A novel variant of ferredoxin-dependent sulfite reductase having preferred substrate specificity for nitrite in the unicellular red alga Cyanidioschyzon merolae

Kohsuke SEKINE*†, Yukiko SAKAKIBARA‡, Toshiharu HASE‡ and Naoki SATO*†

*Center for Structuring Life Sciences, Graduate School of Arts and Sciences, University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan, †Department of Life Sciences, Graduate School of Arts and Sciences, University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan, and ‡Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita-shi, Osaka 565-0871, Japan

INTRODUCTION

In the biological resource cycle, the reduction of nitrite to ammonium and sulfite to sulfide are catalysed by NiR (nitrite reductase) and SiR (sulfite reductase) respectively. Both of these enzymes are classified as assimilatory and dissipatory types by their role within the cell and their structure [1–3]. Although assimilatory NiRs and SiRs in plants and cyanobacteria have a common set of prosthetic groups, a sirohaem group and a [4Fe–4S] cluster, the two enzymes are distinguished by their electron donors and subunit composition. The SiRs of many bacteria, such as that of Escherichia coli, utilize NAD(P)H as an electron donor and exist as a heteromultimer of haem-containing and flavin-containing subunits, whereas the NiRs and SiRs of photosynthetic organisms utilize Fd (ferredoxin) as an electron donor and exist as a single-subunit enzyme [4]. The haemoprotein of NAD(P)H-dependent SiR and Fd-dependent NiR/SiR share a high sequence homology.

The assimilatory NiR and SiR are members of a superfamily of oxidoreductases that reduce inorganic oxides by using six electrons. However, the two enzymes are clearly distinguished by their preferred substrates [5]. X-ray crystal structures are now available for three enzymes: the haemoprotein subunit of E. coli SiR (sirohaem-containing subunit accepting electrons from a flavoprotein) [6–8], Mycobacterium SiR [9] and spinach (Spinacia oleracea) NiR [10]. Swamy et al. [10] found that residues Lys217 of the E. coli haemoprotein, and the corresponding residues Asn226 and Arg149 in spinach NiR, are involved in substrate binding through electrostatic interactions and planarity of the sirohaem group and the two substrates. However, when Lys278 of ZmSiR (maize (Zea mays) SiR), corresponding to Lys217 of the E. coli haemoprotein, was replaced by an asparagine residue, the nitrite-reducing activity of the resulting mutant remained at the wild-type level [4]. So far, the critical residues that contribute to the difference in substrate selectivity remain unclear.

SiR and NiR are both localized to chloroplasts or plastids in plants and algae. In flowering plants, a single species of SiR is present in leaf chloroplasts and root plastids, whereas different Fds act as electron donors in the leaves and roots [11]. Chloroplasts or plastids are the sole site of reduction of both sulfite and nitrite in plants and algae. This is consistent with the presence of a chloroplast- or plastid-targeting signal in all SiRs and NiRs that have ever been identified by genomic analysis. Another interesting feature of SiR is its DNA-binding activity [12–14]. In pea (Pisum sativum) chloroplasts, SiR was found as a major protein in isolated nucleoids [12,14]. SiR causes tight packing of nucleoids and thus represses transcription, but this repression can be relieved by the artificial removal of SiR by heparin in vitro [13]. The physiological releaser of SiR remains unknown. The binding of SiR to nucleoids depends on plant species. In pea and the moss Physcomitrella patens, SiR is bound to nucleoids [15], whereas the majority of SiR is localized in the stroma in Arabidopsis thaliana (thale cress) and maize [14], although ZmSiR definitely has a high affinity for

Key words: Cyanidioschyzon merolae, ferredoxin, hot-spring red alga, nitrite reductase (NiR), substrate specificity, sulfite reductase (SiR).

Abbreviations used: CmSiRA, Cyanidioschyzon merolae sulfite reductase A; CmSiRB, Cyanidioschyzon merolae sulfite reductase B; Fd, ferredoxin; FNR, ferredoxin-NADP+ reductase; NiR, nitrite reductase; SiR, sulfite reductase; SyNiR, Synechocystis sp. PCC6803 nitrite reductase; ZmSiR, Zea mays (maize) sulfite reductase.

1 To whom correspondence should be addressed (email naokisat@bio.c.u-tokyo.ac.jp).
DNA. By contrast, NiR is not localized to the nucleoids in any plants that have been analysed.

*Cyanidioschyzon merolae* is a unicellular red alga living in acidic hot springs. Its completely sequenced genome ([16,17]; http://morolae.biol.s.u-tokyo.ac.jp/) encodes two SiR homologues, termed CmSiRA (*C. merolae* sulfite reductase A; also known as CMJ117C) and CmSiRB (*C. merolae* sulfite reductase B; also known as CGM021C), but, curiously, no gene encoding an NiR has been found. Since *C. merolae* grows on nitrate as the sole nitrogen source, an enzyme that reduces nitrite into glutamine and glutamic acid have been identified. As most known SiRs have a low nitrite-reducing activity, CmSiRA and CmSiRB are candidates for being the nitrite-reducing enzymes. To investigate this hypothesis, we prepared recombinant CmSiRB and characterized its catalytic activities using sulfite or nitrite as a substrate. These properties were compared with those of typical plant-type NiR and SiR, namely SyNiR (the NiR of the freshwater cyanobacterium *Synechocystis* sp. PCC6803 NiR; also known as slr0898) and ZmSiR. The results suggested that CmSiRB is a novel type of enzyme in the Fd/NiR/SiR family.

**Experimental**

**Culture conditions**

*C. merolae* 10D [18] was grown in 2 x Allen’s medium (ammonium medium) [19,20] or in nitrate medium, in which 20 mM (NH₄)₂SO₄ was replaced by 20 mM Na₂SO₄ and 40 mM KNO₃ at pH 2.5. The culture was illuminated by continuous illumination at 50 μE·m⁻²·s⁻¹ provided by fluorescent tubes (50 W) at 42°C with aeration by 1% CO₂ in air. *Synechocystis* was grown in the BG-11 medium [21] supplemented with 5 mM NaHCO₃ and 0.25 mM Hepes/NaOH (pH 7.5) under continuous illumination provided by fluorescent tubes (50 μE·m⁻²·s⁻¹) at 30°C with aeration by 1% CO₂ in air.

**Phylogenetic analysis**

Sequence data of SiRs and NiRs were obtained from the Gclust database ([22–24]; http://gclust.c.u-tokyo.ac.jp/) (Clusters 510 and 7037, version 2007-10, dataset ALL95.4). Additional sequences were obtained from GenBank. Some CobG sequences in Cluster 5094, which are putative cobalamin-biosynthesis proteins, were also added as the closest relatives and used as an outgroup. Amino acid alignment was prepared using MAFFT software version 6.531b [25]. Data manipulation and conversion involving removal of sites having gaps in more than 20% sequences were performed by the SISEQ software version 1.59 [26]. The alignment [82 OTUs (operational taxonomical units), 458 total sites, among which 452 sites carry phylogenetic signals] was used to infer the phylogeny by the Bayesian Inference method using the MrBayes software version 3.1.2. Phylogenetic reconstruction was also done by the Maximum Likelihood method using the TreeFinder software version November 2007 [27] with the WAG model with ‘Empirical’ option. Neighbour-joining analysis was performed using the MEGA software version 4 [28], with the JTT model.

**Preparation of recombinant proteins**

Recombinant ZmSiR [4,29], PetF protein (an Fd homologue) from the cyanobacterium *Plectonema boryanum* [30] and leaf-type FNR (Fd:NADP⁺ reductase) [31] were prepared using an E. coli expression system as described previously. Cysteine synthetase was purified from the leaves of *Brassica rapa* (field mustard) as described previously [32]. Recombinant CmSiRB and SyNiR were overexpressed in *E. coli* cells. The coding regions of the CmSiRB gene and the *synir* gene were amplified by PCR with the following sets of primers: 5'-GATGAAATTCCGACCCACCCGGGTTCGACCGACC-3' and 5'-GATTCTAGACTACGATACGGCGCTGCCGACGC-3' for the gene CmSiRB, and 5'-GGCTTCAATAGGGGTAATATAA- TTGAG-3' and 5'-CCCGAGCTTCATCAACGCCGACGC-3' for the gene *synir*. The amplified DNA fragments were inserted into an expression vector, pTrc99A, with the *E. coli* cysG (sirohaem synthase) gene. For CmSiRB, the first 17 amino acids were not included in the construct, because they represent the transit peptide, as predicted by TargetP software [33]. The recombinant protein was expected to bear an additional sequence at its N-terminus, namely Met-Glu-Phe, which is encoded by the cloning vector. The recombinant enzymes were purified by a method similar to that used for the preparation of ZmSiR. Bacterial cells suspended in 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl₂, 1 mM EDTA and 0.5% (v/v) 2-mercaptoethanol were disrupted by sonication (using a Branson Sonifier model 450 at level 9 of 50% duty cycle for 3 min) and soluble proteins were collected by centrifugation at 10,000 g for 15 min). The resulting extract was treated with DEAE-cellulose in a batchwise manner to remove acidic substances such as nucleic acids. The unbound fraction containing CmSiRB or SyNiR was fractionated by (NH₄)₂SO₄ precipitation between 30 and 60% saturation. The precipitate was dissolved in 50 mM Tris/HCl, pH 7.5, and desalted by passing it through a Sephacel G-15 column equilibrated with the same buffer. Both enzymes were purified by subsequent anion-exchange and hydrophobic column chromatography using the resins Resource Q and phenyl-Sepharose and 0–0.5 M NaCl and 30–0% saturation. SyNiR was further purified by Fd-Sepharose affinity chromatography.

**Measurement of enzymatic activities**

NiR and SiR activities were measured using a reduction system involving an electron transfer from NADPH to Fd via FnR [11]. Each assay mixture contained 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 200 μM NADPH, 20 μM Fd, 1 μM FnR, a suitable concentration of NiR or SiR and a set of various concentrations of (NH₄)₂SO₄, (NH₄)₂S₂O₇ or Na₂SO₄. The reaction was initiated by the addition of NADPH, and NADPH oxidation was monitored by the decrease in ΔA₅₄₀ at room temperature (~25°C) [11]. For assays measuring a decrease of the substrate or increase of the product, the reaction mixture was incubated for 5 min at 30°C in the presence of an NADPH-regeneration system, 5 mM glucose 6-phosphate and 1 unit of yeast glucose-6-phosphate dehydrogenase (Oriental Yeast). The reaction was started by the addition of the substrates to 2 mM. The decrease in nitrite was measured by colorimetric reaction with N-1-naphthyl ethylenediamine after diazotization with sulfanilamide [11] The sulfide was quantitatively converted into cysteine by cysteine synthetase in the presence of excess O-acetylsersine and resulting cysteine was measured by the acidic ninhydrin reaction [11]. The kinetic parameters Kₘ and Vₘₐₓ were estimated by using a non-linear weighted least-squares method [34]. The computation was done with a perl script written essentially to the algorithm therein.

**Immunoblot analysis**

The *C. merolae* cells grown in the ammonium medium were centrifuged at 2900 g. The pellet was washed and resuspended with 4 mM KH₂PO₄ (pH adjusted to 2.5 by H₂SO₄). The seed
cell suspension was inoculated into the ammonium medium and the nitrate medium to a final attenuation ($D_{730}$) of 0.1. The cells were then grown to a $D_{730}$ of 0.5. Aliquots of the cultures were centrifuged at 22,000 g for 1 min, and then the pellet was resuspended in 1% (w/v) SDS sample buffer. The protein was analysed by SDS/PAGE and immunoblotting as described previously [13]. The polyclonal antiserum raised against synthetic peptides KARASTRLSKS and QRYWDGPAPA, corresponding to portions of CmSiRA (647–658) and CmSiRB (667–678) respectively, were used as primary antibodies.

Nitrite consumption by intact cells

The rate of nitrite consumption by intact cells was measured by the rate of uptake of nitrite in the assay medium as described previously [35], with the following modifications. The cells were collected by centrifugation at 2900 g for 20 min and resuspended to a final $D_{730}$ of 0.25 in 4 mM KH$_2$PO$_4$/0.2 M sorbitol, pH 4.2 (for C. merolae), or 4 mM KH$_2$PO$_4$, pH 7.5 (for Synechocystis). The incubation temperature was 42°C (for C. merolae) or 30°C (for Synechocystis). A 10–15 ml sample in a Petri dish was pre-incubated for 20 min with shaking under an illumination of 50 μE·m$^{-2}$·s$^{-1}$ before the reaction was started. Aliquots of 1 ml were dispensed into multiwell culture plates, and then the reaction was started by the addition of 0–500 μM NaN$_3$. Aliquots of the reaction mixtures at the start of incubation or after 30 min incubation were centrifuged at 22,000 g for 1 min to remove the cells. The concentration of nitrate was measured by diazotization of sulfanilamide, which was then allowed to react with N-(1-naphthyl)ethylenediamine for colorimetric determination [11]. The kinetic parameters $K_m$ and $V_{max}$ were estimated using the non-linear weighted least-squares method.

RESULTS

CmSiRB has structural features typical of SiR

NiRs and SiRs both contain sirohaem as a prosthetic group, and an amino acid sequence similarity is found throughout the proteins (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/423/bj4230091add.htm). However, the two enzymes have some distinct regions. We found a number of regions that are conserved in SiRs, including CmSiRB, but are lacking in NiRs. The similarity of CmSiRB to other SiRs and NiRs are 55–65 and 29–41%, respectively. X-ray crystallographic studies on the haemoprotein subunit of E. coli SiR [6–8] and the spinach NiR [10] have revealed key amino acid residues contributing to the active centres. The four cysteine residues involved in the binding of the [4Fe-4S] cluster and sirohaem are conserved in CmSiRB (Cys$^{334}$, Cys$^{540}$, Cys$^{580}$ and Cys$^{584}$) as well as in the NiRs and SiRs. Two arginine residues, one histidine residue and two lysine residues that are considered to be involved in substrate binding are largely conserved in the SiRs. They are Arg$^{153}$, His$^{192}$, Arg$^{222}$, Lys$^{112}$ and Lys$^{314}$ in CmSiRB. Although both arginine residues and the first lysine residue are conserved in NiRs and SiRs, the histidine residue and the second lysine residue are replaced by an arginine residue and an asparagine residue respectively in NiRs.

Phylogenetic analysis of NiRs and SiRs in bacteria and plants (Figure 1) revealed that the two enzymes form clearly defined monophyletic clades. Dissimilatory SiRs belong to a different protein family and are not related to the NiRs and SiRs that are discussed in the present study. In both NiRs and SiRs, the plant enzymes appear to have originated from their cyanobacterial counterparts. Cyanobacterial and plant SiRs are monophyletic and sister to other bacterial enzymes. The two putative SiRs of C. merolae are highly related and are diversified after the separation of the red algae from the other algae. There is no homologue of plant and cyanobacterial NiR in C. merolae. The phylogenetic analysis as well as the sequence analysis clearly showed that the two enzymes in C. merolae are within the clade of SiRs rather than that of NiRs. The results also indicate that the plant and cyanobacterial SiRs and NiRs are clearly distinct enzymes that were probably split before the diversification of cyanobacteria.

Redox activity of CmSiRB

We prepared recombinant CmSiRB using the E. coli co-expression system with the E. coli cysG gene [32], which encodes a uroporphyrinogen III methyltransferase involved in the synthesis of sirohaem. UV–visible absorption spectrometry (see Supplementary Figure S2 at http://www.BiochemJ.org/bj/423/bj4230091add.htm) showed that the recombinant CmSiRB had absorption maxima at 382 and 583 nm, which are attributed to the sirohaem group, as observed for a typical NiR and SiR. The contribution of the [4Fe-4S] cluster to the absorption spectra was not evident. This indicates that the recombinant CmSiRB is assembled with the prosthetic groups as a holoenzyme. The absorption maximum of CmSiRB (583 nm) was closer to that of ZmSiR (581 nm) than that of SyNiR (573 nm). This is also an indication that CmSiRB is more similar to SiR than it is to NiR. It was difficult to express CmSiRA as a holo form in E. coli cells, and CmSiRA was not analysed in the present study.

To investigate the enzymatic properties of CmSiRB we tried initially to measure Fd-dependent NiR and SiR activities by using Fd reduced with dithionite [36], but negligible activity was detected. In this method, oxidative breakdown of dithionite results in significant accumulation of sulfite in the assay mixture. The effect of sulfite on the enzymatic activity was found to be critical for the assay of CmSiRB as described below. Therefore we applied an NADPH/FNR/Fd reduction system for the supply of reducing equivalents to CmSiRB as established in [11].

This electron-transfer system was reconstructed by the addition of NADPH and FNR to the reaction mixture instead of dithionite and thus the enzyme reaction was monitored by following the decrease in $A_{400}$ due to the oxidation of NADPH. The activity of CmSiRB for the reduction of nitrite and sulfite was measured and compared with those of ZmSiR and SyNiR. A significant rate of NADPH oxidation was found without added nitrite or sulfite, owing to a leak of electrons to some oxidants, such as oxygen, from the system. However, we could detect the activity of the enzymes with this system, as listed in Table 1. ZmSiR showed an approx. 5-fold higher activity for sulfite than for nitrite, whereas SyNiR showed negligible sulfite-reducing activity. CmSiRB showed a relatively high nitrite-reducing activity, that is, approx. seven times higher than that of ZmSiR and approx. four times lower than that of SyNiR, but very little sulfite-reducing activity. In the presence of sulfite, the nitrite-reducing activity of CmSiRB was remarkably lowered to 13%. Such a phenomenon was not observed for SyNiR, which is indicative of a specific inhibition of CmSiRB by sulfite. It is noteworthy that we often observed that the rate of NADPH oxidation by CmSiRB was slightly, but reproducibly, lower in the presence of sulfite than in the absence of any substrate. This suggested the possibility that sulfite affects the electron-transfer process from Fd to CmSiRB or an auto-inhibition of CmSiRB to bypass electrons to O$_2$ present in the assay mixture. Therefore a low activity of CmSiRB in the sulfite reduction, if any, was not correctly measured by this assay system.

Direct measurement of sulfite and nitrite reduction by CmSiRB

Enzymatic activity was directly measured by quantification of substrate used in the assay in the presence of an...
Figure 1  Phylogenetic tree of assimilatory SiRs and NiRs

Selected cobalamin-biosynthetic enzyme (CobG) sequences were taken as an outgroup. This tree was estimated by the Bayesian Inference method using the MrBayes software version 3.1.2. The MCMC (Markov chain Monte Carlo) run was performed 2,000,000 times with the following parameters: nst = 6; rates = invgamma; aamodelpr = fixed(WAG); samplefreq = 200; and burnin = 3000. The average S.D. of split frequencies was 0.010461. The branches supported by high clade credibility values (≥ 0.95) are shown by thick lines. The numbers on each split indicate confidence levels in percentage obtained by maximum likelihood (ML) analysis by TreeFinder and neighbour-joining (NJ) analysis by MEGA (Molecular Evolutionary Genetics Analysis). The bar in the upper left corner is the distance scale.

NADPH-regeneration system. Figures 2(A) and 2(B) show substrate-dependence of nitrite and sulfite reduction respectively. CmSiRB was found to have both nitrite- and sulfite-reducing activity, which had not been detected by the NADPH oxidation system. The kinetic parameters determined by non-linear regression analysis are listed in Table 2. The $K_m$ values for nitrite of CmSiRB (221 μM) and ZmSiR (416 μM) were higher than that of SyNiR (37 μM). As expected, the $K_m$ value for sulfite of ZmSiR was low (74 μM), and that of CmSiRB was much lower (8.7 μM). These results indicated that, in terms of $K_m$, CmSiRB has a two orders of magnitude higher affinity for sulfite than for nitrite. By contrast, the $k_{cat}$ value of CmSiRB for sulfite was two orders of magnitude lower than that for nitrite. The $k_{cat}$ for nitrite of CmSiRB was approx. 4-fold higher than that of ZmSiR and approx. 4-fold lower than that of SyNiR, whereas the $k_{cat}$ for sulfite of CmSiRB was 52-fold lower than that of ZmSiR. This indicated that CmSiRB has a relatively high nitrite-reducing activity and a low sulfite-reducing activity compared with general SiRs. It should be noted, however, that CmSiRB definitely had a sulfite-reducing activity, whereas SyNiR showed undetectable

© The Authors. Journal compilation © 2009 Biochemical Society
levels of sulfite reduction. In this respect, CmSiRB is a variant type of SiR, having enhanced ability of nitrite reduction and weakened ability of sulfite reduction. In fact, the nitrite-reducing activity of CmSiRB was inhibited by sulfite (Figure 2A), whereas that of SyNiR was not affected (see Supplementary Figure S3 at http://www.BiochemJ.org/bj/423/bj4230091add.htm). The apparent \( K_m \) of CmSiRB in the presence of 125 \( \mu M \) sulfite was very high (918 \( \mu M \)), and the apparent \( k_{cat} \) of CmSiRB in the presence of sulfite was similar to that in the absence of sulfite (Table 2). This indicates that sulfite competitively inhibits the nitrite reduction by CmSiRB.

**Role of CmSiRB in intact cells**

We confirmed the characteristics of CmSiRB described above *in vivo*. Accumulations of CmSiRA and CmSiRB in *C. merolae* cells grown in medium containing either ammonium or nitrate as a sole nitrogen source were examined by immunoblotting analysis. CmSiRA was detected in both media, whereas CmSiRB was detected only in the nitrate medium (Figure 3). This suggests that CmSiRB is nitrate-inducible and may be involved in nitrate assimilation.

The rate of nitrite consumption by the intact cells was measured. In this assay, the pH of the assay medium for *C. merolae* was adjusted to 4.2, because nitrite was lost as nitric oxide at a low pH, such as 2.5, of the normal growth medium. The assay medium contained sorbitol to compensate for osmotic balance at this elevated pH, as described previously [37]. Figure 4(A) shows that the rate of nitrite consumption by the cells grown in the nitrate medium was three times higher than that of cells grown in the ammonium medium. This increase could be accounted for by the expression of CmSiRB (Figure 3). Figures 4(B) and 4(C) show substrate-dependence of the rate of nitrite consumption by *C. merolae* and *Synechocystis* respectively. The kinetic parameters determined by non-linear regression analysis are listed in Table 3. The apparent \( K_m \) value for nitrite of *C. merolae* cells was 34 \( \mu M \), whereas that of *Synechocystis* was 11 \( \mu M \). When sulfite was added to the assay medium, the rate of nitrite consumption in *C. merolae* was significantly decreased, whereas that in *Synechocystis* was not affected. The \( K_m \) value for nitrite consumption was drastically increased by sulfite, but the apparent \( V_{max} \) value was affected.

**Table 1** NADPH oxidation rate of FNR–Fd-coupled CmSiRB, ZmSiR and SyNiR

Each value is the mean ± S.E.M. for three replicate experiments. The concentration of the substrates was 2 \( mM \). Statistical analysis by Student's t test indicated that all values in each column were significantly different at \( P < 0.05 \), except the pairs indicated by \( a \), \( b \) and \( c \).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>CmSiRB</th>
<th>ZmSiR</th>
<th>SyNiR</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>49 ± 1.6</td>
<td>43 ± 2.9</td>
<td>40 ± 2.6 ( a )</td>
</tr>
<tr>
<td>( \text{NO}_2^- )</td>
<td>262 ± 3.1</td>
<td>75 ± 9.7</td>
<td>942 ± 4.6 ( b )</td>
</tr>
<tr>
<td>( \text{SO}_3^{2-} )</td>
<td>28 ± 0.9</td>
<td>189 ± 8.1</td>
<td>42 ± 4.6 ( b )</td>
</tr>
<tr>
<td>( \text{NO}_2^- + \text{SO}_3^{2-} )</td>
<td>76 ± 5.4</td>
<td>186 ± 8.7</td>
<td>928 ± 13 ( c )</td>
</tr>
</tbody>
</table>

**Table 2** Kinetic parameters of CmSiRB, ZmSiR and SyNiR

\( K_m \) and \( k_{cat} \) values and the individual S.D. values were estimated from the data from three replicate experiments by using a non-linear weighted least-squares method. The sulfite-reducing activity of SyNiR was below the detection limit, and no kinetic parameters were obtained. The values in parentheses are apparent \( K_m \) and \( k_{cat} \) values for nitrite of CmSiRB in the presence of 125 \( \mu M \) sulfite. Statistical analysis by Student's t test indicated that all values in each column were significantly different at \( P < 0.05 \), except the pair indicated by \( a \).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>( K_m (\mu M) )</th>
<th>( k_{cat} ) (mol of product · min(^{-1}) · mol of enzyme(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>CmSiRB</td>
<td>221 ± 36 ( \mu M )</td>
<td>8.7 ± 1.6 ( \mu M )</td>
</tr>
<tr>
<td>+ Sulfite</td>
<td>(918 ± 203) ( \mu M )</td>
<td>243 ± 13 ( \mu M )</td>
</tr>
<tr>
<td>ZmSiR</td>
<td>416 ± 92 ( \mu M )</td>
<td>74 ± 5.6 ( \mu M )</td>
</tr>
<tr>
<td>SyNiR</td>
<td>37 ± 6.3 ( \mu M )</td>
<td>1077 ± 53 ( \mu M )</td>
</tr>
</tbody>
</table>
Table 3  Kinetic parameters of the apparent rate of nitrite consumption by intact cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Apparent $K_m$ ($\mu$M)</th>
<th>Apparent $V_{\text{max}}$ ($\mu$M·min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. merolae</td>
<td>34 ± 4.0</td>
<td>0.98 ± 0.037</td>
</tr>
<tr>
<td>+Sulfite</td>
<td>(141 ± 29)</td>
<td>(0.84 ± 0.082)</td>
</tr>
<tr>
<td>Synechocystis</td>
<td>11 ± 4.3$^3$</td>
<td>0.45 ± 0.052$^2$</td>
</tr>
<tr>
<td>+Sulfite</td>
<td>(11 ± 4.8)$^3$</td>
<td>(0.49 ± 0.061)$^2$</td>
</tr>
</tbody>
</table>

Figure 3 Immnoblotting analysis of CmSiRA and CmSiRB in C. merolae cells

Cell extract containing equal amounts of protein (50 $\mu$g) from the cells grown in the nitrate medium and the ammonium medium was loaded in each lane. The immunodetection was performed using antibodies against CmSiRA and CmSiRB.

only to a small extent. This suggests that sulfate is acting as a competitive inhibitor against nitrite consumption. These in vivo observations are consistent with the molecular characteristics of CmSiRB and SyNiR (Figure 2 and Supplementary Figure S3).

DISCUSSION

Unique properties of CmSiRB

The present paper reports a novel property of CmSiRB, which is structurally a sirohaem-containing sulfite reductase, but acts as a nitrite-reducing enzyme in vitro (Table 2). This enzyme has a high affinity for sulfite but virtually no sulfite-reducing activity, whereas it has a high catalytic-centre activity (‘turnover number’) for nitrite reduction, in spite of a rather low affinity for nitrite. In this respect, CmSiRB is unique among various SiRs and NiRs. There has been no report of the effects on enzymatic activities of NiR and SiR by a combination of nitrite and sulfate. In the present study, the nitrite-reducing activity of SyNiR was not affected by the presence of sulfate, owing to its strict substrate specificity. On the other hand, SiR is known to have a low nitrite-reducing activity. The results obtained in the present study are therefore not completely incompatible with the notion of a wide substrate range for SiR. However, the fact that sulfite acts as a competitive inhibitor of nitrite reduction in CmSiRB is a novel aspect of this enzyme.

It is clear that NiR and SiR share a common origin before the diversification of bacteria (Figure 1). However, the two enzymes belong to different clades and are clearly distinguished by their substrate specificity (Table 2). With respect to the sequence features (Figure 1 and Supplementary Figure S1), CmSiRB is definitely a member of the SiR family. It diverged from CmSiRA within the red-algal clade. The fact that CmSiRB has a strong affinity for sulfite is consistent with this origin (Figure 2 and Table 2). Transition from SiR to NiR or vice versa has not, to our knowledge, been previously reported. Therefore the case of CmSiRB is special in the evolution of this group of enzymes (Figure 1).

Physiological role of CmSiRB as a nitrite-reducing enzyme

There was a mystery with respect to the physiological enzyme that reduces nitrite within the C. merolae cells, because there is no NiR-family protein that is encoded by the complete C. merolae genome. However, the CmSiRB gene, which is mapped...
between the two genes related to nitrate assimilation, the gene for a nitrate transporter (CMG018C) and the gene for nitrate reductase (CMG019C), was a candidate for the gene encoding a nitrite-reducing enzyme. The in vitro enzymatic activity of CmSiRB supports the notion that it is a candidate for the physiological nitrite-reducing enzyme in vivo. The accumulation of CmSiRB within the cells grown in the nitrate medium also supports this possibility (Figure 3).

The kinetic analysis of nitrite consumption in intact cells supports the notion that CmSiRB is the major enzyme involved in physiological nitrite reduction (Table 3). The high nitrite-reducing activity in the nitrate-grown cells suggests an important role of CmSiRB in nitrite reduction (Figure 4A). The competitive inhibition of nitrite consumption by sulfite in C. merolae cells also supports the major role of CmSiRB in nitrite reduction in vivo (Figure 4B).

However, the kinetic parameters of CmSiRB in vitro and those of nitrite consumption of intact cells were significantly different. The apparent $K_m$ value of nitrite consumption in C. merolae cells (Table 3) was approximately one order of magnitude lower than the value obtained with purified enzyme (Table 2). There are several explanations for this discrepancy. A naive intuition may be that the enzyme produced in E. coli cells might not be fully active because of various problems in heterologous expression. However, the apparent high affinity obtained with intact cells may be explained by the difference between the pH of the medium and that of the cytoplasm. The pH of the assay medium was 4.2, whereas the intracellular pH of C. merolae is assumed to be neutral or alkaline (it is known that the stromal pH rises above 8.0 in plant chloroplasts). Assuming the equilibrium of NO$_2^-$ and HNO$_2$ ($pK_a = 3.29$ at $25^\circ$C), free acid present in the acidic medium enters the cells (neutral molecules are known to freely penetrate cell membranes), but, after neutralization within the cell (cytoplasm or chloroplast), NO$_2^-$ is kept within the cells. A simple calculation predicts that the accumulation ratio of nitrite within the cells with respect to within the medium is inversely proportional to the ratio in proton concentration in the cells and in the medium. Thus, the actual intracellular concentration of nitrite may be higher than that in the medium, and this is the reason the apparent $K_m$ value of nitrite consumption by C. merolae cells was comparable with the value for Synechocystis.

We also noted that the activity of nitrite consumption by the ammonium-grown cells was not negligible (Figure 4A), despite virtually no expression of CmSiRB. Because the activity increased with incubation time, the activity might be rapidly induced after the start of incubation with nitrite. Another possibility is that CmSiRA might also have a certain level of nitrite-reducing activity, as other SiRs do. However, it is difficult to produce an active recombinant CmSiRA in E. coli cells, although we repeated the experiment with different constructs. This could be due to DNA-binding activity that is detected in various SiRs, such as pea SiR [14]. Since the cells of C. merolae live in sulfate-rich hot springs, nitrite reduction by CmSiRB is expected to be repressed by intracellular sulfite. C. merolae might have another system of nitrite reduction that is resistant to sulfite. We still do not know whether this is CmSiRA or some other protein.

Comparison of CmSiR B with NiR and SiR

The results of the present study are interesting in the light of structural determinants of substrate specificities of NiR and SiR. In a previous study [4], the mutant ZmSiRs, whose Arg$^{193}$ was replaced by a glutamic acid residue (R193E) and by an alanine residue (R193A), were analysed. R193E showed a limited, but significantly increased, affinity for nitrite, which resulted in a remarkable increase in nitrite-reducing activity. In contrast, R193A showed a remarkable increase in the affinity for nitrite, but the nitrite-reducing activity was not significantly changed. In addition, both mutant ZmSiRs completely lost their sulfite-reducing activity. These results suggest that a single mutation can cause significant changes in substrate specificity and could be a clue to the explanation of the substrate preferences of CmSiRB. In this respect, the corresponding residue, Arg$^{232}$, is conserved in CmSiRB. Other residues must be responsible for the altered substrate specificity of CmSiRB. This fact indicates that the cause of difference in substrate specificity between NiR and SiR is not as simple as a difference in a single amino acid residue. On the basis of a comparison between the three-dimensional structures of SiR haemoprotein and Fd:NiR, Swamy et al. [10] discussed the structural reason for the difference in substrate binding. For example, Ile$^{241}$, Val$^{242}$, Thr$^{142}$ and His$^{238}$ in spinach NiR are replaced by Ala$^{352}$, Ala$^{354}$, Asn$^{116}$ and Asp$^{229}$ in E. coli SiR haemoprotein, which contribute to the backbone shifts and charge distribution on the distal side of the sirohaem group. The first two residues are replaced by threonine and proline in CmSiRB as in all land-plant SiRs in maize, tobacco (Nicotiana tabacum) and Arabidopsis (Supplementary Figure S1). Thr$^{142}$ in spinach NiR is conserved in CmSiRB as in all NiRs and SiRs except E. coli SiR haemoprotein. His$^{238}$ in spinach NiR is replaced by aspartic acid in CmSiRB, as in all SiRs. These examples suggest that the structure of CmSiRB may be very similar to Fd-dependent SiRs, but the special enzymatic properties of CmSiRB cannot be explained by these residues. However, some other residues of CmSiRB are different from the conserved ones. Cys$^{230}$ and Ser$^{231}$ are neighbours of Arg$^{232}$, which is one of the basic residues contributing to the substrate binding. At the position of Cys$^{230}$ in CmSiRB, aliphatic amino acids such as leucine and isoleucine are conserved in other Fd:SiRs, including CmSiRA, and an asparagine residue is conserved in NiRs. At the position of Ser$^{231}$, an asparagine residue and a valine residue are conserved in SiRs and NiRs respectively. Ile$^{232}$ is replaced by a proline residue in all other SiRs, including CmSiRA. This position corresponds to the end of an a-helix in E. coli SiR haemoprotein and is close to the specific region in SiRs lacking in NiRs. Structural analysis of CmSiRB will shed light on the determinants of its unusual substrate specificity.

AUTHOR CONTRIBUTION

Kohsuke Sekine, Toshiharu Hase and Naoki Sato designed the experiments. Kohsuke Sekine performed most of the experiments and analysed the data. Naoki Sato performed the phylogenetic analysis. Yukiko Sakakibara and Toshiharu Hase undertook the preparation of ZmSiR, SyNiR, FNR and PetF. Kohsuke Sekine and Naoki Sato wrote the paper.

FUNDING

This work was performed under the Cooperative Research Program of the Institute for Protein Research, Osaka University and supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT) [grant numbers 17018010, 18017005, 20017006, 16GS0304 (Grants-in-Aid for Scientific Research to N.S.)]; and the Japan Science Society [grant number 21-429 (Sasakawa Scientific Research Grant to K.S.).]

REFERENCES


© The Authors Journal compilation © 2009 Biochemical Society

© The Authors. Journal compilation © 2009 Biochemical Society
SUPPLEMENTARY ONLINE DATA
A novel variant of ferredoxin-dependent sulfite reductase having preferred substrate specificity for nitrite in the unicellular red alga Cyanidioschyzon merolae

Kohsuke SEKINE*, Yukiko SAKAKIBARA‡, Toshiharu HASE‡ and Naoki SATO†

*Center for Structuring Life Sciences, Graduate School of Arts and Sciences, the University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan, †Department of Life Sciences, Graduate School of Arts and Sciences, the University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan, and ‡Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita-shi, Osaka 565-0871, Japan

† To whom correspondence should be addressed (email naokisat@bio.c.u-tokyo.ac.jp).

© The Authors Journal compilation © 2009 Biochemical Society
The sequence of CmSiRB is compared with those of SyNiR, spinach NiR (SoNiR; GenBank accession no. X07568), Arabidopsis thaliana NiR (AtNiR; GenBank accession no. NM_127123), Lotus japonicus NiR (LjNiR; GenBank accession no. AJ293240), CmSiRB, CmSiRA, ZmSiR, tobacco SiR (NtSiR; GenBank accession no. D83583), A. thaliana SiR (AtSiR; GenBank accession no. NM_120541), Synechocystis sp. PCC6803 SiR (SySiR; CyanoBase accession no. slr0963) and E. coli SiR haemoprotein (EcCysI; GenBank accession no. AAA27047). Shading in light grey, dark grey and black indicate the residues conserved in the NiRs, those conserved in the SiRs (with or without CmSiRs and EcCysI) and those conserved in all of the sequences respectively. The unaligned C- and N-terminal extensions are not shown. The amino acid residues supposedly contributing to the binding of the substrate are indicated by /H17006. The cysteine residues predicted to be ligands for the [4Fe-4S] cluster and the sirohaem group are indicated by arrows (↓). Asterisks (*) indicate the residues of CmSiRB that are different from the residues conserved in either the four NiRs or the four SiRs. Crosses (+) indicate the residues of SyNiR reportedly contributing to the backbone shifts and charge distribution on the distal side of the sirohaem group [1].

Figure S1  Comparison of amino acid sequences of selected SiRs and NiRs

<table>
<thead>
<tr>
<th>Protein</th>
<th>Accession No.</th>
<th>Shading</th>
<th>Residues</th>
<th>Cysteine Ligands</th>
<th>Backbone Shifts and Charge Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>SyNiR</td>
<td></td>
<td>Light grey</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SoNiR</td>
<td>X07568</td>
<td>Dark grey</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AtNiR</td>
<td>NM_127123</td>
<td>Black</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LjNiR</td>
<td>AJ293240</td>
<td>Light grey</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CmSiRB</td>
<td></td>
<td>Dark grey</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CmSiRA</td>
<td></td>
<td>Black</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZmSiR</td>
<td></td>
<td>Light grey</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NtSiR</td>
<td>D83583</td>
<td>Dark grey</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AtSiR</td>
<td>NM_120541</td>
<td>Black</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SySiR</td>
<td>slr0963</td>
<td>Light grey</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EcCysI</td>
<td>AAA27047</td>
<td>Dark grey</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CmSiRB</td>
<td></td>
<td>Black</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SyNiR</td>
<td></td>
<td>Light grey</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SoNiR</td>
<td>X07568</td>
<td>Dark grey</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AtNiR</td>
<td>NM_127123</td>
<td>Black</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LjNiR</td>
<td>AJ293240</td>
<td>Light grey</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CmSiRB</td>
<td></td>
<td>Dark grey</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CmSiRA</td>
<td></td>
<td>Black</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZmSiR</td>
<td>D83583</td>
<td>Light grey</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NtSiR</td>
<td>NM_120541</td>
<td>Dark grey</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AtSiR</td>
<td>NM_120541</td>
<td>Black</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SySiR</td>
<td>slr0963</td>
<td>Light grey</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EcCysI</td>
<td>AAA27047</td>
<td>Dark grey</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

© The Authors. Journal compilation © 2009 Biochemical Society
A sulfite reductase having increased nitrate reductase activity

**Figure S2** UV-visible absorption spectra of purified recombinant CmSiRB, ZmSiR and SyNiR

The absorption maxima of the α band of sirohaem are indicated by arrows. An arrowhead indicates the Soret band.

**Figure S3** Inhibition of nitrite reduction of CmSiRB (A) and SyNiR (B) by sulfite

The assay was carried out in the presence of 1 mM NaNO₂ and the presence or absence of various concentrations of Na₂SO₃, as in Figure 3 of the main text. The activity is expressed as a percentage of the reaction rate in the absence of Na₂SO₃.

**REFERENCE**


Received 14 April 2009/2 July 2009; accepted 21 July 2009
Published as BJ Immediate Publication 21 July 2009, doi:10.1042/BJ20090581