999). Consequently, the glycolate oxidase appeared to be well protected against photodamage and its turnover in leaves seemed to be very slow. In the present work we have reinvestigated the conditions for the photoinactivation of glycolate oxidase *in vitro* and examined which factors might contribute to its protection *in vivo*. The results indicate that photoprotection of glycolate oxidase in leaves substantially depends on the presence of catalase and requires that antioxidative reductants are available within the peroxisomal compartment.

Material and Methods

Plant material and growing conditions

For the assay of enzyme activities in leaves, experiments were performed with leaf sections from 6-day-old rye seedlings (*Secale cereale* L., cv. Halo). Seeds were surface-sterilized by a 10-min vacuum infiltration and about 30 min soaking in a freshly prepared filtered solution of 3% (w/v) calcium hypochlorite-chloride, thoroughly washed with demineralized H_2O and grown at 22 °C in glass-covered plastic boxes on filter paper (Macherey and Nagel Mn 218), moistened with a modified Knop's nutrient solution (Feierabend and Schrader-Reichhardt, 1976). Continuous white

light of 96 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) was provided by fluorescent tubes (Osram L 36W/36 Nature and Philips TL 40W/47 de Luxe in alternating sequence).

For the preparation of glycolate oxidase and for immunoblotting assays leaves of 14-day-old pea plants (*Pisum sativum* L., cv. Kleine Rheinländerin) were used. After a 2 h presoaking in H_2O peas were planted in Vermiculite, moistened with H_2O. After 5 days, seedlings were supplied with a modified Knop's nutrient solution. For enzyme preparations, plants were grown at 27 °C with a 16 h photoperiod. Actinic light of 200 μmol m^{-2} s^{-1} PAR was provided by Osram HQI-T 2000 W lamps. For immunoblotting, plants were grown at 22 °C in continuous white light of 96 μmol m^{-2} s^{-1} PAR.

Experimental treatments and light exposure

Segments of 5 cm were excised from the middle of the primary leaves of 6-day-old rye seedlings and each further divided into two halves. For the treatment with inhibitors segments obtained from 10 leaves were floated in petri dishes of 5 cm diameter on 10 ml of nutrient solution in the absence or presence of 35.5 μM cycloheximide, 2 mM 3-amino-1,2,4-triazole, or 35.5 μM cycloheximide + 2 mM 3-amino-1,2,4-triazole and kept for up to 48 h at 25 °C in light of 500 μmol m^{-2} s^{-1} PAR. White light was provided by Osram HQL 250 W high-pressure mercury lamps. Blue and red light were provided by 75 W Osram halogene lamps in combination with the broad-band blue light filter BG 23 (350–550 nm) or the cut-off filter RG2 (>630 nm 50% transmission) from Schott & Gen. (Mainz, Germany). In addition infrared filters KG3 were applied.

In order to deplete the endogenous content of reduced glutathione, complete primary leaves of 5-day-old rye seedlings were excised and incubated for 24 h at 22 °C in light of 96 mol m^{-2} s^{-1} PAR on nutrient solution in the presence of 10 mM D, L-buthionine sulfoximine. The pH of the solutions was adjusted to 7.0. Control leaves were kept on nutrient solution without the inhibitor. After these 24 h preincubation periods 5-cm segments were excised from treated leaves and incubated for further 24 h in white light, as described above, in the presence or absence of 10 mM D, L-buthio-

nine sulfoximine or 2 mM 3-amino-1,2,4-triazole at 25 °C.

For the *in vitro* inactivation experiments 1.5 ml each of a partially purified solution of glycolate oxidase (0.7–1.0 μmol s^{-1} ml^{-1}) from pea leaves in 50 mM triethanolamine buffer, pH 7.8, were irradiated in a water bath at 25 °C with blue light of 800 μmol m^{-2} s^{-1} PAR. Blue light was provided by 75 W Osram halogene lamps in combination with a broad-band blue light filter BG 23 (350–550 nm; Schott & Gen., Mainz, Germany) and an infrared filter KG 3. Where indicated, catalase (from bovine liver), superoxide dismutase (from horseradish), or glutathione peroxidase (from bovine erythrocytes) were added during the irradiations. For the removal of O$_2$ the solution was extensively flushed with N$_2$ in test tubes that were sealed with rubber caps before exposure to blue light.

Preparation of cell-free extracts

Segments of rye leaves were homogenized under ice-cold conditions with a mortar and pestle and with the addition of some acid-washed sea sand with 50 mM K-phosphate buffer, pH 7.5. For each extraction, homogenates from segments of 10 leaves were adjusted to a final volume of 5 ml. Homogenates were centrifuged for 8 min at 10,000×g and 4 °C. The resulting supernatants were used for the enzyme assays.

Immunoblotting

For immunoblotting, total homogenates were prepared by grinding frozen leaflets from the fourth leaf of pea seedlings with liquid nitrogen, extraction with 50 mM K-phosphate buffer, pH 7.5, and 20 min centrifugation at 27,000×g and 4 °C. The supernatants were used for polyacrylamide gel electrophoresis in the presence of SDS, and immunoblotting, as previously described (Hertwig *et al.*, 1992). A rabbit antiserum against glycolate oxidase from spinach leaves (Nishimura *et al.*, 1983) was used for immunodetection.

Preparation of glycolate oxidase

For the rapid partial purification the method of Betsche *et al.* (1986) was applied and modified. The procedure is based on the observation that at low pH and low ionic strength glycolate oxidase binds to the membrane fraction in leaf homogenates and can be pelleted. For one preparation 70–90 g leaves from 14-day-old pea plants were homogenized under ice-cold conditions with a two-fold volume of 50 mM morpholinopropanesulfonic acid (MOPS)-KOH buffer, pH 6.0, containing 10 mM dithioerythritol in a Waring blendor. The homogenate was passed through 4 layers of muslin and one layer of Miracloth and centrifuged for 15 min at 10,000×g and 4 °C. The sediment which contained about 80% of the total glycolate oxidase activity was washed by resuspension with grinding medium and recentrifugation under identical conditions. In order to elute the glycolate oxidase, the washed sediment was suspended in about 30 ml of 0.2 M 2-amino-2-(hydroxymethyl) aminomethane (Tris)-HCl, pH 8.0, containing 10 mM dithioerythritol and 0.4 M KCl. Membranes were pelleted by 15 min centrifugation at 10,000×g and 4 °C. The supernatant containing the glycolate oxidase activity was desalted by chromatography on a Sephadex G-25 coarse column. The column was equilibrated and eluted with 50 mM Tris-HCl, pH 8.5. Fractions containing glycolate oxidase were combined and protein precipitating between 30 and 50% (NH$_4$)$_2$SO$_4$ saturation was collected by 15 min centrifugation at 27,000×g and 4 °C. The precipitate was resuspended in 10 ml of 25 mM triethanolamine buffer, pH 7.8, and desalted by chromatography on a column with Sephadex G-25 coarse. The column was equilibrated and eluted with 25 mM triethanolamine buffer, pH 7.8, and glycolate oxidase containing fractions were combined. Residual catalase activity and contaminating Chl were removed by the batch-wise addition of a suspension (5 ml) of DEAE-Sepharose Cl-6B in 25 mM triethanolamine buffer, pH 7.8. After 15 min incubation with occasional stirring the DEAE-Sepharose Cl-6B gel was removed by 15 min centrifugation at 10,000×g and 4 °C. If necessary, further DEAE-Sepharose Cl-6B suspension was added, until most of the catalase activity and all traces of Chl were removed and the supernatant appeared colorless. In typical preparations glycolate oxidase was approximately 20-fold enriched with a yield of about 13%, relative to the initial total extract, and had a specific activity in the range of 100 nmol s^{-1} (mg protein)$^{-1}$. The preparations were largely depleted of pea catalase.

Any residual catalase activity was totally photoinactivated within 1 h during light exposures or inhibited by the addition of 2 mM aminotriazole and 2 mM Na-azide during dark incubations.

Analytical methods

Total chlorophyll (Chl) was determined from 80% (v/v) acetone extracts according to Arnon (1949). Total protein was determined according to Gerhardt and Beevers (1968) after precipitation in 10% (w/v) trichloroacetic acid and washing the precipitate with 5% trichloroacetic acid.

Enzyme activities were assayed spectrophotometrically at 25 °C. Activities of catalase (EC 1.11.1.6) and glycolate oxidase (EC 1.1.3.1) were assayed, as previously described (Streb and Feierabend, 1996). The contents of reduced and oxidized glutathione were determined after separation by HPLC according to published procedures (Streb and Feierabend, 1996). The contents of H_2O_2 was assayed by its reaction with a titanium(IV)-4(2-pyridylazo)resorcinol complex according to Patterson *et al.* (1984), as described by Streb and Feierabend (1996).

The data presented are mean values from at least three independent experiments, except where indicated otherwise. Standard errors of the mean are indicated.

Results

Photoinactivation of glycolate oxidase in leaves

During a 24 h exposure of sections of mature rye leaves to a moderate light intensity of 500 µmol m^{-2} s^{-1} PAR the activity of the light-sensitive enzyme catalase was largely depleted by photoinactivation when its replacement by *de novo* synthesis was prevented by the presence of the translation inhibitor cycloheximide (Fig. 1A). While the increase of glycolate oxidase activity, that was observed in untreated control leaves, was also blocked by cycloheximide, only a minor decline of this enzyme occurred within 24 h in the presence of the inhibitor, relative to its initial activity. These observations indicate that, in contrast to catalase, the inactivation or turnover of glycolate oxidase must have been low under these conditions. However, the decline of glycolate oxidase was enhanced during a subsequent additional

24 h light exposure in the presence of cycloheximide, when catalase activity had been largely eliminated (Fig. 1B). Complete inhibition of catalase activity by application of the catalase inhibitor aminotriazole was accompanied by a marked decline of glycolate oxidase activity in white light. Aminotriazole had no direct inhibitory effect on glycolate oxidase *in vitro*. The decline of glycolate oxidase activity in leaves was further enhanced when, in addition to the application of aminotriazole, protein synthesis was inhibited by cycloheximide and the potential replacement of inactive enzyme by *de novo* synthesis was suppressed (Fig. 1B). The elimination of catalase by treatment of leaves with aminotriazole or cycloheximide increases the oxidative stress in the tissue, as demonstrated in previous work (Streb and Feierabend, 1996). This was indicated by an enhanced degradation of Chl. However, the photoinactivation of glycolate oxidase clearly preceded the decline of the Chl content in all treatments and thus represented an early event of photooxidative damage in treated leaves (Fig. 1C). When the leaf sections were exposed to either blue or red light during incubation with aminotriazole and cycloheximide the declines of the glycolate oxidase activity and of the Chl content were retarded (Fig. 1E and F), relative to the white light exposures of equal photon flux PAR (Fig. 1B and C). However, in both blue and red light glycolate oxidase was inactivated with almost equal rates in leaf sections from rye seedlings (Fig. 1E).

A comparable inactivation of glycolate oxidase, as in rye leaves, was also observed when pea leaves were incubated in the presence of aminotriazole and cycloheximide in white light of 500 µmol m^{-2} s^{-1} PAR, except that the decline was slower and only about 65% of the initial activity were lost within 48 h. Immunoblotting assays with an antiserum against spinach glycolate oxidase indicated that in pea leaves the loss of enzyme activity was accompanied by a decline of the amount of the enzyme polypeptide which was detected in a similar apparent molecular weight range as the spinach protein (not shown). The antiserum did not cross-react with glycolate oxidase from rye.

Photoinactivation of glycolate oxidase in vitro

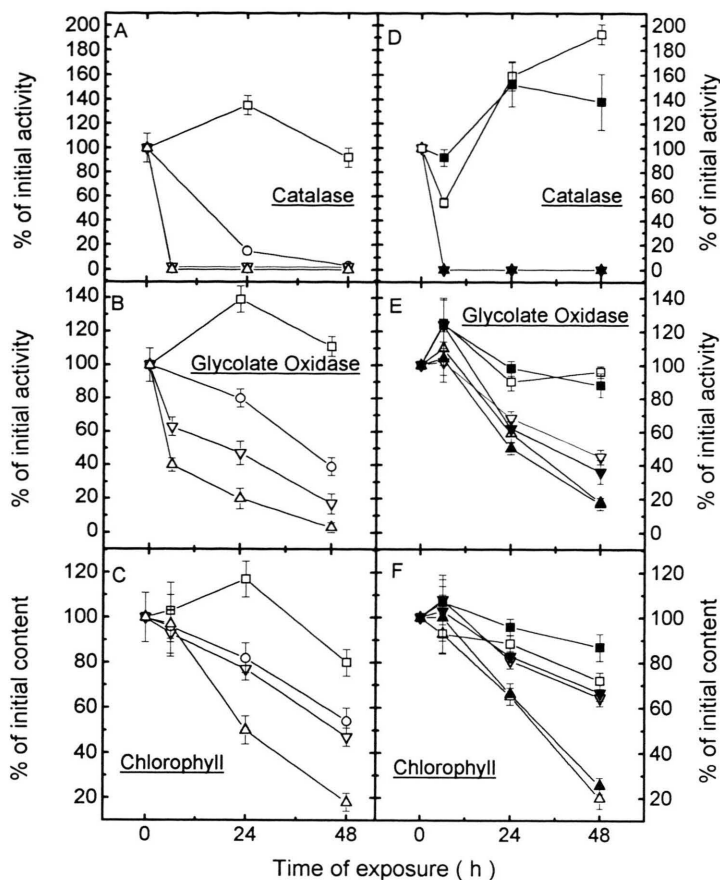A potential protective role of catalase, as suggested by the observations with catalase-depleted

Fig. 1. Changes of the activities of glycolate oxidase, catalase and of the total Chl content in excised sections from rye leaves during a 48 h exposure to white (A–C), or red and blue (D–F) light of 500 µmol m^{-2} s^{-1} PAR in the presence or absence of inhibitors. □ or ■, untreated control on nutrient solution; ○, +35.5 µM cycloheximide; ▽ or ▼, +2 mM aminotriazole; △ or ▲, +2 mM aminotriazole and 35.5 µM cycloheximide. Measurements are expressed as% of values for leaf sections before treatments. In D–F open symbols represent irradiations with red light, dark symbols represent irradiations with blue light. Before treatments leaf sections had a catalase activity of 0.82 ± 0.1 µmol s^{-1} per section, a glycolate oxidase activity of 0.54 ± 0.6 nmol s^{-1} per section, and a Chl content of 42.5 ± 5.1 µg per section.

leaves, was investigated *in vitro* with a partially purified preparation of glycolate oxidase from pea leaves, for which a rapid purification procedure was available. In order to investigate photoinactivation *in vitro*, samples of the partially purified glycolate oxidase were irradiated for 3 h with blue light of 800 µmol m^{-2} s^{-1} PAR. Any remnants of pea catalase contained in the glycolate oxidase preparation were totally inactivated within the first hour of light exposure. Under these conditions a slow inactivation of glycolate oxidase was observed without the addition of FMN. Addition of FMN greatly enhanced the blue light-induced

inactivation of glycolate oxidase, while the enzyme was stable in darkness (Fig. 2A). Photoinactivation of glycolate oxidase increased with the concentration of FMN but was saturated above 5–10 µM FMN (Fig. 2C). This corresponded well to the dependency of the glycolate oxidase activity on the concentration of added FMN which also saturated between 5–10 µM (Fig. 2B). This close correlation suggested that photoinactivation was mediated by enzyme-bound FMN. At higher FMN concentrations the rate of photoinactivation of glycolate oxidase decreased (Fig. 2C). All following light exposures were performed in the
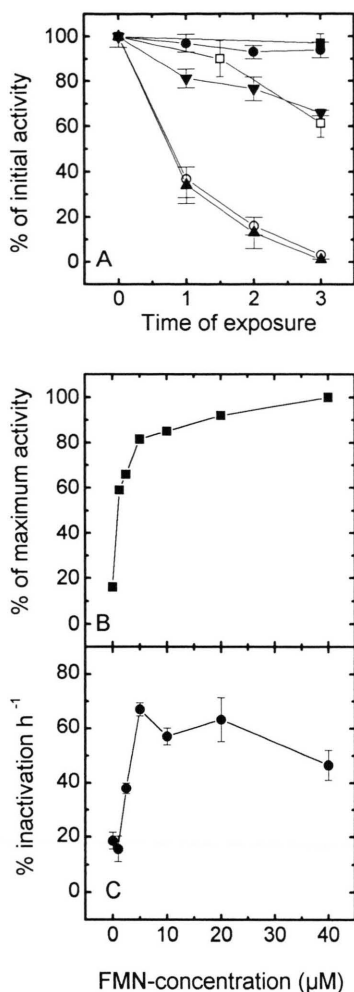
Fig. 2. Time course of the inactivation of partially puri-
fied glycolate oxidase from pea leaves in blue light (A)
and comparison of the FMN-dependency of the acitvity
(B) and the blue light-induced inactivation (C) of gly-
colate oxidase. **A:** Change of the activity of glycolate
oxidase in light in the absence of added FMN (▼), in
the presence of 20 μM FMN (○), in the presence of 20 μM
FMN and 0.3 mM glycolate (▲), or in the presence of
20 μM FMN in the absence of O$_2$ (□). ●, control in the
presence of 20 μM FMN and aerobic conditions in dark-
ness; ■, control in the presence of 20 μM FMN in the
absence of O$_2$ in darkness. **B:** Dependency of glycolate
oxidase activity on the FMN-concentration of the assay
medium (■). **C:** Decline of glycolate oxidase activity
during 1 h exposure to blue light in the presence of dif-
ferent concentrations of FMN (●).

presence of 20 μM FMN. The time courses of gly-
colate oxidase photoinactivation in the absence or
presence of its substrate glycolate were identical.
Some photoinactivation of glycolate oxidase was

also observed in the absence of O$_2$, was, however,
greatly retarded, relative to aerobic conditions
(Fig. 2A). Removal of O$_2$ caused a strong light-
independent inhibition of glycolate oxidase (ca.
65%) that was not readily reversible after aera-
tion. Anaerobic photoinactivation was assayed for
the residual activity.

Inactivation of glycolate oxidase in blue light
was greatly retarded in the presence of purified
catalase from bovine liver. *Vice versa* the photoin-
activation of the catalase was strongly enhanced
by the presence of glycolate oxidase and FMN
(Fig. 3B, see 5 μmol s^{-1} ml^{-1} catalase incubations).
Catalase photoinactivation was accelerated to the
same extent by FMN alone without glycolate oxi-
dase (data not shown). The protective action of
catalase increased with increasing concentration
and was totally abolished when catalase was inhib-
ited by aminotriazole (Fig. 3A). The inactivation
of catalase by blue light can be largely prevented
by the addition of a low concentration of ethanol
(Cheng *et al.*, 1981). Ethanol had no direct effect
on glycolate oxidase. However, protection of gly-
colate oxidase in the presence of catalase was
markedly improved when catalase was stabilized
by the addition of ethanol (Fig. 3). Nevertheless,
also the presence of a very high and stable catalase
activity did not provide complete protection
against photoinactivation of glycolate oxidase. Ad-
dition of a high concentration of bovine serum al-
bumin retarded the photoinactivation of glycolate
oxidase only slightly, indicating that unspecific
protection by the presence of a high protein con-
centration was only of minor relevance (Fig. 3A).

The protection by catalase suggested that H$_2$O$_2$
was formed when glycolate oxidase was irradiated
with blue light, although its substrate glycolate was
not present. This was confirmed by the assay of
H$_2$O$_2$ (Fig. 4B). The amount of H$_2$O$_2$ produced in
blue light of 800 μmol m^{-2} s^{-1} PAR in the absence
of glycolate accounted for 39% of that produced
by the same amount of enzyme in the presence of
glycolate in darkness. However, the rate of inacti-
vation of glycolate oxidase in the absence of sub-
strate in light was higher than in the presence of
substrate in darkness. The presence of H$_2$O$_2$ was
inhibitory to glycolate oxidase. However, the rate
of inactivation was not proportional to the existing
H$_2$O$_2$ concentration. The rate of inactivation in
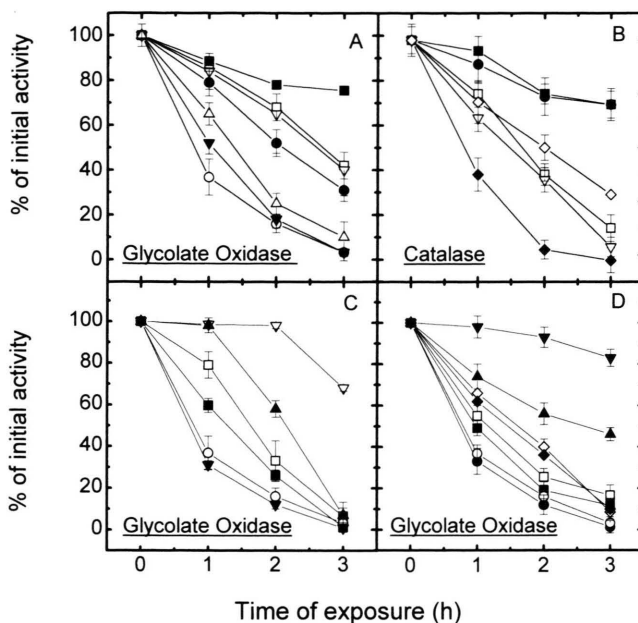darkness in the presence of 5 mM H$_2$O$_2$, a concen-

Fig. 3. Influence of catalase (A and B) and of antioxidants and radical scavengers (C and D) on the inactivation of glycolate oxidase from pea leaves (A, C, D) and catalase from bovine liver (B) during irradiation with blue light in the presence of 20 μM FMN. If not otherwise indicated, catalase was contained in the solution of glycolate oxidase. A and B: ○, control without catalase; △, incubation in the presence of 1 mg ml^{-1} bovine serum albumin; ◆, incubation with 5 μmol s^{-1} ml^{-1} catalase; ●, incubation with 5 μmol s^{-1} ml^{-1} catalase and 0.5% (v/v) ethanol; ▽, incubation with 150 μmol s^{-1} ml^{-1} catalase; ▼, incubation with 150 μmol s^{-1} ml^{-1} catalase and 2 mM aminotriazole; □, incubation with 300 μmol s^{-1} ml^{-1} catalase; ■, incubation with 300 μmol s^{-1} ml^{-1} catalase and 0.5% (v/v) ethanol. ◇, control with 5 μmol s^{-1} ml^{-1} catalase in the absence of glycolate oxidase and FMN. C: Incubations were performed without further addition (○) or in the presence of 1 mM reduced glutathione (GSH, ■), 1 mM GSH and 166 units ml^{-1} glutathione peroxidase (□), 2.5 mM GSH (▲), 5 mM GSH (▽), or 5 mM oxidized glutathione (▼). D: Incubations were performed without further addition (○) or in the presence of 5 mM dithiothreitol (▼), 5 mM ascorbate (▲), 125 mM mannitol (□), 10 mM L-histidine (◇), 10 mM Na$_2$EDTA (◆), 1% (v/v) dimethylsulfoxide (■), 100 units ml^{-1} superoxide dismutase (●).

tration that was 77 times higher than that observed after 3 h irradiation without glycolate, was much slower than in blue light without glycolate, or even than in darkness in the presence of substrate (Fig. 4A). In darkness, glycolate oxidase was also inactivated when $O_2^{\cdot-}$ was produced by xanthine and xanthine oxidase. The rate of inactivation in darkness in the presence of the $O_2^{\cdot-}$ – producing system was similar as in the presence of glycolate, when H_2O_2 was produced. The inactivation in the presence of xanthine and xanthine oxidase could be markedly mitigated by the addition of superoxide dismutase (Fig. 4A).

In order to examine whether removal of H_2O_2 by a peroxidase had a similar protective effect as the presence of catalase, blue light irradiation of glycolate oxidase was performed in the presence of glutathione peroxidase (Fig. 3C). However,

most remarkably the substrate GSH alone was highly efficient to protect glycolate oxidase from photoinactivation. When the concentration of GSH was increased, glycolate oxidase was protected for a longer period of time. During incubation with glycolate oxidase in blue light GSH was totally oxidized and partially either degraded or firmly bound to some unknown compound (Table I). The H_2O_2 accumulation was increasingly suppressed, as the GSH concentration was increased (Table I). Glycolate oxidase was protected, as long as sufficient reduced GSH was present, and photoinactivation commenced, as soon as the GSH was mostly oxidized (Fig. 3C and Table I). Oxidized GSSG had no effect on the photoinactivation of glycolate oxidase. At a low concentration of 1 mM GSH which only moderately protected glycolate oxidase, the photoinactivation
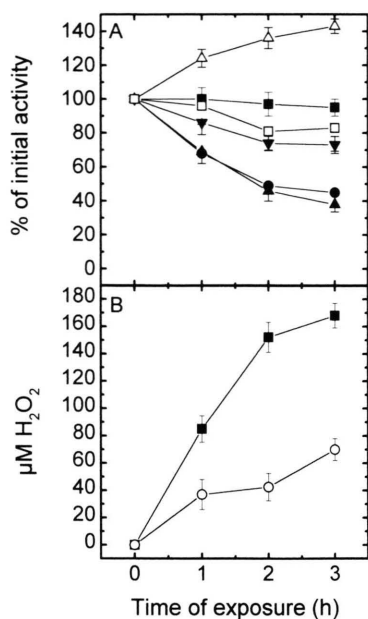
Fig. 4. Changes of glycolate oxidase activity (A) and of the concentration of H_2O_2 (B) during incubation of solutions of partially purified glycolate oxidase from pea leaves in the presence of 20 μM FMN in darkness or in blue light. **A:** Incubations were performed in darkness with the addition of 2 mM aminotriazole and 2 mM Na-azide in order to prevent decomposition of H_2O_2. Further additions were 0.3 mM glycolate (▲), 0.3 mM glycolate and 5 mM reduced glutathione (△), 5 mM H_2O_2 (▼), 150 mM xanthine (■), 0.2 units ml^{-1} xanthine oxidase and 150 mM xanthine (●), or 0.2 units ml^{-1} xanthine oxidase, 150 mM xanthine and 100 units ml^{-1} superoxide dismutase (□). **B:** Incubations were performed in darkness in the presence of 0.3 mM glycolate (■) or in blue light without glycolate (○).

of the latter was slightly more retarded when, in addition, glutathione peroxidase was included in the incubation mixture (Fig. 3C). However, the final extent of protection was determined by the amount of GSH. When photoinactivated glycolate oxidase was incubated for up to 30 min in the presence of 5 mM GSH the enzyme was not reactivated (data not shown). The inactivation of glycolate oxidase observed in darkness in the presence of glycolate was also prevented by GSH (Fig. 4A) and accompanied by a concomitant oxidation of the latter (Table I). In contrast to the light incubations, total glutathione did not decline in darkness (Table I). In darkness GSH induced some slow increase of the activity of the enzyme preparation.

Glycolate oxidase was efficiently protected against photoinactivation by the addition of the thiol-reductant dithiothreitol (Fig. 3D), as in the presence of GSH. However, in darkness glycolate oxidase was only slightly inhibited by relatively high concentrations of reagents reacting with sulfhydryl groups. The inhibition by 0.5 mM iodoacetate amounted to 20%, that by 20 mM sodium arsenate to 26.8%. Protection from photoinactivation by ascorbate was marked, but less efficient than by GSH (Fig. 3D). Photoinactivation of glycolate oxidase was also markedly and to very similar extents retarded by the presence of 10 mM concentrations of L-histidine, EDTA or L-tryptophan which may act as reductants (Foote, 1968; Schmidt and Butler, 1976; Massey *et al.*, 1978) to the sensitizer FMN (Fig. 3D). The curve for L-tryptophan

Table I. Comparison of H_2O_2 formation and GSH oxidation during the inactivation of glycolate oxidase from pea leaves in blue light or in the presence of glycolate in darkness. All experiments were performed in the presence of 20 μM FMN. Where indicated, the incubation mixture contained 100 units superoxide dismutase (SOD) or 166 units glutathione peroxidase ml^{-1} (GSH peroxidase). Dark incubations, in addition to 0.3 mM glycolate and GSH, contained 2 mM aminotriazole and 2 mM Na-azide. The GSH concentrations for experiments in light represent mean values from two independent determinations.

Conditions	H_2O_2 [μM]	Total glutathione [mM]	GSH [mM]	GSSG [mM]
3 h light	65 ± 10	–	–	–
3 h light + SOD	190 ± 25	–	–	–
3 h light + 1 mM GSH	55 ± 8	0.72	0.0	0.36
3 h light + 1 mM GSH + GSH peroxidase	20 ± 7.8	0.7	0.0	0.35
3 h light + 5 mM GSH	5 ± 3.2	3.8	0.6	1.6
Incubation in darkness + glycolate + 5 mM GSH				
0 h	–	5.3 ± 0.5	5.2 ± 0.4	0.05 ± 0.4
3 h	–	5.0 ± 0.3	2.1 ± 0.2	1.4 ± 0.2

is not shown in Fig. 3D since its course was identical to that obtained for the curves for L-histidine or EDTA. Incubation with these compounds caused also a slow increase of the activity of the glycolate oxidase preparation in darkness. Histidine may, in addition, act as singlet oxygen quencher. The presence of superoxide dismutase did not affect the rate of glycolate oxidase inactivation in blue light. Known scavengers of hydroxyl radicals, such as dimethylsulfoxide or mannitol, retarded the course of photoinactivation of glycolate oxidase only slightly. (Fig. 3D).

Photoinactivation of glycolate oxidase in glutathione-depleted leaves

The relevance of GSH for the protection of glycolate oxidase *in vivo* was examined in GSH-depleted rye leaves. It was shown in previous work (Streb and Feierabend, 1999) that the endogenous content of GSH can be greatly eliminated when rye leaves are incubated for 24 h in low light in the presence of 10 mM D, L-buthionine sulfoximine, an inhibitor of the γ-glutamylcysteine synthetase (Meister, 1983). The content of GSH declined to 14% of its level in control leaves (without inhibitor) when buthionine sulfoximine was applied during a 24 h preincubation in low light, and was totally eliminated during a subsequent 24 h exposure to a higher light intensity of 500 μmol m^{-2} s^{-1} PAR. When the inhibitor was applied at the beginning of the high light treatment the GSH content declined to 5% within the 24 h exposure period (Fig. 5A). In leaf sections with diminished GSH contents both catalase and glycolate oxidase were inactivated during the high light exposure (Fig. 5B and C), while only a minor Chl degradation occurred (Fig. 5D). In darkness glycolate oxidase was not inactivated (not shown). The rate of glycolate oxidase inactivation was faster in leaf sections that were depleted of GSH before the high light treatment than in leaf sections that were exposed to buthionine sulfoximine only at the beginning of the high light treatment (Fig. 5C). Similar rates of photoinactivation were observed in leaf sections treated with aminotriazole which inhibited the catalase activity, as in fully GSH-depleted leaves, although the latter contained still high catalase activity during the early phases of the high light exposure. The rate of inactivation of gly-colate oxidase was moderately further enhanced in GSH-depleted leaf sections when, in addition, catalase activity was eliminated by the application of aminotriazole (Fig. 5C).

Discussion

Our present results confirm basic observations by Schmid (1969) that glycolate oxidase is inactivated by blue light *in vitro* and explain why this light-sensitivity is, nevertheless, not usually noticed in leaves. In accord with conclusions drawn by Schmidt (1969), inactivation of glycolate oxidase *in vitro* appears to be mediated by FMN that is bound to the enzyme, since photoinactivation was saturated at a similar FMN concentration as the enzyme activity. Excitation of flavins causes their photoreduction to FMNH$_2$. The FMNH$_2$ was shown to react with O$_2$ giving rise to the production of O$_2^{.-}$ and H$_2$O$_2$ (Massey *et al.*, 1969; Schmidt and Butler, 1976; Massey, 1994). If no reductants are available, other flavin molecules can serve as electron donors for the photoreduction to FMNH$_2$ but suffer from concomitant photodestruction (Schmidt and Butler, 1976). Two alternative mechanisms of inactivation of glycolate oxidase by excited flavins were discussed. Schmid (1969) concluded that the enzyme served as reductant for the excited flavin and was thus oxidatively inactivated without any involvement of ROS (Schmidt and Butler, 1976; Foote, 1968), because in his experiments glycolate oxidase was inactivated also under nitrogen and not protected by the addition of catalase. Excitation of free flavins may also contribute to this type of inactivation since interactions and an exchange of electrons between free and enzyme-bound flavins occur (Massey *et al.*, 1978). Our results provided several evidences that glycolate oxidase was, alternatively, also inactivated by the ROS formed through the excited flavins. In our experiments inactivation of glycolate oxidase was largely suppressed under anaerobic conditions, the enzyme was inactivated by the presence of O$_2^{.-}$ or H$_2$O$_2$, and photoinactivation was mitigated by catalase and several antioxidative compounds both *in vitro* and in leaves. Differences between our results and those of Schmid (1969) may be determined by the lower purity of the enzyme preparation used by Schmid (1969) and by the circumstance that inactivation and as-
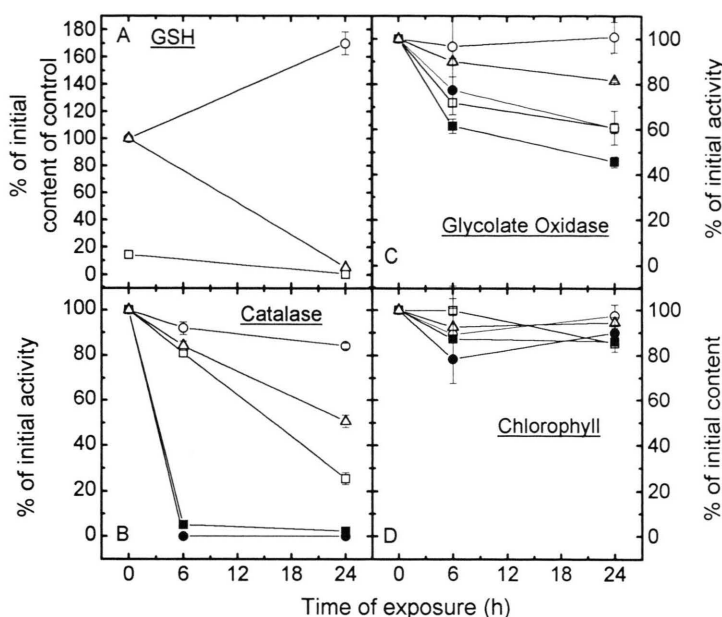
Fig. 5. Changes of the contents of reduced glutathione (A), the activities of catalase (B) and glycolate oxidase (C), and of the total Chl contents (D) in excised sections from rye leaves during a 24 h exposure to white light of 500 μmol m^{-2} s^{-1} PAR in the presence or absence of 10 mM D, L-buthionine sulfoximine and 2 mM aminotriazole. Complete primary leaves of 5-day-old rye seedlings were excised and preincubated for 24 h in low light of 96 μmol m^{-2} s^{-1} PAR in the presence (□, ■) or absence (○, ●, △) of D, L-buthionine sulfoximine contained in nutrient solution. After the preincubation period leaf sections were excised and exposed to 500 μmol m^{-2} s^{-1} PAR light in the absence of inhibitors (○) or in the presence of: ●, aminotriazole after preincubation without inhibitor; △, buthionine sulfoximine after preincubation without inhibitor; □, buthionine sulfoximine after preincubation with buthionine sulfoximine; ■, buthionine sulfoximine + aminotriazole after preincubation with buthionine sulfoximine. The initial glutathione content of the control leaf sections (○) at the beginning of the high light exposure was 7.7 ± 0.5 nmol per section. The initial enzyme activities were 1.2 ± 0.5 μmol s^{-1} per section for catalase, 0.60 ± 0.04 nmol s^{-1} per section for glycolate oxidase after preincubation without inhibitor, and 1.12 ± 0.02 μmol s^{-1} per section for catalase, 0.62 ± 0.06 nmol s^{-1} per section for glycolate oxidase after preincubation with buthionine sulfoximine.

say conditions were not separated in the previous investigation.

With regard to the nature of the effective ROS, our results suggested that H_2O_2 and $O_2^{·-}$ were able to mediate the inactivation of glycolate oxidase. However, inactivation of glycolate oxidase was not proportional to the existing H_2O_2 concentrations and was not affected by superoxide dismutase in light. These discrepancies may indicate that glycolate oxidase was preferentially inactivated by ROS formed directly at the site of the enzyme-bound FMN which is deeply buried in the interior of the enzyme (Lindquist and Brändén, 1989) and therefore presumably out of reach of superoxide dismutase, or that flavin-mediated ROS formation and the direct oxygen-independent reaction with excited oxidized FMN or flavin radicals contributed to its inactivation in varying proportions.

Generally, both types of photoreactions appear to contribute to photosensitized oxidations (Foote, 1968). Reducing agents may scavenge and detoxify the ROS formed by excited flavins or protect sulfhydryl groups of glycolate oxidase. However, the known sequence of spinach glycolate oxidase contains only a single cystein residue which is localized far apart from the active site in a peripheral loop structure close to the carboxy-terminal end (Lindqvist, 1989; Lindqvist and Brändén, 1989). Therefore, intramolecular disulfide formation within a single polypeptide is impossible and could only occur between subunits of the octameric enzyme. Consequently, it is not very likely that reducing compounds, including ascorbate, were primarily or exclusively protecting this single cystein residue. Rather GSH, dithiothreitol or ascorbate may also protect the apoenzyme from reaction

with excited flavins, because they serve as alternative reductants for the FMN. Ascorbate was shown to serve as electron donor to flavins (Schmidt and Butler, 1976). The effects of EDTA, histidine and tryptophan which do not represent sulfhydryl reducing reagents further substantiate that potential reductants for flavins have the ability to protect glycolate oxidase from photoinactivation. EDTA is known to serve as excellent electron donor to FMN (Schmidt and Butler, 1976; Massey *et al.*, 1978). Histidine and tryptophan are also known as reductants for photosensitized reactions and their oxidation appears to be a major cause of deactivation of many enzymes (Foote, 1968).

According to this investigation catalase and GSH represent the most important natural factors for the protection of glycolate oxidase. Catalase can remove the H_2O_2 produced through the photoreduction of FMN but simultaneously appeared to act as a direct scavenger for irradiated glycolate oxidase. Instead of the glycolate oxidase that was protected, the inactivation of catalase was enhanced in a mixture of the two enzymes in light. Since these enzymes are presumably in close contact within the multienzyme complex of the peroxisomal matrix (Heupel *et al.*, 1991) and remain associated even after detergent solubilization of peroxisomes (Heupel and Heldt, 1994), it is quite likely that catalase can exert a direct protective function also *in vivo*. However, in aminotriazole-treated leaves which were depleted of catalase not only its potential protective scavenger function was lost but, in addition, antioxidants, such as ascorbate and GSH, became largely oxidized when the detoxification of H_2O_2 was impaired, particularly when any increases of alternative antioxidative systems were suppressed by cycloheximide (Smith *et al.*, 1985; Streb and Feierabend, 1996). That the stability of glycolate oxidase in leaves strongly depended on the presence of reduced antioxidants was strikingly accentuated by the marked photoinactivation of glycolate oxidase that was induced when the endogenous GSH was depleted by the inhibitor buthionine sulfoximine, although considerable catalase activity was still available. Since the inactivation of glycolate oxidase in leaves treated with aminotriazole and cycloheximide was of similar magnitude in blue and red light, the enzyme was obviously more vulnerable *in vivo* by ROS formed through photosynthetic reactions also in red light, than by direct flavin-sensitized inactivation that occurs only in blue light. Therefore, protection of glycolate oxidase requires a sufficient supply of GSH *in vivo* which needs to be maintained in its reduced state in the leaf peroxisomes. The presence of all enzymes and substrates of the ascorbate-glutathione cycle in peroxisomes was recently demonstrated (Jiménez *et al.*, 1997; Del Rio *et al.*, 1998). Protection of glycolate oxidase or other flavin containing enzymes against photodamage may represent one of the most important functions of this cycle in peroxisomes.

Several of the proteins or enzymes related to photosynthetic metabolism, that are needed in light, such as the reaction center of PSII, catalase and glycolate oxidase, turn out to be particularly sensitive to photoinactivation. While the D1 protein of PSII and catalase depend on continuous repair by *de novo* synthesis, glycolate oxidase, although light-sensitive *in vitro*, appears to be usually well protected by the reduced GSH and by its compartmentation in the close proximity of catalase within the peroxisome. Direct protection by catalase can further increase the light-induced turnover of the latter enzyme. This would, nevertheless, be advantageous for the plant, because it has to replace only the single protein of catalase by *de novo* synthesis which is in a continuous turnover in light anyway (Hertwig *et al.*, 1992), instead of repairing two photoinactivated enzymes.

Arnon D. J. (1949), Copper enzymes in isolated chloroplasts. Plant Physiol. **24**, 1–15.

Aro E.-M., Virgin I. and Andersson B. (1993), Photoinhibition of photosystem II. Inactivation, protein damage and turnover. Biochim. Biophys. Acta **1143**, 113–134.

Asada K. (1994), Production and action of active oxygen species in photosynthetic tissues. In: Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants (Ch. H. Foyer and Ph. M. Mullineaux, eds.). CRC Press, Boca Raton, pp. 77–104.

Betsche T., Eising R., Ruschke M. and Schaller D. (1986), Glycolate oxidase from pea leaves: Rapid purification and some properties. Plant Physiol. **80**, S-83.

Cheng L., Kellog E. W. and Packer L. (1981), Photoinactivation of catalase. Photochem. Photobiol. **34**, 125–129.

Del Rio L. A., Sandalio L. M., Corpas F. J., López-Huertas E., Palma J. M. and Pastori G. M. (1998), Activated oxygen-mediated metabolic functions of leaf peroxisomes. Physiol. Plant. **104**, 673–680.

Feierabend J. and Engel S. (1986), Photoinactivation of catalase *in vitro* and in leaves. Arch. Biochem. Biophys. **251**, 567–576.

Feierabend J. and Schrader-Reichhardt U. (1976), Biochemical differentiation of plastids and other organelles in rye leaves with a high-temperature-induced deficiency of plastid ribosomes. Planta **129**, 133–145.

Feierabend J., Schaan C. and Hertwig B. (1992), Photoinactivation of catalase occurs under both high- and low-temperature stress conditions and accompanies photoinhibition of photosystem II. Plant Physiol. **100**, 1554–1561.

Feierabend J., Streb P., Schmidt M., Dehne S. and Shang W. (1996), Expression of catalase and its relation to light stress and stress tolerance. In: Physical Stresses in Plants. Genes and their Products for Tolerance. (S. Grillo and A. Leone, eds.). Springer, Berlin, Heidelberg, pp. 223–234.

Foote C. S. (1968), Mechanisms of photosensitized oxidation. Science **162**, 963–970.

Foyer Ch. H. (1997), Oxygen metabolism and electron transport in photosynthesis. In: Oxidative Stress and the Molecular Biology of Antioxidant Defenses. (J. G. Scandalios, ed.). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 587–621.

Foyer Ch. H., Lelandais M. and Kunert K. J. (1994), Photooxidative stress in plants. Physiol. Plant. **92**, 696–717.

Gerhardt B. and Beevers H. (1968), Influence of sucrose on protein determination by the Lowry procedure. Anal. Biochem. **24**, 337–339.

Grotjohann N., Janning A. and Eising R. (1997), In vitro photoinactivation of catalase isoforms from cotyledons of sunflower (*Helianthus annuus* L.). Arch. Biochem. Biophys. **346**, 208–218.

Hertwig B., Streb P. and Feierabend J. (1992), Light dependence of catalase synthesis and degradation in leaves and the influence of interfering stress conditions. Plant Physiol. **100**, 1547–1553.

Heupel R. and Heldt H. W. (1994), Protein organization in the matrix of leaf peroxisomes. A multi-enzyme complex involved in photorespiratory metabolism. Eur. J. Biochem. **220**, 165–172.

Heupel R., Markgraf Th., Robinson D. G. and Heldt H. W. (1991), Compartmentation studies on spinach leaf peroxisomes. Plant Physiol. **96**, 971–979.

Jiménez A., Hernández J. A., del Rio L. A. and Sevilla F. (1997), Evidence for the presence of the ascorbate glutathione cycle in mitochondria and peroxisomes of pea leaves. Plant Physiol. **114**, 275–284.

Lindqvist Y. (1989), Refined structure of spinach glycolate oxidase at 2 Å resolution. J. Mol. Biol. **209**, 151–166.

Lindqvist Y. and Brändén C.-I. (1989), The active site of spinach glycolate oxidase. J. Biol. Chem. **264**, 3624–3628.

Massey V. (1994), Activation of molecular oxygen by flavins and flavoproteins. J. Biol. Chem. **269**, 22459–22462.

Massey V., Stankovich M. and Hemmerich P. (1978), Light-mediated reduction of flavoproteins with flavins as catalysts. Biochemistry **17**, 1–8.

Massey V., Strickland S., Mayhew S. G., Howell L. G., Engel P. C., Matthews R. G., Schuman M. and Sullivan P. A. (1969), The production of superoxide anion radicals in the reaction of reduced flavins and flavoproteins with molecular oxygen. Biochem. Biophys. Res. Commun. **36**, 891–897.

Meister A. (1983), Selective modification of glutathione metabolism. Science **220**, 472–477.

Nishimura M., Akhmedov Y. D. and Akazawa T. (1983), Molecular structure and subcellular localization of spinach leaf glycolate oxidase. Photos. Res. **4**, 99–109.

Patterson B. D., McRae E. A. and Ferguson I. B. (1984), Estimation of hydrogen peroxide in plant extracts using titanium (IV). Anal. Biochem. **139**, 487–492.

Schmid G. H. (1969), The effect of blue light on glycolate oxidase of tobacco. Hoppe-Seyler's Z. Physiol. Chem. **350**, 1035–1046.

Schmidt W. and Butler W. L. (1976), Flavin-mediated photoreactions in artificial systems: A possible model for the blue-light photoreceptor pigment in living systems. Photochem. Photobiol. **24**, 71–75.

Shang W. and Feierabend J. (1999), Dependence of catalase photoinactivation in rye leaves on light intensity and quality and characterization of a chloroplast-mediated inactivation in red light. Photosynth. Res. **59**, 201–213.

Smith I. K., Kendall A. C., Keys A. J., Turner J. C. and Lea P. J. (1985), The regulation of the biosynthesis of glutathione in leaves of barley (*Hordeum vulgare* L.). Plant Sci. **41**, 11–17.

Streb P. and Feierabend J. (1996), Oxidative stress responses accompanying photoinactivation of catalase in NaCl-treated rye leaves. Bot. Acta **109**, 125–132.

Streb P. and Feierabend J. (1999), Significance of antioxidants and electron sinks for the cold-hardening-induced resistance of winter rye leaves to photoxidative stress. Plant Cell Environ. **22**, 1225–1237.

Volk S. and Feierabend J. (1989), Photoinactivation of catalase at low temperature and its relevance to photosynthetic and peroxide metabolism in leaves. Plant Cell Environ. **12**, 701–712.