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Glycolate Formation Catalyzed by Spinach Leaf Transketolase Utilizing the Superoxide Radical†

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ABSTRACT: A homogeneous preparation of transketolase was obtained from spinach leaf; the specific enzyme activity was 9.5 μmol of glyceraldehyde-3-P formed (mg of protein)$^{-1}$ min^{-1}, when xylulose-5-P and ribose-5-P were used as the donor and acceptor, respectively, of the ketol residue. Transketolase catalyzed the formation of glycolate from fructose-6-P coupled with the O_2^--generating system of xanthine–xanthine oxidase. The addition of superoxide dismutase (145 units) or 1,2-dihydroxybenzene-3,5-disulfonic acid (Tiron) (5 mM), both O_2^- scavengers, to the reaction system inhibited glycolate formation 72 and 58%, respectively. The reaction was not inhibited by catalase. Mannitol, an ·OH scavenger, and β-carotene and 1,4-diazobicyclo[2.2.2]octane, $^1O_2$ scavengers, showed little or no inhibitory effects. The rate of glycolate formation catalyzed by the transketolase system was measured in a coupled reaction with a continuous supply of KO_2 dissolved in dimethyl sulfoxide, used as an O_2^--generating system. The optimum pH of the reaction was above pH 8.5. The second-order rate constant for the reaction between transketolase and O_2^-, determined by the competition for O_2^- between nitroblue tetrazolium (NBT) and transketolase, was 1.0×10^6 M^{-1} s^{-1}. Transketolase showed an inhibitory effect on the O_2^--dependent reduction of NBT only if the reaction mixture was previously incubated with ketol donors such as fructose-6-P, xylulose-5-P, or glycolaldehyde. The results suggest the possibility that transketolase catalyzes O_2^--dependent glycolate formation under increased steady-state levels of O_2^- in the chloroplast stroma.

It has been well established that chloroplasts of green plants (Mehler, 1951; Asada et al., 1973, 1974a; Asada & Nakano, 1978) and chromatophores from photosynthetic bacteria (Asami & Akazawa, 1977) can reduce molecular oxygen and produce O_2^-. Rates of O_2^- production as high as 10–20 μmol (mg of Chl)$^{-1}$ h^{-1} have been reported[1] (Radmer & Kok, 1976; Radmer et al., 1978; Asami & Akazawa, 1977; Asada & Nakano, 1978). Much higher rates of O_2 reduction [300 μmol (mg of Chl)$^{-1}$ h^{-1}] have been observed with subchloroplast particles (Lien & San Pietro, 1979). However, O_2^- and other active oxygen derivatives such as ·OH, $^1O_2$, and H_2O_2 are known to be toxic to biological systems. It has often been affirmed that superoxide dismutase, which is ubiquitously present in living organisms, scavenges O_2^- and makes its steady-state concentration low, thus constituting an O_2^--detoxifying device (Fridovich, 1975).

Several investigators have reported the damaging photooxidative effect of oxygen on photosynthetic electron transport systems (Avron, 1960; Forti & Jagendorf, 1960; Jones & Kok, 1966), and Asami & Akazawa (1978) have demonstrated that the photooxidative damage to photophosphorylation in *Chromatium vinosum* under high light intensity and an O_2-containing atmosphere appeared to be caused by O_2^-. Radmer & Kok (1976) have shown that O_2 and NADP can be reduced competitively by photosystem I of chloroplasts. Thus, it is conceivable that the rate of O_2 reduction increases under conditions of low CO_2 pressure and high light intensity, which are the environmental conditions believed to be favorable to photorespiration. The mechanism of O_2 reduction needs to be reexamined in relation to glycolate formation, the key metabolic reaction in photorespiration.

Transketolase (EC 2.2.1.1) has long been thought to be involved in photorespiratory glycolate synthesis, since the enzyme catalyzes the formation of glycolate in the presence of artificial oxidants such as ferricyanide and *p*-benzoquinone (Weissbach & Horecker, 1955; Bradbeer & Racker, 1961) (cf. Figure 1).

Shain & Gibbs (1971) theorized that H_2O_2 generated in photosystem I oxidizes the transketolase–glycolaldehyde addition product. Our previous investigation (Asami & Akazawa, 1977) has shown that transketolase catalyzes O_2^--dependent glycolate formation in the presence of fructose-6-P, and we postulated that this subsidiary reaction of transketolase may have a functional role in scavenging O_2^- in the chloroplast stroma. As an extention of this study, we have further explored the mechanism of glycolate formation by the O_2^--requiring

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[1] Abbreviations used: Chl, chlorophyll; crown ether, dicyclohexyl-18-crown-6; DABCO, 1,4-diazobicyclo[2.2.2]octane; NBT, nitroblue tetrazolium; RuBP, ribulose 1,5-bisphosphate; Tiron, 1,2-dihydroxybenzene-3,5-disulfonic acid; TPP, thiamine pyrophosphate.

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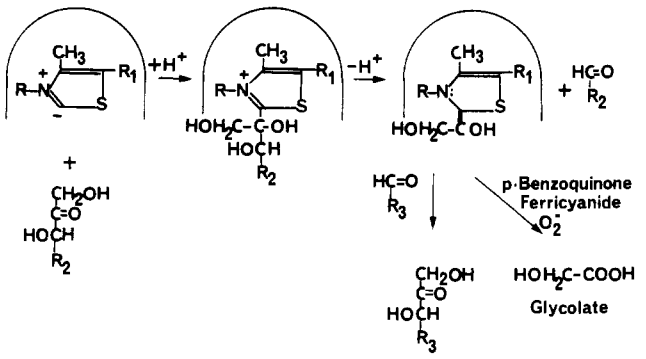

FIGURE 1: Reaction mechanism of formation of glycolate by transketolase reaction in the presence of various oxidants.

transketolase-catalyzed reaction, particularly in terms of the reactivities of the transketolase–glycolaldehyde addition product with O_2^-.

Materials and Methods

Chemicals. D-[U-^{14}C]-Fructose-6-P was purchased from the Radiochemical Centre, Amersham, England. D-Xylulose-5-P (sodium salt) and superoxide dismutase (bovine blood) were the products of Sigma Chemical Co., St. Louis, MO. Xanthine oxidase, glycerol-3-P dehydrogenase, and triose-P isomerase were purchased from Boehringer Mannheim, West Germany. KO_2 was the product of Alfa, France. DEAE-Sephacel was obtained from Pharmacia, Uppsala, Sweden.

Purification of Transketolase. Transketolase from spinach leaf was purified according to the method of Villafranca & Axelrod (1971) with some modifications. One kilogram of fresh spinach leaves were homogenized in a Waring Blendor for 3 min by using 1 L of 25 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA. The whole suspension was centrifuged and the supernatant was subjected to 50% $(NH_4)_2SO_4$ saturation, and the resultant precipitate was discarded by centrifugation. The supernatant fraction was brought to ~65% saturation with $(NH_4)_2SO_4$, and the precipitate collected by centrifugation was dissolved in 10 mL of the homogenizing buffer described above and thoroughly dialyzed against the same buffer (ammonium sulfate I). The dialyzed material was again fractionated by $(NH_4)_2SO_4$ precipitation (57.5–65%). The precipitate was dissolved in 15 mL of homogenizing buffer (ammonium sulfate II) and subjected to enzyme assays. The enzyme solution obtained was placed in a salt–ice bath (−15 °C), and 0.55 volume of cold acetone (−15 °C) was added to the solution dropwise with stirring. The suspension was immediately centrifuged at −15 °C for 10 min at 10000*g* and the precipitate obtained was discarded. An additional 0.5 volume of cold acetone was added to the supernatant fluid and the suspension was centrifuged as described above. The precipitate was dissolved in 5 mL of homogenizing buffer and dialyzed thoroughly against the same buffer (acetone fraction). The enzyme solution was placed on a column of DEAE-Sephacel (1.5 × 30 cm) and eluted with a linear gradient of NaCl from 0 to 0.7 M. The enzyme fractions were precipitated by 65% $(NH_4)_2SO_4$ and kept at 0 °C until used (DEAE-Sephacel). A homogeneous enzyme preparation was obtained as judged from a single band pattern on polyacrylamide gel electrophoresis (pH 8.9) (Davis, 1964) to which 50 μg of enzyme sample was applied (data not shown).

Enzyme Assay Method. Transketolase activity was assayed after the method of Datta & Racker (1961a). The reaction mixture contained the following components in a final volume of 0.5 mL: Tris-HCl (pH 8.0), 50 mM; TPP, 0.75 mM; $MgCl_2$, 10 mM; NADH, 0.175 mM; xylulose-5-P, 1 mM; ribose-5-P, 2 mM; triose-P isomerase, 7 units; α-glycerol-P dehydrogenase, 0.6 unit. The reaction was started by adding xylulose-5-P.

Table I: Purification of Spinach Leaf Transketolase

	total protein (mg)	total act. (μmol min^{-1})	sp act. (μmol mg^{-1} min^{-1})	yield (%)
crude extract	16750	1173	0.07	100
ammonium sulfate I	1008	650	0.65	55.4
ammonium sulfate II	328	325	0.99	27.7
acetone fraction	69	250	3.62	21.3
DEAE-Sephacel	17	164	9.48	14.3

Transketolase-catalyzed glycolate formation was assayed by the method of Asami & Akazawa (1977). The reaction mixture contained the following components in a total volume of 0.5 mL: Tris-HCl (pH 8.0), 50 mM; $MgCl_2$, 10 mM; TPP, 1.5 mM; [U-^{14}C]fructose-6-P at various concentrations, 283 μCi/μmol; purified transketolase, 0.5–1.0 unit. The reaction mixture was coupled with the following O_2^--generating systems: (1) xanthine (0.2 mM) and xanthine oxidase (20 μg); (2) KO_2 dissolved in a dimethyl sulfoxide solution containing 0.5% crown ether (Ohnishi et al., 1977). The concentration of O_2^- was measured polarographically by the evolution of O_2. KO_2 in a solution of dimethyl sulfoxide was infused into the reaction mixture at a rate of 20 μL min^{-1} by using a motor-driven microfeeder (Hirata & Hayaishi, 1975). In both assay systems, the reaction was terminated by adding 400 μL of boiling ethanol. Aliquots (50 μL) of the reaction mixture were subjected to high-voltage paper electrophoresis (pH 5.0; 1.5 kV; 80 min) to separate [^{14}C]glycolate (Asami & Akazawa, 1974). The radioactivity in the glycolate region was determined by a liquid scintillation spectrometer using a nonaqueous scintillator system, details of which have been reported previously (Asami et al., 1977).

Determination of Reactivity of the Transketolase–Glycolaldehyde (C_2) Addition Product with O_2^-. The transketolase–glycolaldehyde addition product was prepared according to the method of Datta & Racker (1961b) by incubating 5 units of transketolase for 10 min at room temperature with fructose-6-P, 3 mM; TPP, 1.5 mM; $MgCl_2$, 10 mM; and Tris-HCl (pH 7.8), 60 mM, in a total volume of 0.2 mL. The assay mixture contained the following in a total volume of 1 mL: Tris-HCl (pH 7.8), 50 mM; EDTA, 0.1 mM; NBT, 0.03 mM; fructose-6-P, 3 mM; TPP, 1.5 mM; $MgCl_2$, 10 mM; xanthine, 0.2 mM; xanthine oxidase (20 μg) in the presence or absence of the transketolase–glycolaldehyde addition product. In the control reaction system, fructose-6-P was eliminated from both incubation and assay mixtures. The reaction was started by the addition of xanthine oxidase, and the reduction of NBT was determined from the initial absorbance change at 560 nm.

Results

Purification of Transketolase. The results of the purification of transketolase from spinach leaf following the procedures under Materials and Methods are summarized in Table I. The yield of the enzyme at the final purification step was 14% with a specific activity of 9.5 μmol of glyceraldehyde-3-P formed (mg of protein)$^{-1}$ min^{-1} [cf. 7.5 μmol (mg of protein)$^{-1}$ min^{-1} by Villafranca & Axelrod (1971)]. During the purification step, enzyme activity was not detectable in fractions other than the isolated enzyme preparation, excluding the possible presence of the isozymic form. The final enzyme

Table II: Effect of Scavengers for Active Oxygens on Transketolase-Catalyzed Glycolate Formation[a]

additions	rel act. (glycolate formation) (%)
none	100
catalase (1000 units[b])	112
superoxide dismutase (145 units[c])	28
superoxide dismutase (290 units[c])	30
Tiron (5 mM)	42
mannitol (20 mM)	85
β-carotene (0.1 mM)	85
β-carotene (1 mM)	94
DABCO (1 mM)	104
DABCO (5 mM)	101

[a] Transketolase-catalyzed glycolate formation coupled with xanthine–xanthine oxidase was assayed as described under Materials and Methods, except that the concentration of [U-^{14}C]fructose-6-P used was 0.03 mM (283 μCi/μmol). The activity of glycolate produced in the control system without addition of any component was 99 568 cpm/tube. β-Carotene and DABCO were used as acetone solutions. [b] One unit is 1 μmol of H_2O_2 decomposed/min. [c] The enzyme activity unit is defined based on the paper by McCord & Fridovich (1969).

preparation did not contain RuBP carboxylase activity.

O_2^--Coupled Glycolate Formation Catalyzed by Transketolase. Our previous work (Asami & Akazawa, 1977) has shown that glycolate formation by yeast transketolase coupled with the xanthine–xanthine oxidase system is strongly inhibited by superoxide dismutase. However, the nature of the active oxygen(s) involved in the reaction system needed to be more clearly identified. We therefore tested the effects of various types of scavengers for active oxygen molecules on glycolate formation catalyzed by the spinach transketolase coupled with xanthine–xanthine oxidase, and the results are shown in Table II. In accordance with our previous findings, catalase did not show any effect, whereas superoxide dismutase and Tiron (5 mM) inhibited the reaction 72 and 58%, respectively. Mannitol (20 mM), a scavenger of ·OH, as well as β-carotene and DABCO, scavengers of $^1O_2$, did not show appreciable effects on the reaction. The overall results strongly indicate the functional importance of O_2^- rather than other molecular species of active oxygen in the glycolate formation by spinach transketolase.

The rate of glycolate formation was determined as a function of the concentration of fructose-6-P. A hyperbolic saturation curve was obtained as shown in Figure 2. The K_m (fructose-6-P) and the maximum reaction rate were determined to be 0.6 mM and 30 nmol (mg of protein)$^{-1}$ min^{-1}, respectively, at pH 8.0. Lilley et al. (1977) found the concentration of hexose phosphate (including fructose-6-P) in spinach chloroplasts to be ~4 mM.

The pH dependence of the glycolate formation by the O_2^--coupled spinach transketolase system is shown in Figure 3. In this case, KO_2 dissolved in dimethyl sulfoxide was slowly infused into the reaction mixture at a constant rate of 68 nmol of O_2^- min^{-1} through a motor-driven microfeeder. As can be seen, the rate of glycolate formation increased drastically at above pH 7.5. These results may be partly ascribed to the stability of O_2^- at alkaline pH values (Behar et al., 1970).

It has been established that the transketolase–glycolaldehyde (C_2) addition product can be formed by incubating transketolase with Mg^{2+}, TPP, and ketol donors such as fructose-6-P, xylulose-5-P, or glycolaldehyde (Datta & Racker, 1961b). We therefore measured the reactivity of the transketolase–glycolaldehyde addition product with O_2^- using the competition for O_2^- between NBT and the transketolase–glycolaldehyde addition product. The method was basically that of Asada

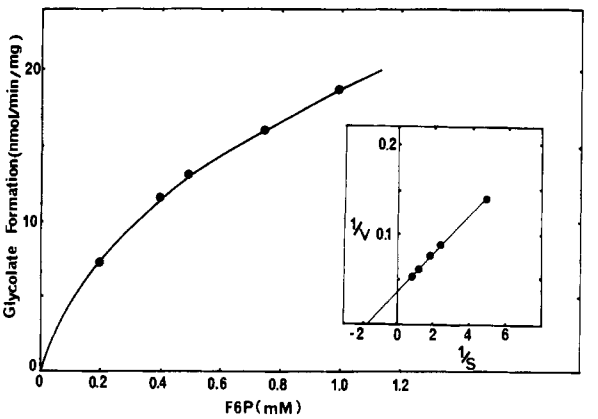

FIGURE 2: Glycolate formation catalyzed by spinach leaf transketolase coupled with the xanthine–xanthine oxidase system, as a function of the concentration of fructose-6-P. The experimental conditions were the same as those shown in Table I, except that the concentrations of fructose-6-P were varied as indicated and the specific radioactivity of D-[U-^{14}C]fructose-6-P was 18.5 μCi/μmol.

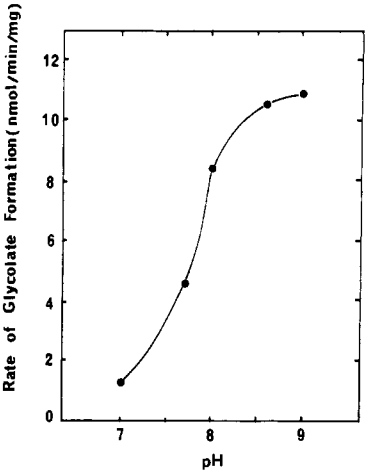

FIGURE 3: pH dependence of glycolate formation by spinach leaf transketolase coupled with a constant supply of O_2^- by KO_2 infusion. The assay procedures are given under Materials and Methods. Incubation was carried out at 25 °C for 2 min.

et al. (1974b). When the reduction of NBT is measured in the presence of the O_2^- scavenger, the initial rate of the O_2^--induced reaction should follow eq 1

$$V/v = 1 + \frac{k_s[\text{transketolase–glycolaldehyde}]}{k_n[\text{NBT}]} \quad (1)$$

in which V and v are the reduction rates of NBT in the absence and presence of the transketolase–glycolaldehyde addition product, respectively, and k_n is the second-order rate constant between NBT and O_2^- [5 × 10^4 M^{-1} s^{-1} at pH 7.8] (Halliwell, 1976). k_s is the second-order rate constant between the transketolase–glycolaldehyde addition product and O_2^-. As presented in Figure 4, the reduction rate of NBT induced by O_2^- generated by xanthine–xanthine oxidase was markedly inhibited by transketolase which was previously incubated with 3 mM fructose-6-P and TPP; the greater the amount of transketolase added, the more prominent the inhibitory effect. As shown in Table III (experiment I), although the inhibitory effect was almost negligible in the absence of fructose-6-P in the assay system, the addition of fructose-6-P and other ketol donors such as xylulose-5-P and glycolaldehyde showed a marked inhibitory effect. The inhibitory effect was also dependent on the concentration of fructose-6-P (experiment II). The relationship between transketolase and the reduction rate of NBT in the presence and absence of fructose-6-P is shown

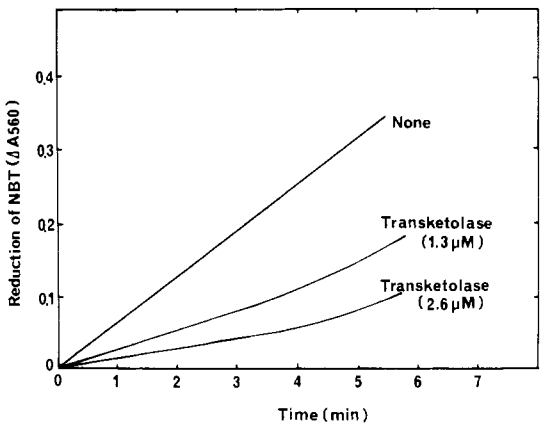

FIGURE 4: Inhibitory effect of transketolase on O_2^--induced reduction of NBT. Basic experimental procedures are described under Materials and Methods. Prior to the incubation, purified spinach transketolase (5 units) was incubated for 10 min with fructose-6-P (3 mM), TPP (1.5 mM), and $MgCl_2$ (10 mM) in 0.2 mL of 60 mM Tris-HCl (pH 7.8). The assay mixture contained Tris-HCl (pH 7.8) (50 mM), EDTA (0.1 mM), NBT (0.03 mM), fructose-6-P (3 mM), TPP (1.5 mM), xanthine (0.2 mM), xanthine oxidase (20 μg), and the indicated amounts of the transketolase preparation which was previously incubated as described above. The reaction was started by adding xanthine oxidase, and the rate of reduction of NBT was monitored by measuring the absorbance change at 560 nm.

Table III: Effect of Transketolase and Substrates on Reduction Rate of NBT by the Superoxide Radical[a]

additions	A_{560}/min	inhibn (%)
Experiment I		
none	0.053	
transketolase (1.2 μM)	0.050	6
fructose-6-P (3 mM)	0.054	−2
transketolase plus fructose-6-P (3 mM)	0.035	34
transketolase plus glycolaldehyde (3 mM)	0.032	40
transketolase plus xylulose-5-P (0.5 mM)	0.037	30
Experiment II		
transketolase (2.0 μM)	0.049	
transketolase plus fructose-6-P (1 mM)	0.035	25
transketolase plus fructose-6-P (2 mM)	0.028	40
transketolase plus fructose-6-P (3 mM)	0.025	47

[a] Experimental conditions are essentially the same as those given for Figure 2 except that transketolase was incubated with several substrates of various concentrations to produce the transketolase–glycolaldehyde addition product.

in Figure 5. The reciprocal ratio of NBT reduction in the presence of transketolase and fructose-6-P to that in the absence of fructose-6-P (V/v) was proportional to the concentration of transketolase. From the slope of V/v, the second-order rate constant of the transketolase–glycolaldehyde addition product with O_2^- was estimated to be 1.0×10^6 M^{-1} s^{-1}.

Discussion

Glycolate formation by spinach transketolase coupled with an O_2^--generating system of xanthine–xanthine oxidase was markedly inhibited by the O_2^- scavengers, superoxide dismutase and Tiron, whereas scavengers for H_2O_2, ·OH, and $^1O_2$ were found to be ineffective. These results suggest the functional importance of O_2^- in the overall reaction. The maximal inhibition by superoxide dismutase observed in the present study was 72%, which is comparable to the inhibition (85%) determined in our previous experiments using a yeast transketolase (Asami & Akazawa, 1977). These results indicate that 15–30% of glycolate formation catalyzed by the transketolase system may proceed through an O_2^--independent

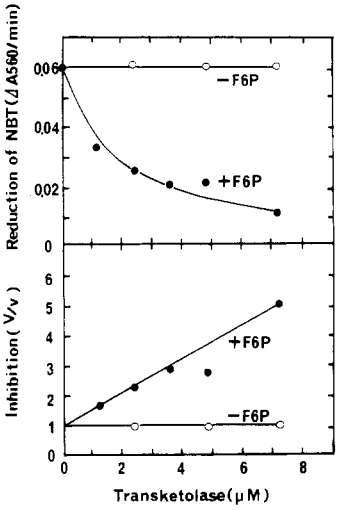

FIGURE 5: Inhibitory effect of spinach leaf transketolase on O_2^--induced reduction of NBT. Basic experimental procedures were the same as those given in Figure 3. Absorbance change at 560 nm was measured with or without addition of fructose-6-P (3 mM) in either the enzyme preincubation or the assay mixtures.

Table IV: Reactivities of Some Chloroplastic Stroma Components with O_2^-

components	rate constant (M^{-1} s^{-1})	concn in chloroplasts (M)	ref
Cu,Zn-superoxide dismutase	2.0×10^9	8×10^{-6}	Asada et al. (1977)
transketolase	1.0×10^6	1.5×10^{-4}	this work
ascorbic acid	2.7×10^5	2.5×10^{-3}	Asada et al. (1977)
glutathione	6.7×10^5	3.5×10^{-3}	Asada et al. (1977)

pathway. The specific activity of glycolate formation by the O_2^--coupled transketolase was 30 nmol (mg of protein)$^{-1}$ min^{-1} under saturating concentrations of fructose-6-P (Figure 2). The ratio of the specific activity of glycolate formation to that of the ordinary transketolase activity was ~1:300. Since the activity of transketolase in intact chloroplast preparations purified by Percoll density gradient centrifugation [cf. Takabe et al. (1979a)] was determined to be 350 μmol (mg of Chl)$^{-1}$ h^{-1} (data not shown), the capacity of transketolase for producing glycolate can be estimated to be ~1 μmol (mg of Chl)$^{-1}$ h^{-1}. Thus, the activity of transketolase-catalyzing glycolate formation is ~1 order of magnitude smaller than that of RuBP oxygenase operating in chloroplasts under atmospheric conditions [~10 μmol (mg of Chl)$^{-1}$ h^{-1}] (Bahr & Jensen, 1974; Jensen & Bahr, 1977).

There are, however, several O_2^--scavenging components in chloroplast stroma, as listed in Table IV. It is generally accepted that Cu,Zn-superoxide dismutase is a most potent scavenging agent for O_2^- ($k = 2 \times 10^9$ M^{-1} s^{-1}); its concentration in chloroplasts is estimated to be 8×10^{-6} M, assuming the concentration of Chl to be 25 mM (Asada et al., 1977). On the other hand, the concentration of transketolase can be estimated to be 1.5×10^{-4} M by using the specific activity of the purified enzyme preparation in Table I and the molecular weight (100 000) reported by Villafranca & Axelrod (1971). Therefore, the O_2^--scavenging effect of transketolase is ~1% of that of superoxide dismutase. Glutathione and ascorbic acid have been known to react with O_2^- (Asada et al., 1977). The O_2^--scavenging effects of ascorbic acid and glutathione are about 4 and 14%, respectively, of that of superoxide dismutase, although these compounds need a reduction system for supporting their constant scavenging activities. Transketolase as well as ascorbic acid and glutathione may thus function as

secondary O_2^- scavengers in chloroplast stroma.

It will be noted that the second-order rate constant of transketolase is higher than that of either glutathione or ascorbic acid. When the steady-state concentration of O_2^- is increased under high light intensity and low CO_2 concentration, transketolase may react more readily with O_2^- and produce glycolate, thereby increasing the contribution of this system to O_2^- detoxification.

Since glycolate is a two-electron oxidation product of glycolaldehyde, there must be a radical intermediate in the oxidation reaction between O_2^- and the transketolase–glycolaldehyde addition product. It has been reported that the oxidation reaction of the transketolase–glycolaldehyde addition product by ferricyanide accompanied progressive inactivation (Christen et al., 1976). The overall reaction mechanism of the transketolase–glycolaldehyde addition product or of 1,2-dihydroxyethyl-TPP with O_2^- needs to be elucidated by future investigations.

It has been reported that with several photosynthetic organisms placed in an $^{18}O_2$-containing atmosphere, one atom of ^{18}O is incorporated into the carboxyl group of the glycolate molecule and the enrichment of ^{18}O is almost 100% (Andrews et al., 1971; Dimon & Gerster, 1976; Lorimer et al., 1977, 1978; Takabe et al., 1979b). The mechanism of this O_2-dependent formation of glycolate in photosynthetic systems can be readily explained on the basis of the RuBP oxygenase reaction (Lorimer et al., 1973). However, Lorimer et al. (1978) have reported that ^{18}O can possibly be incorporated into glycolate (~15–20% enrichment) by the transketolase reaction in the presence of washed spinach thylakoids placed under illumination. Undoubtedly, further elucidation of the nature of ^{18}O incorporation in connection with the O_2^--dependent glycolate formation by the transketolase reaction would lead to a better understanding of this phenomenon.

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