AlgR2 is an ATP/GTP-dependent protein kinase involved in alginate synthesis by *Pseudomonas aeruginosa*

(protein phosphorylation/two-component sensory transduction/cystic fibrosis infection)

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**ABSTRACT** The exopolysaccharide alginate is a major virulence factor in the pathogenicity of *Pseudomonas aeruginosa* infecting the lungs of cystic fibrosis (CF) patients. Alginate synthesis by *P. aeruginosa* is believed to occur in response to environmental signals present in the CF lung. Transcription of a critical alginate biosynthetic gene, *algD*, is triggered by environmental signals and is known to be controlled by regulatory proteins AlgR1, AlgR2, and AlgR3. AlgR1 is a member of the family of response regulators of the phosphorylation-dependent two-component bacterial signal transduction systems. In this report, we describe the characterization of AlgR2 as the kinase involved in phosphorylation of AlgR1. AlgR2, an 18-kDa soluble protein undergoes rapid autophosphorylation in the presence of either ATP or GTP and transfers the phosphate to AlgR1. AlgR2 retains high affinity for both ATP and GTP with an apparent *K_m* of 137 and 249 nM, respectively, for phosphorylation by these two substrates. ADP and GDP exhibit competitive inhibition with an apparent *K_i* of 94 and 314 nM, respectively, during phosphorylation by ATP and 481 and 273 nM during phosphorylation by GTP. AlgR1 and AlgR2 can be isolated in the form of an 80-kDa complex that is capable of undergoing phosphorylation and intracomplex phosphotransfer *in vitro*. A 16-kDa AlgR2 analog, capable of autophosphorylation in the presence of ATP or GTP and transferring the phosphate moiety to AlgR1, has been characterized in *Escherichia coli*.

Protein phosphorylation is a well known process by which environmentally induced gene regulation is mediated in bacteria (1). Phosphorylation is used as a transient mechanism for modulating regulatory proteins, known as response regulators in the two-component sensory transduction systems (1, 2). This transient modification often enables response regulators to activate or repress the transcription of specific genes. Response regulators, together with the protein kinases responsible for their phosphorylation, constitute the essential features of the two-component systems that are emerging as part of numerous regulatory networks involved in bacterial signal transduction (3). The exopolysaccharide alginate is synthesized by *Pseudomonas aeruginosa* as a major virulence factor in the lungs of patients suffering from cystic fibrosis (CF). Transcriptional activation of a critical alginate biosynthetic gene, *algD*, is induced by environmental signals such as high osmolarity, nitrogen or phosphate starvation, and ethanol-induced membrane perturbation (4). Interestingly, some of these conditions are characteristic of the CF lung environment.

The *algD* gene is located at one end of the alginate biosynthetic gene cluster. The enzyme encoded by *algD* is GDPmannose dehydrogenase (GDPmannose:NAD^+ 6-oxido-reductase, EC 1.1.1.132), which catalyzes the virtually irreversible oxidation of GDP mannose to the alginate precursor GDP mannuronic acid. Regulated expression of *algD* is therefore considered a key step in alginate biosynthesis. Studies on the expression of *algD* revealed that a regulatory gene *algR1* is essential for transcriptional activation of *algD* (4). AlgR1 activates transcription from the *algD* promoter by binding to specific 14-mer DNA sequences at two sites centered at -382 and -458 positions with respect to the transcriptional start site of *algD* (5). AlgR1 is homologous to the response regulators of the two-component systems. About 120 amino acids at the amino terminus of AlgR1 demonstrate homology with other members of the response regulator family. Significant homology exists around the critical aspartate (Asp-7, -8, and -54) residues with similar acidic residues in other members of the response regulator class such as CheY or Spo0A (6, 7).

Expression of a second gene, *algR2*, is necessary for the AlgR1-mediated transcriptional activation of *algD* (8). However, AlgR2, an 18-kDa protein, does not share any significant sequence homology with the family of kinases of the two-component systems. In addition, the kinases of this family are in general of higher molecular mass (2, 9). Thus, understanding the functional role of AlgR2 was limited until recently when it was shown that phosphorylation of AlgR1 occurs via that of AlgR2 (10). In this paper, we present data on the characterization of AlgR2. AlgR2 undergoes autophosphorylation in the presence of either ATP or GTP, and the phosphate group acquired by the protein is then transferred to AlgR1. Several characteristics of AlgR2 make it a unique member of the two-component systems.

**MATERIALS AND METHODS**

**Purification of AlgR2.** AlgR2 was purified from the cell-free extract of *P. aeruginosa* strain 8822, which is a spontaneous nonmucoid revertant of the CF isolate strain 8821. The crude extract was first heated at 57.5°C for 4 min at pH 5.0. The precipitate was removed by centrifugation. The supernatant was then subjected to hydrophobic interaction column chromatography using a TSK phenyl-5-PW HPLC column from Bio-Rad. Washout fractions containing AlgR2 were pooled, desalted, and loaded onto a Mono Q FPLC ion exchange column from Pharmacia. AlgR2 was eluted in fractions corresponding to 300 mM NaCl using a 0–500 mM NaCl linear gradient. The fraction showing AlgR2 activity was subjected to gel-filtration chromatography using a Superose 12 FPLC column from Pharmacia. AlgR2 eluted as a monomer according to its elution volume. Approximately 90% pure AlgR2 was obtained at this stage as judged by silver nitrate-stained SDS/PAGE analysis.

**Abbreviation:** CF, cystic fibrosis.

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Phosphorylation Assay. The phosphorylation assay was performed at room temperature in a 25- to 150-μl reaction volume containing 50 mM Tris·HCl (pH 8.0) and 5 mM MgCl₂. The reaction was initiated by the addition of various concentrations of [γ-32P]ATP or [α-32P]GTP (5000 Ci/mmol; 1 Ci = 37 GBq; Amersham) and terminated by the addition of 4× SDS loading buffer containing 125 mM Tris·HCl (pH 6.8), 4% SDS, 20% glycerol, 10% mercaptoethanol, and 0.01% bromophenol blue. Electrophoresis of the samples was carried out at 45–50 mA for 3–3.5 hr with 13% SDS/polyacrylamide gels. The gels were dried and exposed overnight to Kodak X-Omat AR films. Radioactivity of the phosphorylated protein bands was quantitated by an Ambis 2D beta scanner.

Isolation of AlgR1–AlgR2 Protein Complex. AlgR1 was isolated in a complex with AlgR2 using a previously reported procedure (10). The cell-free extract from P. aeruginosa strain 8822 was heated at 57.5°C (pH 5.0) for 30 sec and the precipitate was removed by centrifugation. In the supernatant only two proteins, identified as AlgR1 and AlgR2 (10), were detectable by the phosphorylation assay. The supernatant was subjected to gel-filtration chromatography using a Superose 12 FPLC column. Elution buffer (50 mM Tris·HCl, pH 8.0/5 mM MgCl₂) containing 1 M NaCl was used to minimize nonspecific aggregation of proteins. The fraction corresponding to a mass of ~80 kDa contained both AlgR1 and AlgR2 as detected by the phosphorylation assay.

Determination of pH Stability of Phosphoproteins. Stability of the phosphorylated forms of AlgR1 and AlgR2 was determined by an established procedure (11, 12). In the case of AlgR1, the AlgR1–AlgR2 complex was used as the protein source. Purified AlgR2 was used to determine the stability of AlgR2 phosphate. The protein samples were first phosphorylated at pH 8.0 (for 10 min in the case of AlgR1 and for 10 sec in the case of AlgR2) to the maximal level by incubation at room temperature in the presence of 16 nM [γ-32P]ATP. The reaction was terminated by the addition of 2 vol of 1.5× SDS loading buffer adjusted to pH 1.0, 4.0, 7.0, 10.0, and 12.0 with either HCl or NaOH. The samples were incubated for 10 min at 50°C and then subjected to SDS/PAGE analysis. The level of phosphorylation of the protein samples was determined by scanning the gel. The control sample was maintained at pH 8.0 at 0°C after the phosphorylation reaction.

Isolation of AlgR1 and the AlgR2 Analog from Escherichia coli. E. coli strain MV 1184 containing the plasmid pJK66R1, which contains the algR1 gene under the control of the tac promoter (5), was used as a source of AlgR1 and the AlgR2 analog. The cell-free extract was subjected to heparin-agarose column chromatography and eluted with a linear gradient of 0.1–1.2 M NaCl. AlgR1 was eluted corresponding to 0.7 M NaCl and contained ~60% pure AlgR1 as judged by SDS/PAGE analysis. As previously reported, a 16-kDa E. coli protein, capable of phosphorylating AlgR1, was copurified by this procedure (10).

RESULTS

Autophosphorylation of AlgR2. We previously reported that AlgR1 is phosphorylated via AlgR2 using ATP as the phosphate donor in partially purified preparations containing these two proteins (10). However, it was unclear whether AlgR2 is an autophosphorylating kinase that transfers its phosphate to AlgR1 or whether it is a phosphotransfer agent that requires a protein kinase for its phosphorylation. We therefore embarked on studying the properties of purified AlgR2.

Purified AlgR2 was incubated with [γ-32P]ATP or [α-32P]-ATP for 10 sec and the incorporation of radiolabel was compared. As shown in Fig. 1, a much higher level of radioactivity was detected in the case of incubation with [γ-32P]ATP. This indicates that phosphorylation of AlgR2 is primarily responsible for its radioactivity. However, trace amounts of radioactivity were observed due to incubation with [α-32P]ATP. This is attributable to the formation of a transient intermediate during the process of autophosphorylation. It also suggests that phosphorylation of AlgR2 results from its direct interaction with ATP. A control experiment in which bovine serum albumin was incubated with [α-32P]ATP showed no incorporation of radioactivity in bovine serum albumin, suggesting that the low level of radioactivity in AlgR2 resulted from a specific interaction. Partially purified preparations from the AlgR2+ mutant strain 8882 showed a low level of phosphorylation of the 18-kDa or any other protein band when incubated with [γ-32P]ATP. This indicates that AlgR2 is the 18-kDa protein kinase undergoing autophosphorylation.

Kinetics of Autophosphorylation and Phosphotransfer. To examine the kinetics of autophosphorylation of AlgR2, we first conducted a time course experiment. As shown in Fig. 2A, the maximum level of phosphorylation was achieved within 10 sec of incubation with [γ-32P]ATP. There was a subsequent loss of phosphate followed by a steady state at ~40% of the maximum level. It was previously shown that phosphorylated AlgR2 can transfer its phosphate to AlgR1 (10). Therefore, the time course experiment was repeated with the 80-kDa complex containing AlgR1 and AlgR2. As shown in Fig. 2B, phosphorylation of AlgR1 is concomitant with the loss of phosphate from AlgR2. In addition, the loss of phosphate from AlgR2 was significantly higher in this case as compared to that in the case of purified AlgR2 (Fig. 2A).

The substrate specificity of AlgR2 for ATP and the competitive inhibition by its analogs was studied by Michaelis-Menten kinetics. From the results of this experiment (Fig. 3A), the apparent Kₘ for the phosphorylation of AlgR2 by ATP was estimated at 137 nM. ADP inhibited the reaction in a competitive manner with an apparent Kᵢ of 94 nM. Interestingly, GDP was also found to be a strong inhibitor. The kinetics of its inhibition appears to be competitive with an apparent Kᵢ of 314 nM. This observation led us to test GTP as an alternative substrate by monitoring the incorporation of 32P in AlgR2 during incubation with [γ-32P]GTP. Kinetics of phosphorylation by GTP (Fig. 3B) revealed that AlgR2 retains high affinity for GTP as a phosphate donor with an
Fig. 2. Time course experiment monitoring the phosphorylation of AlgR2 and AlgR1. (A) The level of phosphorylation of purified AlgR2 was analyzed by an Ambis 2D beta counter and plotted against the corresponding incubation time. (Inset) Autoradiograph of the samples analyzed by SDS/PAGE. Lanes 1–5, samples incubated with 16 nM [γ-32P]ATP for 10 sec, 30 sec, 1 min, 1 hr, and 2 hr. (B) Phosphorylation of AlgR2 and AlgR1 in the AlgR1–AlgR2 complex in the presence of 16 nM [γ-32P]ATP is shown as a function of incubation time. (Inset) Equivalent to that in A.

apparent $K_m$ of 245 nM. However, ATP with a lower $K_m$ is the preferred substrate. ADP and GDP were found to competitively inhibit the phosphorylation of AlgR2 by GTP ($K_i = 481$ and 273 nM, respectively). These results indicate that ADP is a stronger inhibitor than GDP when ATP is used as the phosphate donor. In contrast, GDP is a stronger inhibitor than ADP when GTP is used as the phosphate donor. The competitive inhibition by both ADP and GDP and their differential nature of inhibition of phosphorylation by ATP and GTP suggest that ATP and GTP have separate nucleotide binding sites but share a common site of phosphorylation.

Amino Acid Residues Involved in Phosphorylation of AlgR1 and AlgR2. The stability of phosphorylated AlgR1 and AlgR2 at different pH values was used to determine the nature of the amino acid-phosphate bonds. As shown in Fig. 4, AlgR1 phosphate was unstable at both low and high pH values. However, phosphorylated AlgR1 was relatively stable at near physiological pH. This is characteristic of acyl phosphates, suggesting the possible involvement of aspartate residue(s) in the phosphorylation of AlgR1. The stability of AlgR2 phosphate increased with pH. The lability of phosphorylated AlgR2 toward acidic pH and its stability toward basic pH is indicative of N-linked phosphates, suggesting the possible involvement of a histidine residue in the phosphorylation of AlgR2.

A Functional Analog of AlgR2 in E. coli. It was previously observed (10) that AlgR1, when hyperproduced in E. coli (using the plasmid pJK66R1), can be phosphorylated by a 16-kDa analog of AlgR2 in the presence of ATP. Results shown in Fig. 5 demonstrate that the AlgR2 analog can phosphorylate AlgR1 by using either ATP or GTP as the phosphate donor. However, the rates of phosphorylation and subsequent phosphotransfer are higher with ATP than with GTP as the phosphate donor. From pH stability analysis of the phosphorylated E. coli AlgR2 analog, it appears that, like P. aeruginosa AlgR2, this protein also has a histidine residue involved in its phosphorylation (data not shown).

Fig. 3. Kinetics of AlgR2 phosphorylation by ATP (A) and GTP (B). Initial rate of AlgR2 autophosphorylation ($V$) was measured as a function of ATP or GTP concentration ($S$) and was calculated as the average net rate of phosphorylation per sec within the first 10 sec of reaction. The phosphorylation reaction and quantitation of radioactivity were carried out as described. The radioactivity monitored in cpm was converted to the amount of 32P using [γ-32P]ATP and [γ-32P]GTP as standards. $V$, $*$, and $\bullet$, Phosphorylation reactions carried out with no inhibitor, 400 nM ADP, and 1 μM GDP, respectively.

DISCUSSION

Alginate synthesis by P. aeruginosa is almost exclusively associated with its infection of the CF-affected lung. The alginate genes are turned on in response to a unique set of environmental conditions present in the diseased lung. The role of AlgR1 as an environmentally responsive positive regulator of alginate biosynthetic gene transcription was known from previously reported work (4). However, AlgR1, as a member of the response regulator family, was also believed to require a kinase for its phosphorylation and functional activity. Previous studies have shown that AlgR2 is essential for alginate synthesis by P. aeruginosa (8). Thus, characterization of AlgR2 as the kinase, capable of phosphorylating AlgR1, provides strong evidence of the importance of phosphorylation of AlgR1 in alginate synthesis. A model describing the putative roles of phosphorylated AlgR1 and other regulatory proteins in activating transcription from the algD promoter is presented in Fig. 6. It should be noted that the extracts used for phosphorylation of AlgR2 and AlgR1 were obtained from P. aeruginosa strain 8822, a spontaneous nonmucoid (alginate negative) revertant of the mucoid CF isolate strain 8821. Our results therefore suggest that nonmucoid cells, in which the algD promoter is activated by
environmental signals, are capable of phosphorylating both
AlgR2 and AlgR1. At this point it is not clear whether
phosphorylated AlgR1, as shown in Fig. 6, is the active form
in which it regulates alginate synthesis or whether it under-
goes further modification in the genotypically mucoid strains
of _P. aeruginosa_ isolated from the CF lung.

The properties of AlgR2 reported in this paper reveal some
interesting features. First, its 18-kDa molecular mass is unusually
low for a kinase of the two-component systems. Autophosphor-
ylation of AlgR2 occurs extremely rapidly with the maximum
level reached within 10 sec of incubation with ATP. This may
reflect a potential for rapid change in the state of phosphorylation
of AlgR2. In addition, results shown in Fig. 2A suggest that the
level of AlgR2 phosphorylation may be modulated via a coupled
phosphorylation/dephosphorylation process.

Results obtained with the 80-kDa AlgR1–AlgR2 protein
complex indicate that AlgR1 can be phosphorylated via an
intracomplex transfer of phosphate. Involvement of protein
complexes in bacterial signal transduction has been recently
reported (15). However, our results showing the formation of
a complex by AlgR1 and AlgR2 provide evidence for such a

![Fig. 4](image)

**Fig. 4.** Analysis of pH stability of AlgR1 (○) and AlgR2 (▲)
phosphates. In the case of AlgR1 phosphate, the AlgR1–AlgR2
complex was incubated with [γ-^-32P]ATP for 10 min at room tem-
perature to allow maximal phosphorylation. The reaction was stopped
by the addition of SDS loading buffer and then incubated at 50°C at
various pH values for 10 min. The protein samples were analyzed by
SDS/PAGE and scanned for radioactivity. AlgR2 phosphate in this
complex was extremely unstable, presumably because of a higher
rate of phosphotransfer. Stability of AlgR2 phosphate was analyzed
by using the purified protein.

complex formation by the kinase and the response regulator.
This observation is consistent with the notion that these two
proteins may have specific affinity for each other, thereby
facilitating transient modification of the response regulator. It
is, however, possible that other proteins besides AlgR1 and
AlgR2 are also present in the complex.

The kinetics of autophosphorylation of AlgR2 have several
interesting aspects. The apparent _K_m for phosphorylation by
ATP is unusually low. For example, the _K_m for the phospho-
rylation of CheA, the soluble kinase involved in bacterial
chemotaxis, is ~2000-fold higher than that for AlgR2 (1). This

![Fig. 5](image)

**Fig. 5.** Phosphorylation of AlgR1 (○) in _E. coli_ by the AlgR2
analog (▲) in the presence of 32 nM ATP or GTP. The partially
purified AlgR1–AlgR2 analog was used to monitor the phosphory-
lation reaction. ATP and GTP were used as the phosphate donors in
A and B, respectively. Note that because of the extremely rapid rate
of autophosphorylation by the AlgR2 analog, the first data point (10
sec) represents maximal incorporation of ^32P.

![Fig. 6](image)

**Fig. 6.** Model depicting phosphorylation ofAlgR2 and AlgR1 and activation of the _algD_ promoter via a DNA looping mechanism. The overall
process of alginate regulation is known to require participation of at least two other proteins, AlgR3 and a _P. aeruginosa_ analog of the cyclic
AMP receptor (CRP) protein. AlgR3 is highly homologous to eukaryotic histone H1 and is postulated to be involved in DNA bending and in
stabilizing the highly supercoiled _algD_ upstream region (13). The _E. coli_ CRP protein binds the _algD_ upstream region at ~362 and its analog
in _P. aeruginosa_ is believed to be involved in looping out the _algD_ upstream region to facilitate the contact between AlgR1 molecules (bound
at ~382 and ~458 regions of the _algD_ upstream DNA) and RNA polymerase holoenzyme at the promoter region (14). It is likely that these
proteins act in coordination with one another to optimally regulate the _algD_ promoter.
result indicates a very high affinity of AlgR2 for ATP. Competitive inhibition exhibited by ADP and GDP with $K_i$ values in the 100–300 nM range suggest that phosphorylation of AlgR2 by ATP depends on the ratio of intracellular concentration of ATP to those of ADP and GDP. It is also evident that AlgR2 is capable of undergoing phosphorylation at a concentration of ATP several orders of magnitude below that of exponentially growing cells (16).

Members of the kinase family of the two-component systems are not known to utilize GTP as a phosphate donor (1, 2). Thus, autophosphorylation of AlgR2 in the presence of GTP appears to be an unusual property of this enzyme. Involvement of GTP in phosphorylating AlgR2 may have special physiological significance. GTP is required for the formation of GDP mannone, which is an alginate precursor (17). In addition, intracellular GTP concentration is sensitive to the growth conditions of the cell (18). In *Bacillus subtilis*, a drop in the intracellular GTP concentration caused by starvation conditions leads to efficient sporulation (19). Starvation caused by nitrogen and phosphate limitation is one of the signals leading to AlgR1−AlgR2-mediated activation of the algD promoter in *P. aeruginosa* (20). Therefore, concentration of GTP may be sensed as an internal signal via GTP-dependent phosphorylation of AlgR2. The ability of both ATP and GTP to phosphorylate AlgR2 may suggest that alginate gene regulation may be responsive to environmental as well as intracellular signals reflecting the nutritional status of the cell. Inhibition by ADP and GDP suggests that the extent of phosphorylation of AlgR2 depends on the ratio of ATP and GTP to ADP and GDP concentrations.

Experiments conducted to determine the pH stability of phosphorylated AlgR1 and AlgR2 suggest that the involvement of specific amino acid residues in phosphorylation follows the pattern established by studies on the other members of the two-component systems. In all the kinases characterized thus far, a histidine residue is found to be the site of phosphorylation while an aspartate residue is phosphorylated in the response regulators (1, 2, 7). The increase in stability of AlgR2 phosphate with an increase in pH implicates histidine as the possible phosphorylated residue. Instability of AlgR1 phosphate at both low and high pH and its relative stability at physiological pH suggests aspartate as the potential phosphorylated residue. Based on amino acid sequence homology, Asp-54 in AlgR1, equivalent to Asp-57 in CheY and Spo0A (6, 7), appears to be the site of AlgR1 phosphorylation. However, further experimentation involving site-directed mutations is necessary for the definitive identification of the site(s) of phosphorylation.

Identification of an AlgR2 analog in *E. coli* raises interesting questions about its physiological role. Like AlgR2, this low molecular mass protein also undergoes ATP/GTP-dependent phosphorylation and can functionally substitute for AlgR2 in phosphorylating AlgR1. Our results suggest a broader role of AlgR2-like protein kinases in bacterial signal transduction. However, thus far nothing is known about the protein(s) receiving phosphate from this analog of AlgR2 in *E. coli*. Further research involving mutants of the corresponding gene(s) may shed light on the biological function of this interesting protein.

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