Annals of Microbiology, **56 (1)** 53-56 (2006)

Essential residues for DNA binding activity of ManR from *Anabaena* sp. PCC 7120

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Received 7 June 2005 / Accepted 16 December 2005

Abstract - Cyanobacterial ManR is a member of the OmpR family of response regulator that regulates the expression of the *mntABC* and *mntH* in response to Mn^{2+} signals. Single-alanine substitutions of I204, L207 and R208 residues of the ManR, which constituted the DNA recognition helix, were obtained by the overlap extension method of PCR. EMSA was used to detect the complexes of the proteins of ManR mutants I204A, L207A, R208A, and the DNA fragment of promoter region of the *mntH* gene from *Anabaena* sp. PCC 7120. Results showed the formation of the complexes of the proteins of ManR mutants and DNA could not be detected, indicating that the mutagenesis of the residues I204, L207 and R208 in the ManR HTH domain could lead to the elimination of DNA binding activity of the ManR. Homologous analysis showed that residues I204, L207 and R208 of the ManR are also conservative in the α helix 3 region of effector domain of other proteins of OmpR/PhoB subfamily, indicating that they are essential residues for DNA binding activity. No significant alteration between wild type and mutant proteins was detected by Far-UV CD spectra at the secondary structure level.

Key words: ManR; DNA-binding; *Anabaena* sp. PCC 7120; cyanobacteria.

INTRODUCTION

Manganese (Mn) is required for the growth and survival of most living organisms, especially for the production of molecular oxygen by cyanobacteria, plants and algae (Yocum and Pecoraro, 1999). Mn^{2+}-sensing occurs widely in bacteria and influences both Mn^{2+} homeostasis and genes expression involved in the oxidative stress response (Jakubovics and Jenkinson, 2001). A two-component Mn^{2+}-Sensing System (ManS-ManR) has been found in *Synechocystis* sp. PCC 6803 that regulates the expression of *mntCAB* operon (Yamaguchi *et al.,* 2002; Ogawa *et al.,* 2002). In *Anabaena* sp. PCC 7120 we have shown that a 19-bp DNA sequence composed of two direct repeats of the consensus sequence (T/A)ATGA(G/A)A(A/G) is the specific recognition site for ManR (Huang and Wu, 2004). And the conservative two direct repeats were also found in the promoter regions of genes coding for the homologous proteins of MntCAB and MntH from cyanobacteria, indicating that the expression of both *mntABC* and *mntH* were regulated by a two-component Mn^{2+}-Sensing System containing ManR in cyanobacteria (Huang and Wu, 2004).

ManR is a member of the winged helix OmpR subfamily of response regulators based on sequence similarities of the C-terminal effectors domains. It has been reported that eight residues important for DNA binding were clustered between Val202 and Arg210 by alanine scanning mutagenesis of the PhoP α loop and α helix 3 region of PhoPC (Val190 to E214) (Chen *et al.,* 2004).

Homologous analysis shown that residues I206, L209 and R210 in PhoP from *Bacillus subtilis* are also conservative in the effector domain of the ManR (corresponding to I204, L207 and R208, respectively) and other proteins of OmpR/PhoB subfamily (Chen *et al.,* 2004). Studies described here were initiated to identify the essential residues of the α helix 3 region of effector domain required for ManR DNA binding. Mutant proteins resulting from alanine scanning mutagenesis of the ManR were constructed with intact ManR protein for *in vitro* functional analyses. We found that the complexes of the proteins of ManR mutants I204A, L207A and R208A and the DNA fragment of promoter region of the *mntH* gene from *Anabaena* sp. PCC 7120 were never formed, indicating that the mutagenesis of the residues I204, L207 and R208 in the ManR HTH domain could lead to the elimination of DNA binding activity of the ManR.

MATERIALS AND METHODS

Bacterial strains. The *Escherichia coli* strain DH5α was used as host strain for cloning. The *E. coli* strain BL21 (DE3) (Novagen) was used as host for protein expression. *E. coli* was routinely grown aerobically in Luria-Bertani medium at 37 °C with 50 μg ml^{-1} kanamycin.

Construction of plasmids. DNA manipulations were performed according to the descriptions of Sambrook *et al*. (1989). pET28a-*manR* was produced by connecting the 711 bp *Bam*H I-*Xho* I fragment of *manR* from *Anabaena* sp. PCC 7120 with an *E. coli* expression vector pET28a (Novagen) (Huang and Wu, 2004).

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Site-directed mutagenesis by PCR. Mutations in the *manR* gene were generated by the overlap extension method of PCR, which was described by Mikaelian and Sergeant (1992). PCR was carried out with a couple of proper primers designed as follows (positions are shown in italics):
ManRu (5′-GCGCGGATCCATGCTTACCTCAAGCAGC-3′)
ManRd (5′-GCGCCTCGAGTTATTCAACTAAATTTGT-3′)
M204d5 (5′-CATTA*gcc*AGATTGCTACGCCGTAAAATCG-3′)
M204u3 (5′-CGATTTTACGGCGTAGCAATCT*ggc*TAATG-3′)
M207d5 (5′-CATTAATCAGATTG*gca*CGCCGTAAAATCG-3′)
M207u3 (5′-CGATTTTACGGCG*tgc*CAATCTGATTAATG-3′)
M208d5 (5′-CATTAATCAGATTGCTA*gcc*CGTAAAATCG-3′)
M208u3 (5′-CGATTTTACG*ggc*TAGCAATCTGATTAATG-3′) which contain mismatched nucleotides designed to have one replaced amino acid reside at the HTH DNA binding domain. The plasmid pET28a-*manR* was used as the template DNA in the first round of PCR. A couple of the first round PCR products were then offered to the second round of PCR. Thus obtained PCR products were digested by two proper restriction enzymes and then connected into the same restriction sites of the *manR* gene in pET28-*manR*, resulting in a site-directed mutation in *manR*. The absence of mutations was verified by DNA sequencing, which was carried out by the dideoxy termination method. The plasmid containing a site-directed mutation in *manR* was transferred to *E. coli* strain BL21 (DE3) to expression the mutant ManR proteins.

Overexpression and purification of protein. The ManR protein of wild type was overexpressed and purified according to Huang and Wu (Huang and Wu, 2004).

Overnight cultures of *E. coli* BL21 (DE3) with the corresponding mutant plasmids were diluted 1:100 in Luria-Bertani medium in the presence of 50 μg ml^{-1} kanamycin. Expression of protein was induced with IPTG (100 μM final concentration) at an optical density of 0.6 at 600 nm. The expression of protein was confirmed by SDS-PAGE and Coomassie staining of the proteins.

The bacterial pellet was resuspended in the buffer of 20 mM phosphate, 0.5 M NaCl, and 10 mM imidazole, pH 7.4 supplemented with 1 mM PMSF. Cells were broken by sonication, and the cell lysate was centrifuged at 12, 000 rpm for 30 min. The purification process was performed on a ÄKTA purifier with a HiTrap Chelating HP column (Amersham Pharmacia Biotech) according to the manufacturer. His-tagged protein was eluted in the elution buffer containing 20 mM phosphate, 0.5 M NaCl, and 500 mM imidazole, pH 7.4.

Electrophoretic Mobility Shift Assays (EMSA). Protein-DNA complexes were detected by EMSA using purified protein. A DNA fragment that corresponded to the region from positions -47 to +140 upstream of the translation initiation site of *mntH* gene was used as the DNA probe, which was prepared by PCR amplification using the primers: 5′-TTTCTGGGATTATGAAAAGAATATGAGAATATTTCATGG-3′, 5′-CCAGGGTCTATGTATCCAAC-3′. DNA-protein reaction mixture (20 μl) typically containing 100 ng DNA fragment and various concentrations of purified protein, were incubated in binding buffer: 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM KCl and 1 mM DTT. Each reaction mixture was incubated at 25 °C for 20 min and then loaded onto a 6% nondenaturing polyacrylamide gel. Electrophoresis was performed at 25 °C in TAE buffer (40 mM Tris-acetate and 1 mM EDTA). Gels were stained with ethidium bromide for visualization of DNA bands.

Circular dichroism measurement. Far-UV CD spectra of ManR proteins of wild type and mutants were measured on a Jasco J-715 (Japan) spectropolarimeter at Room temperature. The spectra were recorded using 1 mm path length cells. Protein concentrations of 0.5 mg/ml were used for far-UV CD spectra measurements. All the spectra were cumulative averages of 10 repeated scans.

RESULTS AND DISCUSSION

Site-directed mutagenesis of the ManR

In order to identify the important role of three residues I204, L207 and R208 in the ManR HTH domain, we used the overlap extension method of PCR to obtain single-alanine substitutions of I204, L207 and R208 residues of the ManR. PCR products obtained were digested by two *Bam*H I-*Xho* I restriction enzymes and then connected into the same restriction sites of the plasmid pET28, resulting in pET28-I204A, pET28-L207A and pET28-R208A with single site-directed mutation of I204, L207 and R208 amino acids residues, respectively.

Purification of ManR mutants

The proteins of ManR mutants were overexpressed as 6His-tagged protein in *E. coli* strain BL21 (DE3) with the corresponding plasmids. Fig. 1A shows the protein profile from bacterial soluble extracts generated before and after induction of the recombinant proteins of ManR mutants with IPTG and after purification procedure. SDS–PAGE analysis of bacterially expressed proteins of ManR mutants revealed the presence of a predominant band of the recombinant protein in presence or absence of IPTG (Fig. 1A), while the *E. coli* BL21 (DE3) strain with pET28a used as control. The proteins of ManR mutants were purified by an affinity chromatography as described under materials and methods. The proteins of ManR mutants were 95% pure, as analysed by SDS–PAGE (Fig. 1B).

DNA binding activity of ManR mutants

To identify residues that might be directly involved in the protein-DNA interaction, three residues I204, L207 and R208 were mutagenized by PCR and the formation of the complexes of the proteins of ManR mutants with the DNA fragment of promoter region of the *mntH* gene from *Anabaena* sp. PCC 7120 were detected by EMSA. The ManR protein of wild type was used as positive control. A band shift caused by the binding of the ManR protein of wild type to the promoter region of *mntH* was observed (Fig. 2). But binding of all of the proteins of ManR mutants I204A, L207A and R208A to the DNA fragment of promoter of the *mntH* were never detected (Fig. 2), indicating that the mutagenesis of the residues I204, L207 and R208 in the ManR HTH domain could lead to the elimination of DNA binding activity of the ManR. In other words, all of the residues I204, L207 and R208 were essential for DNA binding activity of the ManR. It is very interesting that the DNA probe was decreased significantly with the increasing of the concentrations of mutant proteins I204A and L207A though specific protein-DNA complex was not found, different to that of mutant protein R208A. The specific protein-DNA complex was also not found, but the DNA probe was not decreased with the increasing of the concentration of mutant protein R208A. Our results showed that the decrease of DNA probe in the case of I204A and

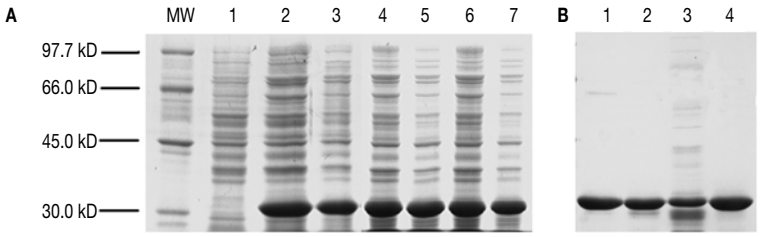

FIG. 1 – Overexpression and purification of the proteins of ManR mutants I204A, L207A and R208A in *Escherichia coli*. Samples were analysed by SDS-PAGE (12% acrylamide). A: lanes 2, 4, 6 and lanes 3, 5, 7 contain crude extracts from *E. coli* BL21 (DE3) with the corresponding mutant plasmids cells in the absence or in the presence of IPTG, respectively; lane 1, crude extract from *E. coli* BL21 (DE3)/pET28a in the presence of IPTG. B: lanes 1, 2, 3 and 4, the purified His-tagged ManR proteins of wild type, I204A, L207A and R208A. MW, molecular size markers.

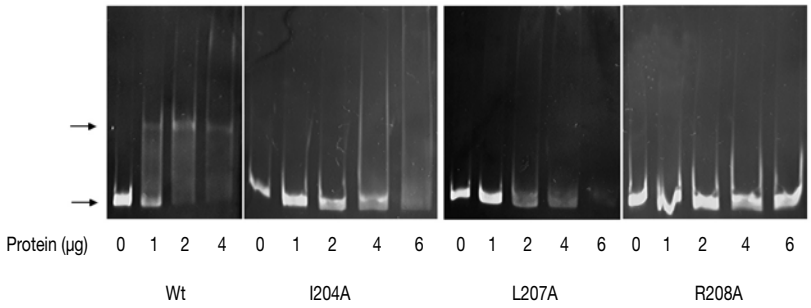

FIG. 2 – DNA binding activity of the mutants of ManR. EMSA experiments were performed in the absence or in the presence of purified proteins of the proteins of ManR mutants I204A, L207A and R208A. Wt, the ManR protein of wild type.

L207A was not due to DNA degradation (Fig. 3). Therefore, the residues I204, L207 and R208 in the ManR HTH domain might have different roles in DNA binding activity of the ManR.

It was reported that eight residues important for DNA binding were clustered between Val202 and Arg210 in *Bacillus subtilis* PhoP (Chen *et al.,* 2004). These residues are in the highly conservative sequence that is part of the DNA recognition helix in the proteins of OmpR/PhoB subfamily. From Fig. 4 we could find that residues I206, L209 and R210 in *Bacillus subtilis* PhoP are also conservative in the ManR (corresponding to I204, L207 and R208, respectively). In addition, mutageneses of these residues also lead to the elimination of DNA binding activity of PhoP. We also found that these three residues are also conservative in other proteins of OmpR/PhoB subfamily (Chen *et al.,* 2004). Therefore, these three residues must have important roles in DNA binding activity of the proteins of OmpR/PhoB.

Circular dichroism measurement

Far-UV CD spectra were utilized to examine the secondary structure changes of ManR proteins of wild type and mutants. The far-UV CD spectra results (Fig. 5) revealed that there was no significant alteration of the proteins of ManR mutants at the secondary structure level. It might be that there were no secondary structure changes caused by mutagenesis of I204, L207 and R208 amino acid residues or that the changes were too little to detect by Far-UV CD spectra.

Collectively, in this study, we set out to identify three

FIG. 3 – Un-related DNA fragment of *sll0789* from *Synechocystis* sp. PCC 6803 incubated in the presence of mutant proteins of ManR was used to detect the stability of DNA probe. EMSA experiments were performed with 6 μg purified mutant proteins, respectively, I204A (lane 1), L207A (lane 2) and R208A (lane 3).

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7120-ManR   EPPNSNVIAA LIRLLRRKIE VGKELP-LIH TVYGKGYRFG TN-LVE....
6803-ManR   SPPSSNVLAA LVRLLRRKIE QPNAPR-LIN SVYGKGYCFE AN----....
PhoP        FAGDTRIVDV HISHLRDKIE NNTKKPIYIK TIRGLGYKLE EPKMNE....
OmpR        YSAMERSIDV QISRLRRMVE EDPAHPRYIQ TVWGLGYVFV PDGSKA....
PhoB        VYVEDRTVDV HIRRLRKALE PGG-HDRMVQ TVRGTGYRF- --STRF....
DrrD        DEVFSDVLRS HIKNLRKKVD KGFKKK-IIH TVRGIGY--- VA-RDE....
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FIG. 4 – Alignment was performed with amino acid sequences from *Anabaena* sp. PCC 7120 (7120-ManR), *Synechocystis* sp. PCC 6803 ManR (6803-ManR), *Bacillus subtilis* PhoP (PhoP), *Escherichia coli* OmpR (OmpR), *E. coli* PhoB (PhoB) and *Thermotoga maritime* DrrD (DrrD) using the program DNASIS 2.5 (Hitachi Software Engineering Co., Ltd). The conservative amino acid residues were shown by dark gray boxes.

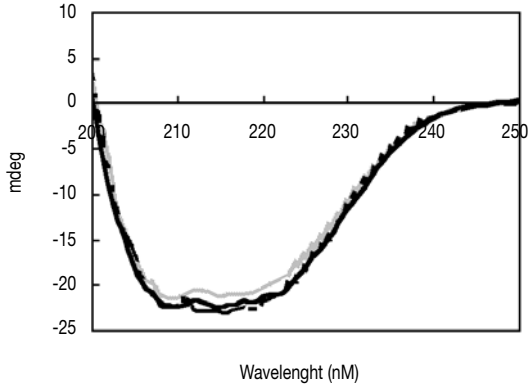

FIG. 5 – Far UV-CD spectra of ManR proteins of wild type and mutants. Curves "———", "▬ ▬ ▬" and "·····" represent the CD spectra of ManR proteins of wild type and mutants I204A and R208A measured at room temperature.

essential residues I204, L207 and R208 in the ManR HTH domain required for DNA binding activation. To do this, we used the overlap extension method of PCR to obtain single-alanine substitutions of I204, L207 and R208 residues of the ManR, which constituted the DNA recognition helix. The complexes of the proteins of ManR mutants I204A, L207A, R208A, and the DNA fragment of promoter region of the *mntH* gene from *Anabaena* sp. PCC 7120 were detected by EMSA. Results demonstrated that protein-DNA complexes were never found, indicating that the mutagenesis of the residues I204, L207 and R208 in the ManR HTH domain could lead to the elimination of DNA binding activity of the ManR. Far-UV CD spectra were utilized to examine the secondary structure changes of ManR proteins of wild type and mutants. However, there was no significant alteration of the proteins of ManR mutants at the secondary structure level.

Acknowledgements

This work was supported by the NSFC projects 30070065, 40272054 and NSFC key project 40332022.

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