Evidence for redox regulation of the transcription factor NtcA, acting both as an activator and a repressor, in the cyanobacterium Anabaena PCC 7120

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INTRODUCTION

Anabaena PCC 7120 is a filamentous cyanobacterium capable of aerobic nitrogen fixation by forming specialized cells, heterocysts, at intervals along the filaments [1]. The differentiation of photosynthetic vegetative cells into heterocysts is a complex process that involves a number of morphological and biochemical changes and is regulated by both external cues and intercellular communication. Heterocysts lack oxygen evolution via photosystem II and provide the microaerobic environment required for the oxygen-labile nitrogenase catalysing the reduction of di-nitrogen to ammonia [2]. The differentiation of vegetative cells into heterocysts is accompanied by global changes in gene expression and affects a large number of genes [2,3]. Some genes, such as the *rbcLS* operon (encoding the large and small subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase) [2,4] and *gor* (encoding glutathione reductase) [5] are turned off or become down-regulated during heterocyst differentiation. In contrast, other genes that are related to or directly involved in heterocyst differentiation, nitrogen fixation and the subsequent ammonium assimilation, i.e. *xisA* (encoding a site-specific recombinase expressed during heterocyst differentiation) and *ntcA* (encoding NtcA itself). However, the mechanism by which NtcA serves as a critical regulator for such diverse processes is not understood.

NtcA has been identified as a nitrogen-responsive regulatory protein required for nitrogen assimilation and heterocyst differentiation in cyanobacteria. It is proposed that NtcA functions through the formation of DNA–protein complexes with its specific target sequence within the promoter regions of the regulated genes. In vitro, NtcA of *Anabaena PCC 7120* binds to upstream regions of the genes whose products are involved in nitrogen assimilation, but also to the upstream region of *rbcLS* (carbon-fixation gene), *xisA* (encoding a site-specific recombinase expressed during heterocyst differentiation) and *ntcA* (encoding NtcA itself). However, the mechanism by which NtcA serves as a critical regulator for such diverse processes is not understood.

With the use of electrophoretic mobility shift assays, NtcA from *Anabaena PCC 7120* was here shown to interact with the promoter sequence of the *gor* gene, encoding glutathione reductase, thereby providing a novel example of NtcA’s acting as a repressor, previously found only for the *rbcLS* gene. Furthermore we demonstrate that the binding of DNA by NtcA is regulated in vitro by a redox-dependent mechanism involving cysteine residues of the NtcA protein. These findings suggest that NtcA is a transcriptional regulator that responds not only to the nitrogen status but also to the cellular redox status, a function that might be particularly significant during heterocyst differentiation.

**MATERIALS AND METHODS**

**Partial purification of NtcA**

*Anabaena PCC 7120* grown in BG-11 medium (3 litres) without combined nitrogen were harvested at late exponential growth phase (D_{680} = 0.5) by centrifugation at 3000 g for 5 min. The cells were suspended in 10 ml of buffer A [50 mM Tris/HCl (pH 7.5)/1 mM EDTA/2 mM dithiothreitol (DTT)/0.5 mM Triton X-100/1 mM PMSF/10% (v/v) glycerol], then added

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**Abbreviations used:** CRP, cAMP receptor protein; diamide, azodicarboxylic acid bis(dimethylamidine); DTT, dithiothreitol; NEM, N-ethylmaleimide.

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The protein concentration was determined with Bio-Rad Protein Assay (PMSF, Tris) linear gradient of 0.1–1.0 M (NH₄)₂SO₄ in buffer A. The column was washed with the equilibrating buffer and eluted with a 50 ml linear gradient of 0.1–1.0 M (NH₄)₂SO₄ in buffer A. Fractions (2 ml) were collected and dialysed against 2.5 litres of 50 mM Tris/HCl (pH 7.5)/0.1 mM EDTA/2 mM DTT/0.5 mM PMSE/10% (v/v) glycerol. All steps were performed at 4°C. The protein concentration was determined with Bio-Rad Protein Assay (Bio-Rad Laboratory).

DNA fragments for mobility shift assays

Both the gor-fragment containing the sequence from −153 to +39 (with respect to the translation start site) [16] and the glnA-fragment containing the sequence from −314 to −92 [8] were isolated by PCR with primers (custom synthesized by Operon Technologies, Alameda, CA, U.S.A.) carrying sequences identical with the 5' and 3' sequences of the fragments. The primers also contained flanking restriction enzyme sites for cloning. The PCR products were first cloned into pGEM 3Zf(+) (Promega), and then cut with XbaI and AatI. The fragments were end-labelled on both strands with [γ-³²P]dCTP for mobility shift assays. A segment of the gor gene composed of 224 bp (bases +105 to +328) [16] was used as the unrelated DNA fragment.

Gel mobility shift analysis

Labelled DNA fragments (1 ng) were incubated with 8 μl of partly purified protein extract in binding buffer [4 mM Tris/HCl (pH 8.0)/12 mM Hepes (pH 7.9)/12% (v/v) glycerol/60 mM KCl/0.5 mM EDTA/5 mM MgCl₂] containing 0.1 μg of BSA and 0.5 μg of poly(dI-dC) in a final volume of 20 μl with different concentrations of DTT. After incubation at room temperature for 20 min, the reaction mixtures were separated by electrophoresis on a 5% (w/v) native polyacrylamide gel. The gels were dried and autoradiographed. Treatment with the thiol-oxidizing agent azodicarboxylic acid bis(dimethylamide) (diamide) (Sigma) was performed by incubating the protein extract in binding buffer containing 5 mM DTT for 1 h at 37°C in the presence or absence of 1 mM diamide before adding DNA fragments. For thiol modification, the protein extract was incubated in the binding buffer containing 5 mM DTT with the thiol-modifying agent N-ethylmaleimide (NEM) (Sigma), before or after adding DNA fragments.

RESULTS AND DISCUSSION

Specific binding of NtcA to gor promoter fragment

Our previous studies showed that the upstream non-coding region of Anabaena PCC 7120 gor gene contains three putative Escherichia coli sigma-70 promoters (−10 and −35 hexamer sequences) and one NtcA-binding site that overlaps with the middle promoter [16]. The middle promoter was used only during growth on ammonium but not on other nitrogen sources such as nitrate and dinitrogen [16]. These results indicated that NtcA's binding to its target sequence might prevent RNA polymerase from binding to the middle promoter under growth on nitrate or on dinitrogen (i.e. nitrogen-fixing conditions). To confirm our previous results that NtcA acts as a repressor of the gor gene, we examined the interaction of NtcA with the gor promoter fragment of Anabaena PCC 7120. NtcA was partly purified from the cyanobacterium by heparin–Sepharose CL 6B column chromatography [10]. Each column fraction from a 0.1–1.0 M (NH₄)₂SO₄ elution gradient was assayed by electrophoretic mobility shift with a 192 bp gor promoter fragment containing the bases from −153 to +39 (with respect to the translation start site) [16] of the gene. This fragment contains a single NtcA recognition site: TGTGCAACTGACA. The presence of a DNA-binding protein was observed in fractions 15–23 (Figure 1). The binding buffer contained DTT at a concentration (1 mM) previously used under the standard conditions of 15–20 min incubation time [10,14]. To confirm that the mobility shift was due to binding of NtcA, competitive binding experiments were performed under the same conditions with the glnA promoter fragment (bases −314 to −92). The latter also contains a single NtcA recognition site and is known to have a relatively high NtcA binding affinity [8,14]. A 50-fold excess of the unlabelled glnA fragment and a 100-fold excess of the unlabelled gor fragment was required to compete out effectively the labelled gor fragment binding to NtcA. A 100-fold excess of an unlabelled, unrelated DNA fragment did not significantly interfere with the formation of the NtcA-gor complex (Figure 2).

These results indicated a specific binding affinity of NtcA for gor and that the affinity is weaker for glnA. Previous studies have similarly demonstrated different binding affinities of NtcA for different promoter targets [14]. The binding affinity of NtcA might be affected by the middle unconserved nucleotide sequences and flanking regions of the NtcA-binding site.

The results presented here, together with our previous primer extension studies of gor [16] suggest that NtcA is a repressor of gor in Anabaena PCC 7120 under nitrogen-fixing conditions. Low levels of glutathione reductase as well as of ribulose-1,5-bisphosphate carboxylase/oxygenase (the product of rbcLS) specifically in heterocysts have been reported previously [4,5], and so far gor and rbcLS are the only genes found to be negatively controlled by NtcA. As for gor, NtcA has been suggested to function as a repressor as of rbcLS by blocking the initiation of its transcription [14]. In this case the binding site of NtcA directly follows the TATA box and covers the transcription start site. Therefore, like other members of the CRP family, NtcA can act both as an activator and a repressor of the target genes, depending on the location of the target sequence in relation to the promoter [18]. Figure 3 shows the NtcA-binding sequences of one of the complementary DNA strands (even though the actual binding occurs to double-stranded DNA). Interestingly, the recognition sites of NtcA within the gor and rbcLS promoters show a higher sequence similarity to each other than to recognition sites of promoters positively regulated by NtcA (Figure 3). Even within the middle unconserved NtcA-binding region, six out of ten nucleotides are identical. It might be that these nucleotide sequences are critical for the distinction of NtcA as an activator or as a repressor. Comparisons of the kinetic and thermodynamic parameters of these NtcA-binding sequences are now needed to understand their functional significance.

NtcA requires reducing conditions to promote specific binding of NtcA by DNA

The complex formed by NtcA with the gor promoter fragment shifted only a small proportion of the labelled gor fragment, indicating a low binding affinity under the conditions used (Figure 1). However, increasing amounts of the NtcA-containing heparin–Sepharose fractions did not increase the proportion of shifted fragment (results not shown). This suggested that there
Regulation of transcription factor NtcA in Anabaena

Figure 1 NtcA purification and binding of NtcA in vitro to a gor promoter fragment containing a single NtcA recognition site

(a) Elution profile of heparin–Sepharose CL 6B column chromatography. A crude lysate of Anabaena PCC 7120 cells was loaded on the column and eluted with a linear 0.1–1.0 M (NH₄)₂SO₄ gradient. Fractions (approx. 2 ml) were collected. (b) An 8 µl portion of each fraction was assayed for protein concentration and binding affinity for a gor promoter fragment of 192 bp [13]. The positions of the free DNA fragments and the NtcA–DNA complexes are indicated by arrows.

Figure 2 Competition of the Anabaena PCC 7120 glnA, gor promoter fragments and unrelated DNA fragment (UF) for NtcA binding to radioactively labelled gor fragment

The fold excess of unlabelled competitor fragment added, on a molar basis, is indicated above the lanes. A 7 µl sample of fraction number 18 (Figure 1) was used for the binding assay as described in the legend to Figure 1, except that different amounts of the competitors were added. The glnA competitor contained 234 bp of the glnA promoter region including the NtcA recognition site [6]. The unlabelled gor competitor was identical with the labelled gor fragment. The unrelated DNA fragment (UF) was a segment of gor containing 224 bp, bases +105 to +328 [13].

Figure 3 Alignment of NtcA recognition sequences of the gor, rbcLS, glnA and ntcA genes from Anabaena PCC 7120

NtcA recognition sequences of the four genes are aligned 5’ to 3’ with respect to the open reading frame. Numbers indicate the first and last nucleotides in the sequence and are identified in relation to the translation start site set as +1.

<table>
<thead>
<tr>
<th>Consensus</th>
<th>gor</th>
<th>rbcL</th>
<th>glnA</th>
<th>ntcA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TGT-N (50×10) -ACA</td>
<td>TGTT.GCACACTGACA</td>
<td>TGTTGACAAGTTACA</td>
<td>GGTATCATTAAGACAGAC</td>
</tr>
<tr>
<td>fold excess</td>
<td>-70</td>
<td>-56</td>
<td>-495</td>
<td>-135</td>
</tr>
<tr>
<td>competitor</td>
<td>50× g/l</td>
<td>100× g/l</td>
<td>100× UF</td>
<td>50× gor</td>
</tr>
<tr>
<td>fragment</td>
<td></td>
<td></td>
<td></td>
<td>150× UF</td>
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are additional requirements for optimal NtcA/DNA interactions. The increased NtcA-dependent transcription in heterocysts [14] suggested that NtcA might be subject to intracellular signals. One major difference between heterocysts and the photosynthetic vegetative cells is that heterocysts offer a microaerobic environment for the oxygen-labile nitrogen fixation process. We therefore hypothesized that NtcA might be subject to redox control. To test this hypothesis, the binding capacity of NtcA to the gor promoter fragment (negatively regulated by NtcA) and the glnA promoter fragment (positively regulated by NtcA) in the presence or absence of the reducing agent DTT was analysed (Figure 4). In the presence of 1 mM DTT, NtcA shifted only part of the labelled gor fragments, as shown in Figures 1 and 4. However, with increasing concentrations of DTT and the same incubation time (approx. 20 min), the affinity of NtcA was markedly enhanced for both the gor and the glnA promoter.
fragments. In fact, the gor fragment was completely shifted in the presence of 5 or 10 mM DTT. The proportion of shifted glnA also increased when the DTT concentration was increased (Figure 4). These results demonstrated that higher levels of DTT than those used in DNA binding assays (1 mM DTT) are required for efficient binding of NtcA to DNA. It is not likely that the reducing agent is needed to dissociate aberrant NtcA aggregates because increased amounts of DTT improved the NtcA–DNA interaction for both dimeric and polymeric NtcA (Figure 4). The enhanced NtcA-binding affinity for both positively and negatively regulated promoters suggests that a low redox potential does not affect the distinction between different target sequences but rather represents a general requirement for binding of NtcA to DNA. Increased DNA affinity under reducing conditions is a typical character of other redox-responsive transcriptional factors such as AP-1 [19], E2 of papilloma virus [20] and USF [21]. These factors require high concentrations of DTT, typically 5–10 mM, for efficient DNA binding in vitro.

Furthermore interactions between NtcA and DNA might also be affected by conformational changes and/or by polymerization states of the NtcA protein, because two shifted bands were observed with both the glnA fragment (Figure 4) and the gor fragment (in an experiment parallel to that shown in Figure 1). Most of the CRP family members form dimers when they interact with DNA, each monomer binding a single half-site [18]. Previous DNase I protection experiments showed that regions protected by NtcA from Anabaena PCC 7120 covered the whole recognition site for the target sequences tested [14]. This indicated that NtcA is also a dimeric protein. Therefore the lower shifted bands of glnA (Figure 4) might be caused by a dimeric NtcA–DNA interaction, and the up–shifted bands by a polymeric NtcA–DNA interaction. A small proportion of polymeric complexes is often observed in such experiments in vitro [10,14], but such complexes might not have any physiological significance. High concentrations of DTT did not block NtcA polymerization (Figure 4), indicating that the formation of intracellular disulphide bonds is not the cause of the polymerization.

**Figure 4** Redox regulation of NtcA binding to the gor and glnA promoters from Anabaena PCC 7120 in vitro

The gor and glnA promoter fragments were assayed for NtcA binding in the absence or presence of increasing concentrations of DTT. A 7 µl sample of fraction number 18 (Figure 1) was used for the binding assays.

**Figure 5** Thiol-group dependence of the redox regulation of NtcA binding by DNA in vitro

For oxidation of the thiol group, a 7 µl sample of protein fraction number 18 (Figure 1) was incubated without (lane 1) or with (lane 2) diamide at 37 °C for 1 h before addition of the binding buffer and the labelled gor fragment. For the thiol modification, a 7 µl sample of protein fraction number 18 (Figure 1) was incubated with NEM for 1 h at room temperature, before (lane 4) or after (lane 5) addition of the binding buffer and the gor fragment. Lane 3 shows the control binding reaction without NEM added. The binding buffer used contained 5 mM DTT.

**Figure 6** Schematic diagram illustrating the location of cysteine residues in three cyanobacterial NtcA proteins

The numbers refer to the positions of amino acid residues in NtcA of Anabaena PCC 7120 (sequences are from [12]).

**Free thiol groups of cysteine residues as targets of the redox effect**

Because redox regulation is usually mediated through the thiol groups of cysteine residues, the effects of a thiol-oxidizing agent and a thiol-alkylating agent on the binding of Anabaena PCC 7120 NtcA by DNA were investigated. Figure 5 shows that treatment of the NtcA protein extract with diamide, a thiol-oxidizing agent, resulted in the inhibition of DNA binding (lane 2), implicating that thiol groups are targets for the redox regulation. DNA binding was also inhibited by preincubation of the NtcA protein extract with the thiol-modifying agent NEM (lane 4). In contrast, incubation of the protein extract with gor promoter fragment before NEM treatment protected the complex from inactivation by NEM (lane 5). This suggests that one or more unmodified NtcA cysteine residues are required for DNA binding. It is not likely that thiol groups of other proteins in the extract enhanced the binding of NtcA. If this had been the case, preincubation of the NtcA protein extract with the binding buffer containing 5 mM DTT would already have activated NtcA, and the NtcA binding would not have been affected any further by the incubation with thiol-oxidizing and thiol-alkylating agents.

NtcA of Anabaena PCC 7120 contains two cysteine residues [12]: Cys-157 and Cys-164 (numbering based on the Anabaena sequence), which are both located close to the N-terminus of the helix–turn–helix DNA-binding motif (Figure 6). The first cysteine residue is conserved in the three cyanobacterial strains investi-
Redox-sensing mechanisms vary. In centres of many redox-sensitive transcriptional regulators, the investigation. Although cysteine residues are present in the redox of the six cysteine residues of OxyR is critical for redox sensing. In contrast with NtcA, the oxidized form of OxyR functions as an activator [22]. The E. coli strain Salmonella typhimurium, OxyR responds to oxidative stress [21] but only one of the six cysteine residues of OxyR is critical for redox sensing. In contrast with NtcA, the oxidized form of OxyR functions as an activator [22]. The E. coli Fnr protein does not exist only in the heterocystous cluster of the CRP family member, which allows the use of electron acceptors other than oxygen under anaerobic conditions, requires the cysteine clusters for anaerobic responses [23]. Some transcriptional regulatory proteins, like NtcA of Anabaena PCC 7120, require free thiol residues for binding of DNA or the activation of transcription, such as the eukaryotic regulators AP-1 [19] and USF [20].

Functional consequence of NtcA action

Taken together, our results demonstrate the capability of NtcA to regulate gene expression by a redox-dependent mechanism. Oxidizing conditions might cause modifications of NtcA, thereby affecting its potential to recognize its cognate DNA target. These results explain why NtcA purified from heterocysts (offering reducing conditions) exhibited a stronger binding affinity for glnA than NtcA purified from vegetative cells (producing oxygen endogenously in photosynthesis) [14]. This might also explain the stronger activation of NtcA in heterocysts, in which NtcA down-regulates the expression of certain genes, those not supporting nitrogen fixation, while it up-regulates those involved in nitrogen fixation. We also propose that NtcA belongs to the category of the responsive regulators detected in other nitrogen-fixing organisms, such as nifLA in certain eubacteria [24] and fixK in Rhizobium [25]. These respond not only to the external nitrogen status but also to the cellular redox status. Even if the present study suggests that NtcA can respond to both external nitrogen signals and to reducing agents, it is likely that NtcA, like other transcription factors, requires additional proteins to act as co-inducers to fulfil its regulatory functions in vivo.

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REFERENCES


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