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THE MECHANISM OF PHOTOSYNTHETIC SULFATE REDUCTION BY ISOLATED CHLOROPLASTS

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SUMMARY

The reduction of sulfate by isolated spinach chloroplasts was studied. A reconstituted system of broken chloroplasts and of chloroplast extract reduced sulfate to sulfite in the light when ADP, $NADP^+$, ferredoxin and glutathione were added. The chloroplast extract reduced sulfate to sulfite in the dark if supplemented with ATP and with reduced glutathione. Neither ferredoxin nor NADPH were needed for this reduction in the dark.

A sulfite reductase was purified from spinach leaves. Broken chloroplasts and sulfite reductase reduced sulfite to sulfide in the light when ferredoxin was added. $NADP^+$ was not required for this reduction.

The results suggest that in chloroplasts a sulfate activated by ATP (phosphoadenosine phosphosulfate) is reduced to sulfite by a sulfhydryl compound and that sulfite is reduced to sulfide by a ferredoxin-dependent sulfite reductase.

INTRODUCTION

The mechanism of sulfate reduction has been studied extensively in microorganisms[1,2]. However, there is little information about the details of sulfate reduction by cell-free extracts from higher plants. ASADA[3], TAMURA *et al.*[4] and MAYER[5] have described a methyl viologen-dependent sulfite reductase in spinach. ASAHI[6] has obtained a photosynthetic sulfate reduction by isolated chloroplasts. His system, however, worked only with rather low rates and only after adding sulfate activating enzymes from yeast. SCHIFF and co-workers[7,8] have shown the reduction of sulfate to thiosulfate by extracts from Chlorella. We have recently reported[9] that isolated whole chloroplasts from spinach in the light reduce sulfate to sulfide incorporated in cysteine with a rate of 3 μmoles/h per mg chlorophyll. More details on the mechanism of the photosynthetic sulfate reduction, particularly on the cofactor requirements of the photosynthetic reduction of sulfate to sulfite and of sulfite to sulfide by broken chloroplasts, is now reported.

Abbreviations: APS, adenosine phosphosulfate; PAP, phosphoadenosine phosphate; PAPS, phosphoadenosine phosphosulfate.

METHODS

Whole chloroplasts from spinach were prepared according to JENSEN AND BASSHAM[10], while broken chloroplasts were prepared either by breaking whole chloroplasts in a dilute buffer or by the method of ALLEN *et al.*[11]. A chloroplast extract with about 3 mg protein per ml was made by breaking chloroplasts from 30 g of spinach prepared according to JENSEN AND BASSHAM[10] in 7 ml of 0.015 M Tris–HCl buffer (pH 7.8). The reduction of radioactive sulfate was followed by adding trichloroacetic acid after the incubation period and by distilling the volatile radioactivity formed in a $CdSO_4$ solution which precipitated sulfide but not sulfite according to the method of WILSON *et al.*[12]. Sulfite was trapped by a second KOH trap.

Sulfite reductase was prepared from spinach leaves and from a chloroplast extract[9,13]. 1.5 kg of spinach leaves were homogenized in 1.7 l of 0.01 M Tris–HCl buffer (pH 8.0) and were filtered through cheese-cloth. The extract was fractionated by adding acetone of —10°. The fraction obtained from 45–70 % acetone was collected by centrifugation, was resuspended in 0.02 M Tris–HCl buffer (pH 7.8) and was dialyzed. Protein which precipitated during dialysis was discarded. The supernatant was loaded onto a DEAE-cellulose DE-52 column pretreated with 0.05 M Tris–HCl buffer (pH 7.8), and the column was eluted with 0.05 M Tris–HCl buffer (pH 7.8). The chloride concentration is indicated in Table IV. The fractions obtained were concentrated by adding $(NH_4)_2SO_4$ up to a concentration of 70 %, by resuspending and by dialyzing the precipitate in 0.01 M Tris–HCl buffer (pH 7.8).

RESULTS

As previously shown, whole chloroplasts are able to reduce sulfate in the light[9]. Sulfite, sulfide, cysteine and other S-containing compounds are formed. By breaking the whole chloroplasts, it is possible to separate the reduction of sulfate to sulfide into two processes: the reduction of sulfate to sulfite and of sulfite to sulfide.

A reconstituted system containing the broken chloroplasts and the chloroplast extract reduces sulfate to sulfite in the light if it contains the phosphorylating system ADP + P_i, ferredoxin, $NADP^+$ and glutathione. While in light for 30 min, 6 nmoles

TABLE I

PHOTOREDUCTION OF SULFATE BY BROKEN CHLOROPLASTS AND BY THE CHLOROPLAST EXTRACT

The complete system contained in 3 ml (in μmoles): 100 Tris–HCl buffer (pH 7.8); 40 $MgCl_2$; 5 ADP; 10 P_i; 0.03 spinach ferredoxin; 2 $NADP^+$; 3 oxidized glutathione; 1 [^{35}S]sulfate (with 172 000 counts/min); 0.8 ml chloroplast extract and broken chloroplasts (P_1S_3) with 0.4 mg chlorophyll. The reaction was run at 20° for 30 min in the light (35 000 lux) under N_2.

Conditions	*Sulfite formed (nmoles)*	*Sulfide formed (nmoles)*
Complete system	6.10	0.1
without ADP and P_i	0.16	—
without ferredoxin	1.00	—
without $NADP^+$	0.51	—
without glutathione	0.10	—
without chloroplast extract	0.00	—

TABLE II

REDUCTION OF SULFATE IN THE DARK BY THE CHLOROPLAST EXTRACT

The system contained 0.8 ml chloroplast extract and the following (in μmoles): 100 Tris–HCl buffer (pH 7.8); 40 $MgCl_2$; 3 ATP; 10 creatine phosphate; creatine phosphokinase (Boehringer) 0.05 mg and 10 μC [^{35}S]sulfate. NADPH was formed by adding 1 μmole $NADP^+$, 10 μmoles glucose 6-phosphate and 0.01 mg glucose-6-phosphate dehydrogenase (Boehringer). 1 h incubation at 30°.

Additions	*Sulfite formed (counts/min)*
—	29
NADPH regenerating system	45
NADPH regenerating system + 3 μmoles oxidized glutathione	3694
3 μmoles oxidized glutathione	509
6 μmoles reduced glutathione	8944

TABLE III

REDUCTION OF SULFATE IN THE DARK IN THE CHLOROPLAST EXTRACT BY A NUMBER OF SULFHYDRYL COMPOUNDS

Conditions as in Table II.

Addition of 10 μmoles	*Sulfite formed (counts/min)*
—	15
Dithioerythritol	40 460
2,3-Dimercaptopropanol	37 800
Cysteine	8 110
Homocysteine	1 930
Cysteamine	11 250
Reduced glutathione	54 400
H_2S	86
Sulfite	1 654

TABLE IV

PURIFICATION PROCEDURE FOR SULFITE REDUCTASE FROM SPINACH LEAVES

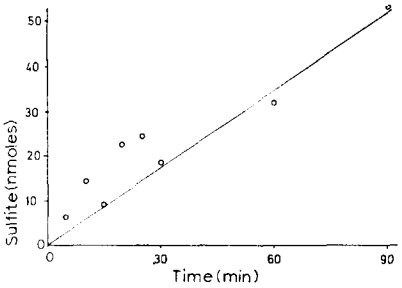
Procedure	*Total protein (mg)*	*Sulfite reduced (counts/min per mg protein)*	*Enzyme units* $\times 10^{-6}$	*Purification factor*	*Yield (%)*
Crude homogenate	10 520	755	7.95	1	100
Acetone precipitate (between 45–70% acetone)	1 460	889	1.3	1.18	16.6
Chromatography on DEAE-cellulose DE-52 with a NaCl gradient					
elution with 0.05 M chloride	165	12 450	2.05	16.5	25.8
elution with 0.1 M chloride	168	2 320	0.39	3.1	4.9
elution with 0.2 M chloride	315	1 110	0.35	1.4	1.4

sulfite and virtually no sulfide is formed in the complete system (Table I). The experiment suggests that ATP is required presumably to activate sulfate to phosphoadenosine phosphosulfate (PAPS) and this is supported by the finding that sulfurylase[6,13] as well as adenosine-phosphosulfate (APS) kinase[13] are present in the chloroplast extract. The experimental results described in Table I further suggest that either NADPH or glutathione is the reducing agent and that ferredoxin is required for the photosynthetic reduction of $NADP^+$. Therefore the effects of NADPH or of reduced glutathione should approximate the light effect. Table II shows that sulfate is reduced by the chloroplast extract alone in the dark if both an ATP regenerating system (ADP, creatine phosphate and creatine-phosphate kinase) and reduced glutathione are added. NADPH cannot replace glutathione which indicates clearly that glutathione is the reducing agent in the sulfate reduction in this reconstituted system. Ferredoxin is not required for the reduction of sulfate in this system in the dark.

Fig. 1 shows the kinetics of the reduction of sulfate by the chloroplast extract in the dark. The rate is constant for at least 90 min and is 30 nmoles at 60 min; however, the sulfate reduction rate obtained with whole chloroplasts is about 3 μmoles/h.

Other sulfhydryl compounds as well as glutathione can support the sulfate reduction. Table III indicates that in addition to glutathione, dithioerythritol and dimercaptopropanol are the most active sulfhydryl compounds. Sulfite formed from the reduction of sulfate is able to reduce sulfate in the chloroplast system to a certain extent. From these experiments one cannot infer which sulfhydryl compound is the natural reducing agent; however, it cannot be a sulfhydryl-containing enzyme since NADPH should be active in the absence of glutathione.

The photoreduction of sulfite to sulfide by chloroplasts has already been documented[9,13], and the properties of a sulfite reductase and the ferredoxin dependency of sulfite reduction have recently been reported[9,13]. Table IV summarizes the procedure for isolating the enzyme from spinach leaves. Sulfite reductase is free from nitrite reductase because these two enzymes separate on the DEAE-cellulose DE-52 column at different chloride concentrations, as shown in Fig. 2. The sulfite reduction



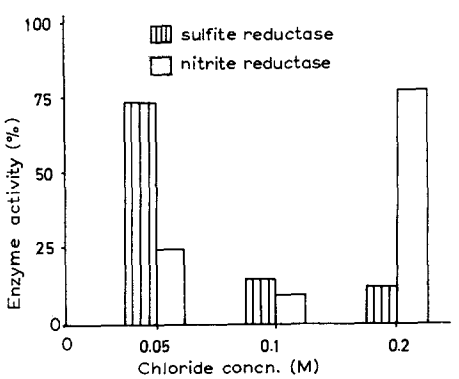


Fig. 1. Kinetics of sulfate reduction by chloroplast extract. Conditions as in Table II with 6 μmoles reduced glutathione.

Fig. 2. Separation of nitrite and sulfite reductase during chromatography on DEAE-cellulose DE-52 and a NaCl gradient.

by sulfite reductase can be coupled to either broken chloroplasts in the light or to NADPH in the dark, and for both systems ferredoxin is required[9,13]. Table V shows that ferredoxins from different organisms replace each other to almost the same extent. In our system, methyl viologen and other carriers are completely inactive as a sulfite reducing agent; our sulfite reductase can use ferredoxin but not methyl viologen, whereas the spinach sulfite reductase described by ASADA *et al.*[4,14] is active with methyl viologen but not with ferredoxin.

In the experiments described above sulfate was reduced to sulfite or sulfite was reduced to sulfide. Both systems can be coupled, and sulfate can be reduced to sulfide by the chloroplast extract and by additional sulfite reductase at the expense of NADPH (Table VI). More sulfide than sulfite is formed in the complete system; however, without dithioerythritol neither sulfite nor sulfide forms showing that NADPH alone cannot reduce sulfate. Without the sulfite reductase, sulfite is the main product, and in the absence of ferredoxin, sulfite accumulates indicating that ferredoxin is required for the reduction of sulfite but not for the reduction of sulfate in the dark (Table VI). In this latter experiment, the chloroplast extract had been freed from ferredoxin by passing the extract through DEAE-cellulose DE-52.

TABLE V

FERREDOXIN REQUIREMENT OF THE PHOTOREDUCTION OF SULFITE BY BROKEN CHLOROPLASTS AND BY SULFITE REDUCTASE

The system contained in 2 ml (in μmoles): 50 Tris–HCl buffer (pH 7.8); 5 ADP; 10 phosphate; 5 $Na_2{}^{35}SO_3$ (215000 counts/min); 4 mg sulfite reductase from spinach and broken chloroplasts (P_1S_3) with 0.15 mg chlorophyll. 30 min light at 20°.

Additions in μmoles	*Sulfite reduced (nmoles)*
0.03 spinach ferredoxin	56
0.03 Euglena ferredoxin	21
0.03 Clostridium ferredoxin	32
0.2 methyl phenazonium methosulfate	0
0.2 methyl viologen	0
0.2 FAD	0

TABLE VI

REDUCTION OF SULFATE TO SULFIDE IN A CELL-FREE SYSTEM

Conditions as in Table II; 4 mg sulfite reductase, 0.03 μmole ferredoxin and 10 μmoles dithioerythritol were added. Complete system contained: chloroplast extract + ferredoxin + NADPH + dithioerythritol + ATP + sulfite reductase.

Conditions	*Sulfite formed (counts/min)*	*Sulfide formed (counts/min)*
Complete system	1639	8346
without dithioerythritol	15	46
without sulfite reductase	8972	1796
without chloroplast extract	23	18
Complete system (chloroplast extract passed before through DEAE-cellulose DE-52)	37	1075
without ferredoxin	875	50

DISCUSSION

The experiments described here show the following results: (1) The chloroplast extract contains the enzymes needed for sulfate reduction. (2) The reduction of sulfate to sulfite as well as the reduction of sulfite to sulfide can be coupled to the electron transport system of broken chloroplasts in the light. (3) In a reconstituted chloroplast system sulfate reduction requires: (a) in the light: ADP, a sulfhydryl compound like glutathione, ferredoxin and $NADP^+$; (b) in the dark: ATP and glutathione (or dithioerythritol) but no ferredoxin or $NADP^+$. (4) Sulfite is a reducing agent in the sulfate reduction though to a lesser extent than is glutathione. (5) Sulfite reduction by an enriched sulfite reductase can be coupled to the electron-transport system of broken chloroplasts in the light or to NADPH in the dark. Both systems require ferredoxin.

These results could be explained by the following reaction sequence:

$$\text{Sulfate} \xrightarrow{\text{ATP}} \text{APS} \xrightarrow{\text{ATP}} \text{PAPS} \qquad (1)$$

$$\text{PAPS} + \text{HS–X} \xrightarrow{\text{Sulfotransferase}} \text{X–S–SO}_3\text{H} + \text{PAP} \qquad (2)$$

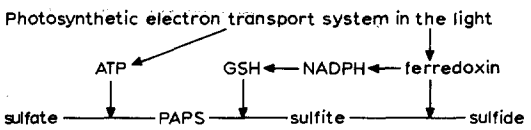
$$\text{X–S–SO}_3\text{H} + \text{HS–X} \xrightarrow{\text{Thiosulfone reductase}} \text{HSO}_3\text{H} + \text{X–S–S–X} \qquad (3)$$

$$\text{X–S–S–X} + \text{NADPH} \xrightarrow{\text{Glutathione reductase}} 2\ \text{X–SH} + \text{NADP}^+ \qquad (4)$$

In reaction sequence 1, sulfate is activated by sulfurylase and APS kinase to PAPS with ATP being consumed. PAPS catalyzed by a sulfotransferase specific for a sulfhydryl group transfers its sulfate group onto a sulfhydryl compound which can be substituted by glutathione in the system *in vitro* (Reaction 2). The thiosulfonate is reduced with another mole of glutathione, *i.e.*, thiolysis, by a thiosulfone reductase to sulfite and oxidized glutathione (Reaction 3). The reversibility of this reaction leads to an isotope exchange between inactive sulfite and active sulfate which explains the reduction of sulfate by sulfite in Table III. In Reaction 4, the oxidized glutathione or the natural reductant is reduced again by NADPH. The photoreduction of $NADP^+$ by the electron transport system requires ferredoxin.

This photosynthetic mechanism of reducing sulfate to sulfide shows great similarities to those proposed for the sulfate reduction by microorganisms[1,2,12,15,16]. Particularly HILZ and co-workers[17–19] have suggested that an intermediate thiosulfonate is formed and thiolysed by a disulfide component like lipoic acid during the reduction of activated sulfate by yeast.

The sulfite formed in the reaction sequence 1–4 then is reduced by sulfite reductase with reduced ferredoxin as the reducing agent. The role of a protein-bound sulfite formed in intact chloroplasts[9,13] during the photoreduction of sulfate remains unclear. It has been described also in microorganisms[20].



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