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In vitro phosphorylation of AlgR, a regulator of mucoidy in *Pseudomonas aeruginosa*, by a histidine protein kinase and effects of small phospho-donor molecules

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Summary

AlgR is a transcriptional regulator of mucoidy in *Pseudomonas aeruginosa*, a critical virulence factor expressed in cystic fibrosis. AlgR belongs to the superfamily of bacterial signal transduction systems, and has been shown to bind to the *algD* promoter, a critical point in the regulation of mucoidy. This protein, like other typical response regulators, contains highly conserved residues known to be critical for the phosphorylation and signal transduction processes. However, a typical second component interacting with AlgR has not been identified. Here we demonstrate that AlgR undergoes phosphorylation *in vitro* when interacting with the well-characterized histidine protein kinase CheA. These results indicate that AlgR is capable of undergoing phosphorylation typical of other two-component signal transduction systems. Moreover, the phosphotransfer reaction between CheA and AlgR was found to be affected by the presence of carbamoyl phosphate, acetyl phosphate, and salts of phosphoramidic acid, recently shown to act as small-molecular-weight phospho-donors in the process of phosphorylation of several response regulators. These findings suggest that AlgR may react with intermediary metabolites such as carbamoyl phosphate and acetyl phosphate, and that these processes may play a role in the control of mucoidy in *P. aeruginosa*.

Introduction

Respiratory infections with mucoid *Pseudomonas aeruginosa* strains are the major cause of high mortality and morbidity in cystic fibrosis (Govan, 1988). Mucoidy is the result of the overproduction and excessive secretion of the exopolysaccharide alginate (Govan, 1988). Transcriptional activation of *algD* (Deretic *et al.*, 1987), a gene encoding GDPmannose dehydrogenase (Deretic *et al.*, 1987), is a *sine qua non* for the establishment of mucoidy (Deretic *et al.*, 1991). AlgR is absolutely required for the transcription of *algD* and binds to three sites within the *algD* promoter (Mohr *et al.*, 1992), two of which are located in a region positioned unusually far upstream from the mRNA start site (Mohr *et al.*, 1990). AlgR is a member of the superfamily of bacterial signal transduction systems (Deretic *et al.*, 1989; Stock *et al.*, 1989b), and contains a conserved *N*-terminal domain typical of response-regulators, as well as a unique *C*-terminal domain (Deretic *et al.*, 1989). A sensory component interacting with AlgR, commonly present in other two-component systems, has not been identified. It has been suggested that the *algQ* gene (Deretic and Konyecsni, 1989; Konyecsni and Deretic, 1990), also known as AlgR2 (Kato *et al.*, 1989), located several kilobases downstream of *algR*, plays a role in this process (Deretic and Konyecsni, 1989; Kato *et al.*, 1989; Konyecsni and Deretic, 1990). However, this relatively small acidic polypeptide does not contain any recognizable histidine kinase motifs (Kato *et al.*, 1989; Konyecsni and Deretic, 1990). The *N*-terminal domain of AlgR has its primary structure closely resembling all members of the class, and it contains the critical residues (corresponding to Asp-12, Asp-13, Asp-57, and Lys-109 of CheY, the paradigm of these types of signal transducers (Stock *et al.*, 1989a,b)) found in all characterized response regulators that undergo phosphorylation (Stock *et al.*, 1989a,b; Bourret *et al.*, 1991). In this work we examined the possibility that AlgR can interact with a typical histidine protein kinase using CheA, a protein involved in the regulation of chemotaxis in *Salmonella typhimurium* (Wylie *et al.*, 1988). In addition, since mucoidy appears to be modulated by a variety of different environmental signals, including growth rate (Terry *et al.*, 1991) and carbon source (DeVault *et al.*, 1991), we also investigated whether AlgR can interact with small-molecular-weight phospho-donors such as carbamoyl phosphate and acetyl phosphate, as has been recently described for CheY and PhoB (Lukat *et al.*, 1992; Wanner and Wilmer-Reisenberg, 1992).

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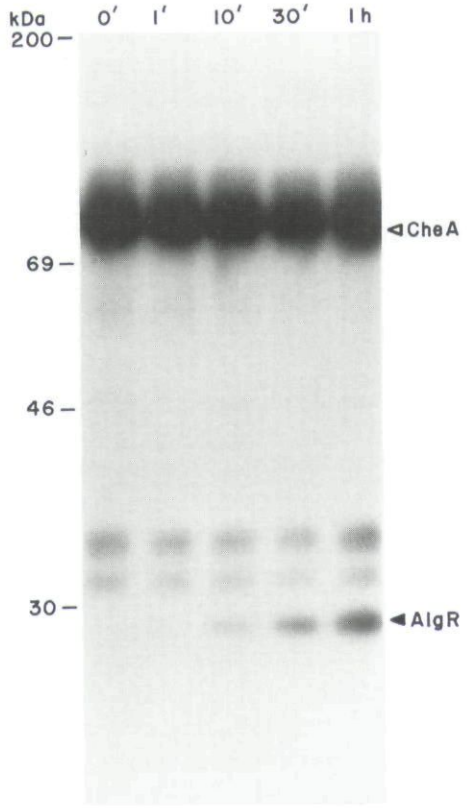

Fig. 1. Time course of the phosphotransfer reaction between CheA and AlgR. CheA was autophosphorylated with [γ-^{32}P]-ATP, as described in the *Experimental procedures*, upon which AlgR was added to the reaction mixture and the mixture incubated at 37°C. Aliquots were taken at the indicated times, and reaction stopped by adding SDS sample buffer. Reaction products were separated by SDS–PAGE electrophoresis and phosphorylated proteins detected by autoradiography. ◁, CheA; ◀, AlgR. Bands seen in addition to CheA and AlgR are degradation products of CheA, and their appearance is independent of AlgR addition.

Results

AlgR undergoes phosphorylation in the presence of autophosphorylated CheA

CheA was autophosphorylated by incubation with [γ-^{32}P]-ATP for 20 min, followed by the addition of AlgR, and the time course of the transfer of phosphate from CheA to AlgR was monitored (Fig. 1). These experiments demonstrated that AlgR can accept phosphate from CheA in what appears to be a typical phosphotransfer reaction between response regulators and their cognate or heterologous histidine protein kinases (Ninfa *et al.*, 1988; Stock *et al.*, 1989b; Bourret *et al.*, 1991). Additional faint bands seen in these reactions come from degradation products of CheA (J. Stock, personal communication). The phosphorylated form of AlgR was stable for several hours, indicating that it was more like Spo0A and OmpR than CheY and PhoB, which have half-lives of their phosphorylated forms measured in hours and minutes, respectively. Further tests indicated that phosphorylated AlgR displayed properties characteristic of proteins phosphorylated at aspartate side chains, since the incorporated ^{32}P could be removed by treatments with 1 M NaOH and 1 M HCl (Stock *et al.*, 1989b; Bourret *et al.*, 1991). The sensitivity of this bond(s) was compared with the removal of label from CheA, which was more sensitive to acidic conditions (Fig. 2A). These experiments demonstrated that AlgR was capable of undergoing a phosphorylation reaction typical of known two-component systems (Stock *et al.*, 1989b; Bourret *et al.*, 1991). The identity of AlgR as the major polypeptide phosphorylated in reaction with CheA was confirmed using monoclonal antibodies raised against AlgR (Fig. 3). This figure also contains additional analysis of polypeptides phosporylated in crude extracts of *P. aeruginosa* (see the *Discussion*).

Phosphorylation of AlgR in the presence of carbamoyl phosphate and acetyl phosphate

Recently, the existence of another pathway for phosphorylation of response regulators was demonstrated (Lukat *et al.*, 1992). It has been shown that CheY (but not

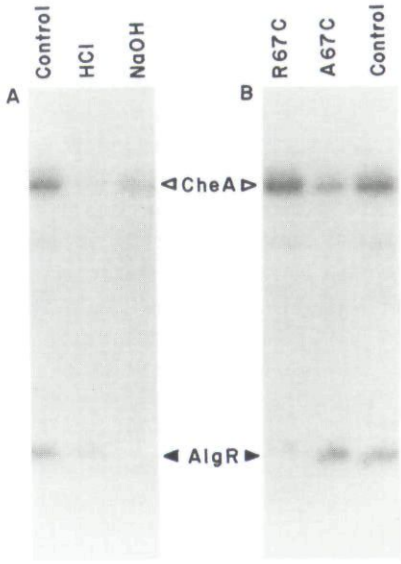

Fig. 2. A. Chemical stability of phosphoryl bonds in phosphorylated AlgR. Phosphorylated AlgR was incubated for 15 min at 37°C with the addition of H_2O (Control lane), HCl to 1 M (HCl lane), and NaOH to 1 M (NaOH lane).
B. Effects of partial heat-denaturation of AlgR and CheA on the phospho-transfer reaction. Lanes: 'R67C', AlgR heated for 15 min at 67°C prior to mixing with autophosphorylated CheA; 'A67C', CheA heated after its autophosphorylation and prior to addition of AlgR; 'Control', no heat denaturation of either component. The duration of the phosphotransfer reaction was 1 h. The reaction products were separated and visualized as in Fig. 1.

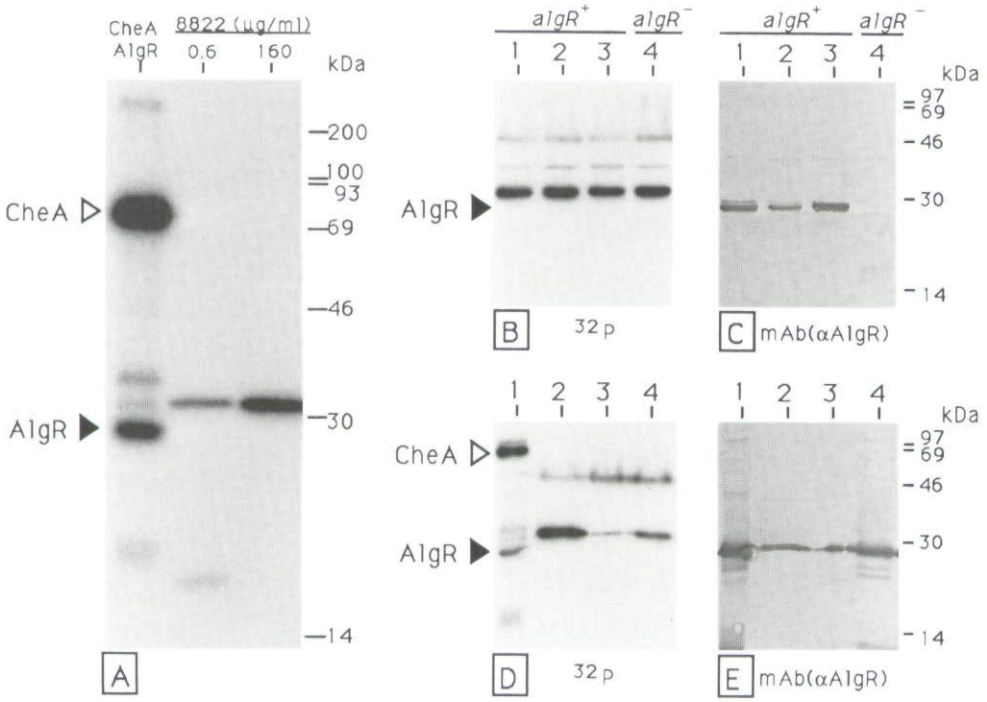

Fig. 3. Identification of phosphorylation products by immunoblotting and autoradiographic analyses.
A. Comparison of CheA–AlgR phosphotransfer products (see the *Discussion*) with the phosphorylation of an uncharacterized polypeptide in crude extracts of *P. aeruginosa* 8822 reported to represent AlgR by Roychoudhury *et al.* (1992a,b). Lanes: 'CheA–AlgR', transfer of ^{32}P phosphate from CheA to AlgR, as described in the *Experimental procedures*; '8822', 0.6 μg ml^{-1}, crude extracts (concentration of protein adjusted to 0.6 μg ml^{-1}) prepared accordinng to Roychoudhury *et al.* (1992a,b) incubated with [γ-^{32}P]-ATP for 5 min at room temperature; '8822', 160 μg ml^{-1}, the same as in lane 8822 0.6, except that the concentration of proteins was 160 μg ml^{-1}. Two polypeptides are seen (lane 8822 0.6 μg ml^{-1}) at positions as reported by Roychoudhury *et al.* (1992b), but the radiolabelled polypeptide above the 30 kDa has a different mobility from that of phosphorylated AlgR.
B and C. Autoradiogram and Western blot, respectively, of the same gel with crude protein preparations (Roychoudhury *et al.*, 1992b) from different strains of *P. aeruginosa*. Lanes: 1, PAO579 (mucoid (*muc-23*) derivative of PAO381); 2, PAO381 (non-mucoid strain); 3, PAO568 (mucoid (*muc-2*) derivative of PAO381, and parental to CDM1/1); 4, CDM1/1 (*algR*$^-$ strain with insertionally inactivated *algR* in the PAO568 background) (Fyfe and Govan, 1980; Mohr and Deretic, 1990; Mohr *et al.*, 1990).
D and E. Autoradiogram and Western blot, respectively, of the same gel with 1, autophosphorylated CheA incubated with AlgR as described in the *Experimental procedures*, 2, 3, and 4, crude extracts of 8822 (160, 0.6 and 0.6 μg ml^{-1}, respectively) prepared and incubated with [γ-^{32}P]-ATP as described by Roychoudhury *et al.* (1992b). Lane 4 has a preparation of partially purified AlgR (50–60% pure AlgR) added to the reaction mixture according to the procedure described by Roychoudhury *et al.* (1992a,b). An increase in the radiolabelling of the band migrating slightly slower than the 30 kDa standard is probably due to the presence of a contaminating protein in this partially purified AlgR preparation.

CheB) can be phosphorylated in the absence of histidine protein kinases by acetyl phosphate and carbamoyl phosphate, cellular intermediates important in carbon utilization and synthesis of both pyrimidine and arginine, respectively. This phenomenon entails that sources for phosphorylation other than typical histidine protein kinases may be used by certain response regulators such as AlgR. Since there is no commercially available radiolabelled carbamoyl phosphate or acetyl phosphate, we employed a competition assay based on monitoring the transfer of ^{32}P phosphate from CheA to AlgR in the presence of these small-molecular-weight compounds. As shown in different panels of Fig. 4, the addition of carbamoyl phosphate and acetyl phosphate efficiently suppressed the transfer of ^{32}P from CheA to AlgR. This suggested that these compounds interacted with AlgR probably by competing with the transfer of the phosphoryl group from CheA to AlgR.

Phosphorylation of AlgR in the presence of phosphoramidates

It has recently been demonstrated that CheY as well as CheB, both homologues of AlgR, can undergo direct phosphorylation in the presence of salts of phosphoramidic acid (Lukat *et al.*, 1992). This suggests that phosphotransfer between histidine protein kinases and response regulators may depend on a more active enzymatic role of response regulators in their interaction with phosphohistidine residues located in kinases (Lukat *et al.*, 1992). To explore this possibility in the case of CheA–AlgR interaction, we partially heat-denatured AlgR or phosphorylated

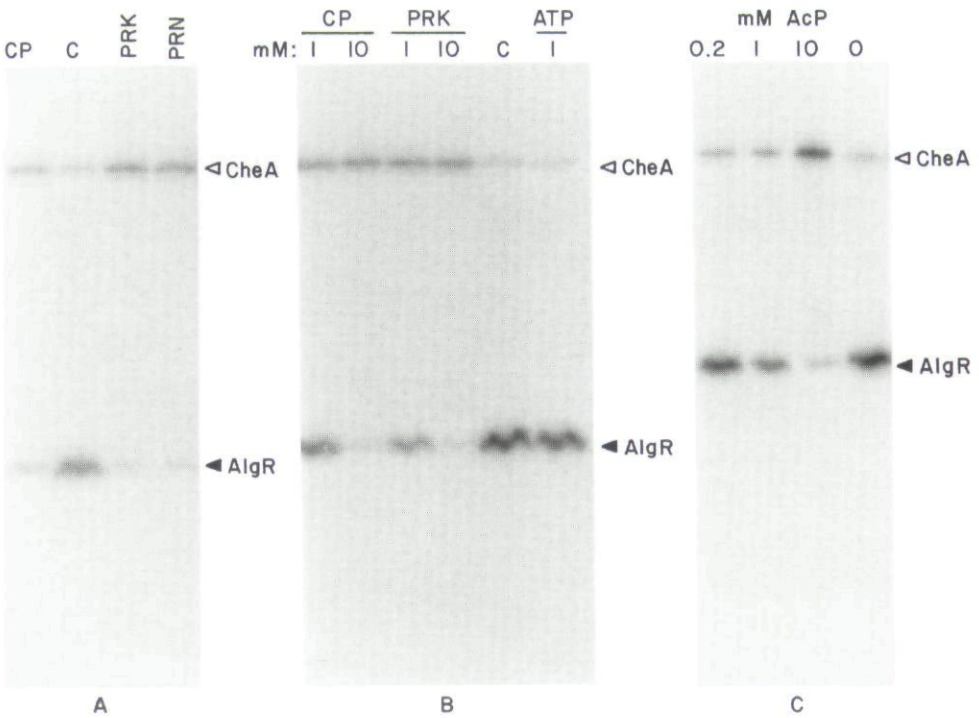

Fig. 4. Competition of carbamoyl phosphate, phosphoramidate, and acetyl phosphate with CheA for phosphotransfer to AlgR.
A. Autophosphorylated CheA was incubated with AlgR in the presence of 10 mM carbamoyl phosphate (lane CP), no additions (control, lane C), 10 mM potassium hydrogen phosphoramidate (lane PRK), or 10 mM ammonium hydrogen phosphoramidate (lane PRN).
B. Autophosphorylated CheA was incubated with AlgR in the presence of different concentrations (1 and 10 mM where indicated) of carbamoyl phosphate (CP), potassium hydrogen phosphoramidate (PRK), ATP, or no additions (lane C).
C. Effects of different concentrations of acetyl phosphate (0.2, 1, 10 mM) on the transfer of phosphate from CheA to AlgR compared with no additions (lane labelled 0). In each case the incubation time was 1 h. The products of phosphorylation reactions were treated as described in the *Experimental procedures*, separated by SDS–PAGE, and visualized by autoradiography.

CheA prior to their mixing, and examined the effects of such treatments on the efficiency of the phosphotransfer reaction (Fig. 2B). These results indicated that heat treatment of AlgR had a more profound effect and significantly reduced the efficiency of the phosphotransfer in contrast to the treatment of CheA, in support of the notion that molecules such as AlgR may play an active enzymatic role in the process of their phosphorylation. To examine further whether AlgR can interact with small-molecular-weight molecules with the chemistry of a phosphoryl bond similar to the phosphoramidate bond in CheA, potassium and ammonium hydrogen salts of phosphoramidic acid were synthesized and used in the competition assay with the CheA–AlgR phosphotransfer reaction (Fig. 4A). Phosphoramidate proved to be as efficient a competitor of the transfer of ^{32}P from CheA to AlgR as were carbamoyl phosphate and acetyl phosphate.

Concentration-dependent effects of carbamoyl phosphate, acetyl phosphate and phosphoramidate

In all three cases of the inhibition of the phosphotransfer reaction by acetyl phosphate, carbamoyl phosphate, and phosphoramidate, the anticipated mechanism of action was that the small-molecular-weight phospho-donors modified AlgR and reduced the number of available acceptor sites or their affinity for the transfer of radiolabelled phosphate from CheA to AlgR. In support of direct phosphorylation and competition for the similar residues,

Fig. 5. Radiolabelling of AlgR with acetyl [^{32}P]-phosphate. AlgR was incubated with acetyl [^{32}P]-phosphate (2 mCi mmol^{-1}) as described in the *Experimental procedures*, and reaction products were analysed by SDS–PAGE and autoradiography. Lanes: 1, acetyl [^{32}P]-phosphate; 2, as lane 1 with the addition of 10 mM EDTA.

preliminary quantitative measurements with acetyl phosphate indicate that this is a competitive inhibition. The concentration-dependence of these processes is shown in Fig. 4, B and C. In addition, acetyl [^{32}P]-phosphate was synthesized according to the method of Stadtman (1957), and it did radiolabel AlgR when incubated with this protein (Fig. 5); however, the specific activity of radiolabelled acetyl phosphate was very low (2 mCi mmol^{-1}) and the detectable incorporation of ^{32}P was far less than in the CheA–AlgR phosphotransfer reaction. In addition, when EDTA was present in the reaction mixture this reduced the radiolabelling of AlgR with acetyl [^{32}P]-phosphate, but did not completely eliminate it.

The results obtained in this study also suggest that AlgR, like CheY (Lukat *et al.*, 1992), may play an active role in its own phosphorylation. Moreover, the finding that carbamoyl phosphate and acetyl phosphate, two important intermediates in bacterial metabolism, can affect the rate of AlgR phosphorylation *in vitro*, points to the possibility that the complex regulation of mucoidy in *P. aeruginosa* (Deretic *et al.*, 1991) may be affected by intracellular concentrations of such small-molecular-weight phosphorylated intermediates.

Discussion

In this work we have examined whether AlgR is capable of undergoing a phosphorylation modification observed with several other studied response regulators (Ninfa *et al.*, 1988; Stock *et al.*, 1989b; Bourret *et al.*, 1991). The results show that AlgR can be phosphorylated in a similar manner. This suggests that a typical histidine protein kinase cognate to AlgR may be present in *P. aeruginosa*, although such a protein has yet to be identified.

Taken together, the results presented here and those of Lukat *et al.* (1992), as well as the recent genetic studies of Wanner and Wilmer-Riesenberg (1992), strongly suggest that the phosphotransfer reactions involving response regulators may be more complicated *in vivo* than originally anticipated. It is possible that several types of macromolecules and small-molecular-weight phosphate donors could interact with a given response regulator, thus broadening the operational definition of the term 'cross-talk', a phenomenon formerly ascribed to interactions among non-cognate sensors and regulators (Ninfa *et al.*, 1988; Stock *et al.*, 1989b).

The role of carbamoyl phosphate and acetyl phosphate may be of significance for the understanding of the environmental modulation of mucoidy and the emergence of mucoid strains in cystic fibrosis (Deretic *et al.*, 1991). Interpretations of phenomena related to environmental modulation of this virulence determinant (Deretic *et al.*, 1991) should now take into account the effects that growth conditions can have on intracellular levels of intermediates such as carbamoyl phosphate and acetyl phosphate. The former is necessary for pyrimidine and arginine biosynthesis, and is also used by *P. aeruginosa* for ATP synthesis under anaerobic conditions via the arginine deiminase pathway (Galimand *et al.*, 1991); the latter is related to the synthesis of acetyl-CoA, a key substrate for the tricarboxylic acid cycle, and is an important intermediate in utilization of pyruvate, glucose, and acetate, as well as in ATP synthesis when oxygen is limiting in some organisms (Knappe, 1987).

It has been reported that slowing the growth rate of *P. aeruginosa* by limitation of different nutrients (Deretic *et al.*, 1991; Terry *et al.*, 1991) can result in activation of *algD* and the emergence of mucoid strains. Exactly what processes and messenger molecules are involved in the transduction of such signals have never been understood. Based on the results reported here, we propose that intermediates such as carbamoyl phosphate and acetyl phosphate, which may vary in concentration depending on the growth rate and nutrition conditions (Lukat *et al.*, 1992; Wanner and Wilmer-Reisenberg, 1992), could affect the signal transduction processes. In such a model, intracellular levels of these intermediates could affect AlgR phosphorylation or its capacity to be phosphorylated, and thereby contribute to the modulation of the alginate system in *P. aeruginosa*. In addition, the ability of simple, small-molecular-weight phospho-donors to interact with AlgR and affect its phosphorylation suggests that perhaps inhibitors of phosphotransfer reactions may be developed in an effort to provide alternative therapeutic strategies for control of the currently intractable *Pseudomonas* infections in cystic fibrosis.

In order to accomplish these goals it will be important to identify protein kinases which interact with AlgR *in vivo*. Of the mutations affecting *algD* transcription, several have been suggested as potentially representing the putative second component (Deretic *et al.*, 1991). The first gene proposed to play such a function was *algQ* (Deretic and Konyecsni, 1989) (also termed AlgR2 (Kato *et al.*, 1989)). However, AlgQ has no discernable similarity with the known histidine kinases (Kato *et al.*, 1989; Konyecsni and Deretic, 1990). While this manuscript was in preparation, Roychoudhury *et al.* (1992a,b) presented evidence indicating that AlgQ (AlgR2) can undergo phosphorylation and transfer the phosphate to a 30 kDa polypeptide in crude extracts of *P. aeruginosa*. Furthermore, Roychoudhury *et al.* (1992b) suggested that the 30 kDa polypeptide was AlgR, and reported that they were monitoring phosphorylation of AlgR (Roychoudhury *et al.*, 1992a,b). Since AlgR in our hands displayed the capacity to undergo phosphorylation typical of the two-component signal transduction systems, we repeated the experiments of Roychoudhury *et al.* (1992b). The results of these studies are shown in Fig. 3. In these experiments, while we could

clearly detect the phosphorylation of a 30 kDa polypeptide (Fig. 3A) with electrophoretic mobility identical to that reported by Roychoudhury *et al.* (1992b), monoclonal antibodies against AlgR did not react with this polypeptide (Fig. 3, C and E). Instead, monoclonal antibody 3H9 recognized AlgR in the same sample as a band located below the 30 kDa polypeptide, at the position which was in agreement with the previously determined M_r of AlgR (27 600 Da); the same antibodies reacted with the CheA-phosphorylated AlgR (Fig. 3E) at a position identical to that of the band seen on autoradiograms (Fig. 3D). Moreover, when an *algR*$^-$ strain was tested, the 30 kDa phosphorylated protein was still present on autoradiograms (Fig. 3B) while AlgR was completely absent in this strain, as judged by Western blot analysis (Fig. 3C). Thus, the phosphorylated 30 kDa polypeptide reported to be AlgR by Roychoudhury *et al.* (1992a,b) is not AlgR and its identity, role, and any relevance to mucoidy are unclear.

We are currently in the process of identifying protein kinase(s) interacting with AlgR *in vivo*, studying how environmental conditions affect intracellular concentrations of carbamoyl and acetyl phosphate, and determining whether these processes modulate phosphorylation of AlgR *in vivo*.

Experimental procedures

Proteins and reagents

AlgR was purified as previously described (Mohr *et al.*, 1991). The purity was estimated to be greater than 95% (Mohr *et al.*, 1991). Purified CheA from *S. typhimurium* was a gift from Dr J. Stock. [γ-^{32}P]-ATP, 6000 Ci mmol^{-1}, was obtained from DuPont NEN. Potassium and ammonium salts of phosphoramidic acid ($KHPO_3NH_2$ and $NH_4HPO_3NH_2$) were synthesized according to the method of Sheridan *et al.* (1971) and the synthesized compounds characterized by mass spectroscopy. Acetyl [^{32}P]-phosphate was synthesized using acetic anhydride and potassium [^{32}P]-orthophosphate (9000 Ci mmol^{-1}; NEN DuPont) according to the method of Kornberg as described by Stadtman (1957), except that one tenth of the recommended volume of 4 N LiOH was used to precipitate the dilithium salt of acetyl phosphate. Acetyl and carbamoyl phosphates were obtained from Sigma Chemical Company.

Phosphorylation of AlgR

CheA was autophosphorylated according to the published procedure (Wylie *et al.*, 1988). After incubation of CheA with [γ-^{32}P]-ATP for 20 min at room temperature, purified AlgR was added so that the final concentrations of components were 100 mM Tris pH 8.0, 5 mM $MgCl_2$, 50 mM KCl, 2.5 μM CheA and 10 μM AlgR. When indicated, solutions of acetyl phosphate, carbamoyl phosphate, and K^+ or NH_4^+ salts of phosphoramidic acid were added to the desired final concentrations. Phosphorylation of AlgR was carried out for 1 h at 37°C, unless specified otherwise. The reaction was stopped by adding SDS sample buffer and placing samples on dry ice. Radiolabelled products were detected by autoradiography after their separation by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Where indicated, the reaction products were treated by adding 50 μg of crude protein to the sample; this removed most of the label associated with proteolytic CheA products but did not affect the amount of ^{32}P incorporated in the AlgR band. In the case of phosphorylation using acidified crude extracts of *P. aeruginosa* the protocols described by Roychoudhury *et al.* (1992b) were followed. AlgR was radiolabelled with acetyl [^{32}P]-phosphate according to the protocol of Lukat *et al.* (1992), using an estimated 2.5-fold higher concentration of acetyl [^{32}P]-phosphate (2 mCi mmol^{-1}).

Monoclonal antibodies against AlgR and Western blotting analysis

Monoclonal antibodies against AlgR were generated as previously described (Deretic *et al.*, 1992), using purified AlgR (Mohr *et al.*, 1991). Hybridomas producing AlgR-reactive antibodies were subcloned by the method of limiting cell dilution and were then cryopreserved. In the case of monoclonal antibody 3H9 the subclass was IgG1. Large-scale production of antibodies was performed from ascites fluid and antibodies purified by chromatography on a protein G sepharose 4 fast-flow column, using the monoclonal antibody Mab Trap G Kit (Pharmacia). Western blots were performed as described before (Deretic *et al.*, 1992), using horseradish peroxidase-conjugated goat anti-mouse IgG (HyClone). Bound antibodies were visualized by reaction with H_2O_2 and diaminobenzidine (Deretic *et al.*, 1992).

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