In Vitro Reconstitution and Characterization of the Rhodobacter capsulatus NtrB and NtrC Two-component System*

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Enhancer-dependent transcription in enteric bacteria depends upon an activator protein that binds DNA far upstream from the promoter and an alternative σ factor (σ^54) that binds with the core RNA polymerase at the promoter. In the photosynthetic bacterium Rhodobacter capsulatus, the NtrB and NtrC proteins (RcNtrB and RcNtrC) are putative members of a two-component system that is novel because the enhancer-binding RcNtrC protein activates transcription of a^54-independent promoters. To reconstitute this putative two-component system in vitro, the RcNtrB protein was overexpressed in Escherichia coli and purified as a maltose-binding protein fusion (MBP-RcNtrB). MBP-RcNtrB autophosphorylates in vitro to the same steady state level and with the same stability as the Salmonella typhimurium NtrB (StNtrB) protein but at a lower initial rate. MBP-RcNtrB phosphorylates the S. typhimurium NtrC (StNtrC) and RcNtrC proteins in vitro. The enteric NtrC protein is also phosphorylated in vivo by RcNtrB because plasmids that encode either RcNtrB or MBP-RcNtrB activate transcription of an NtrC-dependent nifL− lacZ fusion. The rate of phosphotransfer to RcNtrC and autophosphatase activity of phosphorylated RcNtrC (RcNtrC−P) are comparable to the StNtrC protein. However, the RcNtrC protein appears to be a specific RcNtrB−P phosphatase since RcNtrC is not phosphorylated by small molecular weight phosphate compounds or by the StNtrB protein. RcNtrC forms a dimer in solution, and RcNtrC−P binds the upstream tandem binding sites of the glnB promoter 4-fold better than the unphosphorylated RcNtrC protein, presumably due to oligomerization of RcNtrC−P. Therefore, the R. capsulatus NtrB and NtrC proteins form a two-component system similar to other NtrC-like systems, where specific RcNtrB phosphotransfer to the RcNtrC protein results in increased oligomerization at the enhancer but with subsequent activation of a a^54-independent promoter.

The NtrB and NtrC proteins of enteric bacteria form a two-component signal transduction system that has been extensively characterized genetically and biochemically (see Ref. 1 for review). Under conditions of nitrogen limitation, the NtrB sensor kinase autophosphorylates on a specific histidine residue (2−5) and transfers the phosphate to the NtrC response regulator protein on a specific aspartate residue (2, 4, 6). Phosphorylated NtrC (NtrC−P) is a transcriptional activator of genes involved in nitrogen metabolism such as glnA (glutamine synthetase). NtrC−P has enhanced DNA binding activity (7), presumably due to increased oligomerization on the DNA template (8, 9), and an ATPase activity (10, 11), which may also be due to the oligomerization of the NtrC phosphoprotein (12, 13). These properties of oligomerization and ATPase activity are essential for transcriptional activation in vitro (14) and in vivo (15, 16, 17).

Members of this class of proteins share certain properties. (a) They bind to DNA at tandem sites far upstream (−100 bp) of the promoters that they activate (see Ref. 18 for review); (b) they contain an ATP binding motif, and possess ATPase activity (11); and (c) they require a specific σ factor, called σ^54, that binds with the core RNA polymerase at highly conserved promoters (see Refs. 19−21 for reviews). The RcNtrC protein binds to sites over 100 bp upstream of the glnA promoter (22, 23), and DNA looping occurs between the NtrC protein bound at the enhancer and the σ^54−RNA polymerase holoenzyme (which forms a stable closed complex) bound at the promoter (24−27). Interaction between the activated NtrC protein and the σ^54 RNA polymerase holoenzyme, in combination with the ATPase activity of the NtrC protein results in a dramatic stimulation of the expression of the glnA gene (12, 14).

The NtrC protein from the photosynthetic bacterium Rhodobacter capsulatus (RcNtrC) is a novel enhancer-binding protein that does not require the σ^54 factor to activate transcription of the R. capsulatus nifA1, nifA2, and glnB genes (28−32). The promoters of these genes have been defined by lacZ translational fusions and primer extension analysis; they are expressed in strains lacking σ^54 and have no sequence homology to σ^54 promoters. The proteins encoded by the nifA1 and nifA2 genes are themselves transcriptional activators that induce nitrogen fixation (nif) gene expression, using the σ^54 RNA polymerase under conditions of nitrogen and oxygen limitation (29, 33). The glnB gene is part of a glnBA operon; the GlnB protein putatively acts to repress R. capsulatus NtrB (RcNtrB) function under conditions of nitrogen excess (34, and see 28 and 35 for reviews). The RcNtrC protein also binds to sites on the DNA greater than 100 bp upstream from the promoters that it activates. The RcNtrC binding sites have been characterized by extensive deletion analysis of the nifA1 and nifA2 promoters (29, 31). In vitro, DNase I footprinting directly demonstrates that RcNtrC binds to tandem sites of dyad symmetry at the nifA1, nifA2, and glnB upstream regions (31, 32). In addition, the RcNtrC protein has an ATP binding

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1 The abbreviations used are: NtrC−P, phosphorylated NtrC; bp, base pair(s); DTT, dithiothreitol; IPTG, isopropyl-β-D-thiogalactoside; PMSF, phenylmethylsulfonyl fluoride; RcNtrC, R. capsulatus NtrC; RcNtrB, R. capsulatus NtrB; MBP-RcNtrB, maltose-binding protein-R. capsulatus NtrB fusion; StNtrB, S. typhimurium NtrB; StNtrC, S. typhimurium NtrC; NtrB−P, phosphorylated NtrB; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

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motif, which by homology with other ATP-binding proteins is predicted to bind and hydrolyze ATP (36); mutations in this motif prevent transcriptional activation by the RnCt protein in vivo (31).

The RnBt and RnCt proteins are putative members of a two-component system based on sequence homology to the enteric NtrB/NtrC proteins, especially in the regions that are highly conserved in other two-component systems (37). Genetic evidence demonstrates that the R. capsulatus nrb and nrc genes are members of an operon, and both genes are essential for transcriptional activation of nif genes in vivo (38, 39). The present study demonstrates that the RnBt and RnCt proteins comprise a two-component regulatory system, that RnBt - P is a specific substrate for RnCt, and that the phosphorylated RnCt protein has increased DNA binding activity in vitro at RnCt tandem upstream binding sites. The R. capsulatus proteins are compared to their counterparts in enteric bacteria.

**EXPERIMENTAL PROCEDURES**

Bacterial Strains and Plasmids—All strains and plasmids are described in Table 1. The malE-mRNA-binding protein fusion to the RnBt protein was made by polymerase chain reaction (PCR) of the R. capsulatus nrb gene in plasmid pDQ2013 using the upstream oligonucleotide 5'-CCGGATCCATGAACCTGCCCCCGCCGATC-3' and the downstream oligonucleotide 5'-CCCCAAGCTTAAAGCTCCTTCGCCGAC-3'. The 1.2-kb PCR product was cut with BamHI and HindIII and cloned into the pml-c2 vector (New England Biolabs, Beverly, MA) to create an in-frame malE-R. capsulatus nrb fusion (pMBPRb). The pETRb plasmid that contains the R. capsulatus nrb gene directly downstream of an inducible T7 promoter was made by PCR amplification of the R. capsulatus nrb gene using the upstream oligonucleotide 5'-CATGCCATGGACCTGCCCCCGCCGATC-3' and the downstream oligonucleotide for pMBPRb. The PCR fragment that contains nrb was cut with Ncol and HindIII and cloned into pET22B (Novagen, Madison, WI). The PCR-generated genes have been shown to complement R. capsulatus nrb mutants. The plasmid pglnBP12 was constructed by cutting pglkR1218 (that contains the glnBA genes with SacII) of the 300-bp R. capsulatus glnB upstream region, and ligation into pUC18 (21). The maltose-binding protein fusion to the RnCt protein was made by PCR of the nctC gene in plasmid pDQ2013 using the upstream oligonucleotide 5'-CATGCCATGGACCTGCCCCCGCCGATC-3' and the downstream oligonucleotide 5'-CCGGATCCATGAACCTGCCCCCGCCGATC-3'. The 1.2-kb PCR product was cut with BamHI and HindIII and cloned into the pml-c2 vector (New England Biolabs, Beverly, MA) to create an in-frame malE-R. capsulatus nctC fusion (pMBPRc).

**Protein Purification**—The maltose-binding protein fusion on plasmid pMBPRb was overexpressed in Escherichia coli strain TB1 by the addition of 1 mM IPTG for 3 h at 37°C. Cells were sonicated in lysis buffer (10 mM NaPO₄, pH 7, 30 mM NaCl, 10 mM EDTA, 10 mM EGTA, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF) and loaded onto an amylose column (New England Biolabs, Beverly, MA). The column was washed with 20 volumes of column buffer, and the MBP-RnBt protein was eluted by the addition of column buffer and 10 mM maltose.

**Radiolabeled Acetyl Phosphate**—Radiolabeled acetyl phosphate was prepared by the incubation of [γ-32P]ATP (6000 Ci/mol; Amersham) in 25 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 10 mM DTT, and 0.5 mM PMSF and loaded onto an amylose column (New England Biolabs, Beverly, MA). The column was washed with 20 volumes of column buffer, and the MBP-RnBt protein was eluted by the addition of column buffer and 10 mM maltose (21). The RnCt protein was purified as described (31) with the addition of an ion exchange step using a DEAE-cellulose column (Sigma) before gel filtration chromatography.

**DNA sequencing**—The purified MBP-RcNtrB or StNtrB proteins (200 nM dimers) were precipitated at 37 °C for 2 in minute phosphorylation buffer (25 mM Tris-acetate, pH 8.0, 0.1 mM EDTA, 50 mM KCl, 1 mM DTT, and 4% glycerol). 100 μM unlabeled ATP, 0.2 μM [γ-32P]ATP (6000 Ci/mol; Amersham) were incubated with purified MBP-RcNtrB-phosphate (separated away from ATP as described above). The MBP-RcNtrB-phosphate (500 nM) was phosphorylated by incubation with StNtrB (200 nM, dimers) as described above. The MBP-RcNtrB-phosphate (500 nM) was phosphorylated by incubation with StNtrB (200 nM, dimers) as described above. The MBP-RcNtrB-phosphate (500 nM) was phosphorylated by incubation with StNtrB (200 nM, dimers) as described above. The MBP-RcNtrB-phosphate (500 nM) was phosphorylated by incubation with StNtrB (200 nM, dimers) as described above. The MBP-RcNtrB-phosphate (500 nM) was phosphorylated by incubation with StNtrB (200 nM, dimers) as described above. The MBP-RcNtrB-phosphate (500 nM) was phosphorylated by incubation with StNtrB (200 nM, dimers) as described above. The MBP-RcNtrB-phosphate (500 nM) was phosphorylated by incubation with StNtrB (200 nM, dimers) as described above. The MBP-RcNtrB-phosphate (500 nM) was phosphorylated by incubation with StNtrB (200 nM, dimers) as described above. The MBP-RcNtrB-phosphate (500 nM) was phosphorylated by incubation with StNtrB (200 nM, dimers) as described above. The MBP-RcNtrB-phosphate (500 nM) was phosphorylated by incubation with StNtrB (200 nM, dimers) as described above. The MBP-RcNtrB-phosphate (500 nM) was phosphorylated by incubation with StNtrB (200 nM, dimers) as described above. The MBP-RcNtrB-phosphate (500 nM) was phosphorylated by incubation with StNtrB (200 nM, dimers) as described above. The MBP-RcNtrB-phosphate (500 nM) was phosphorylated by incubation with StNtrB (200 nM, dimers) as described above. The MBP-RcNtrB-phosphate (500 nM) was phosphorylated by incubation with StNtrB (200 nM, dimers) as described above.
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Prepared as described (40). Western analysis was performed using ECL detection agents (Amersham, Little Chalfont, United Kingdom). Sensitivitive gels contained SDS only in the running buffer (0.1% SDS); no SDS was present in the sample buffer or the polyacrylamide gels themselves. Protein concentrations for all experiments were determined by BCA assays (Pierce).

RESULTS AND DISCUSSION

Purification and Autophosphorylation of the R. capsulatus NtrB Protein—For in vitro studies on the RcntrB and RcntrC two-component system, both proteins were purified. RcntrB was purified as a maltose-binding protein fusion (MBP-RcntrB) because overexpression of the RcntrB protein alone using an inducible T7 overexpression system (Ref. 41; Novagen) produced only low levels of the RcntrB protein (data not shown). Additionally, a previous report indicated that a MBP-fusion to the E. coli NtrB protein did not affect the autophosphorylation or phosphate transfer activities of NtrB (42). The MBP-RcntrB fusion was made as an N-terminal R. capsulatus ntrb fusion to the C terminus of the malE gene to create pMBPRcB. E. coli cells transformed with pMBPRcB were induced in liquid culture with IPTG to overexpress MBP-RcntrB, which was observed as a doublet that migrates at approximately 80 kDa by SDS-PAGE analysis (Fig. 1, lane 3); the predicted molecular mass for the fusion protein is 78 kDa, and 38 kDa for the RcntrB protein. After ultracentrifugation (Fig. 1, lane 4) of sonicated cell extracts, MBP-RcntrB was purified by affinity chromatography to more than 95% purity by SDS-PAGE analysis (Fig. 1, lane 7). The MBP-RcntrB protein had several major forms that are presumably degradation products of the MBP-RcntrB protein. Attempts to prevent or limit this proteolysis were unsuccessful. Proteolysis occurs in vivo upon overexpression; however, the lower forms did not interfere with the activity of the RcntrB protein in vivo or in vitro (see below).

To determine if the R. capsulatus NtrB protein is a histidine kinase capable of autophosphorylation, MBP-RcntrB was incubated with [γ-32P]ATP at 37 °C. MBP-RcntrB became labeled, indicating that it autophosphorylates (Fig. 2, lane 3). To compare the autokinase activity of RcntrB to the enteric NtrB protein directly, purified S. typhimurium NtrB protein (StntrB) was also incubated with [γ-32P]ATP (Fig. 2, lane 1). Time-course experiments demonstrated that the StntrB protein became labeled more rapidly than the MBP-RcntrB protein, but at maximal phosphorylation both proteins incorporated an equivalent level of label, indicating that both proteins were phosphorylated to the same degree (Fig. 3).

Two-component sensor proteins autophosphorylate on a spe-

Table 1

Bacterial strains and plasmids

| Strain/plasmid | Description | Source/Reference |
|---------------|-------------|-----------------|
| E. coli J M101 (ΔDE3) | F' ara Δ(lac-proAB) rpsL (Str^r) [Δ800lacΔ(lacZ) M15] hsdR (r^− m^−) | Novagen (Madison, WI) |
| pUC118 | Amp, M13 intergenic region | Novagen (Madison, WI) |
| pMBPRcB | Amp, malE-R. capsulatus ntrb fusion under control of lacP | This study |
| pmal-C2 | Amp, malE gene under control of lacP | New England Biolabs (Beverly, MA) |
| pglnBP12 | Amp, 300-bp R. capsulatus glmB upstream region | Novagen (Madison, WI) |
| pDG0103 | Amp, ordered deletion of pRCN102 | This study |
| pRCN2128 | Amp, 2.2-kb EcoRI R. capsulatus glmB fragment in pUC118 | (34) |
| pET21B | Amp, T7 overexpression vector | Novagen (Madison, WI) |
| pETRcC | Amp, R. capsulatus ntrC under control of T7 promoter | (31) |
| pETRcB | Amp, R. capsulatus ntrB under control of T7 promoter | This study |
| pRCN102 | TetR, R. capsulatus ntrR3, ntrB, ntrC, 8.0-kb fragment (pLAFR1) | (62) |
| pDO531 | Tet, K. pneumoniae nifL-lacZ gene | (48) |
cific histidine residue in the conserved C terminus of the protein (6, 44, 45). The RcNtrB protein contains a histidine at position 214 that is completely conserved with the enteric NtrB proteins and other sensor proteins. To demonstrate that the RcNtrB protein is a histidine kinase, purified MBP-RcNtrB–P was blotted directly onto nitrocellulose, washed in 50 mM Tris-HCl, and exposed to neutral, acidic, or basic conditions prior to radiodetection. MBP-RcNtrB–P was sensitive to acidic conditions (a 13-fold loss of signal was observed in the presence of 1 N HCl) and stable in the presence of basic conditions (no loss of signal was observed in the presence of 0.5 N NaOH; data not shown). These results are consistent with the properties of histidinyl-phosphate residues (46, 47), indicating that phosphorylation of the MBP-RcNtrB protein probably occurs on a histidine residue.

The RcNtrB–P Phosphorylates the E. coli NtrC Protein in Vivo and the StNtrC Protein in Vitro—We wanted to determine if the RcNtrB protein phosphorylates the enteric NtrC protein in vivo and to compare the activities of the RcNtrB and the MBP-RcNtrB proteins. The nitrogen-regulated nifL gene from Klebsiella pneumoniae, which is under control of the enteric NtrB/NtrC and is expressed to high levels under nitrogen limiting conditions (48), was used as a reporter for RcNtrB activity. Overexpression plasmids that contained the R. capsulatus ntrB gene (pETRcB), the R. capsulatus malE-ntrB gene (pMBPRcB), the R. capsulatus ntrC gene (pETRcC), and the pma-C2 and pET21B plasmids as controls were transformed into strains that contained the nifL-lacZ fusion on a compatible plasmid (Table I). Two E. coli strains were used in this study: TB1, to induce the expression of the malE and malE-R. capsulatus ntrB genes from the lac promoter, and JM109 (DE3), to induce expression in the pET21B, pETRcB and pETRcC plasmids. Colonies that contained both plasmids were picked onto nitrogen-rich plates to prevent activation by the endogenous E. coli NtrB protein. These plates also contained 0.5 mM IPTG to induce protein expression from both the T7 and the lac promoters (the MBP-RcNtrB protein is overexpressed to approximately 5% of the total cell protein (see Fig. 1 for MBP-RcNtrB), which is presumably sufficient to overcome inhibition by the E. coli GlnB protein of the RcNtrB kinase activity). 5-Bromo-4-chloro-3-indol-β-D-galactopyranoside at 50 μg/ml was added to observe the level of lacZ expression from the nifL gene. The R. capsulatus ntrB gene as well as the R. capsulatus malE-ntrB gene induced expression from the nifL-lacZ fusion to comparable levels (Fig. 4, A and C, respectively), whereas plasmid pET21B, or plasmids that encode RcNtrC, or MBP alone did not induce expression (Fig. 4, B, D, and E, respectively). We conclude that the RcNtrB protein can phosphorylate the E. coli NtrC protein, the transcriptional regulator of the nifL gene, and that the RcNtrB and the MBP-RcNtrB show similar in vivo activities.

Previous experiments have shown that the enteric NtrC protein can be phosphorylated in vitro by a variety of sensor kinases, including the CheA protein (49). To determine if MBP-RcNtrB–P could phosphorylate the enteric NtrC protein, MBP-RcNtrB–P was incubated with the Salmonella typhimurium NtrC protein (StntrC) in vitro. Label disappeared from both major forms of the MBP-RcNtrB protein and appeared in the StntrC protein, indicating that the MBP-RcNtrB–P is a substrate for the StntrC protein (Fig. 3, lane 2). To directly compare the phosphotransfer properties of the RdNtrB to the enteric NtrB protein, both MBP-RcNtrB–P and StntrB–P (at 200 nM dimers) were incubated with the StntrC protein, and the transfer of phosphate was measured. Both MBP-RcNtrB–P and StntrB–P labeled StntrC to a comparable level (Fig. 3, compare lanes 2 and 4). The autophosphatase activity of StntrC–P...
was determined to be the same irrespective of phosphorylation by the MBP-RcNtrB or StNtrB proteins (see below). Thus, the StNtrC protein probably interacts with a highly conserved functional domain of both NtrB proteins (supported by amino acid sequence homology in the region of the conserved histidine).

The MBP-RcNtrB–P Protein Phosphorylates the RcNtrC Protein in Vitro—To determine if the RcNtrC protein can be phosphorylated by RcNtrB, purified MBP-RcNtrB was phosphorylated as described above and incubated with purified RcNtrC (Fig. 1, lane 8). As shown in Fig. 2 (lane 7), the addition of RcNtrC led to the disappearance of label from RcNtrB with its subsequent appearance in RcNtrC. Optimal transfer of phosphate from RcNtrB to RcNtrC was found to occur when RcNtrB was present at approximately one-half the concentration of RcNtrC (Fig. 5A). Increasing StNtrB to equimolar with StNtrC did not improve StNtrC phosphorylation (Fig. 2, lane 10). Optimal phosphorylation of RcNtrC also occurred when the DTT concentration was increased from 1 mM to 10 mM (Fig. 1). Optimal phosphorylation of RcNtrC also occurred when 10 mM DTT was present at exactly the same concentration as the RcNtrC (data not shown). Increasing StNtrB to equimolar with StNtrC did not improve StNtrC phosphorylation (Fig. 2, lane 7) that was stable for at least 1 h (Fig. 2, lane 8). RcNtrC alone did not label in the presence of [γ-32P]ATP under any conditions (Fig. 2, lane 5). To directly compare the phosphorylation of the RcNtrC and StNtrC proteins, we used the MBP-RcNtrB–P protein to phosphorylate both proteins. The RcNtrC and StNtrC proteins were phosphorylated at approximately the same rate by the MBP-RcNtrB protein; the maximal level of RcNtrC–P was comparable to the StNtrC–P (Fig. S5B). Previous work demonstrated that the enteric NtrC–P has an autophosphatase activity that recycles the protein to its unphosphorylated form. Phosphate is also removed from RcNtrC–P by a mechanism called regulated dephosphorylation, which requires both the NtrB and GlnB proteins (Ref. 3). To determine if the RcNtrC–P has an autophosphatase activity, RcNtrC–P was formed and its decay was measured and compared to the decay for the StNtrC–P protein. RcNtrC–P had an autophosphatase activity with a half-life of 2–4 min (Fig. 6) similar to the 3–5 min observed for StNtrC–P (Fig. 6, and see Ref. 3).

To directly address the specificity of the RcNtrC protein for phosphate donors (or kinase proteins), we tested the ability of RcNtrC to be phosphorylated by a variety of substrates. Small molecular weight high energy phosphate compounds (acetyl phosphate, carbamyl phosphate, and phosphoramidate) have been shown to phosphorylate the enteric NtrC protein (50) and other members of the response regulatory protein family in vitro (51). Radiolabeled acetyl phosphate was prepared and incubated with both the StNtrC and RcNtrC proteins for 10 and 40 min; StNtrC–P was detected after 10 min and increased in concentration during the following 40 min; however, no label was incorporated into the RcNtrC protein after 40 min (data not shown). Unlabeled acetyl phosphate (Sigma), carbamyl phosphate (Sigma), and either ammonium or potassium phosphoramidate (each at 20 mM) did not inhibit the phosphorylation of RcNtrC by the MBP-RcNtrB–P (data not shown), whereas these compounds can compete with the enteric

![Graph of the disappearance of phosphate from the RcNtrC protein](image)

**Fig. 6. The autophosphatase activity of the RcNtrC protein.** Graph of the disappearance of phosphate from the RcNtrC–P and StNtrC–P proteins. The y axis refers to the log of the labeled phosphoprotein (in cpm), and the x axis refers to time (in minutes). Open squares denote StNtrC–P, and diamonds denote RcNtrC–P. Autophosphatase assays are described in the text.

![The RcNtrC protein is a dimer](image)

**Fig. 7. The RcNtrC protein is a dimer.** A, the graph represents the elution profile of the purified RcNtrC protein off of a Sephacryl S200-HR (gel filtration) column calibrated by size standards. The x axis represents the fraction number off of the column, and the y axis represents the concentration of purified RcNtrC protein (in μg/μl). Arrows denote the elution of size standards, except for the arrow at approximately 110 kDa that indicates the peak elution fraction of the RcNtrC protein. B, Western analysis of a semi-native 10% SDS-polyacrylamide gel (see "Experimental Procedures") that was transferred to nitrocellulose and probed with antibodies raised against the RcNtrC protein as detected by phosphorescence of protein A peroxidase (ECL kit). The gel was loaded with 20 μl (lane 1), 100 μl (lane 2), or 500 μl (lane 3) of the purified RcNtrC protein. Lane 4 shows a Western analysis of extracts of 10 μg of wild type R. capsulatus (SB1003) probed with antibodies against the RcNtrC protein. Size standards are indicated at left. Western blots were performed as described under "Experimental Procedures."
NtrB–P for enteric NtrC phosphorylation (52). Additionally, DNase I footprinting (see below) of the RcNtrC protein was not enhanced by any of these small molecular weight compounds. The enteric NtrB protein can phosphorylate other response regulatory proteins, including the CheY protein in vitro (49). To determine if the RdNtrC protein could be phosphorylated by the enteric NtrB protein in vitro, phosphorylated StNtrB protein was prepared and incubated in the presence of the RdNtrC protein. No loss of label was observed from the StNtrB–P, and no label was incorporated into the RdNtrC protein (Fig. 2, lane 6). In this respect it is interesting that R. capsulatus ntrB mutants that are not polar on R. capsulatus ntrC are still Nif$^-$ (38), suggesting that cross-talk with other kinases or RdNtrC phosphorylation by small molecular weight compounds does not occur in vivo. This is in contrast with the enteric NtrC protein (53) and consistent with a hypothesis that the phosphorylation domain of RdNtrC may form a structure that makes it less accessible than the domain of the StNtrC protein for other substrate kinases.

Phosphorylation of RdNtrC protein is predicted to occur on an aspartate residue within the conserved N terminus; RdNtrC has an aspartate at residue 53 that is highly conserved between other members of the NtrC class of enhancer-binding proteins (54). Using the same methods described for RdNtrB–P, the RdNtrC–P was determined to be sensitive to base (a 3-fold loss of signal was observed in 0.5 N NaOH) and stable in acid (less than 10% loss of signal was observed in 1 N HCl; data not shown), indicative of a serine, threonine, or aspartate residue (4, 55, 56).

**DNase I Footprinting with the Unphosphorylated and Phosphorylated RcNtrC Protein in Vitro—** During purification, the RdNtrC protein eluted from a gel filtration column in fractions that suggested a native molecular mass of dimers rather than the 54-kDa monomer (31). The enteric NtrC protein naturally forms a dimer in solution (57, 58), and upon activation by phosphorylation, it oligomerizes at specific DNA binding sites (8). To more fully characterize the purified RdNtrC protein, a 95% pure preparation of the RdNtrC protein was loaded onto a gel filtration column (Sephacryl S200-HR, Pharmacia) calibrated by size standards to determine its native size. The majority of the purified RdNtrC protein elutes predominantly at 110 kDa, confirming that it behaves as a dimer (Fig. 7A). The RdNtrC protein was also observed as a dimer by Western blot analysis of semi-native polyacrylamide gels (see “Experimental Procedures”). Antibodies against RdNtrC were able to detect a monomer form (54 kDa), a dimer form (110 kDa), and oligomer forms (>150 kDa) of the RdNtrC protein (Fig. 7B). The dimer form was the predominant species when 20 nM, 100 nM, or 500 nM protein was loaded onto the gel (Fig. 7B). The addition of SDS (1%) to the loading buffer caused greater than 90% of the upper bands to shift to the 54-kDa band (see Fig. 2, lane 8). Additionally, cell extracts of R. capsulatus grown under nitrogen limiting conditions revealed the presence of both the monomer and dimer forms of RdNtrC by Western analysis (Fig. 7B, lane 4). Thus, the RdNtrC protein forms a dimer in vitro and presumably in vivo.

The enteric NtrC protein binds in vitro and in vivo to specific sites on DNA far upstream of promoters (22, 23, 59). Phosphorylation of the enteric NtrC protein stimulates enhancer binding in vitro by 4–20-fold by oligomerization at tandem binding sites (7, 8, 15). We wanted to analyze this property of the RdNtrC protein. DNase I footprinting was performed with the phosphorylated RdNtrC protein to determine if, like the enteric NtrC, RdNtrC–P has enhanced DNA binding activity compared to unphosphorylated RdNtrC. RdNtrC was phosphorylated by the MBP-RdNtrB–P under optimal conditions (various concentrations of RdNtrC were incubated for 15–45 min at 24 °C with 1 μM MBP-RdNtrB protein in the presence of 10 mM DTT) and allowed to bind to an end-labeled DNA probe containing the glnB promoter region. DNase I digests were performed as described under “Experimental Procedures,” and the regions that were protected from digestion were compared to the regions protected by the unphosphorylated RdNtrC protein. Complete protection was observed at the upstream binding sites of the glnB promoter at 160 nM unphosphorylated RdNtrC (Fig. 8A) but at 40 nM with phosphorylated RdNtrC (Fig. 8C). The binding of unphosphorylated RdNtrC was unaffected by the presence of ATP (Fig. 8, compare A and B). This increase in binding is also clearly indicated by the hypersensitive site induced by RdNtrC binding at the tandem sites (Fig. 8, see arrowheads). We conclude that phosphorylation of RdNtrC increases DNA binding by approximately 4-fold, similar to the enteric NtrC system. Similar to the enteric NtrC–P, RdNtrC may show enhanced DNA binding activity due to increased oligomerization at the enhancer (12).
The RcNtrB/RcNtrC proteins form a novel two-component system in which the RcNtrB enhancer-binding protein activates transcription of the RcNtrC protein in vivo (31). The present study shows that the R. capsulatus NtrB and NtrC proteins are members of a two-component system that has been reconstituted and characterized in vitro. The RcNtrB protein is a sensor kinase that auto-phosphorylates on a histidine residue and transfers the phosphate moiety to the response regulator RcNtrC protein. Phosphotransfer between the RcNtrB and RcNtrC proteins is a key signal that fixed nitrogen is being supported by genetic evidence of the Rc. capsulatus ntrB and ntrC genes, as well as homology to the enteric NtrB/NtrC system. The RcNtrC protein is a dimer in solution and RcNtrC-P has a stronger affinity for the tandem binding sites upstream of glmB, potentially due to RcNtrC oligomerization.

The R. capsulatus and enteric NtrB/NtrC system were directly compared. The RcNtrB protein auto-phosphorylates to the same level and stability as the StNtrB protein, although at a lower initial rate. Both proteins are capable of phosphate transfer to the StNtrC protein, but only the RcNtrB protein phosphorylates the RcNtrC protein, indicating that the RcNtrC protein is a more specific phosphatase than the enteric NtrC protein. This specificity is supported by other genetic and biochemical evidence, including the inability of RcNtrC to use small molecular weight phosphate compounds in vitro and probably in vivo. Conditions for the phosphorylation of RcNtrC protein were optimized, facilitating future biochemical studies on this novel transcriptional activation system.

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REFERENCES

1. Swanson, R. V., Alex, L. A., and Simon, M. I. (1994) Trends Biochem. Sci. 19, 485–490
2. Ninfa, A. J., and Magasanik, B. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 5909–5913
3. Keener, J., and Kustu, S. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 4976–4980
4. Weiss, V., and Magasanik, B. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 8919–8923
5. Ninfa, A. J., and Bennett, R. L. (1991) J. Biol. Chem. 266, 6888–6893
6. Sanders, D. A., Gillece-Castro, B. L., Burlingame, A. L., and Koshland, D. E., Jr. (1990) J. Bacteriol. 174, 3117–3122
7. Weiss, V., Claverie-Martin, F., and Magasanik, B. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 5088–5092
8. Porter, S. C., North, A. K., Wedel, A. B., and Kustu, S. (1993) J. Bacteriol. 175, 2258–2273
9. Chen, P., and Retzer, L. J. (1995) J. Bacteriol. 177, 2490–2496
10. Austin, S., and Dixon, R. (1993) EMBO J. 12, 2219–2225
11. Weiss, D., Batut, J., Klose, K. E., Keener, J., and Kustu, S. (1991) Cell 67, 155–167
12. Flaschner, Y., Weiss, D. S., Keener, J., and Kustu, S. (1995) J. Mol. Biol. 249, 700–713
13. Mertke, I., Fiedler, U., and Weiss, V. (1995) J. Bacteriol. 177, 5056–5061
14. Wedel, A., and Kustu, S. (1995) Genes & Dev. 9, 2024–2052
15. Dixon, R., Eydmann, T., Henderson, N., and Austin, S. (1991) Mol. Microbiol.
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