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

Fanyi JIANG*, Bengt MANNERVIK*[1] and Birgitta BERGMAN†[1]

*Department of Biochemistry, Uppsala University, Biomedical Center, Box 576, S-751 23 Uppsala, Sweden, and †Department of Botany, Stockholm University, S-106 91 Stockholm, Sweden

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.

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 dinitrogen 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 development. 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 required for *nif* gene rearrangements in heterocysts) [6], the *nifHDK* operon (encoding subunits of nitrogenase) [7] and *glnA* (encoding glutamine synthetase) [8] are switched on or become overexpressed in heterocysts. A DNA-binding protein, NtcA, has been suggested to be involved in the regulation of these genes.

NtcA belongs to a family of prokaryotic regulatory proteins represented by the cAMP receptor protein (CRP) [9,10]. NtcA was first discovered in the unicellular cyanobacterium *Synechococcus* PCC 7942 [11] but has since been detected in nine cyanobacterial genera of differing morphological complexities [12]. An *ntcA* mutant of *Anabaena* PCC 7120 requires ammonium for growth and is defective in heterocyst formation, indicating that NtcA is required for nitrate assimilation and heterocyst differentiation [13]. NtcA interacts *in vitro* with the promoter regions of several cyanobacterial genes: *glnA*, *xisA*, *nir*, *nifH*, *rbcLS* and *ntcA* itself [13–15]. In most cases binding occurs at the target sequence TGT(N)$_n$ACA ($n = 9$ or 10). Because NtcA is considered to be a key regulator in the control of nitrogen metabolism, it has been difficult to explain its interaction with the *rbcLS* promoter region and repression of *rbcLS* expression [14]. Moreover, NtcA has recently been suggested as a negative regulator of *gor*, a gene that encodes the antioxidant defence enzyme glutathione reductase [16]. This gene represents yet another target for NtcA not associated with nitrogen assimilation or metabolism, suggesting that NtcA might also respond to other regulatory signals.

Redox control is a regulatory mechanism by which many organisms couple external and internal signals to selective gene expression [17]. The intracellular redox status influences the formation of specific protein–DNA complexes whereby transcriptional initiation is controlled.

Here we demonstrate that NtcA interacts with the *gor* promoter *in vitro* and that this interaction might lead to a down-regulation of *gor* of *Anabaena* PCC 7120. Furthermore, to address the possibility that NtcA responds to redox changes, we characterized the DNA-binding properties of NtcA in the presence and the absence of reducing and oxidizing agents as well as thiol-modifying agents.

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 ($D_{750} = 0.3$) 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 % (v/v) Triton X-100/1 mM PMSF/10 % (v/v) glycerol], then added

Abbreviations used: CRP, cAMP receptor protein; diamide, azodicarboxylic acid bis[dimethylamide]; DTT, dithiothreitol; NEM, *N*-ethylmaleimide.

[1] Correspondence may be addressed to either author.

dropwise to liquid nitrogen and ground to a fine powder. More buffer A was added to cell powder to a final volume of 15 ml. The cell lysate was centrifuged at 25000 ***g*** for 10 min, then at 114000 ***g*** for 60 min. The supernatant was loaded on a 3 ml heparin/Sepharose CL6B (Pharmacia Biotech) column, equilibrated with 0.1 M $(NH_4)_2SO_4$ 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 $(NH_4)_2SO_4$ 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 PMSF/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 *Xba*I and *Ava*I. The fragments were end-labelled on both strands with [α-^{32}P]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 $MgCl_2$] 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 $(NH_4)_2SO_4$ 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: **TGT**TGACAACTG**ACA**. 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 *gor* than 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 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

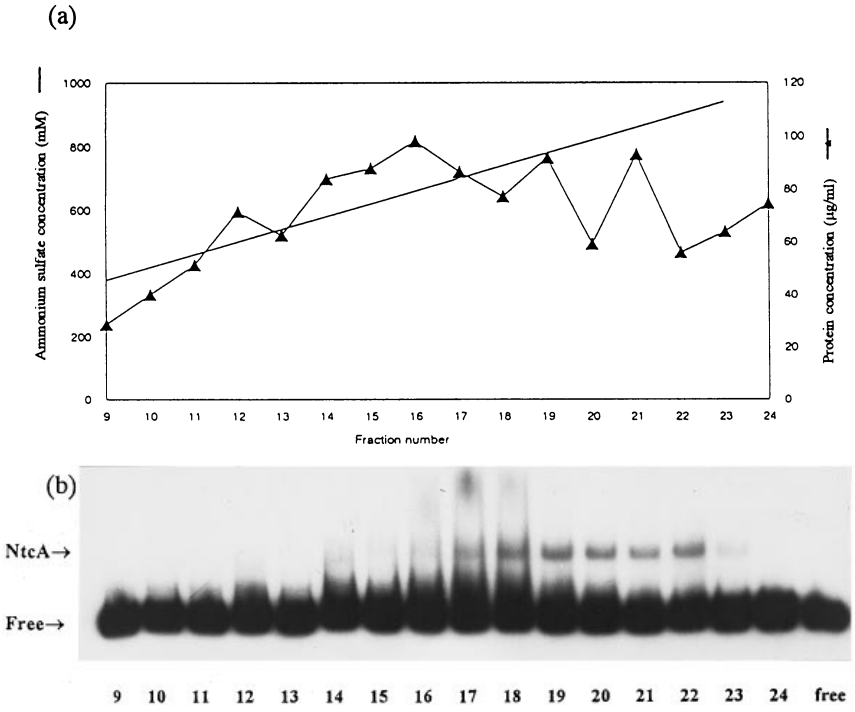

Figure 1 NtcA purification and binding of NtcA *in vitro* to a *gor* promoter fragment containing a single NtcA recognition site

(**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 $(NH_4)_2SO_4$ 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.

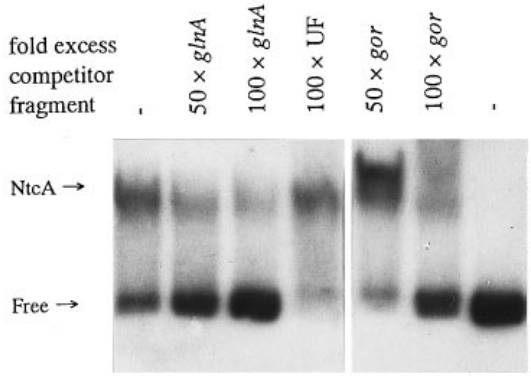

Figure 2 Competition of the *Anabaena* PCC 7120 *glnA, gor* promoter fragments and unrelated DNA fragment (UF) for NtcA binding to radioactively labelled *gor* fragment

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].

Consensus		**TGT**-N(9or10)-**ACA**	
gor	-70	**TGT**T.GACAACTG**ACA**	-56
rbcL	-510	**TGT**GAGACAAGTT**ACA**	-495
glnA	-142	**TGT**AACAAAGACT**ACA**	-123
ntcA	-150	**GGT**ATCATTATG**AACA**	-135

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.

are additional requirements for optimal NtcA/DNA interactions. The increased NtcA-dependent transcription in heterocysts [15] and the enhanced DNA-binding capacity of NtcA purified from 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 fixtion 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

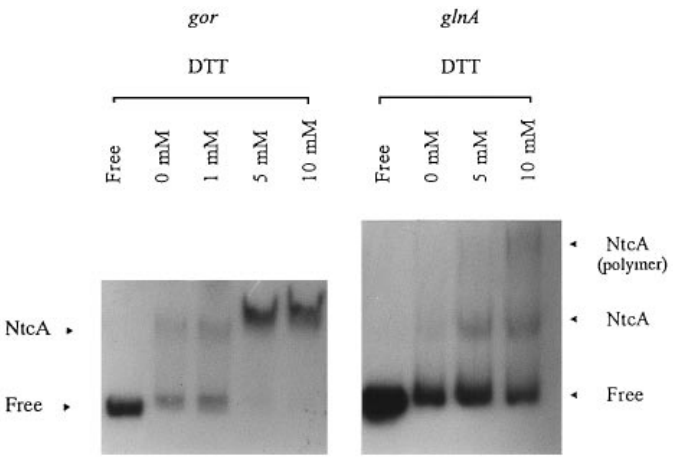

Figure 4 Redox regulation of NtcA binding to the *gor* and *glnA* promoters from *Anabaena* PCC 7120 *in vitro*

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.

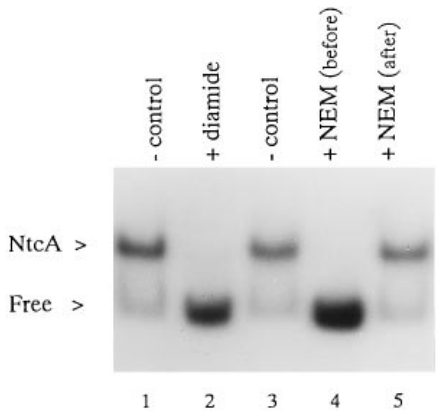

Figure 5 Thiol-group dependence of the redox regulation of NtcA binding by DNA *in vitro*

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.

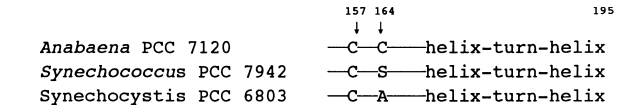

Figure 6 Schematic diagram illustrating the location of cysteine residues in three cyanobacterial NtcA proteins

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

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 [20]. 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 being a dyad symmetrical with the other 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.

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-

gated so far. The second residue exists only in the heterocystous strain *Anabaena* PCC 7120, but not in the unicellular, non-nitrogen-fixing *Synechococcus* PCC 7942 and *Synechocystis* PCC 6803 [12]. The contribution of the individual cysteine residues to DNA binding, and the role of the second cysteine residue in the redox response in NtcA of *Anabaena* PCC 7120, now warrant investigation. Although cysteine residues are present in the redox centres of many redox-sensitive transcriptional regulators, the redox-sensing mechanisms vary. In *E. coli* and *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 (another 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]. The results 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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