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ON THE DECARBOXYLATION OF α-KETO ACIDS BY ISOLATED CHLOROPLASTS

E. F. ELSTNER and A. HEUPEL

Lehrstuhl für Biochemie der Pflanzen, Ruhr-Universität Bochum, D-463 Bochum-Querenburg (Germany)

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SUMMARY

The mechanism of the decarboxylation of α-keto acids by isolated chloroplasts has been studied with the aid of superoxide dismutase and catalase. Using photosynthetic and enzymatic systems, which are known to catalyze peroxidic oxidations, we have been able to demonstrate that both the superoxide free radical ion and H_2O_2 are necessary for maximal rates of decarboxylation. In isolated chloroplasts, an auto-oxidizable electron acceptor as well as an electron donor for Photosystem I are absolute requirements for the decarboxylation. H_2O_2 seems to be the primary oxidant in the decarboxylation of pyruvate or glyoxylate by isolated chloroplasts. A secondary rate of decarboxylation is superimposed on the primary one, mediated by superoxide free radical ion. Mn^{2+} stimulates the decarboxylation probably *via* intermediarily-formed Mn^{3+} in a reaction, which is neither inhibited by catalase nor by superoxide dismutase. A decarboxylation of pyruvate or glyoxylate by isolated chloroplasts in the presence of $NADP^+$ is initiated, as soon as the available $NADP^+$ is fully reduced. In this case, the open-chain electron transport seems to switch from $NADP^+$ to oxygen as the terminal electron acceptor.

INTRODUCTION

In isolated chloroplast fragments, at least three fundamentally different ways have been described for the oxidation of inorganic or organic compounds:

(1) Chloroplast fragments contain oxidases, which catalyze the hydroxylation or oxidation of phenolic compounds[1–3].

(2) Several compounds have been found to act as electron donors for the photosynthetic electron transport system.

(3) Isolated chloroplasts under certain conditions can produce peroxides, *i.e.* H_2O_2 and the superoxide free radical ion[4–8], which can act as oxidants in a catalyzed or non-catalyzed reaction.

The first mechanism is not dependent on the function of the pigment system, the others are strictly light-dependent and follow the characteristics of photosynthetic electron transport systems.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Triquat, 1,1′-trimethylene-2,2′-bipyridylium dibromide.

Kisaki and Tolbert[9] reported on the decarboxylation of glyoxylate by isolated chloroplasts. Their results, however, are not in absolute agreement with either one of the above mechanisms. Recently Zelitsch[10] showed, that a decarboxylation of α-keto acids by isolated chloroplasts only occurs in the presence of 10^{-2} M Mn^{2+}. This reaction was light- and oxygen-dependent. The mechanism of the reaction was not clear, though a function of H_2O_2 was discussed. The inability of high concentrations of catalase to inhibit this reaction, however, makes it impossible to accurately assess the validity of the above assumption.

We wish to report on studies on the mechanism of the photooxidative decarboxylation of α-keto acids in isolated chloroplasts. Superoxide dismutase was used to aid in the determination of the nature of the oxidant. Superoxide dismutase, a copper-containing enzyme isolated from green peas[11], catalyzes the dismutation of superoxide free radical ion into H_2O_2 and O_2 (ref. 12), and has been shown to be an extremely useful tool for studying biochemical oxidations for a possible involvement of the superoxide free radical ion[13].

MATERIALS AND METHODS

Chloroplasts were isolated from spinach leaves as described by Nelson *et al.*[14]. Ferredoxin and ferredoxin:$NADP^+$ reductase were prepared according to the methods of Tagawa and Arnon[15] and Shin *et al.*[16]. $Cytochrome_{552}$ from *Euglena gracilis* and superoxide dismutase from dried green peas were isolated as described[8,11,17]. The isolated superoxide dismutase showed no catalase activity. The number of enzyme units of the superoxide dismutase were determined as described by McCord and Fridovich[12].

Digitonin-fragmented chloroplasts were prepared as described[18], and protein was determined according to Murphy and Kies[19]. Photosynthetic decarboxylations were conducted in cubic Warburg vessels for 15 min in a water bath at 15 °C with illumination (25000 lux) from the bottom. The standard reaction mixture for photosynthetic decarboxylations was 3 ml and contained: chloroplast fragments with 0.2 mg chlorophyll, 80 μmoles Tris buffer (pH 7.6), 5 μmoles NH_4Cl, 1 μmole α-keto acid with a specific activity of about 0.2 Ci/mole and the additions as shown in the tables and figures. 7000 units of catalase and 50 units of superoxide dismutase were added, as indicated. The reactions were terminated by injecting 0.2 ml 25 mM H_2SO_4 through a rubber cap. The radioactive CO_2 was trapped in the center well with 0.2 ml phenethylamine (Packard) by shaking for 15 min in the dark. An aliquot of 0.1 ml was counted in 10 ml Aquasol (N.E.N.) in a liquid scintillation counter.

Catalase, glucose 6-phosphate, glucose-6-phosphate dehydrogenase and $NADP^+$ were purchased from Boehringer, Mannheim, radioactive compounds from the Radiochemical Centre, Amersham.

RESULTS

The light-dependent decarboxylation of α-keto acids by isolated chloroplasts at a pH under 8.0 is very low, as already reported by others[9,10]. This endogenous rate can be stimulated several fold by the addition of Mn^{2+} or by auto-oxidizable electron acceptors as shown in Table I. The rates of decarboxylation in the dark

TABLE I

STIMULATION OF THE DECARBOXYLATION OF PYRUVATE, GLYOXYLATE OR GLYCOLATE WITH ISOLATED CHLOROPLASTS BY AUTO-OXIDIZABLE ELECTRON ACCEPTORS OR BY Mn^{2+}, AND ITS INHIBITION BY DCMU

The reaction mixture contained in 3 ml: chloroplast fragments with 0.1 mg chlorophyll, 80 μmoles Tris buffer (pH 7.6), 5 μmoles NH_4Cl, 1 μmole sodium [1-^{14}C]pyruvate (0.2 Ci/mole) or 1 μmole sodium [1-^{14}C]glyoxylate (0.21 Ci/mole) or 1 μmole sodium [1-^{14}C]glycolate (0.21 Ci/mole). The reaction was carried out for 15 min at 15 °C in the light (25000 lux).

Additions	*nmoles decarboxylated*			
	Glycolate	*Glyoxylate*	*Pyruvate*	*Pyruvate + $2 \cdot 10^{-5}$ M DCMU*
None	2.2	7.3	6.0	0.8
0.2 μmole anthraquinone-2-sulfonic acid	9.0	56	70	0.9
30 nmoles ferredoxin	10	51	40	1.2
10 μmoles $MnCl_2$	7.5	22	40	7.6
10 μmoles $MnCl_2$ + 0.2 μmole anthraquinone-2-sulfonic acid	21	115	210	8.5

TABLE II

DECARBOXYLATION OF PYRUVATE BY DIGITONIN-FRAGMENTED CHLOROPLASTS

The reaction mixture contained in 3 ml: digitonin-fragmented chloroplasts with 0.2 mg chlorophyll, 80 μmoles Tris buffer (pH 7.6), 0.2 μmole Triquat as electron acceptor, 1 μmole sodium-[1-^{14}C]pyruvate (0.25 Ci/mole), an electron donator system with 40 nmoles cytochrome$_{552}$. Electron donator I: 10 μmoles ascorbate, 0.2 μmole diaminodurole. Electron donator II: 10 μmoles glucose 6-phosphate, 20 ng glucose-6-phosphate dehydrogenase; 2 μmoles $NADP^+$, ferredoxin:$NADP^+$ reductase (fraction with 2 mg protein). 15 min light reaction at 15 °C, 25000 lux.

System	*nmoles pyruvate decarboxylated*
Complete, with electron donator I	46
with electron donator I (dark)	2.3
without electron donator	2.2
without cytochrome$_{552}$	9.5
without electron acceptor	26
Complete, with electron donator II	36
with electron donator II (dark)	3.5
without glucose 6-phosphate	1.5
without ferredoxin:$NADP^+$ reductase	1.5
without $NADP^+$	1.6
without cytochrome$_{552}$	7.3
without electron acceptor	14

are negligible (1.5–2 nmoles/mg chlorophyll per h). The rate of decarboxylation of glycolate is low, compared with the rates of pyruvate or glyoxylate. The decarboxylation is enhanced, if both an auto-oxidizable electron acceptor and high concentrations of Mn^{2+} are present at the same time. $2 \cdot 10^{-5}$ M 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) inhibits these reactions.

The possibility, that the light-dependent decarboxylation of α-keto acids could run partially independent of the function of Photosystem II, as one might conclude from the reports by Kisaki and Tolbert[9] and Zelitsch[10], was ruled out by using digitonin-fragmented chloroplasts. The decarboxylation of pyruvate by these particles is dependent on the addition of cytochrome$_{552}$ (substituting for the lost plastocyanine), an artificial electron donor (substituting for Photosystem II activity) and an auto-oxidizable electron acceptor (Table II).

The requirement for an auto-oxidizable electron acceptor can be further demonstrated, by following the kinetics of the decarboxylation of pyruvate by isolated chloroplasts in the presence of ferredoxin and $NADP^+$. As demonstrated by the kinetics, shown in Fig. 1, a maximal and linear rate of decarboxylation is obtained after a lag phase of 5 min. During this 5-min period the available $NADP^+$ becomes reduced. This lag phase in the kinetics of the pyruvate decarboxylation in the presence of ferredoxin and $NADP^+$ is more pronounced, if the chlorophyll concentration is lowered.

The electron transport seems to switch over from $NADP^+$ reduction to an oxygen reduction by the primary electron acceptor as soon as all available $NADP^+$ is reduced (*cf.* ref. 8).

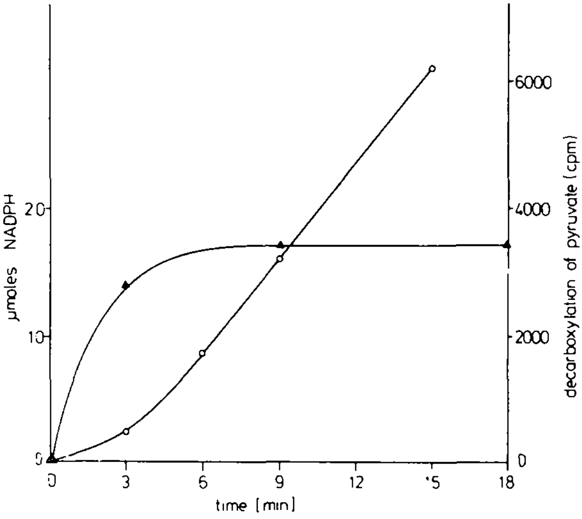
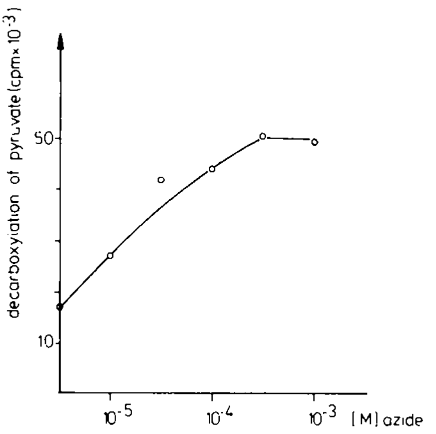


Fig. 1. Kinetics of $NADP^+$ reduction and pyruvate decarboxylation by isolated chloroplasts. The reaction mixture contained in 3 ml: chloroplast fragments with 0.2 mg chlorophyll, 10 nmoles ferredoxin, 2 μmoles $NADP^+$, 5 μmoles NH_4Cl, 1 μmole sodium [1-^{14}C]pyruvate (0.2 Ci/mole), 80 μmoles Tris buffer (pH 7.6). The reaction was run at 25000 lux and 15 °C. The light reactions were terminated at the times indicated by injecting 0.2 ml 25 mM H_2SO_4. ▲—▲, μmoles $NADP^+$; ○—○, cpm $^{14}CO_2$ trapped.



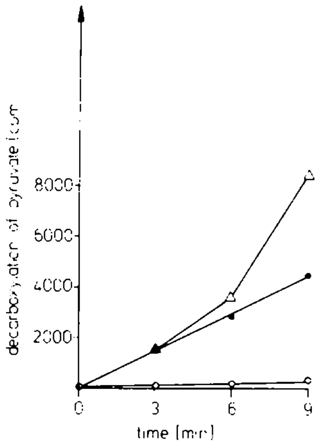


Fig. 2. Influence of azide on the decarboxylation of pyruvate by isolated chloroplasts with anthraquinone-2-sulfonic acid as electron acceptor. For experimental conditions see Table I. ○—○, cpm $^{14}CO_2$ trapped.

Fig. 3. Initial kinetics of the decarboxylation of pyruvate by isolated chloroplasts and its inhibition by superoxide dismutase or catalase. For experimental conditions see Table I. Anthraquinone-2-sulfonic acid was used as electron acceptor. cpm $^{14}CO_2$ trapped: △—△, without addition; ●—●, with superoxide dismutase; and ○—○, with catalase.

It has been shown, that the product of the auto-oxidation of reduced ferredoxin or a reduced quinone-type acceptor is H_2O_2 (refs 5, 6). As shown by Fig. 2, the decarboxylation of pyruvate by isolated chloroplasts with anthraquinone-2-sulfonic acid as electron acceptor increases with increasing amounts of azide. The observation that azide only showed a stimulating effect and no inhibition up to a concentration of 10^{-3} M, suggested, that H_2O_2 might be the oxidant in a reaction, which is not catalyzed by a peroxidase. The stimulating effect can be explained by an inhibition of the endogenous catalase activity of isolated chloroplasts. One might, therefore, expect that the addition of catalase to the above system would cause an inhibition of the decarboxylation. Along with catalase, the influence of superoxide dismutase was also investigated. As shown (Fig. 3) by the initial kinetics of the decarboxylation of pyruvate by isolated chloroplasts with anthraquinone-2-sulfonic acid as electron acceptor, a linear rate of decarboxylation is obtained in the presence of superoxide dismutase. The addition of catalase inhibits this reaction almost completely. In the absence of superoxide dismutase, a second rate of decarboxylation seems to be superimposed on the linear kinetic of decarboxylation, apparently caused by the superoxide free radical ion.

As demonstrated by the results presented in Table III, the decarboxylation of pyruvate in the presence of higher than 10^{-3} M Mn^{2+} seems to proceed by an entirely different reaction mechanism, compared with the one in the presence of the peroxide-producing system alone. In the presence of Mn^{2+}, the inhibitory effect of catalase is converted into a stimulation of decarboxylation. There is only little if any influence of superoxide dismutase in the presence of Mn^{2+}. The stimulating effect of catalase, however, is abolished by the addition of superoxide dismutase.

TABLE III

INHIBITION OF DECARBOXYLATION OF PYRUVATE WITH ISOLATED CHLOROPLASTS AND DIFFERENT ELECTRON ACCEPTORS BY SUPEROXIDE DISMUTASE OR CATALASE IN THE PRESENCE OR ABSENCE OF Mn^{2+}

Experimental conditions as in Table I.

System	*Additions*	*nmoles pyruvate decarboxylated*
0.2 μmole anthraquinone-2-sulfonic acid as electron acceptor	–	92
	Catalase	6
	Superoxide dismutase	52
	Catalase + superoxide dismutase	0.5
30 nmoles ferredoxin as electron acceptor	–	40
	Catalase	1
	Superoxide dismutase	25
	Catalase + superoxide dismutase	0
Without electron acceptor *plus* 10 μmoles $MnCl_2$	–	37
	Catalase	20
	Superoxide dismutase	35
	Catalase + superoxide dismutase	17
0.2 μmole anthraquinone-2-sulfonic acid as electron acceptor *plus* 10 μmoles $MnCl_2$	–	140
	Catalase	220
	Superoxide dismutase	150
	Catalase + superoxide dismutase	160

DISCUSSION

Pyruvate has been chosen in most of the experiments shown under Results as a representative α-keto acid for studying the mechanism of light-dependent decarboxylations with isolated chloroplasts. As demonstrated in the figures and tables, the role of the pigment system of chloroplasts in this reaction is to provide the oxidants, which are responsible for the decarboxylation. Both H_2O_2 and superoxide free radical ion seem to be involved.

The observation, that catalase and superoxide dismutase inhibit (*i.e.* approximately 95% and 40%, respectively) the decarboxylation of pyruvate with isolated chloroplasts in the presence of an auto-oxidizable electron acceptor implies a mechanism, where H_2O_2 and $O_2^{\cdot -}$ act more or less cooperatively.

As shown by the kinetics of the initial phase of the decarboxylation of pyruvate with isolated chloroplast in the presence of anthraquinone-2-sulfonic acid as electron acceptor (Fig. 1), the reaction which is dependent on the superoxide free radical ion, seems to be superimposed on the one, involving H_2O_2. The latter reaction is apparently not catalyzed by a peroxidase. Though it has been shown, that a peroxidative activity is present in isolated chloroplasts[20], the commonly described peroxidase (EC 1.11.1.7) seems to be associated with cell fractions other than chloroplasts[3].

In the presence of higher than 10^{-3} M Mn^{2+}, catalase and superoxide dismutase show less or no inhibition of the decarboxylation. Catalase stimulates the decarboxylation of pyruvate by isolated chloroplasts in the presence of higher than 10^{-3} M Mn^{2+} and an auto-oxidizable electron acceptor. Apparently neither H_2O_2 nor superoxide free radical ion participate in the decarboxylation in the presence of both an auto-oxidizable electron acceptor and Mn^{2+}. In the case of glyoxylate decarboxylation by isolated chloroplasts in the presence of Mn^{2+}, Zelitsch[10] could show, that carboxyl-labeled glyoxylate yields radioactive CO_2, while a label in C-2 of glyoxylate can be identified as formic acid after the light reaction. Conclusions, concerning the mechanism of the decarboxylation of α-keto acids by our systems can be drawn, as soon as the nature of the oxidation products under the above conditions have been identified and more kinetic details are available.

A possible physiological role of the reactions described by the above investigations might be seen in photorespiration. As demonstrated, the decarboxylation of an α-keto acid by isolated chloroplasts in the presence of $NADP^+$ starts as soon as all available $NADP^+$ is reduced. This might well be the case in the intact chloroplast under conditions, where photorespiration is observed.

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