Ferredoxin-dependent Sulfite Reductase from Spinach Leaves

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Sulfite reduction, observed in chlorophyllous tissues, is generally considered to be the final step of assimilatory sulfate reduction in which sulfite is reduced to the level of sulfide according to the following equation.¹⁻⁹⁾ $\text{SO}_3^{2-} + 6\text{e}^- + 6\text{H}^+ \rightarrow \text{S}^{2-} + 3\text{H}_2\text{O}$ This step of reaction is catalyzed by the enzyme, sulfite reductase (SiR).^{2,4,9)}

Most information on the plant-type SiR has been derived from experiments using artificial dye, reduced methyl viologen ($\text{MV}^\cdot$) as electron donor.¹⁰⁾ Recently, however, photoreduced ferredoxin ($\text{Fd}^\cdot$) has been reported to be a physiological electron donor in this reductase system.^{11,12)} Although $\text{MV}^\cdot$ -SiR [hydrogen-sulfide: (acceptor) oxidoreductase, EC 1.8.99.1] of *Allium odorum*,²⁾ *Spinacea oleracea*⁴⁾ and *Porphyra yezoensis*⁹⁾ have been highly purified and extensively characterized, none of these purified enzymes have been demonstrated to be able to couple with the reduced ferredoxin.

Apart from these $\text{MV}^\cdot$ -SiRs, we wish to report the purification of ferredoxin-dependent SiR ($\text{Fd}^\cdot$ -SiR) [hydrogen-sulfide: ferredoxin oxidoreductase, EC 1.8.7.1.] from green tissue.

Spinach chloroplast-grana were prepared as described by Shin.¹³⁾ Ferredoxin was purified from spinach leaves as described by Tagawa and Arnon¹⁴⁾ ($A_{422}/A_{277} = 0.45$). Ferredoxin-Sepharose was prepared according to Shin and Oshino.¹⁵⁾ The assay method for $\text{Fd}^\cdot$ -SiR was essentially the same as described in the previous paper¹¹⁾ with slight modifications. The reaction mixture included, in a final volume of 2.4 ml, grana heated at 55°C for 5 min containing 200 μg chlorophyll, SiR preparation, 400 μg of ferredoxin, 100 μmol of Tris- H_2SO_4 buffer, pH 8.0, 25.5 μmol of sodium ascorbate, 0.2 μmol of 2,6-dichlorophenol indophenol and 7.2 μmol of sodium sulfite. The reaction was carried out in Warburg vessels at 37°C under strict anaerobiosis. The mixture was illuminated by a 300-W tungsten lamp providing approximately 20,000 lux. The reaction was initiated by adding sodium sulfite from the first side arm, and terminated by adding 0.1 ml of 12 N H_2SO_4 from the second side arm. Then, the

mixture was shaken for 30 min to allow the evolved H_2S to be completely absorbed into 0.2 ml of 15% NaOH in the center well, which was then analyzed for sulfide. Sulfide was determined according to Siegel.¹⁶⁾ One unit of $\text{Fd}^\cdot$ -SiR is defined as the amount which causes the formation of 1 μmol of sulfide per min under the assay conditions described. $\text{MV}^\cdot$ -SiR activity was assayed by the method described by Asada *et al.*¹⁰⁾ and one unit of the enzyme is defined as the amount catalyzing the oxidation of 1 μmol of $\text{MV}^\cdot$ per min under the assay conditions described. To get crude extract, spinach leaves were homogenized in the same volume of TGE buffer (50 mM Tris- H_2SO_4 (pH 8.0), 10% (v/v) glycerol, 1 mM EDTA) in a Waring blender. From the crude extract described, $\text{Fd}^\cdot$ -SiR was purified by acetone fractionation, DEAE-cellulose chromatography, Sephadex G-200 gel filtration and ferredoxin-Sepharose affinity chromatography.

The result of chromatography on DEAE-cellulose column are shown in Fig. 1. Two distinct peaks of SiR (Fractions I and II in the figure) were eluted at chloride concentration of 200 mM in a highly reproducible manner. There is a profound difference in electron donor specificity between these two SiRs. Namely Fraction I reacted both with $\text{MV}^\cdot$ and $\text{Fd}^\cdot$, while Fraction II reacted principally or perhaps only with $\text{MV}^\cdot$. To isolate $\text{Fd}^\cdot$ -SiR, only Fraction I was applied to the column of Sephadex G-200 and finally to the affinity column of ferredoxin-Sepharose.

FIG. 1. Separation of Two SiRs by Chromatography on DEAE-cellulose. The preparation (2.94 g/68 ml) obtained from acetone fractionation was applied to a column of DEAE-cellulose (4.2 $\times$ 56 cm) previously equilibrated with TGE buffer (50 mM Tris- H_2SO_4 (pH 8.0), 10% (v/v) glycerol, 1 mM EDTA). The stepwise elution was carried out. Fractions of 16.8 ml were collected.

A, TGE buffer; B, 200 mM NaCl (in TGE buffer). The tubes in brackets were pooled for the gel filtration of $\text{Fd}^\cdot$ -SiR. $\circ$ — $\circ$, $\text{Fd}^\cdot$ -SiR activity; $\bullet$ — $\bullet$, $\text{MV}^\cdot$ -SiR activity; $\square$ — $\square$, protein.

FIG. 2. Elution Profile of Fd·-SiR from Ferredoxin-Sepharose Column.

Enzyme solution (79.2 mg/32 ml) obtained from Sephaedx G-200 gel filtration was applied to a column of ferredoxin-Sepharose (2×1.9 cm) previously equilibrated with TGE buffer. The stepwise elution was carried out. Fractions of 2.8 ml were collected.

A, TGE buffer; B, 50 mM NaCl (in TGE buffer). The tubes in brackets were pooled as purified Fd·-SiR. ○—○, Fd·-SiR activity; ●—●, MV·-SiR activity; □—□, protein.

FIG. 3. Absorption Spectrum of Purified Spinach Fd·-SiR.

Absorption spectra were measured in a Hitachi 556 recording spectrophotometer. The concentration of enzyme (ferredoxin-Sepharose fraction) was 1.54 mg of protein per ml. The protein is in TGE buffer. The insert shows the absorption beyond 500 nm in greater detail.

TABLE I. SUMMARY OF THE PURIFICATION OF Fd·-SiR

Fraction	Total protein (mg)	Fd·-SiR		MV·-SiR		A/B
		Total activity (A) (units)	Specific activity (units/mg-protein)	Total activity (B) (units)	Specific activity (units/mg-protein)	
0~50% acetone ^a	6860	199	0.029	74.7	0.0109	2.66
DEAE-cellulose	410	43.8	0.107	8.88	0.0217	4.93
Sephadex G-200	98.6	28.8	0.292	5.77	0.0585	4.99
Ferredoxin-Sepharose	3.32	16.9	5.09	2.82	0.849	5.99

^a Starting from 8 kg of fresh spinach leaves.

As shown in Fig. 2, Fd $\cdot$ -SiR was eluted as a single peak. Although this purified Fd $\cdot$ -SiR was not yet homogeneous as judged by polyacrylamidegel electrophoresis, the final specific activity of 5.09 μ mol of sulfide produced/min per mg of protein was measured with sulfite as substrate. The absorption spectrum of this enzyme showed three major peaks at 278, 378 and 584 nm, and minute peaks were also observed at 458 and 488 nm (Fig. 3). This spectrum is rather different from that shown by spinach MV $\cdot$ -SiR reported previously.¹⁰⁾ The nature of the visible spectrum suggests the presence of siroheme, which has been detected in enterobacterial NADPH-SiR¹⁷⁾ and also in spinach nitrite reductase.¹⁸⁾ Photoreduced ferredoxin was the most effective electron donor for this purified Fd $\cdot$ -SiR. Methyl viologen, chemically reduced by zinc granule, could replace reduced ferredoxin, but the efficiency was much lower (Table). Now, it remains to be established if Fd $\cdot$ -SiR and MV $\cdot$ -SiR have basically different protein structures, or if the latter enzyme is an artifact derived from native Fd $\cdot$ -SiR in an early step of purification.

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