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Mutational analysis of the substrate specificity of plant sulfite reductase

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Introduction

Plants have ferredoxin (Fd)-dependent sulfite reductase (SiR: E.C.1.8.7.1) and nitrite reductase (NiR: E.C.1.7.7.1) for assimilation of inorganic sulfur and nitrogen, respectively. These enzymes contain common prosthetic groups, one [4Fe-4S] cluster and one siroheme, and have partially overlapped substrate specificity, both catalyzing a six-electron reduction of sulfite and nitrite to sulfide and ammonia, respectively. SiR shows a strong preference for sulfite over nitrite (Krueger and Siegel, 1982). However, the structural basis for such substrate discrimination and the mechanism by which multiple electrons are transferred via the [2Fe-2S] cluster of Fd and the two redox centers of SiR for the complete reduction of the substrates, have been little investigated. *E. coli* has an another type of SiR utilizing NADPH as an electron donor for its catalysis (Siegel *et al*., 1973). This bacterial SiR, unlike the plant enzyme, is composed of catalytic hemoprotein subunit and electron-supplying flavoprotein subunit. X-ray crystallographic studies of the hemoprotein subunit have revealed the precise redox center structure; four cysteines function as ligands to the [4Fe-4S] cluster, one of the cysteines also serves as an axial ligand to the siroheme, and an extensive hydrogen-bonding network is formed among positively charged amino acid side chains, ordered water molecules, siroheme carboxylates and the substrate sulfite (Crane *et al*., 1995, 1997a, b; Fig. 1). This redox center structure is considered to be crucial for facilitating the six-electron reduction of the substrates without releasing intermediates during the catalytic reaction. These cysteines and basic residues are strictly conserved in all plant SiRs, suggesting that the configuration of the redox centers and coordination of the substrate anion in the active center are very similar between the two types of SiR (Yonekura-Sakakibara *et al.,* 1998; Nakayama *et al*., 2000). We tried site-directed mutagenesis of maize SiR focusing these basic residues, by using *E. coli* expression system (Ideguchi *et al*., 1995) and report here that drastic changes in the substrate preference for sulfite and nitrite is induced by a single amino acid substitution.

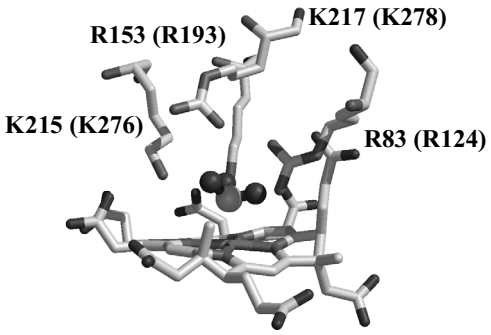

Fig. 1. Three dimentional structure of *E. coli* SiR active site. Four basic amino acids are conserved and form hydrogen bond lattice between sulfite and carboxylates of siroheme. Amino acids in parenthesis are corresponding residues of maize SiR.

Materials and methods

PCR and plasmid construction for mutant SiR

To substitution of Arg-124, Arg-193, Lys-276, Lys-278 of maize SiR for glutamine, glutamate, or alanine, mutagenetic oligonucleotides were designed and synthesized, systematically. These primers were used in PCR with a maize SiR cDNA, as a template (Ideguchi *et al*., 1995). Amplified DNA fragments were digested with appropriate restriction enzymes. Resulting fragments were inserted into the corresponding region of maize SiR cDNA, whose expression plasmid was constructed using pTrc99A vector (Amersham Pharmacia Biotech) carrying *E. coli CysG* (Ideguchi *et al*., 1995). DNA sequences were determined by dye-terminator sequencing.

Purification of SiRs and spinach NiR

WT and mutant SiRs were purified from transformed *E. coli* cells by ammonium sulfate fractionation, gel-filtration and anion exchange chromatography. As a final step of purification, Fd-immobilized resin column was employed. Purified SiRs were concentrated and stored at –80 ˚C. Preparation of NiR from spinach leaves was followed the methods as described previously (Vega and Kamin, 1977).

Spectroscopic and enzymatic analysis of mutant SiRs

Spectroscopic measurements were performed with UV-2500PC (Shimadzu). Concentaration of SiR was determined with molecular coefficient value ε_{580} = 17,800 cm^{-1} M^{-1}.

Enzymatic assay of SiR was carried out using with maize non-photosynthetic Fd (type III) and root-type ferredoxin $NADP^+$ reductase (FNR) as a reductant supply system as described previously (Yonekura-Sakakibara *et al*., 2000). A total 600 µl reaction mixture contained 80 nM SiR, 10 nM FNR, and 10 µM Fd. Reaction was initiated by the addition of 200 µM NADPH in the presence of sulfite, nitrite or hydroxylamine. The oxidization of NADPH to $NADP^+$ was recorded for 60 sec in terms of absorbance at 340 nm.

Results and discussion

Characterization of purified SiRs

We carried out SDS-PAGE and immunoblotting, and confirmed expression of R193E, R193A, K276Q, K278N, and K278Q in soluble fraction of *E. coli* cells as holo-form with the two prosthetic groups assembled. These mutant SiRs were purified nearly to homogeneity (Fig. 1). Significant differences between mutants and WT were revealed by UV-visible spectroscopy. The α peaks of all mutants were similar to that of WT. However, in some mutants, the soret and charge transfer bands were shifted or vanished (data not shown). The secondary structures of all mutants except for K278Q were confirmed to be essentially same as that of WT by CD spectroscopy (data not shown). These results implied that substitution of

single basic amino acid caused electrostatic changes around siroheme of mutant SiRs, without disrupting protein conformation.

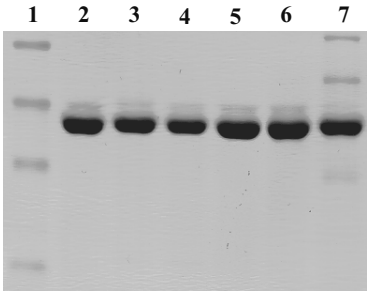

Fig. 2. SDS-PAGE analysis of recombinant maize SiR and its mutants. All mutants shown in this figure were purified as a holo-enzyme. Lane 1, pre-stained protein marker (New England Biolab); lane 2, WT; lane 3, R193E; lane 4, R193A; lane 5, K276Q; lane 6, K278N; lane 7, K278Q.

Assay of mutant SiRs in reduction of sulfite, nitrite and hydroxylamine

Enzymatic assay of mutant SiRs was carried out using an electron donation system of NADPH, root-type FNR and Fd (Yonekura-Sakakibara *et al*., 2000). SiR activity was monitored as decrease of absorbance derived from consumption of NADPH at 340 nm as presented in Fig. 3. WT showed high activities with sulfite, nitrite and hydroxylamine (Fig. 3A). Although R193E showed a high nitrite and hydroxylamine reduction activity, this mutant had no activity for sulfite (Fig. 3B). K276Q had activity for neither sulfite nor nitrite (Fig. 3C). Other mutant R193A, K276Q and K278N also lost sulfite reduction activity (data not shown).

As Fd S46G has a higher redox potential (-167 mV) than Fd WT (-345 mV), this Fd mutant could not function as an efficient electron donor to SiR for reduction of sulfite and nitrite. However, hydroxylamine reduction proceeded significantly with support of this Fd mutant. We examined effect of sulfite and nitrite on S46G supported reduction of hydroxylamine. The activity of WT, R193A and K276Q was competitively inhibited with μmole concentrations of sulfite, nitrite, and both the substrates, respectively. These results implied that mutant SiRs even lacking activity of sulfite and/or nitrite reduction still retained ability to bind these substrates.

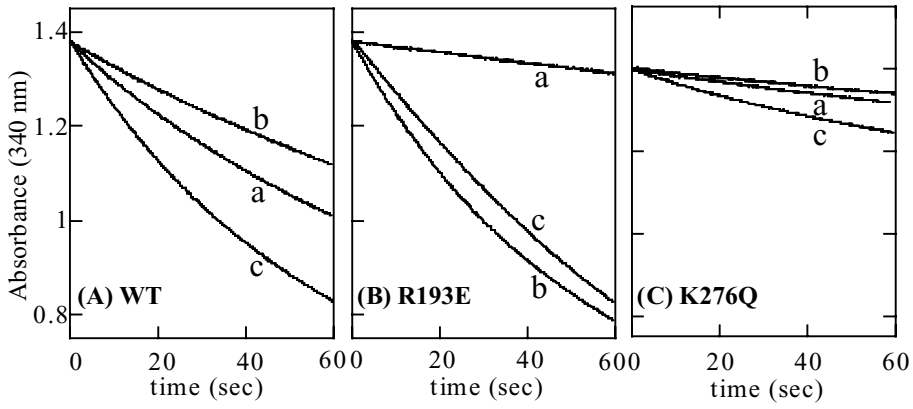

Fig. 3. Spectroscopic assay of SiR WT (A), R193E (B), and K276Q (C) activity in the NADPH-dependent sulfite reduction system. The reaction mixture contained SiR (80 nM), Fd (10 μM), FNR (10 nM), NADPH (200 μM) and substrate (500 μM each). Sulfite (a), nitrite (b) and hydroxylamine (c) reduction were monitored in terms of the decrease of absorbance at 340 nm. WT showed the activities for all substrates, while R193E did not reduce sulfite, but did nitrite and hydroxylamine. K276Q had negligible activity for both sulfite and nitrite.

Comparison of kinetic property

A summary of kinetic measurements of WT and mutants of SiR together with that of spinach NiR is shown in Table 1. Only SiR WT showed sulfite reduction activity with a low *K*m value (3.3 μM) and other mutants and NiR had no sulfite reductase activity (data not shown). In the case of nitrite reduction, mutants differed considerably from WT. For example, *k*cat for nitrite reduction of R193E was greater than that of WT, but those of K278N and R193A were decreased. R193A acquired a high affinity for nitrite (*K*m = 2.2 μM) equivalent with the authentic NiR, although its *k*cat value was one fiftieth.

Table 1. Kinetic parameters of SiR for sulfite and nitrite**.**

Substrate	**SO_3^{2-}**	**NO_2^-**				
Enzyme	**WT**	**WT**	**R193E**	**R193A**	**K278N**	**NiR**
***K*m (μM)**	**3.3**	**440**	**310**	**2.2**	**560**	**2.4**
***k*cat (e^-/min/siroheme)**	**460**	**460**	**910**	**180**	**110**	**11000**

General Conclusion

We established an overexpression system of plant SiR in *E. coli* and carried out site-directed mutagenesis at the siroheme distal region. Resulting mutants were classified into three groups; class 1, affinity for nitrite and sulfite similar to WT, but no reduction of sulfite; class 2, higher affinity for nitrite than WT with malfunction of sulfite reduction; class 3, nitrite and sulfite can bind, but not are reduced. These results showed that side chain structures of R193, K276 and K278 are crucial for substrate binding and reduction.

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