2-Oxoglutarate increases the binding affinity of the NtcA (nitrogen control) transcription factor for the *Synechococcus glnA* promoter

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Received 12 November 2001; revised 18 December 2001; accepted 19 December 2001

First published online 11 January 2002

Edited by Ulf-Ingo Flügge

Abstract The cyanobacterial NtcA global nitrogen regulator belongs to the catabolite activator protein (CAP) family and activates transcription of nitrogen assimilation genes in response to nitrogen step-down. The binding affinity of NtcA towards a DNA fragment carrying the promoter of the *glnA* gene from *Synechococcus* sp. PCC 7942, analyzed in vitro by band-shift assay, was increased five-fold by 2-oxoglutarate in the presence of Mg^{2+} ions. The 2-oxoglutarate effect peaked at about 0.6 mM, a rather physiological concentration for this compound under nitrogen-limiting conditions, and could be partially reproduced by 3-oxoglutarate but not by oxaloacetate or glutamate. These results suggest 2-oxoglutarate as a signal of the C to N balance of the cells to regulate NtcA activity and provide a new example of regulation in the versatile CAP family of proteins.

Key words: *glnA*; Nitrogen regulation; NtcA; 2-Oxoglutarate; *Synechococcus* sp.

1. Introduction

The NtcA transcription factor is a key element for nitrogen control in cyanobacteria [1,2]. When the cells are incubated in the absence of ammonium, NtcA activates the transcription of genes encoding proteins that are involved in the assimilation of nitrogen sources alternative to ammonium or needed for a more efficient assimilation of ammonium itself [1,2]. Whereas the structure of the DNA sites to which NtcA binds is well established [2], the mechanism(s) through which this regulatory system senses the nitrogen status of the cell is unknown. In some cyanobacteria [2], including the unicellular strain *Synechococcus* sp. PCC 7942 [3], the *ntcA* gene is auto-regulatory pointing to a modulation of the activity of the NtcA protein as a possible mechanism of response to the availability of ammonium.

NtcA belongs to the catabolite activator protein (CAP) family of bacterial transcriptional regulators. These proteins are characterized by bearing close to their C-terminus a helix–turn–helix motif for interaction with DNA [4], and their DNA binding and transcription activation activities are modulated through protein motifs present in their N-terminal parts. Different proteins in this family are involved in the regulation of quite diverse metabolic processes. In addition to CAP, which is a regulator of carbon/energy source assimilation, some examples of proteins belonging to this family are FNR (fumarate and nitrate respiration regulatory protein) that regulates anaerobic metabolism [5], CooA that regulates carbon monoxide metabolism in *Rhodospirillum rubrum* [6], DNR and similar proteins (DnrD, NNR, NnrR) that regulate denitrification in several bacteria [7–10], and FixK that regulates nitrogen fixation in *Sinorhizobium meliloti* [11]. The mechanisms through which these proteins sense the relevant cellular metabolic conditions are also diverse. CAP is an allosteric protein modulated by cAMP, which signals the carbon/energy status of the cell and whose binding to a pocket within the β-roll structure carried in the N-terminal half of the protein increases CAP affinity for specific binding sites in CAP-dependent promoters [4]. FNR bears close to its N-terminus a Fe–S cluster that responds to the presence of oxygen permitting, under anaerobic conditions, the formation of FNR dimers that bind to their target DNA sequences to activate transcription [5]. CooA contains a heme group, which upon reduction under anaerobic conditions can bind CO resulting in stabilization of a dimeric form of the protein that allows sequence-specific DNA binding and transcription activation [6]. The denitrification regulatory DNR-like proteins appear to respond to NO as a physiological signal molecule, but binding of NO to these proteins has not yet been established [9,10]. On the other hand, *fixK* is regulated at the level of gene expression by a two-component regulatory system so that the cellular level of the FixK protein determines FixK-dependent transcription [11]. All these examples illustrate the versatility of this family of transcription factors to regulate, in response to a variety of environmental cues, expression of genes involved in different cellular processes.

The best characterized bacterial nitrogen control system is the NtrB–NtrC two-component regulatory system of the enterobacteria, in which phosphorylated NtrC is an activator of transcription from σ^{54}-dependent promoters and NtrB is a kinase/phosphatase of NtrC whose activity is modulated by the P_{II} (*glnB* gene product) protein [12]. The action of P_{II} is itself regulated by uridylylation catalyzed by the uridylyl transferase/uridylyl removing enzyme. The activity of this complex regulatory system responds to the nitrogen and carbon/energy status of the cell, which is signaled by glutamine, 2-oxoglutarate and ATP [13,14]. Whereas 2-oxoglutarate is the carbon skeleton used for the incorporation of nitrogen, glutamine is a principal product of nitrogen assimilation, and both of them are metabolites of the glutamine synthetase–glutamate synthase cycle for nitrogen assimilation. In the uni-

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0014-5793/02/$22.00
PII: S0014-5793(02)02219-6

cellular cyanobacterium *Synechococcus* sp. PCC 7942, the P_{II} protein, which is essential for the post-translational inhibition by ammonium of nitrate uptake [15], is regulated by phosphorylation in a process in which 2-oxoglutarate and ATP play key roles [16,17]. These data suggest that 2-oxoglutarate and ATP may be involved in signaling the nitrogen status of the *Synechococcus* cells.

In this article, we report that 2-oxoglutarate and Mg^{2+} ions influence the binding in vitro of NtcA to the promoter of the *Synechococcus* sp. PCC 7942 *glnA* gene, a well-characterized NtcA-activated promoter [3].

2. Materials and methods

2.1. Purification of NtcA

The NtcA recombinant protein from *Synechococcus* sp. PCC 7942 was purified from extracts of induced cells of *Escherichia coli* DH5α (pCSI26) [3], which overexpressed NtcA from the *trc* promoter, a synthetic isopropyl β-D-thiogalactopyranoside (IPTG)-inducible promoter. *E. coli* DH5α cells carrying plasmid pCSI26 were grown overnight in LB medium supplemented with 100 μg ampicillin ml^{-1}. 30 ml of this saturated culture were used to inoculate 500 ml of fresh medium that was incubated at 37°C with shaking (200 rpm) for 90 min. IPTG was then added to a final concentration of 1 mM and the cells were further incubated for 2 h under the same conditions. Cells were harvested at $10\,000\times g$ for 5 min at 4°C and resuspended in cold buffer A, containing 20 mM sodium phosphate (pH 7.0), 0.2 M NaCl and 10% (v/v) of glycerol, at a ratio of 5 ml of buffer A per g cells. The cells were then disrupted by four cycles of 30 s of sonication, with a Branson 250 sonifier at 60 W, followed by 30 s of incubation on ice. Soluble fractions were obtained after two successive centrifugations at $31\,000\times g$ for 30 min and $142\,000\times g$ for 90 min, both at 4°C. Cell-free extracts from the cells of 1–2 l of induced culture were applied to a 4-ml heparin–Sepharose column equilibrated with buffer A and washed with 10 column volumes of the same buffer, at a flow of 0.2 ml min^{-1}, and then eluted with a 0.2 to 1 M NaCl gradient, followed by 10 ml of buffer A supplemented with 1 M NaCl, at 0.06 ml min^{-1}. NtcA was eluted at a salt concentration of 0.85 M. Fractions containing NtcA were mixed, diluted with 20 mM sodium phosphate and 10% glycerol to decrease salt concentration, and subjected again to the same chromatography process. This protocol rendered electrophoretically homogeneous NtcA protein.

2.2. Band-shift assays

A 0.35-kb DNA fragment containing the promoter from the *Synechococcus* sp. PCC 7942 *glnA* gene, corresponding to the *glnA* promoter fragment IV in [3], was isolated by restriction of pCSI38 (fragment IV cloned in pBluescript SK^+ [3]) with *Eco*RI and *Xho*I and end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (Boehringer Mannheim). The reaction was carried out in the buffer supplied by the manufacturer, with 0.5–1 U μl^{-1} of enzyme and 9×10^5 Bq of [γ-^{32}P]ATP. After an incubation of 1 h at 37°C, labeled DNA was precipitated with ethanol, and non-incorporated nucleotides were removed by consecutive washes with 70% (v/v) ethanol. Finally, the DNA was resuspended in 10 mM Tris–HCl and 0.1 mM EDTA (pH 7.0) buffer.

Binding assays were performed essentially as described previously [3], using an extract (0.1 μg of total protein added to a 20 μl-reaction mixture) from IPTG-induced *E. coli* (pCSI26) or purified NtcA protein (see above) at about 4 nM and target DNA (see above) at 0.2 to 1 nM, in a buffer that contained 0.1 μg μl^{-1} of herring sperm DNA, 0.25 μg μl^{-1} of bovine serum albumin, 8% glycerol, 12 mM HEPES–NaOH (pH 8), 4 mM Tris–HCl (pH 8), 60 mM KCl, 1 mM dithiothreitol, and the additions indicated in each experiment (nucleotides were added as their lithium salts). Images of radioactive gels were visualized and quantified with an InstantImager detector for β particles (Packard).

To calculate K_d values, a 161-bp DNA fragment containing the *Synechococcus glnA* promoter region was prepared by polymerase chain reaction using the primers glnA5 (5′-CGC CTG CAA GAT TTC GTT AC-3′) and glnA6 (5′-CAC AAC CAG GAA CTG AAG AC-3′) and pCSI38 as template. Band-shift assays were carried out with 1.5 nM DNA fragment (labeled as described above) and 1–500 nM of purified NtcA protein. The percentage of the radioactivity retarded in each lane with respect to the total radioactivity loaded was plotted against the concentration of NtcA used. By fitting to a simplified version of the Hill equation [18], the K_d value was calculated as the concentration of NtcA protein effecting retardation of half of the amount of the labeled DNA used.

3. Results

NtcA is a positive-acting transcription factor for the expression of the *glnA* gene under nitrogen-limiting conditions. The effect of 2-oxoglutarate as a putative positive effector for binding of NtcA to a DNA fragment carrying the *Synechococcus glnA* promoter was tested in band-shift assays, both in the presence and absence of 5 mM $MgCl_2$ and 100 μM ATP, using extracts of an *E. coli* strain overproducing NtcA or a purified NtcA protein preparation. The results obtained indicated that, in the presence of $MgCl_2$/ATP, 2-oxoglutarate stimulated the binding of NtcA such that addition of 0.1 or 1 mM 2-oxoglutarate resulted in a percent retarded fragment higher than that obtained in the absence of 2-oxoglutarate or of $MgCl_2$/ATP. ATP was however found not to be required for the positive effect of 2-oxoglutarate since this effect could be observed when $MgCl_2$/ATP was substituted by $MgCl_2$ alone or by combinations of $MgCl_2$/ADP, $MgCl_2$/CTP, $MgCl_2$/GTP or $MgCl_2$/UTP (not shown). The effect of 2-oxoglutarate and $MgCl_2$ on the binding of NtcA to the target DNA fragment was then characterized using the purified NtcA protein.

The effect of $MgCl_2$ was tested at concentrations of 1 to 5 mM, and a concentration-dependent inhibition and stimulation of binding was observed in the absence and presence of 2-oxoglutarate, respectively (Fig. 1). Results in Fig. 1 additionally show that neither the negative effect of $MgCl_2$ alone nor the positive effect of 2-oxoglutarate/$MgCl_2$ could be reproduced at similar levels when $MgCl_2$ was substituted by $CaCl_2$ or by $MnCl_2$.

Some metabolites were tested at different concentrations for their capability to substitute for 2-oxoglutarate as a positive effector for NtcA binding to the *glnA* promoter in the presence of $MgCl_2$. As shown in Fig. 2, no significant stimulation of the binding was observed with either glutamate or oxalo-

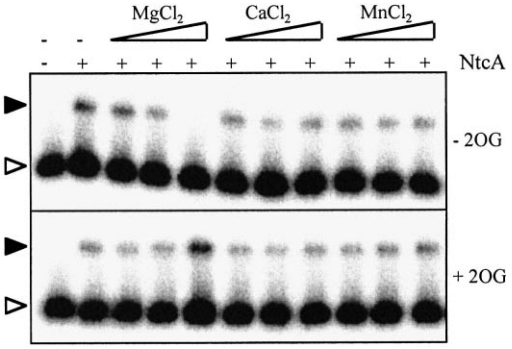

Fig. 1. Effect of $MgCl_2$, $CaCl_2$, and $MnCl_2$ on the binding of NtcA to the *Synechococcus glnA* promoter in the presence and absence of 2-oxoglutarate. Band-shift assays were performed with purified NtcA protein in the presence of 0, 1, 2 or 5 mM of the indicated divalent cation salt and, when indicated, 1 mM 2-oxoglutarate (2OG). The left-most sample in each panel is labeled DNA fragment without any addition. Open triangles point to the free DNA fragment; closed triangles point to the NtcA–DNA complex.

acetate, whereas a lower stimulation, as compared to that obtained with 2-oxoglutarate, was observed with 3-oxoglutarate. Results presented in Fig. 2 additionally show that the maximal effects of 2-oxoglutarate and 3-oxoglutarate were obtained at a concentration of about 0.6 mM. Glutamine, added at 0.01, 0.1 or 1 mM, had no significant effect on the binding of NtcA tested under the same conditions (not shown).

The K_d of the NtcA–DNA complex was calculated in the presence and absence of $MgCl_2$ and/or 2-oxoglutarate (Fig. 3). K_d corresponds to the concentration of NtcA that determines equal concentrations of free and NtcA-bound DNA fragment. K_d values of 52 (in the absence of $MgCl_2$ or 2-oxoglutarate), 40 (in the presence of 0.6 mM 2-oxoglutarate), 37 (in the presence of 5 mM $MgCl_2$), and 10 (in the presence of both $MgCl_2$ and 2-oxoglutarate) nM were obtained (figures are the mean of two independent determinations with similar results).

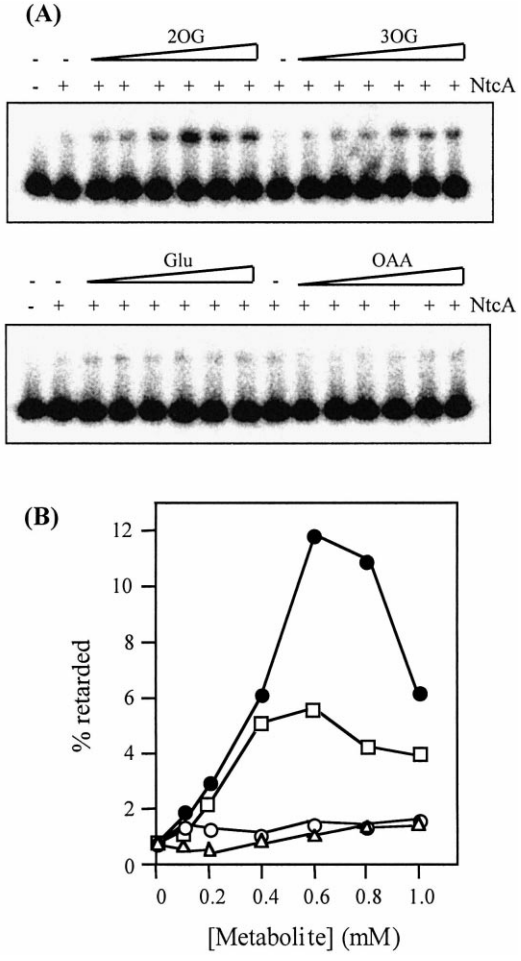

Fig. 2. Effect of different concentrations of 2-oxoglutarate, 3-oxoglutarate, glutamate or oxaloacetate on the binding of NtcA to the *Synechococcus glnA* promoter. A: Band-shift assays performed with purified NtcA protein in the presence of 5 mM $MgCl_2$ and 0, 0.1, 0.2, 0.4, 0.6, 0.8 or 1 mM of the indicated metabolite (2OG, 2-oxoglutarate; 3OG, 3-oxoglutarate; Glu, glutamate; OAA, oxaloacetate). The left-most sample in each panel is labeled DNA fragment without any addition. B: Quantification of the results from A. Solid circles, 2-oxoglutarate; squares, 3-oxoglutarate; open circles, glutamate; triangles, oxaloacetate. The range of metabolite concentrations used in this experiment was tested in three independent experiments with similar results, i.e. the maximal 2-oxoglutarate and 3-oxoglutarate effects were observed in every case at a concentration of 0.6 mM.

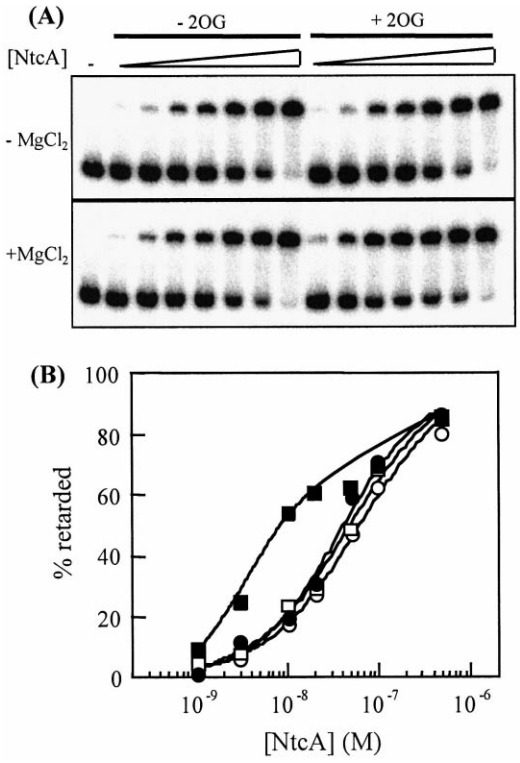

Fig. 3. Determination of the K_d for the NtcA–DNA (*Synechococcus glnA* promoter) complex. A: Band-shift assays were performed with 1, 3, 10, 20, 50, 100, or 500 nM of purified NtcA protein in the presence, as indicated, of 0.6 mM 2-oxoglutarate (2OG) and/or 5 mM $MgCl_2$. The left-most sample in each panel is labeled DNA fragment without any addition. B: Quantification of the results from A. Open circles, no additions; solid circles, $MgCl_2$ added; open squares, 2-oxoglutarate added; solid squares, $MgCl_2$ and 2-oxoglutarate added.

Thus, the presence of $MgCl_2$ and 2-oxoglutarate together in the band-shift assay determines a five-fold increase in the binding affinity of NtcA for the *Synechococcus glnA* promoter. No negative effect of $MgCl_2$ was observed on the K_d of the NtcA–DNA complex. Although we did not pursue the reason for this discrepancy with the results shown above, it should be noted that the assay for K_d determinations involved the use of significantly higher amounts of protein preparation. High protein concentrations may also lead to protein saturation effects hindering the observation of the 2-oxoglutarate effect (Fig. 3).

4. Discussion

The results presented above indicate that 2-oxoglutarate can act as a positive effector of binding of the NtcA transcription factor to the promoter of an NtcA-dependent gene, *glnA* of *Synechococcus* sp. PCC 7942. The lack of effect of another dicarboxylic acid, oxaloacetate, and of other metabolites that are substrates/products of the glutamine synthetase–glutamate synthase cycle, namely glutamate and glutamine, argue in favor of a specific 2-oxoglutarate effect. Consistent with this notion is also the observation that 3-oxoglutarate partially reproduced the effect. 2-Oxoglutarate effects on the signal transduction P_{II} protein are synergic with ATP [13,17], but a similar ATP (or nucleotide) dependence has not been found for the 2-oxoglutarate effect on

NtcA binding to DNA. An effect of a nucleotide might have been expected because NtcA may bear in its N-terminal half a β-roll structure similar to the one that constitutes the cAMP-binding pocket in CAP [2]. cAMP was also tested and found not to affect NtcA binding to DNA under our experimental conditions (not shown). This β-roll structure appears to be conserved in the CAP family proteins, many of which are however not known to bind a nucleotide.

Investigation of a putative nucleotide effect revealed, however, positive and negative effects of $MgCl_2$ on NtcA binding to DNA in the presence and absence of 2-oxoglutarate, respectively. These effects may be ascribed to Mg^{2+} ions since neither $CaCl_2$ nor $MnCl_2$ could effectively replace $MgCl_2$. The basis for the negative Mg^{2+} effect is unknown, but it is conceivable that Mg^{2+} ions shield negative DNA charges, in a way that cannot be paralleled by Ca^{2+} or Mn^{2+} ions, resulting in a requirement for a more specific or efficient NtcA binding. It should be noted that the tested Mg^{2+} concentrations (1–5 mM) are in the physiological range of concentrations for this cation. On the other hand, our results have shown that the positive effect of 2-oxoglutarate requires Mg^{2+} ions, and that a concentration-dependent positive effect of Mg^{2+} ions is manifest in the presence of 2-oxoglutarate. These observations indicate synergy of 2-oxoglutarate and Mg^{2+} in stimulation of NtcA binding to the *Synechococcus glnA* promoter. However, we are not aware of any evidence for changes in the intracellular concentration of Mg^{2+} in response to the nitrogen regime and, therefore, it is possible that Mg^{2+}, although being required, has not a true regulatory action on NtcA.

Because cyanobacteria lack a 2-oxoglutarate dehydrogenase [19], the main metabolic role for 2-oxoglutarate in these microorganisms is incorporation of nitrogen through the glutamine synthetase–glutamate synthase cycle [20]. Therefore, 2-oxoglutarate is considered to be particularly well suited to act as a C to N balance signal in cyanobacteria [21]. Indeed, incorporation of $^{14}CO_2$-derived ^{14}C into 2-oxoglutarate has been shown to respond to the availability of nitrogen in *Synechococcus* sp. (formerly known as *Anacystis nidulans*). After 15 min of $^{14}CO_2$ fixation, an intracellular pool of about 200 μM labeled 2-oxoglutarate is built in the absence of any nitrogen source, and a 13-fold lower pool is found in the presence of combined nitrogen [22]. These and other available data [23] indicate that the physiological range of 2-oxoglutarate concentrations in cyanobacteria incubated under nitrogen-limiting conditions is close to the concentrations of 2-oxoglutarate that exert an effect on NtcA binding to the *glnA* promoter, about 0.6 mM 2-oxoglutarate. The fact that this effect exhibits a 2-oxoglutarate concentration optimum is reminiscent of the cAMP interaction with CAP, in which a CAP dimer with only one bound cAMP molecule represents the species with maximal affinity and specificity for DNA [4]. It should be noted that NtcA appears also to behave as a dimer [2].

The results presented in this work contribute to the knowledge of the versatility of the CAP family of transcriptional regulators, since NtcA appears to respond to 2-oxoglutarate, a putative effector that has not been found before to be involved in the modulation of proteins of this family. We have shown that 2-oxoglutarate increases the affinity of binding of NtcA to a target promoter, but other points of action of this effector on NtcA-dependent transcription activation cannot been ruled out. Although other modes of regulation of the activity of the NtcA protein are also possible, a four- to five-fold increase in binding affinity may explain at least in part the increase in the use of the *glnA* promoter that takes place in *Synechococcus* cells immediately upon ammonium withdrawal, a physiological situation under which the *Synechococcus* cells would contain low levels of NtcA [3].

Acknowledgements: This work was supported by grant PB98-0481 from the Ministerio de Ciencia y Tecnología (Spain).

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