Scavenging Systems for Reactive Carbonyls in the Cyanobacterium *Synechocystis* sp. PCC 6803

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To elucidate the scavenging systems of sugar- and lipid-derived reactive carbonyls (RCs) in the cyanobacterium *Synechocystis* sp. PCC 6803 (S. 6803), we selected proteins from S. 6803 based on amino-acid (AA) sequence similarities with proteins from *Arabidopsis thaliana*, and characterized the properties of the GST-fusion proteins expressed. Slr0942 catalyzed the aldol-keto reductase (AKR) reaction scavenging mainly sugar-derived RCs, methylglyoxal (MG). Slr1192 is the medium-chain dehydrogenase/reductase (MDR). It catalyzed the AKR reaction scavenging several lipid-derived RCs, acrolein, propionaldehyde, and crotonaldehyde. Slr0315 is a short-chain dehydrogenase/reductase (SDR), and it catalyzed only the reduction of MG in the AKR reaction. Slr0381 catalyzed the conversion of hemithioacetal to S-lactoylglutathione (SLG) in the glyoxalase (GLX) I reaction. Slr1019 catalyzed the conversion of SLG to glutathione and lactate in the GLX2 reaction. GLX1 and GLX2 compose the glyoxalase system, which scavenges MG. These enzymes contribute to scavenging sugar- and lipid-derived RCs as scavenging systems.

Key words: aldol-keto reductase (AKR); cyanobacteria; medium-chain dehydrogenase/reductase (MDR); short-chain dehydrogenase/reductase (SDR); glyoxalase (GLX)

Sugar catabolic and anabolic metabolism are essential processes in living organisms, but undesirable reactions and by-products can lead to cell dysfunction. D-Glucose, the initial substrate of glycolysis, auto-oxidizes to produce reactive oxygen species (ROS),1,5 and it reacts with proteins non-enzymatically under physiological conditions to form glycated proteins.2,3 Amadori rearrangements of sugars also can produce methylglyoxal (MG), glyoxal (GLO), and 3-deoxyglucosone (3-DG). Moreover, MG is also formed by spontaneous degradation of triosephosphates glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP), intermediates of glycolysis.4,5 These sugar-derived dicar-bonyls induce modifications of proteins that can inactivate protein function.6–8 Furthermore, the ROS produced by autoxidation of D-glucose peroxidize free and membrane-bound unsaturated fatty acids, and produce α,β-unsaturated carbonyls such as acrolein, crotonaldehyde, and trans-4-hydroxy-2-nonenal (HNE).9,10 These lipid-derived α,β-unsaturated carbonyls have an ability to modify proteins and nucleic acids.6,11,12 All of the reactive carbonyls (RCs) just mentioned can inactivate physiological functions, and thus cause sugar toxicity in cells.

Most organisms possess scavenging systems for RCs alleviating sugar toxicity. First, aldol-keto reductases (AKR) reduce aldehyde- and ketone-groups of RCs to the corresponding alcohols with NAD(P)H as electron donors.13–15 Second, medium-chain dehydrogenase/reductases (MDR), and third, short-chain dehydrogenase/reductases (SDR), reduce RCs, with NAD(P)H as electron donors.16–18 Fourth, in the glyoxalase system, glyoxalase I (GLX1) and glyoxalase II (GLX2) cooperatively convert MG to β-lactate with reduced glutathione (GSH).19,20 The widespread distribution in organisms of these scavenging pathways, the AKR-, MDR-, SDR-, and GLX-systems, suggests that detoxification and control of RCs are essential in alleviating sugar toxicity.

Photosynthetic autotrophs convert CO₂ to sugars by means of light energy, and accumulate photosynthates to high concentrations in their cells. Thus photosynthetic organisms are exposed to higher levels of sugar-toxicity than heterotrophs. For example, when photosynthesis was stimulated under high [CO₂], carbonylated proteins accumulated in the cells, indicating that the production of RCs was enhanced, leading to protein modification.21 In higher plants, RCs, including MG, GLO, acrolein and other RCs, impair physiological functions.22–25 Acrolein inhibited CO₂-dependent O₂ evolution in isolated chloroplasts through inactivation of Calvin-cycle enzymes.25 Recently, Shimakawa et al. (2013) found that acrolein directly inactivated photosystem II under
illuminations. Expression of the genes involved in the AKR, MDR, SDR, and GLX pathways of RC detoxification is stimulated by abiotic stresses such as drought and salt when RCs accumulate in plant cells. In addition, enzymes that detoxify lipid-derived \(\alpha,\beta\)-unsaturated carbonyls have been found in Arabidopsis thaliana and cucumber. 2-Alkenal reductase (AER; EC 1.3.1.74) and alkenal/alkenone reductase (AOR) catalyze the reduction of lipid-derived RCs such as acrolein, crotonaldehyde, and hexanal.

In the present study, we investigated RC scavenging systems in cyanobacteria, oxygenic photosynthetic eubacteria. Higher plant chloroplasts are endosymbiotic descendants of cyanobacteria. In cyanobacteria, glycolysis and photosynthesis proceed in the same compartment, the cytosol. Consequently, the danger of sugar toxicity is probably even more profound in cyanobacteria than in higher plants. Here we expect cyanobacteria to possess efficient scavenging systems for RCs. Studies of the mutant cyanobacterium Synechococcus sp. PCC 7002, which is deficient in AKR, have indicated that AKR is involved in the detoxification of MG, but the details are lacking. In this study, we selected proteins from the cyanobacterium Synechocystis sp. PCC 6803 (\(S.\) 6803) that might function in the scavenging of RCs, based on amino-acid (AA) sequence similarities with proteins of known function in higher plants, and characterized their enzymatic properties.

Materials and Methods

Cloning and expression of the recombinant \(S.\) 6803 AKR, MDR, SDR, and GLX proteins. Total RNA was isolated with an RNaseasy Plant Mini Kit (Qiagen, Valencia, CA). cDNA was synthesized with a Takara cDNA Synthesis Kit (Takara, Shiga, Japan). The coding regions of Synechocystis AKR, MDR, SDR, and GLX, and of Arabidopsis AKR4C9, CAD4, and CAD5, were obtained with KOD-FX Neo (Toyobo, Osaka, Japan), and were subcloned via Bam HI into pGEX4T-3 (GE Healthcare, Buckinghamshire, UK) with In-Fusion HD Cloning Kit (Takara). The primers for these genes are listed in Supplemental Table 1 (see Biosci. Biotechnol. Biochem. Web site). Recombinant proteins were produced in BL21 (DE3, Agilent Technologies, Wilmington, DE) host cells. Overnight cultures of the transformed BL21 cells in Luria-Bertani broth were used to inoculate (0.1%) fresh Luria-Bertani broth, which was then incubated at 37\(^\circ\)C for 30 min. The lysate was loaded into GSTrap FF columns (GE Healthcare). Unbound proteins were removed by washing with PBS buffer. Then the recombinant GST-fusion proteins were eluted with elution buffer (50 mM Tris–HCl, 10 mM GSH, 1 mM PMSF, pH 7.4). The eluted solution was loaded onto a PD-10 column (GE Healthcare) that was eluted with GSH-free elution buffer to remove GSH. The purified recombinant protein was quantified by Pierce 660 nm Protein Assay (Thermo Scientific, Waltham, MA).

Expression and purification of recombinant GST-fusion proteins of the Arabidopsis AKR4C subfamily. Recombinant proteins of AKRs (AKR4C8, AKR4C9, AKR4C10, and AKR4C11) belonging to the AKR4C subfamily of Arabidopsis thaliana were expressed in Escherichia coli and prepared for the enzyme assay, as described in Saito et al.

Enzyme assays. The activities of \(S.\) 6803 AKR, MDR, and SDR and of Arabidopsis CAD4 and CAD5 in the reduction of RCs were determined in 1 mL reaction mixture (1 mL) that contained 50 mM potassium phosphate (pH 7.0), 0.15 mM NADPH, arbitrary amounts of the recombinant proteins, and various concentrations of substrates. RCs-dependent oxidation of NADPH was followed by monitoring the decrease in absorbance at 240 nm, assuming an absorption coefficient of 6.22 mM\textsuperscript{-1} cm\textsuperscript{-1}. The activity of GLX1 (GLX1 reaction activity) was determined in a reaction mixture (1 mL) that contained 50 mM sodium phosphate (pH 7.0), arbitrary amounts of the recombinant proteins, and various concentrations of SLG. The rate of hydrolysis was followed by measuring the decrease in absorbance at 240 nm. An \(K_{\text{m}}\) of 3.1 mM\textsuperscript{-1} cm\textsuperscript{-1} was used to calculate the GLX2 activity rate. Furthermore, the 3-DG reduction activity of the recombinant GST-fusion proteins of the Arabidopsis AKR4C subfamily were determined in a reaction mixture (1 mL) that contained 50 mM sodium phosphate (pH 7.0), 0.2 mM NADPH, arbitrary amounts of the recombinant proteins, and various concentrations of 3-DG. The 3-DG-dependent oxidation of NADPH was followed by monitoring the decrease in absorbance at 340 nm, assuming an absorption coefficient of 6.22 mM\textsuperscript{-1} cm\textsuperscript{-1}.

Sequence analysis. Sequence alignments were determined by ClustalX and the phylogenetic tree was constructed by the UPGMA algorithm with Phyloidendron (http://labio.bio.indiana.edu/treepapp/treeprint-form.html).

Results and Discussion

Characterization of the AKR, Slr0942

AKRs, monomers of 35 to 40 kDa, form a protein superfamily of 14 families. In the AKR family, the \(\alpha,\beta\)-barrel structures and NAD(P)H-binding sites are highly conserved. AKR substrate specificities are determined by three loop structures (loops A, B, and C). Several homologs of AKR, including Slr0942, Slr1503, and Slr0545, were found in the genome of \(S.\) 6803. Phylogenetic trees including \(S.\) 6803 AKRs, SakR1 (Synechococcus sp. PCC7002 AKR), and At2g37770 (Arabidopsis thaliana AKR4C9), were analyzed (Fig. 1a). At2g37770 belongs to the AKR4C subfamily and catalyzes the reduction of RCs, and SakR1 reduces MG with NADPH. The amino-acid (AA) sequence of Slr0942 showed homologies of 17.5, 14.4, 41.6, 20.6% with Slr0545, Slr1503, At2g37770, SakR1 respectively. As described below, Slr0942 showed RC-reducing activity, but on the other hand neither Slr1503 nor Slr0545 showed any RC-reducing activity (data not shown). Next, the AA sequence of Slr0942 was compared to those of AKR4C9 and SakR1 in a multiple sequence alignment constructed with ClustalX (Fig. 1b). Slr0942 carries the AKR1-motif often found in the AKR4C subfamily. Several AA residues related to the catalytic reaction are conserved in the AKR4C subfamily, and are present in Slr0942, Asp52, Tyr57, Lys86, and His119. Furthermore, the AA residues functioning in NADPH binding to the catalytic sites in AKRs are...
also conserved in Slr0942, except for Gln279. On the other hand, the AA sequences relating to loops A, B, and C, which determine substrate specificity, were different in the three proteins. Consequently, the substrate specificity of Slr0942 was predicted to differ from those of AKR4C9 and SakR1.

Next, the AA sequence of Slr0942 was compared to those of Slr1503 and Slr0545 in a multiple sequence alignment constructed with ClustalX (Fig. 1c). In the AKR1 motif, several AAs, G, D, and Y, are often found, similarly to At2g37770 and SakR1. The catalytic sites in AKR reaction are also often found, similarly to At2g37770 and SakR1. Although several NADPH-binding motives were found, the sequences of AA were different from each other. Furthermore, the AA sequences relating to loops A, B, and C were different between them. That is, these different sequences in the NADPH-binding site and three loops must be the reason neither Slr1503 nor Slr0545 showed any RC-reducing activity.

We isolated gene slr0942 from the cDNA prepared from the mRNAs expressed in S. 6803 and overproduced Slr0942 as a GST-fusion protein in E. coli (Supplemental Fig. 1). We evaluated the AKR activity of recombinant Slr0942 (Table 1), and found that it had a lower \( K_m \) for MG than AKR in the AKR4C subfamily.28,39) The ratio \( k_{cat}/K_m \) was also larger in Slr0942 than in the AKR4C subfamily. Slr0942 reduced 3-DG, but its \( k_{cat}/K_m \) was lower than those in the AKR4C subfamily (Supplemental Table 1). Slr0942 also reduced DHAP and GAP, two intermediates of the Calvin-cycle and glycolysis. Furthermore, we found that the most reactive \( \alpha,\beta \)-unsaturated carbonyl, acrolein,9) was reduced by Slr0942. On the other hand, neither Slr1503 nor Slr0545 showed any RC-reducing activity (data not shown) although both show homology with At5g53580 (48%) and At1g06690 (42%) of A. thaliana. Recombinant At5g53580 expressed in yeast catalyzed the NADPH-mediated reduction of pyridoxal to pyridoxine.45) We conclude that Slr1503 and Slr0545 have evolved in functionally different directions than Slr0942.

Slr0942 showed an optimum temperature of 60 °C for the reduction of MG (Supplemental Fig. 2a), and an optimum pH of 5 (Supplemental Fig. 2b).

While MG and acrolein are substrates for AKRs, these compounds can modify AA residues of AKRs and inactivate them.39) MG at 1 mM inactivated enzymes of

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**Table 1.** Kinetic Parameters for the Reduction of Sugar- and Lipid-Derived RCs, d-Glucose, and Triose Phosphates by Recombinant Slr0942

<table>
<thead>
<tr>
<th>Substrates</th>
<th>( K_m ) (mM)</th>
<th>( k_{cat} ) (min(^{-1}))</th>
<th>( k_{cat}/K_m ) (min(^{-1}) mM(^{-1}))</th>
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<tr>
<td>MG</td>
<td>0.096 ± 0.005</td>
<td>351 ± 4.5</td>
<td>3.640</td>
</tr>
<tr>
<td>GLO</td>
<td>10.8 ± 2.3</td>
<td>1,049 ± 109</td>
<td>98</td>
</tr>
<tr>
<td>3-DG</td>
<td>0.52 ± 0.09</td>
<td>226 ± 14</td>
<td>439</td>
</tr>
<tr>
<td>DHAP</td>
<td>1.2 ± 0.17</td>
<td>117 ± 4.2</td>
<td>94</td>
</tr>
<tr>
<td>GAP</td>
<td>4.7 ± 0.27</td>
<td>289 ± 6.5</td>
<td>62</td>
</tr>
<tr>
<td>d-Glucose</td>
<td>1,359 ± 437</td>
<td>600 ± 116</td>
<td>0.44</td>
</tr>
<tr>
<td>Acrolein</td>
<td>1.7 ± 0.44</td>
<td>1,078 ± 91</td>
<td>624</td>
</tr>
<tr>
<td>Propionaldehyde</td>
<td>3.6 ± 0.63</td>
<td>694 ± 41</td>
<td>195</td>
</tr>
<tr>
<td>Crotonaldehyde</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
</tr>
</tbody>
</table>

NADPH (0.15 mM) was used as electron donor. nd, not determined. All experiments were repeated 3 times with independent batches of recombinant proteins. Data are means ± SD.
the AKR4C subfamily to 80% of their original activities. Similarly, acrolein at 1 mM inactivated AKR4C8 to 40%, AKR4C9 to 60%, AKR4C10 to 80%, and AKR4C11 to 40% of their original activities.39 Slr0942 retained its activity in the presence of acrolein and MG at up to 1 mM (Supplemental Fig. 2c, d). These results indicate that Slr0942 was more tolerant of RCs than the AKR4C subfamily enzymes, and this can be explained by the differences in the AA sequences of Slr0942 and AKR4C9.

Characterization of the MDR, Slr1192

MDRs are found in at least eight protein families, including dimeric alcohol dehydrogenases (ADH, originally detected in animals/plants) and cinnamyl alcohol dehydrogenases (CAD, originally detected in plants).17) In ADHs and CADs, reductase reactions are preferred over dehydrogenase reactions, due to higher affinities for the aldehyde groups of RCs than for the alcohol groups.30,46 Recent studies indicate that CAD catalyzes the last step in the biosynthesis of the monomeric precursors of lignin, the main constituent of secondary plant cell walls.47,48 In contrast to A. thaliana, S. 6803 possesses only one MDR with an ADH motif, Slr1192.49 The AA sequence of Slr1192 is similar to those of the CAD proteins in A. thaliana, which form a subfamily of the MDR family. The CAD subfamily includes At1g19450 (CAD4 of Arabidopsis thaliana), and At4g34230 (CAD5 of Arabidopsis thaliana). Shaded box, Zn-containing ADH signature. Squared-line box, Zn2 structural motif respectively.

We isolated the slr1192 gene from S. 6803 and overproduced a GST-fusion construct of Slr1192 in E. coli (Supplemental Fig. 1) to evaluate its activity (Table 2). Slr1192 showed lower K_m values for MG, acrolein, and propionaldehyde than CAD4 or CAD5 (Supplemental Table 2). The K_m for acrolein was close to the value observed in soluble fractions prepared from S. 6803,26 indicating that the acrolein reduction activity of the total soluble fraction was due mainly to the activity of Slr1192.

It has been reported that acrolein was scavenged by two enzymatic reactions.50,51 As for the first one, the hydrogenation of the α,β-unsaturated bond catalyzed by AOR produced propionaldehyde, and as second one, the reduction of the aldehyde moiety catalyzed by AKR produced the allyl alcohol, propenol. If Slr1192 catalyzes the AOR reaction, propionaldehyde is produced. On the other hand, if Slr1192 catalyzes the AKR reaction, propenol is produced and accumulates. Slr1192 showed the reduction activities of both acrolein and propionaldehyde (Table 2). We tested whether Slr1192 catalyzes both the AOR and the AKR reaction. If Slr1192 catalyzes the hydrogenation of acrolein to propionaldehyde in the AOR reaction and sequentially the reduction of the aldehyde moiety of propionaldehyde to propenol in the AKR reaction, we predicted that Slr1192 catalyzes the conversion of acrolein to propenol, with the following stoichiometry:

$$2 \text{NADPH} + \text{acrolein} + 2 \text{H}^+ \rightarrow 2 \text{NADP}^+ + \text{propenol}$$

In the event, acrolein and NADPH were consumed at equimolecular amounts in the reaction catalyzed by the GST-fusion construct of Slr1192 (data not shown), and we concluded that Slr1192 scavenged acrolein by catalyzing the AKR reaction, as follows:

$$\text{NADPH} + \text{acrolein} + \text{H}^+ \rightarrow \text{NADP}^+ + \text{propenol}$$

The optimum temperature for the reduction of acrolein by Slr1192 was 60 °C (Supplemental Fig. 3a), and the optimum pH was 6 (Supplemental Fig. 3b).

### Table 2. Kinetic Parameters for the Reduction of Sugar- and Lipid-Derived RCs, D-Glucose, and Triose Phosphates by Recombinant Slr1192

<table>
<thead>
<tr>
<th>Substrates</th>
<th>K_m (mM)</th>
<th>k_cat (min⁻¹)</th>
<th>k_cat/K_m (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG</td>
<td>0.26 ± 0.07</td>
<td>1.32 ± 0.10</td>
<td>5.081</td>
</tr>
<tr>
<td>GLO</td>
<td>31 ± 0.1</td>
<td>1.53 ± 0.97</td>
<td>49</td>
</tr>
<tr>
<td>3-DG</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
</tr>
<tr>
<td>DHAP</td>
<td>vh</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>GAP</td>
<td>vh</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>d-Glucose</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
</tr>
<tr>
<td>Acrolein</td>
<td>0.16 ± 0.02</td>
<td>3.07 ± 0.14</td>
<td>19.741</td>
</tr>
<tr>
<td>Propionaldehyde</td>
<td>0.053 ± 0.01</td>
<td>1.383 ± 0.15</td>
<td>26.012</td>
</tr>
<tr>
<td>Crotonaldehyde</td>
<td>0.02 ± 0.004</td>
<td>1.574 ± 0.76</td>
<td>77.478</td>
</tr>
</tbody>
</table>

NADPH (0.15 mM) was used as electron donor. nd, not determined. vh, very high; could not be fitted with any accuracy to the Michaelis-Menten equation. Experiments were repeated 3 times with independent batches of recombinant protein. Data are means ± SD.
Slr1192 activity remained in the presence of acrolein and MG (Supplemental Fig. 3c, d), indicating that Slr1192 was not attacked by the RCs, similarly to Slr0942.

CAD is involved in the synthesis of aromatic compounds, e.g., coniferyl-, p-coumaryl-, and sinapyl-alcohols, which are intermediates in lignin biosynthesis in higher plants.52) This pathway is unique to higher plants, bacteria, and fungi. 17) Thus Slr1192 evidently functions in contexts other than RC detoxification. 49) We isolated the CAD4 and CAD5 genes from A. thaliana and overexpressed them as GST-fusion proteins in E. coli to study their enzymatic activities. Differently from Slr1192, CAD4 and CAD5 did not reduce MG, GLO, or acrolein (Supplemental Table 2). On the other hand, CAD4 and CAD5 reduced 3-DG, crotonaldehyde, and cynnamylaldehyde. The scavenging activities of MG and GLO by CADs apparently became lost in higher plants in favor of a role in the lignin biosynthesis pathways.

Characterization of the SDR, Slr0315
SDR is a multimeric non-metallo-oxidoreductase that has a classical Rossmann-fold motif for NAD(P)H binding, in which an α/β-folding pattern with a central β-sheet is flanked by 2–3 α-helices from each side.53) SDR and MDR perhaps evolved from a common ancestor protein. 54) A large number of homologs of SDR have been found in the genome of S. 6803. 55) Based on AA sequence homologies with At3g04000, 31) we selected Slr0315, Sll5079, and Slr0886 as candidates for functional SDRs. A phylogenetic tree including Sll5079, Slr0886, Slr0315, and At3g04000 was analyzed (Fig. 3a). The AA sequence of Slr0315 showed homologies of 20.9, 26.6, and 25.8% with At3g04000, sll5079, and Slr0886 respectively. The AA sequence of Slr0315 was compared to those of Slr0579, Slr0886, and At3g04000 in a multiple sequence alignment constructed with ClustalX (Fig. 3b). The typical active site (155-Y-x-x-x-K (Slr0315)) was often found, indicating that these proteins belong to the classical SDR family.53,55)

We isolated gene slr0315 from S. 6803 and overproduced the gene product as a GST-fusion protein in E. coli (Supplemental Fig. 1), to study its activity (Table 3). Slr0315 showed a high $k_{cat}/K_m$ for MG as compared to that of At3g04000, 31) but the $k_{cat}/K_m$ values for RCs other than MG were low in Slr0315. Kramm et al. (2012) suggested that Slr0315 functions as a clavaldehyde dehydrogenase. 55) Clavaldehyde dehydrogenase catalyzes the transformation of clavaldehyde to clavulanic acid at the final step of clavulanic acid biosynthesis.56,57) Similarly, Slr0315 may act on unknown substrates under physiological conditions. On the other hand, Sll5079 and Slr0886 did not show any RC-reducing activity in our tests (data not shown).

Slr0315 showed a broad optimum temperature range from 30 to 50 °C (Supplemental Fig. 4a) and an optimum pH of 7 for the reduction of MG (Supplemental Fig. 4b). In contrast to Slr0942 and Slr1192, Slr0315 was inactivated by 1 mM MG to 80% of its original activity (Supplemental Fig. 4c). Furthermore, Slr0315 activity was reduced to 60% by 0.25 mM acrolein (Supplemental Fig. 4d), and to 5% by 1 mM acrolein. The acrolein effect on Slr0315 was more pronounced than that on Arabidopsis enzymes AKR4C8 and AKR4C11. 39) The active site of Slr0315, YxxxK, contains Lys, which might be the main target in modification by acrolein.58)
Characterization of the GLX system, Slr0381 and Sll1019

At the initial step of the GLX system, GSH reacts with MG to produce hemithioacetal (HA), which then is converted to S-D-lactoylglutathione (SLG) by GLX1. GLX2 catalyzes the hydrolysis of SLG to D-lactate with regeneration of GSH. The genome of S. 6803 contains one GLX1, Slr0381, and two GLX2s, Sll1019 and Slr1259, while 11 GLX1s and five GLX2s are present in A. thaliana. The AA sequence of Slr0381 showed 49.6% homology with that of At1g11840, one of the GLX1s in A. thaliana, which contains three GLX motifs. The AA sequence of Sll1019 showed 36.6% homology with At3g10850, which encodes a cytosol-localized protein with high GLX2 activity. Furthermore, the AA sequence of Sll1019 showed 14.7% homology with Slr1259. Slr1259 showed homology with At1g53580 (44%), a structural GLX2 that shows no GLX2 activity but rather that of a sulfur dioxygenase. We isolated slr1259 from S. 6803 and overproduced Slr1259 as a GST-fusion protein in E. coli. Slr1259 did not exhibit GLX2 activity (data not shown). AA sequence of Sll1019 was compared to those of Slr1259 and At3g04000 in a multiple sequence alignment constructed with ClustalX (Fig. 5b). The Glyoxalase II (metallo-β-lactamase) motif was often found in these proteins. The box shows the AAs required for the substrate binding, which were conserved. The dotted line box shows the AAs required for Zn$^{2+}$-binding, which differed among the three proteins. These must be the reason why Slr1259 did not show GLX2 activity.

Next, we analyzed the gene products of slr0381 and sll1019 in a similar way (Supplemental Fig. 1) and characterized their substrate specificities and kinetic parameters (Table 4). The $K_m$ for HA of Slr0381 (GLX1) resembled the values obtained for humans, yeast, and E. coli. Similarly, Sll1019 (GLX2) showed $K_m$ and $k_{cat}$ for SLG similar to enzymes from humans, E. coli, and A. thaliana. These results indicate that GLX1 and GLX2 in S. 6803 possess affinities for HA and SLG respectively that do not differ much from those found in homologous enzymes of other organisms.

The optimum temperature for the GLX1 reaction catalyzed by Slr0381 was 70°C (Supplemental Fig. 5a). We could not determine maximum pH, because the non-

<table>
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<th>Substrate</th>
<th>$K_m$ (mM)</th>
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<th>$k_{cat}/K_m$ (min$^{-1}$ mM$^{-1}$)</th>
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<tr>
<td>HA</td>
<td>0.39 ± 0.1</td>
<td>351 ± 27</td>
<td>908</td>
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<th>Substrate</th>
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<tr>
<td>SLG</td>
<td>0.13 ± 0.02</td>
<td>18,271 ± 986</td>
<td>146,164</td>
</tr>
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</table>

a. Kinetic parameters for the conversion of hemithioacetal to S-D-lactoylglutathione by recombinant Slr0381 protein. Experiments were repeated 3 times with independent batches of recombinant protein. Data are means ± SD. b. Kinetic parameters for the hydrolysis of S-D-lactoylglutathione by recombinant Sll1019 protein. Experiments were repeated 3 times with independent batches of recombinant protein. Data are means ± SD.

![Fig. 4. Sequence Alignment of Slr0381 and At1g11840 (GLX1 of Arabidopsis thaliana). Shaded boxes, Glyoxalase I-1 and Glyoxalase I-2 motifs respectively. Squared-line box, substrate-binding site. Dotted-line box, Zn$^{2+}$ or Ni$^{2+}$-binding sites.](image)

![Fig. 5. Sequence Analysis of Amino Acids.](image)
enzymatic reaction of MG with GSH forming HA showed pH dependence. On the other hand, SII1019 showed an optimum temperature of 70 °C for the GLX2 reaction (Supplemental Fig. 6b). The pH dependence of this reaction showed no clear optimum (Supplemental Fig. 6b).

Slr0381 and SII1019 were inactivated by 1 mM MG to 70% and 80% respectively of the original activities (Supplemental Figs. 5b and 6c), and by 1 mM acrolein to 60% and 50% respectively of the original activities (Supplemental Figs. 5c and 6d). The substrate-binding site of GLX1, including Arg9, Phe39, Asn60, and Phe112, is highly conserved in Slr0381 (Fig. 4). On the other hand, the substrate-binding site of GLX2, including Arg146, Phe148, Tyr177, Arg251, and Lys254, is highly conserved in SII1019 (Fig. 5b). The Arg and Lys residues are the main targets in modification by MG and acrolein. MG reacts directly with Arg residues to form hydroimidazolone $N_2$-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine residues. Furthermore, the GLX1 of humans, E. coli, and S. 6803 contain metal-binding sites that are composed mainly of His and Glu residues (Fig. 4). The GLX2s of humans, yeast, A. thaliana and S. 6803 contain a highly conserved metal-binding domain, THxHxDH (Fig. 5b). Acrolein reacts with His residues to form $N_2$-(3-propanal)histidine. We concluded that acrolein attacked the His residues of the metal binding sites of Slr0381 and SII1019, resulting in the observed reductions of enzyme activity.

Conclusions

In the genome of cyanobacterium S. 6803, we found several genes that encode enzymes involved in the scavenging of sugar- and lipid-derived RCs: AKR (Slr0942), MDR (Slr1192), SDR (Slr0315), GLX1 (Slr0381), and GLX2 (SII1019). Slr0381 and SII1019 appear to represent a glyoxalase system for the detoxification of MG, whereas Slr1192 appears to scavenge lipid-derived RCs: AKR and CAD in our model cyanobacterium. Slr1192 has stronger activity in reducing lipid-derived RCs than AER and AOR in higher plants (Table 2). It might represent the original enzyme scavenging lipid-derived RCs in photosynthetic organisms. The physiological functions of RC-scavenging systems in vivo can be clarified by the use of mutants that are deficient in one or more scavenging enzymes in the future.

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