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Chemical Modification of Residue of Lysine, Tryptophan, and Cysteine in Spinach Glycolate Oxidase

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Abstract: Spinach glycolate oxidase was subjected to a series of chemical modifications aimed at identifying amino acid residues essential for catalytic activity. The oxidase was reversibly inactivated by treatment with pyridoxal 5'-phosphate (PLP). The inactivation by PLP was accompanied by the appearance of an absorption peak of around 430 nm, which was shifted to 325 nm upon reduction with $NaBH_4$. After reduction, the PLP-treated oxidase showed a fluorescence spectrum with a maximum of around 395 nm by exciting at 325 nm. The substrate-competitive inhibitors oxalate and oxaloacetate provided protection against inactivation of the oxidase by PLP. These results suggest that PLP inactivates the enzyme by forming a Schiff base with lysyl residue(s) at an active site of the oxidase. The enzyme was also inactivated by tryptophan-specific reagent N-bromosuccinimide (NBS). However, competitive inhibitors oxalate and oxaloacetate could not protect the oxidase significantly against inactivation of the enzyme by NBS. The results implicate that the inactivation of the oxidase by NBS is not directly related to modification of the tryptophanyl residue at an active site of the enzyme. Treatments of the oxidase with cysteine-specific reagents iodoacetate, silver nitrate, and 5,5'-dithiobis-2-nitrobenzoic acid did not affect significantly the activity of the enzyme.
Key words: glycolate oxidase, chemical modification, pyridoxal 5'-phosphate, N-bromosuccinimide.

The FMN-dependent glycolate oxidase (glycolate: oxygen oxidoreductase EC 1.1.3.1) catalyzes the oxidation of hydroxyacids to the corresponding keto acids with molecular oxygen as an electron acceptor. The oxidase is found in the peroxisomes of mammalian liver and kidney (Shuman and Massey, 1971), and is believed to be involved in the metabolic production of oxalate by the oxidation of glycolate through glyoxylate (Riao and Richardson, 1973). In green plants, glycolate oxidase is one of the key enzymes in photorespiration where it oxidizes glycolate to glyoxylate. The initial step of photorespiration is catalyzed by oxygenation of ribulose-1,5-bisphosphate carboxylase/oxygenase, which produces phosphoglycolate from ribulose-1,5-bisphosphate. The first step of the glycolate pathway is the oxidation of glycolate to glyoxylate. Since net photosynthesis is drastically reduced due to active photorespiration, an effective control of this pathway might be one possible way to increase the efficiency of photosynthesis (Miziorko and Lorimer, 1983). A detailed knowledge of the enzymes involved in the photorespiration is required to be able to work out procedures for an effective control of photorespiration *in vivo*.

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Glycolate oxidase is generally isolated as a tetramer or octamer composed of identical subunits of Mr of approximately 40 KDa (Lindqvist and Bränden, 1985). Lindqvist and Bränden (1985) determined the tertiary structure of the oxidase from spinach by X-ray crystallographic analysis. The amino acid sequence of the spinach oxidase has been determined from peptide sequencing (Cederlund *et al.*, 1988), and deduced from the DNA sequence of cDNA clone (Volokita and Sommerville, 1987). In our laboratory we have shown that an arginyl and a histidyl residue are located at an active site of the oxidase by chemical modification (Lee and Choi, 1986; Lee *et al.*, 1987), and confirmed that Arg-257 and His-254 are essential for catalytic activity by site-directed mutagenesis (Son *et al.*, 1994). In this work we have carried out a series of chemical modifications of glycolate oxidase aimed at identifying functional amino acid residues.

Materials and Methods

Materials

Potassium glycolate, flavin mononucleotide (FMN), potassium oxalate, potassium oxaloacetate, pyridoxal 5'-phosphate, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), iodoacetate, silver nitrate, N-bromosuccinimide, sodium

borohydride, protamine sulfate, DEAE-cellulose, and Sephadex G-25 were purchased from Sigma Chemical Co. (St. Louis, USA). Sodium 2,6-dichlorophenol-indophenol was obtained from the Aldrich Chemical Co. (Milwaukee, USA). Biogel A 1.5 was a product of Bio-Rad (Richmond, USA). All other chemicals were of the reagent grade from commercial sources.

Purification and assay of glycolate oxidase

Glycolate oxidase was purified from spinach leaves essentially according to Kerr and Groves (1975). The procedure includes the step of precipitation at pH 5.3, ammonium sulfate fractionation, protamine sulfate precipitation, DEAE-cellulose, and agarose chromatography. The purity of the enzyme was checked by SDS polyacrylamide slab gel electrophoresis and the UV-VIS absorption spectrum. Enzyme activity was measured using the dye reduction method of Frigerio and Harburg (1958). Each assay solution contained 0.1 M potassium phosphate (pH 8.0), 2 mM FMN, 20 mM glycolate, 35 μM 2,6-dichlorophenol-indophenol, and enzymes. The reaction was followed by a decrease in the absorbance at 610 nm due to reduction of 2,6-dichlorophenol indophenol. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as the standard.

Chemical modification

The reaction of glycolate oxidase with pyridoxal 5′-phosphate was carried out in 0.1 M potassium phosphate buffer, pH 8.0, in the dark as much as possible. The concentrations of PLP were determined spectrophotometrically in 0.1 N NaOH using the molar extinction coefficient of 5,800 $M^{-1}cm^{-1}$ at 300 nm and 6,600 $M^{-1}cm^{-1}$ at 388 nm, respectively (Sober, 1970).

Modification reactions were initiated by adding the reagent to the enzyme solution which had been preincubated for 10 min at 20℃. Aliquots of reaction mixtures were removed at a time interval and assayed immediately for residual enzymatic activity. Further modification or reactivation was not found during the activity assay. Activity is expressed as the ratio of the activity of the modified enzyme, V, to that of control Vc, multiplied by 100. The reduction of PLP-treated enzymes was carried out by the addition of $NaBH_4$, and the mixture was allowed to stand for 30 min at 4℃. The reversibility of PLP inactivation upon removal of excess PLP and dilution was determined by first inactivating the enzymes with PLP. Then the inactivated enzyme was freed from PLP by gel filteration over a Sephadex G-25 centrifuge column, and then diluted 20 times with 0.1 M phosphate buffer. The enzyme solution was periodically assayed for activity over a period of incubation at 20℃ and compared with that of the control.

The other modification reactions of the oxidase with other reagents were carried out as described in the reactions with PLP. The conditions of the reactions are indicated in the figure and table legends.

Spectroscopic measurement

Electronic absorption spectra were recorded on a Beckman DU-70 spectrophotometer or Simadazu recording spectrophotometer UV-240 at 20℃. For the fluorescence emission measurement, a Hitachi F-3000 fluorescence spectrophotometer with a microcomputer was used. Experimental conditions for absorption and emission measurements are described the in Figure legends.

Results

Purification of glycolate oxidase

Glycolate oxidase was purified from spinach leaves essentially according to Kerr and Groves (1975). In sodium dodecyl sulfate polyacrylamide slab gel electrophoresis, a single clear protein band was evident (not shown). The apparent molecular weight of the subunit of the oxidase was approximately 40 KDa in agreement with a reported value by Hall *et al.* (1985). The absorption spectrum of FMN bound to glycolate oxidase in Tris-HCl buffer, pH 8.3, showed two broad bands at approximately 450 nm and 340 nm in the region of 300~500 nm (not shown), which is in agreement with a previous report (Choi *et al.*, 1990). The spectrum of free FMN at pH 8.3 consists of two peaks at 375 nm and 450 nm in the near UV and visible region, respectively.

Lysine modification with pyridoxal 5′-phosphate

Incubation of glycolate oxidase with amine-specific reagent PLP in 0.1 M potassium phosphate buffer, pH 8.0, and at 20℃ caused a time-dependent decrease of activity, and the inactivation was a function of the concentration of PLP (Fig. 1). Over the time of incubation, the control enzyme sample retained full activity.

The inactivation of the oxidase by PLP was reversible; the enzymatic activity was partially (from 15% to 65%) restored by removing unreacted PLP and then diluting the enzyme sample 20-fold with 0.1 M phosphate buffer (Fig. 2). The treatment of PLP-modified enzyme with $NaBH_4$, however, rendered the inactivation irreversible (not shown).

The different spectrum of the PLP-treated glycolate oxidase against PLP showed a positive peak around 430 nm and a negative one around 380 nm (Fig. 3). The positive peak of 430 nm is characteristic of an

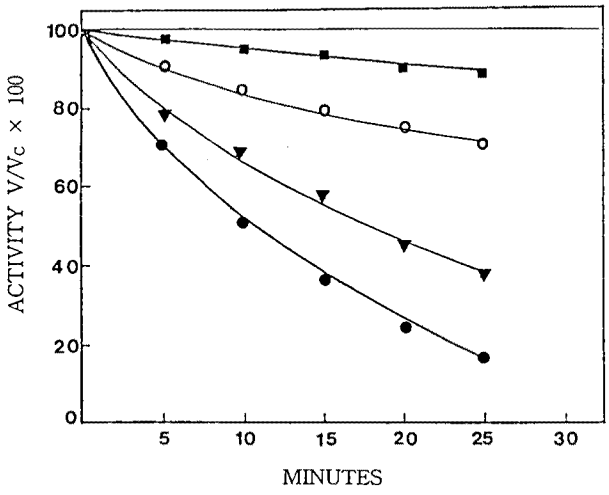

Fig. 1. Time course of inactivation of glycolate oxidase from spinach by pyridoxal 5'-phosphate. The oxidase (2 μM) was incubated with 1 mM (■), 2 mM (○), 5 mM (▲), and 10 mM (●) pyridoxal 5'-phosphate at 20℃, and at specified intervals aliquots were withdrawn and assayed immediately for residual activity.

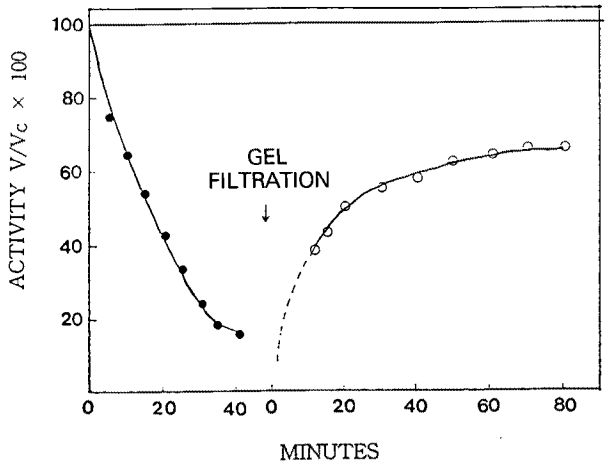

Fig. 2. Time course of reversal of inactivation of glycolate oxidase upon dilution of the PLP-treated enzyme. The enzyme (10 μM) after incubation with 10 mM PLP at 20℃ for 40 min was separated from excess PLP on two Sephadex G-25 centrifuge column, then immediately diluted 20-fold with 0.1 M potassium phosphate buffer (pH 8.0). The arrow indicates the time at which sample was applied to the column. The native enzyme of the control retained full activity during inactivation and reactivation.

aldimine between the formyl group of PLP and an amine. The negative peak of 380 nm is due to absorption of PLP. The PLP concentration in an incubation mixture shoud be decreased by reacting with amine groups in the enzyme. A similar spectrum was observed in the reaction of PLP with Saccharopine dehydrogenase (Ozawa Fujioka, 1980). As expected from such a Schiff base, the reduction of PLP-treated enzyme with $NaBH_4$ caused the appearance of a new peak at 325 nm with the disappearance of the 430 nm (Fig. 4A).

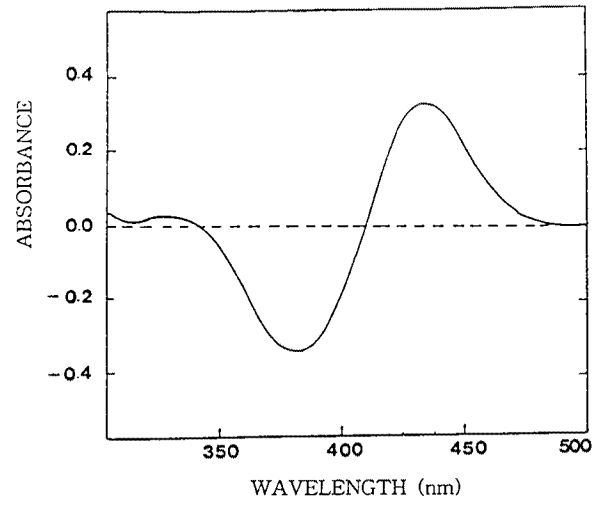

Fig. 3. Difference spectrum of pyridoxal 5'-phosphate treated glycolate oxidase against pyridoxal 5'-phosphate. The enzyme (2 μM) was incubated with 10 mM PLP in 0.1 M potassium phosphate (pH 8.0) at 25℃ for 30 min. The difference spectrum was obtained by recording the absorbance of PLP-treated oxidase against 10 mM PLP in the buffer.

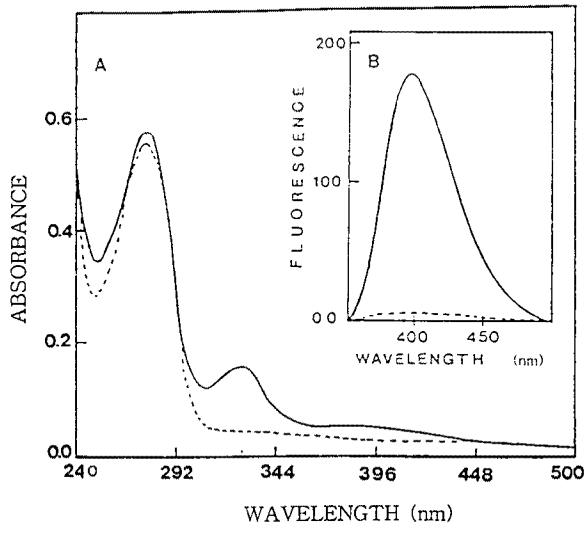

Fig. 4. UV-Vis absorption spectra (A) and fluorescence emission spectra (B) of the native and the PLP-$NaBH_4$-treated enzyme. The enzyme (10 μM) was incubated with 10 mM PLP in 0.1 M potassium phosphate buffer (pH 8.0) for 30 min at 25℃. After the addition of $NaBH_4$ (0.75 mM), the mixture was allowed to stand at 4℃ for 30 min, and then dialyzed against 0.1 M phosphate buffer (pH 8.0) in the dark. Absorption and fluorescence spectra of native (·····) and the reduced (——) were recoded at the same protein concentration. The fluorescence excitation wavelength was 325 nm.

Furthermore when excited at 325 nm, this modified enzyme gave a fluorescence spectrum with a maximum at 395 nm (Fig. 4B).

Together, these results suggest that PLP inactivates the oxidase by forming Schiff bases with ε-amino groups of lysyl residues or the N-terminal amino group

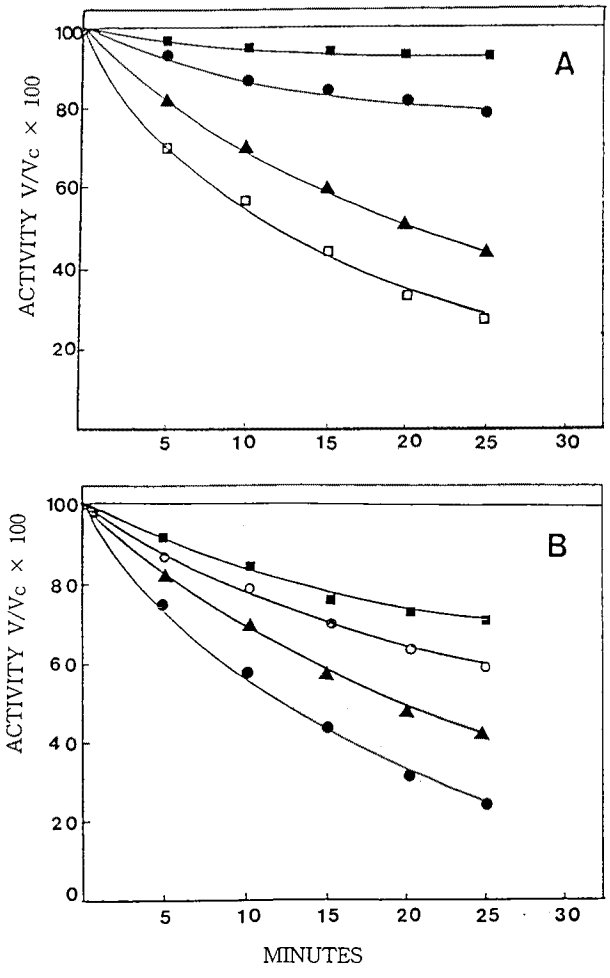

Fig. 5. Protective effect of competitive inhibitors on the inactivation of glycolate oxidase by pyridoxal 5′-phosphate. The oxidase was incubated with 10 mM PLP in the presence of 0 mM (□), 0.1 mM (▲), 4 mM (●), and 10 mM (■) oxalate (A), and in the presence of 0 mM (●), 2 mM (▲), 5 mM (○), and 10 mM (■) oxaloacetate (B).

in the enzyme.

Protection by competitive inhibitors against PLP-inactivation

The effect of competitive inhibitors on the inactivation of the oxidase by PLP was examined by incubating glycolate oxidase with PLP in the presence of inhibitors. As shown in Fig. 5(A) and (B), the substrate-competitive inhibitors oxalate and oxaloacetate effectively protected the oxidase against inactivation by PLP, and the extent of protection was a function of the concentration of the inhibitor. As expected from their K_i values of 0.8 mM and 3.9 mM for oxalate and oxaloacetate, respectively (Chae, 1993), oxalate protected the enzyme more effectively against inactivation than oxaloacetate did. If the inactivation of the enzyme by PLP occurs due to the modification of lysyl residue at the active site of the enzyme, the inactivation might be prevented by

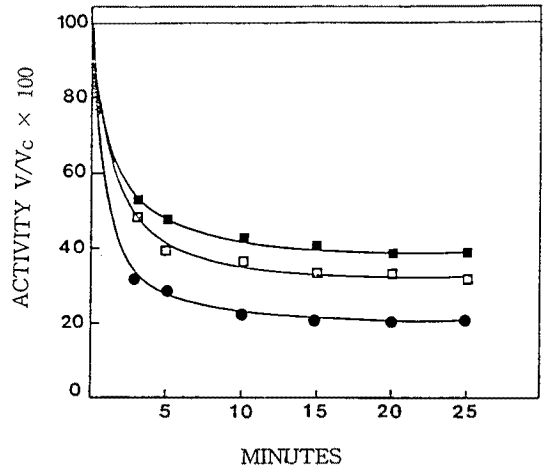

Fig. 6. Protective effects of competitive inhibitors on the inactivation of glycolate oxidase by N-bromosuccinimide. The oxidase (2 μM) was incubated with 7 mM NBS in the presence of 0 mM inhibitors (●), 10 mM oxaloacetate (■), and 10 mM oxalate (□).

substrate or substrate-competitive inhibitors (Cohen, 1970).

The data for protection indicates that PLP inactivates the enzyme by reacting with the amino group of lysyl residue of the active site of the enzyme. X-ray crystallographic data for the spinach oxidase showed that the N-terminal amino group is located far from the active site (Lindqvist and Branden, 1985).

Tryptophan modification with N-bromosuccinimide

Glycolate oxidase was also inactivated by treatment with tryptophan-specific reagent NBS. However, the inactivation did not follow pseudo-first-order kinetics with an excess modifying reagent (Fig. 6). The enzyme was rapidly inactivated by incubation with 7 mM NBS within 5 min, but the inactivation did not proceed further significantly by longer incubation. These data indicate that the enzyme is only partially inactivated by tryptophan modification. If modification by NBS occurs at the active site of the enzyme, this modification might be prevented by substrate or competitive inhibitors. The competitive inhibitors of the enzyme oxalate and oxaloacetate did not provide significant protection against inactivation by NBS even at the saturation level (Fig. 6). These results suggest that the inactivation of the enzyme by NBS is not due to modification of the tryptophan residue at the active site of the enzyme.

Cysteine modification with cystein-specific reagents

In the oxidation of glycolate to glyoxylate by glycolate oxidase, a cysteine residue could be a candidate for an acid-base catalyst. Treatments of the oxidase with cysteine-specific reagents, iodoacetic acid (40 mM),

Table 1. Inactivation of glycolate oxidase by iodoacetate, silver nitrate, and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB)

Time (min)	Activity, V/Vc 100		
	Iodoacetate 40 mM	Silver nitrate 1 mM	DTNB 20 mM
0	100	100	100
5	93	94	93
10	93	93	93
15	89	93	87
20	89	90	87
25	87	89	87

The oxidase (2 μM) was incubated with sulfhydryl-specific reagents, iodoacetate (40 mM), silver nitrate (1 mM), and DTNB (20 mM).

silver nitrate (1 mM), and 5,5′-dithiobis 2-nitrobenzoic acid (20 mM) caused only a slight decrease of enzyme activity (Table 1). Thus, any cysteine residue is not likely involved in the catalytic reaction.

Discussion

Spinach glycolate oxidase was reversibly inactivated by incubation with pyridoxal 5′-phosphate. The inactivation results from the formation of a Schiff base with lysine residue(s) of the enzyme. The treatment of the oxidase with PLP caused a time-dependent loss of activity, and the inactivation was a function of PLP concentration. The enzymatic activity of PLP-treated enzyme was partially restored by removing untreated PLP and then diluting the enzyme sample. However, reduction of PLP-modified enzyme with borohydride rendered the inactivation irreversible. The enzyme modified by PLP showed UV and visible absorption properties which are similar to those obtained for amine model compounds (Anderson *et al.*, 1966). Borohydride reduction of the modified enzyme lead to the appearance of absorption and fluorescence properties characteristic of reduced Schiff-base linkage (Cleland, 1977).

Pyridoxal 5′-phosphate is a chemical modifying reagent which is highly specific for amino groups (Lundblad and Noyes, 1984; Eyzaguirce, 1987). It is useful for the modification of lysine residues of proteins because of solubility, selectivity of reaction, spectural properties of the modified residue, reversibility of reaction, and the establishment of stoichiometry by reduction of the Schiff base formed between PLP and lysine with borohydride (Lundblad and Noyes, 1984).

If the inactivation of an enzyme by a specific reagent occurs due to modification of active site amino acid residues, the substrate and substrate-competitive inhibitors will protect the enzyme against inactivation (Cohen, 1970). Competitive inhibitors of glycolate oxidase oxalate and oxaloacetate protected the enzyme effectively against the inactivation of the oxidase by PLP. The saturation level of oxalate protected the enzyme almost completely against inactivation by PLP.

Together, pyridoxal 5′-phosphate inactivated spinach glycolate oxidase by forming a Schiff base with a lysine residue at the active site of the enzyme. PLP was able to form a Schiff base with the N-terminal amino group. However, inactivation is not likely due to modification of the N-terminal amino group, since X-ray crystallographic studies showed that, the N-terminal is located far from the active site (Lindqvist and Branden, 1985).

Cationic amino acids such as lysine and arginine are frequently found at the active site of enzymes that act on anionic substrate and cofactors. Since the substrate glycolate and cofactor FMN for glycolate oxidase contain an anionic carboxyl group and phosphatase group, respectively, the enzyme might contain lysine and/or arginine residues at the active site of the enzyme. In our laboratory we have shown that an arginine residue is located at the active site of the enzyme as determined by chemical modification (Lee and Choi, 1986), and confirmed Arg-257 to be essential for catalytic activity (Son *et al.*, 1984). Based on X-ray crystallographic studies of the oxidase, Lindqvist and Brändén (1989) suggested that Arg-257 is important for binding of the substrate, and Lys-230 may interact with cofactor FMN. Thus, it is strongly suggested that PLP inactivates the oxidase by forming a Schiff base with the Lys-230 residue.

N-Bromosuccinimide, a tryptophan-specific reagent, also inactivated glycolate oxidase. However, competitive inhibitors oxalate and oxaloacetate could not protect the enzyme effectively against inactivation by NBS. Even the saturation level of oxalate provided only 20% protection against inactivation by NBS, while the same level of oxalate provided more than 60% protection against inactivation by PLP. Thus, the inactivation of the oxidase by NBS is not likely due to modification of active site tryptophan residue(s). The change of conformation and/or of interaction between subunits induced by tryptophan modification may be the cause of the inactivation of the enzyme. Sulfhydryl groups seem to play a less important role in the catalytic function of the oxidase. None of three different kinds of sulfhydryl-reagents, iodoacetate, silver nitrate, or 5,5′-dithiobis-2-nitrobenzoic acid inactivated the enzyme significantly.

Glycolate oxidase is one of the key enzymes in photorespiration of green plants. Since photosynthetic productivity is drastically reduced by active photorespira-

tion especially in C-3 plants, an effective control of photorespiration might be one possible way to increase the efficiency of photosynthesis. It was shown that inhibition of photorespiration with biochemical inhibitors of glycolate oxidase increases net photosynthesis by 50% or more for a short period (Zelitch, 1966). It is required to elucidate the nature and role of the amino acid residues within the active site for a rational design of specific inhibitors of the oxidase.

Acknowledgement

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