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Site-directed mutagenesis of cysteine to serine in a superoxide responsive transcriptional regulator SoxR

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Abstract

SoxR protein in *Escherichia coli*, which is a transcriptional activator for the transcription of *soxS*, contains four cysteine residues at positions 119, 122, 124 and 130. These cysteines have been separately mutated into serine by the site-directed mutagenesis. The wild type and the mutant SoxR proteins were expressed in *E. coli* JM109 with pKK223-3 based expression vectors containing a *tac* promoter system. Purified four cysteine-to-serine mutant SoxR proteins do not contain the iron–sulfur cluster though the wild type SoxR expressed in this system contains a [2Fe–2S] cluster, which shows that all of the four cysteine at positions 119, 122, 124 and 130 are the ligand of the [2Fe–2S] cluster in SoxR.

Keywords: SoxR; [2Fe–2S] cluster; Site-directed mutagenesis; Transcriptional regulator

1. Introduction

Iron–sulfur clusters are one of the popular prosthetic groups in metalloproteins which are involved in redox reactions [1]. Although the most popular function of iron–sulfur clusters is electron transfer, non-redox functions of iron–sulfur clusters have been reported [2]. For example, the iron–sulfur cluster in aconitase [3] and endonuclease III [4] has a function of a Lewis acid with providing the substrate binding site, and has a structural role, respectively.

Recently, a novel iron–sulfur proteins have been discovered, which function is the regulation of gene expression [5–11]. Iron-responsive element binding protein, which is expressed ubiquitously in various tissues and species [5,6], and FNR in *Escherichia coli* [7,8] contain a [4Fe–4S] cluster. A transcriptional regulator SoxR in *E. coli* has been reported to contain a [2Fe–2S] cluster [9–11].

SoxR controls the expression of *soxRS* regulon in *E. coli* which includes the genes responding to the oxidative stress, especially superoxide stress, such as the gene encoding Mn-containing superoxide dismutase (Mn-SOD), DNA repair endonuclease IV, glucose-6-phosphate dehydrogenase, the *micF* antisense RNA which suppresses synthesis of the OmpF outer

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membrane porin, ferredoxin (flavodoxin): NADPH oxidoreductase, and an oxidatively stable fumarase (FumC) [12,13].

The [2Fe–2S] cluster in SoxR has been suggested to act as a superoxide sensor and to regulate the activity of SoxR as a transcriptional activator by changing the oxidation state of the cluster [9,10,14–19]. The microenvironment around the iron–sulfur cluster and the ligand of the cluster are important factors to adjust the redox properties of the [2Fe–2S] cluster. However neither the ligand of the [2Fe–2S] cluster nor the molecular structure of SoxR is not obvious. In this work, we prepared four mutant SoxR proteins, in which cysteine residue was replaced by serine by site-directed mutagenesis, to determine the ligand of the [2Fe–2S] cluster in SoxR.

2. Experimental

2.1. Materials and instrumentation

CM-Sepharose, Sephacryl S-100, HiTrap Heparin column, and pKK223-3 were obtained from Pharmacia Biotech. TA Cloning kit and Chameleon double-stranded site-directed mutagenesis kit were provided by Invitorogene and Stratagene, respectively. A protein assay kit by the method of Bradford and molecular weight marker for SDS-PAGE were obtained from Bio Rad. The synthetic oligonucleotides were supplied by Curuachem. YM30 membranes were obtained from Amicon.

The UV-Visible absorption spectra and CD spectra were measured on a Hitachi U-3500 and JASCO J-720, respectively. DNA sequencing analysis was carried out by an ABI 373A.

2.2. Plasmids and strains

Two oligonucleotides (a, 5′-GAGGTAAAGCGACATATGGAAAAGAAATTACCCCGC-3′; b, 5′-GCGGGATCCAGAGAAAGACAAAGACC-3′), which were designed according to the DNA sequence of *soxR* [20], were used as primers in a polymerase chain reaction (PCR) to synthesize *soxR* gene. The chromosomal DNA isolated from *E. coli* JM109 was used as the template DNA in the PCR. The PCR product was cloned into pCRII-vector by TA Cloning kit to form pCRSoxR plasmid. The DNA fragment containing *soxR* gene excised from pCRSoxR by *Eco*RI was ligated into *Eco*RI-digested pKK223-3 to form the SoxR expression vector, pKKSoxR. The direction and the nucleotide sequence of *soxR* in pKKSoxR were confirmed by DNA sequencing analysis.

The mutant *soxR* genes were prepared by a Chameleon double-stranded site-directed mutagenesis kit with pKKSoxR as the template plasmid according to the manufacturer's protocol. These constructs were sequenced to ensure that no replication errors and desired mutation were introduced.

Strains were grown in LB medium, and ampicillin (50 μg/ml) was added when appropriate. For overproduction of the wild type and mutant SoxR proteins, *E. coli* JM109 was used as a host cell. The cells were grown at 37°C until they reached $OD_{600} \sim 0.8$, and then incubated 20 h at 20°C with 1 mM IPTG (isopropyl-b-D-thiogalactopyranoside) to induce the expression of SoxR. The cells were collected by centrifugation and were stored at −80°C after washing with 50 mM KH_2PO_4–NaOH buffer (pH 7.6).

2.3. Purification of proteins

The cells were resuspended in buffer A (50 mM KH_2PO_4–NaOH buffer (pH 7.6)) with 4 mM $MgCl_2$ and deoxyribonuclease I (10 μg/ml), and were disrupted by sonication. Cell debris were removed by centrifugation (33 000 $\times g$, 20 min, 4°C). The supernatants were applied to a CM-Sepharose column (2.6 × 35 cm) preequilibrated with buffer A. After washing the column with 3-bed volume of buffer A, adsorbed proteins were eluted with a linear gradient of NaCl from 0 to 1 M at flow rate of 1

ml/min. The eluted solutions were collected in 10 ml of fractions. The fractions containing SoxR, which were checked by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), were combined and concentrated to about 2 ml by ultrafiltration with a YM30 membrane. The concentrated sample was applied on a Sephacryl S-100 column (1.6×93 cm) preequilibrated with buffer B (50 mM KH_2PO_4–NaOH buffer (pH 7.6) containing 0.2 M NaCl), and eluted with buffer B at flow rate of 0.15 ml/min. The fractions containing SoxR were combined and applied to a 1 ml of HiTrap Heparin column at flow rate of 0.5 ml/min. The column was washed with 10 ml of buffer B and then 10 ml of buffer A containing 0.35 M NaCl. The adsorbed proteins were eluted with buffer A containing 0.5 M NaCl at flow rate of 0.5 ml/min. The eluted solutions were collected in 1.25 ml of fractions. The fractions containing SoxR were combined and concentrated by ultrafiltration with a YM30 membrane. The purified SoxR was dissolved in buffer B. The protein concentration was determined by the method of Bradford with a protein assay kit using bovine gamma globulin as a standard.

3. Results and discussion

The expression system constructed in this work successfully worked and the recombinant SoxR proteins were expressed in soluble fraction of *E. coli* cells. When the soluble extracts prepared by sonication were directly loaded on a cation-exchange column (CM-Sepharose), both of the wild type and mutant SoxR proteins were adsorbed on a CM-Sepharose column. The recombinant SoxR proteins were further purified by a gel filtration and a HiTrap Heparin column to nearly homogeneous state as shown in Fig. 1.

The UV-Visible absorption spectra of the wild type SoxR and SoxR C124S, in which cysteine at position 124 was replaced by serine, are shown in Fig. 2. The wild type SoxR at oxidized state revealed four absorption maxima

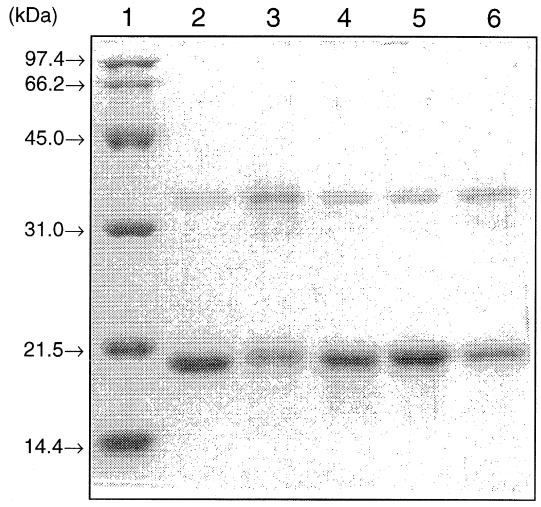

Fig. 1. SDS-PAGE of purified SoxR proteins. Lanes (1) molecular weight marker, (2) wild type SoxR, (3) SoxR C119S, (4) SoxR C122S, (5) SoxR C124S, and (6) SoxR35y C130S.

in the visible region as shown in Fig. 2(a), which is characteristic for iron–sulfur proteins containing [2Fe–2S] cluster. This characteristic spectrum shown in Fig. 2(a) indicates that the recombinant SoxR was expressed as a holo-form containing an iron–sulfur cluster as a prosthetic group in the expression system described in this work. Oxidized SoxR revealed an induced CD spectrum in the visible region as shown in Fig. 3(a), which was similar to that of some [2Fe–2S] type ferredoxins [21–23]. The induced CD spec-

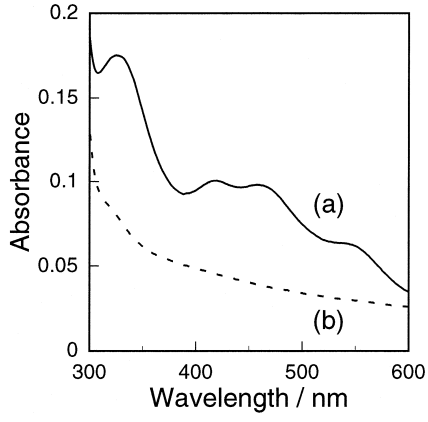

Fig. 2. Electronic absorption spectra of (a) the wild type SoxR (17 μM) and (b) SoxR C124S (17 μM) in 50 mM KH_2PO_4–NaOH buffer (pH 7.6) containing 0.2 M NaCl.

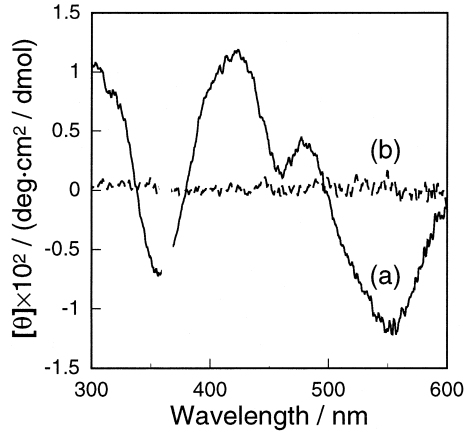

Fig. 3. CD spectra of (a) the wild type SoxR (17 μM) and (b) SoxR C124S (17 μM) in 50 mM KH_2PO_4–NaOH buffer (pH 7.6) containing 0.2 M NaCl.

trum also indicate the existence of the [2Fe–2S] cluster in SoxR.

SoxR C124S showed no absorption maxima in the visible region unlike the wild type SoxR as shown in Fig. 2(b). No induced CD signals in the visible region were also observed in the case of SoxR C124S as shown in Fig. 3(b). Other mutants (SoxR C119S, C122S, and C130S) revealed the identical results to SoxR C124S for UV-Visible and CD spectra. These results show that the mutant SoxR proteins prepared in this work do not contain the [2Fe–2S] cluster, which suggest that all of the four cysteine residues at position 119, 122, 124 and 130 ligate the [2Fe–2S] cluster in SoxR. The possibility that the disruption of the [2Fe–2S] cluster is caused by the second effect of the mutation cannot be ruled out. In this case, the mutation may cause local conformational change to disrupt the [2Fe–2S] cluster though the mutated cysteines are not the ligand of the [2Fe–2S] cluster. If this is the case, at least one ligand of the [2Fe–2S] cluster will be another amino acid than cysteine. However, the coordination of another amino acid than cysteine to the [2Fe–2S] cluster in SoxR seems to be unlikely because the reported EPR spectrum of SoxR [11,15,17] resembles that of adrenodoxin and putidaredoxin, in which all of the ligands to the [2Fe–2S] cluster are cysteine [11], but not to that of Rieske-type iron–sulfur cluster which has two histidines and two cysteines as the ligand of [2Fe–2S] cluster. Serine residue could not substitute for cysteine residue as the ligand of the [2Fe–2S] cluster in the case of SoxR though the replacement of the ligand of iron–sulfur clusters from cysteine to serine have been reported in some ferredoxins [24–29]. In the case of SoxR, the replacement of one cysteine by serine at any position 119, 122, 124, or 130 causes disruption of the [2Fe–2S] cluster to form apo-SoxR in vivo.

The arrangement of the cysteine residues ligating the [2Fe–2S] cluster in SoxR is unique compared with the plant type ferredoxins containing the typical [2Fe–2S] cluster, in which four cysteines are arranged in the pattern Cys-X_4-Cys-X_2-Cys-X_{29}-Cys [30]. In the plant type ferredoxins, the first and the second cysteines in the above motif ligate the same iron ion in the [2Fe–2S] cluster. In the case of SoxR, the four cysteines are arranged in the pattern Cys-X_2-Cys-X-Cys-X_5-Cys [20], in which only one amino acid residue intervene between the second and the third cysteines. The distance between the third and fourth cysteine is also short compared with the case of plant type ferredoxins. The spectroscopic measurements of the cysteine-to-serine mutant SoxR described above

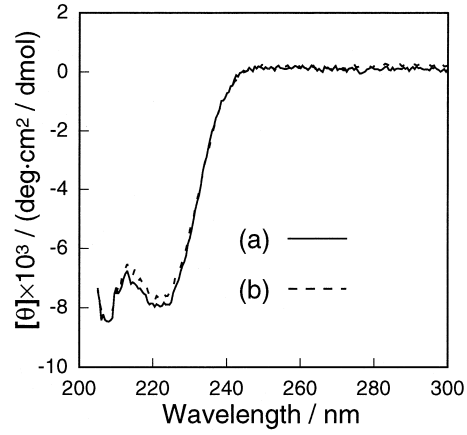

Fig. 4. CD spectra of (a) the wild type SoxR (0.47 μM) and (b) SoxR C124S (0.55 μM) in 50 mM KH_2PO_4–NaOH buffer (pH 7.6) containing 0.2 M NaCl.

show that all of the four cysteine residues are ligands of the [2Fe–2S] cluster in SoxR. The arrangement of the last three cysteines in the above motif of SoxR may cause the conformational strain.

The CD spectra in UV-region of the wild type and the mutant SoxR proteins were measured to determine whether the existence of the iron–sulfur cluster affect the secondary structure of SoxR or not. The CD spectra of the mutant SoxR proteins will be different from that of the wild type SoxR if the iron–sulfur cluster in SoxR plays somewhat a structural role. The results are shown in Fig. 4 indicating that no difference was observed between the wild type SoxR and SoxR C124S. Other three mutants (SoxR C119S, C122S, and C130S) showed the same results as SoxR C124Snnn. These results show that the iron–sulfur cluster does not play an important role in the structural function in SoxR.

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