The Enhancement of CO_2 Fixation in Isolated Chloroplasts by Low Sulfite Concentrations and by Ascorbate

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The enhancement of CO_2 fixation in isolated, intact spinach chloroplasts by ascorbate or by low sulfite concentrations (<1 mM) is strongly reduced or even abolished by the addition of superoxide dismutase (SOD). By the use of $^{35}SO_3^{2-}$ it is demonstrated that the rate of sulfate formation is much lower than the sulfite induced increase in CO_2 fixation. This indicates that the superoxide radical is the chain initiating event; and, in parallel to ascorbate (Elstner and Kramer, 1973), the $HSO_3^{\cdot}$ radical, acting as a Hill reagent for photosystem I, is reduced to sulfite in turn. The inhibitory action of sulfite at concentrations >1 mM is not relieved by SOD, since this effect is mainly based on a competitive inhibition of ribulosediphosphate carboxylase.

SOD itself stimulates the CO_2 fixation, if the reaction is started after 3 min of pre-illumination. This effect is discussed with respect to factors linked with the isolation procedure.

Introduction

It has been known for a long time that low SO_2 doses (<0.2 ppm) cause an increase in plant productivity. Formerly, this was attributed to the improvement in sulfur supply [1]. However, incubation of isolated spinach chloroplasts with low sulfite concentrations (<1 mM) demonstrated that $^{14}CO_2$ fixation was enhanced [2]. As soon as a threshold concentration of about 1 mM sulfite was reached, CO_2 fixation was inhibited competitively with respect to bicarbonate [3].

In contrast to total CO_2 fixation, the Hill-reaction maintained a plateau even up to the concentration of 5 mM tested [2]. A similar increase in CO_2 fixation of isolated chloroplasts was reported to be caused by ascorbate [4, 5].

There is good evidence that the sulfite oxidation is induced by the electron transport system of the chloroplast [2, 6]. The initiation of the oxidative chain reaction is coupled to the monovalent reduction of oxygen to the superoxide anion. Thus the enhanced ferricyanide reduction as well as the uptake of O_2 can be abolished by the addition of SOD [6]. The same is true for the ascorbate mediated enhancement of photophosphorylation [7].

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Abbreviations: SOD, superoxide dismutase.

The following experiments were concerned with three questions. Firstly they had to establish whether the superoxide radicals, generated during photosynthetic oxygen reduction [8] were the cause only for the ascorbate mediated photophosphorylation [7] or the sulfite stimulated electron transport in a reconstituted system [6, 2] or whether they finally result in an enhanced CO_2 fixation of intact chloroplasts.

Secondly they had to show whether the inhibition of CO_2 fixation by sulfite concentrations >1 mM is independent of superoxide radicals. Finally, the sulfite oxidation in intact, illuminated chloroplasts had to be measured directly using $^{35}SO_3^{2-}$, in order to compare the rates of sulfite oxidation and the enhancement of CO_2 fixation.

Materials and Methods

Spinach was grown at 17 °C in a Heraeus incubator (20 000 lx; 10 hours light, 14 hours dark). The chloroplasts were isolated according to Jensen and Bassham [9]. Structural integrity of the chloroplasts was ascertained by the use of phase contrast and electron microscopy. About 70% of the chloroplasts were class I according to Spencer and Unt [10]. The absolute fixation rates varied depending on the stage of the plant material, but the relative effects of the treatments were not affected. The results presented in Figs 1–3 were obtained using spinach plants grown for 4–6 weeks following transplantation. Under these conditions the results within each series (with at least 4 experiments)

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were uniform. The fixation rates (~ 15 μmol/mg chlorophyll·h) were much lower than from spinach grown in the field, but this was compensated by their uniformity.

The incubation medium (solution C according to l. c. [9]; final vol. 1.5 ml) was 5 mM with respect to sodium bicarbonate and contained 10 μCi $NaH^{14}CO_3$, and chloroplasts (chloroplyll $30-40$ μg/ml). A total volume of 0.1 ml of sulfite or ascorbate was added; 3.3 μg SOD were used. This is 8 times the concentration needed for 90% inhibition of adrenochrome formation, assayed according to Misra and Fridovich [11].

The chloroplasts were illuminated with Agraphot BM (500 W; about 15 000 lx) at 16 °C. The reaction was started by the addition of $NaH^{14}CO_3$ and stopped by 2 ml acetic acid (80%) and 4 ml methanol. Aliquots were dried, redissolved in 1 ml H_2O and counted in a scintillation counter.

Chlorophyll determinations were made according to Arnon [12]. Sulfite oxidation was followed by incubation of isolated chloroplasts ($25-45$ μg chlorophyll/ml) in solution C (l. c. [9]) at an illumination of 26 000 lx. After 3 min of preillumination 0.1 ml of $NaHSO_3$ was added to the reaction medium to obtain the sulfite concentration indicated (spec. activity 1 μCi/0.5 μmol). The final volume was 1.0 ml. The reaction was stopped by adding 1 ml of 2.5 N HCl. After centrifugation, 0.5 ml of 0.5 M $BaCl_2$ solution was added and the mixture was stored for about 17 hours under N_2 at 4 °C. Under these conditions only the sulfate but not the sulfite ion is precipitating. After centrifugation, the precipitate was washed 3 times with 0.2 ml of 70% methanol, plated, and counted in a Geiger-Müller proportional counter. The data were corrected for background ($Ba^{35}SO_4$ at t_0).

HEPES, MES,and SOD were from Sigma, sodium [^{14}C]bicarbonate and [^{35}S]sulfate from Amersham Buchler. All other chemicals were analytical grade from Merck.

Results

a. The effect of SOD on the enhancement of CO_2 fixation by ascorbate or by low sulfite concentrations

In chloroplasts, pre-illuminated for 3 min, 1 mM ascorbate or 0.25 mM sulfite enhanced photosynthetic CO_2 fixation (Fig. 1). The sulfite dependent increase in CO_2 fixation was completely abolished by SOD. Ascorbate increased the CO_2 fixation even more, and this effect was only partially reversed. Under these conditions of pre-illumination, SOD alone also stimulated CO_2 fixation.

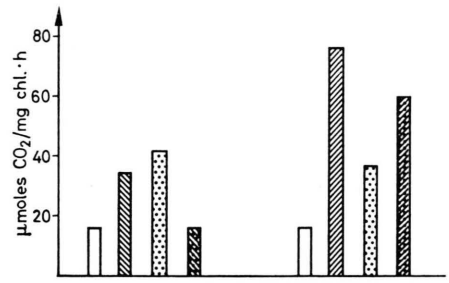

Fig. 1. CO_2 fixation by isolated, pre-illuminated spinach chloroplasts.

, control;

, with 0.25 mM sulfite;

, with 3.3 μg SOD;

, with 0.25 mM sulfite + 3.3 μg SOD;

, with 1 mM ascorbate;

, with 1 mM ascorbate + 3.3 μg SOD.

The reaction was started after 3 min of pre-illumination by addition of $NaH^{14}CO_3$ and stopped after 6min illumination.

Without pre-illumination, the stimulation by ascorbate or by sulfite, as well as by SOD alone, was much less marked (Fig. 2). The reduction of sulfite or ascorbate mediated enhancement by SOD was only minimal or even missing.

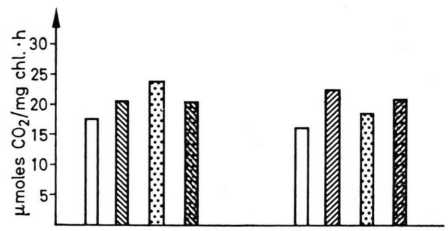

Fig. 2. CO_2 fixation by isolated spinach chloroplasts without pre-illumination. For legends see Fig. 1. The reaction was started from the dark by addition of $NaH^{14}CO_3$ and stopped after 6 min of illumination.

b. The effect of SOD on the inhibition of CO_2 fixation at high sulfite concentrations

In agreement with the above results the fixation rates of chloroplasts were increased by SOD alone and no enhancement was observed at low sulfite concentrations at its presence (Fig. 3). At inhibito-

ry sulfite concentrations, SOD did not prevent the decrease of CO_2 fixation. There was no effect of pre-illumination on the sulfite dependent inhibition.

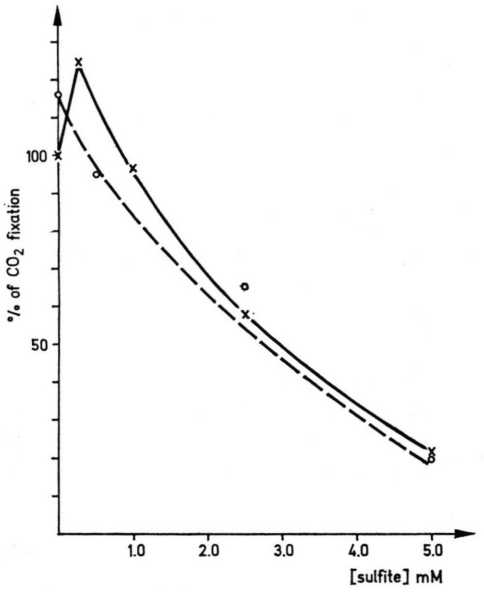

Fig. 3. Action of different concentrations of sulfite on CO_2 fixation of illuminated chloroplasts. ×———×, sulfite; ○– – –○, sulfite + SOD (3.3 μg).

c. *The oxidation rate of sulfite in illuminated chloroplasts*

In intact chloroplasts the rate of sulfate formation in the dark was very low (Fig. 4). It was enhanced by illumination. The variability of the oxidation rate may be due to the variability in oxidative capacity, and, to some extend, to the variability in the precipitation reaction. Thus statistical treatment of the data was necessary. Nevertheless a clear cut dependence of sulfate formation on sulfite concentration was demonstrated (Fig. 4, insert). A precise determination of sulfate formation at stimulatory sulfite concentrations (0.25 – 0.5 mM) was, however, impossible as in this range approximately 4 μmol of sulfate/mg chlorophyll·h were formed (see inset Fig. 4).

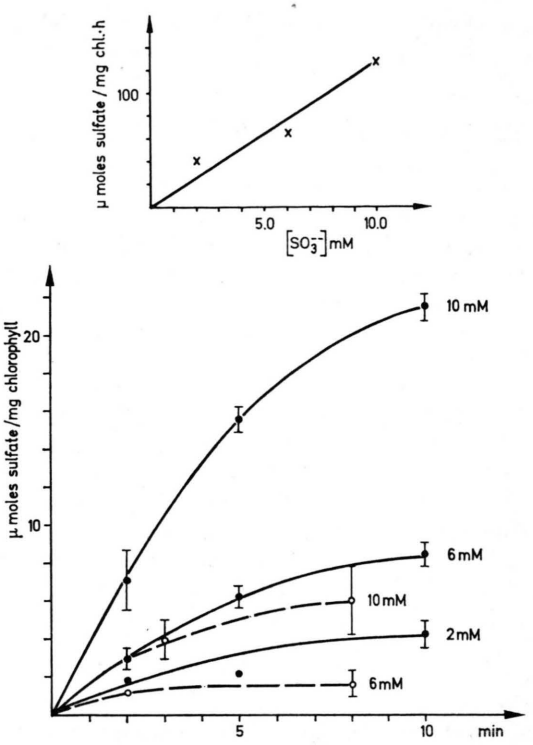

Fig. 4. Sulfate formation in isolated spinach chloroplasts at different concentrations of $Na_2{}^{35}SO_3$. ●———●, illuminated chloroplasts; ○– – –○, chloroplasts kept in the dark. ⊢ S.E.

Discussion

The experiments demonstrate that the superoxide radical initiates a sulfite or ascorbate mediated enhancement of CO_2 fixation in intact chloroplasts, as shown earlier for the Hill-reaction [6] and the photophosphorylation [7]. Even though during the isolation procedure, the ascorbate in solution A (see l. c. [9]) may only act as a protective agent, the results agree with the proposal of Elstner and Kramer [7] that during illumination ascorbate together with the superoxide radical maintains photophosphorylation under NADPH saturated conditions; and seemingly, low sulfite concentrations are acting in the same way. The fact that maximum $O_2^{\cdot-}$ generation, in turn, is dependent on NADPH saturation may explain the need for pre-illumination to obtain a marked stimulation by ascorbate or by sulfite.

In chloroplasts, which were isolated under non-isosmotic conditions, the rate of sulfite oxidation drastically exceeds the enhancement of the Hill-reaction [7], whereas in intact chloroplasts, used in the experiments described above, the sulfate formation does not reach stoichiometric amounts compared with the enhancement of CO_2 fixation. This is a further indication that sulfite is acting in analogy to ascorbate (see Fig. 1 in l. c. [7]): it is oxidized by the superoxide radical to form the $HSO_3^{\cdot}$ radical, which in turn, acts as an electron acceptor for photosystem I, being reuced to sulfite

again. The $^{35}SO_4^{2-}$ accumulation in relatively high amounts, following fumigation with $^{35}SO_2$ [13] may be due to a sulfite oxidase in the plasma.

The failure of SOD to relieve the inhibition of CO_2 fixation at sulfite concentrations >1 mM strongly indicates that the inhibition is not linked to a radical chain reaction; rather it is consistent with the view that it is caused by competition between sulfite and CO_2[2], that is, by competitive inhibition of ribulosediphosphate carboxylase with respect to HCO_3^-[14].

Incidentally, the experiments revealed a marked increase in CO_2 fixation by SOD itself, especially if the reaction was started after 3 min of preillumination. At the present state of experiments two explanations my be offered. Firstly, some SOD of the stroma may have been lost during isolation, leaving mainly the enzyme which is bound to the lamellae [15, 16]; and the bulk of superoxide radicals, arising after saturation of the NADPH pool [8] can only be removed by addition of excess SOD. Secondly, broken chloroplasts, which comprise about 30% of the chloroplast population produce $O_2^{\cdot -}$ radicals; these, in turn, impair the CO_2 fixation of the total preparation. This effect may be eliminated by the addition of SOD.

The variability of ascorbate mediated enhancement of CO_2 fixation [5], which is also valid for sulfite, may be intimately connected with the variable leakage of stromal SOD. Constitutive differences in different plant species or differences during development may be of importance for differing SO_2 tolerance and will be subject of further investigation.

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