Recombination of formation at a fairly high frequency (GTP hydrolysis follows first order kinetics, with a maximum velocity (V_{\text{max}}) of 1900 pmol of P_i produced per min per mg of protein and a K_m for GTP of 9.6 ìM at 37 °C).

The pilA gene of Neisseria gonorrhoeae encodes the response regulator of a two-component regulatory system that controls pilin gene expression. Examination of the primary sequence of PilA indicates that the protein contains at least two functional domains. The N-terminal region has a proposed helix-turn-helix motif thought to be involved in DNA binding. This region also contains the residues that are presumed to form the acidic pocket involved in phosphorylation by PilB, the sensor kinase of the system. The C terminus of the protein has extensive homology to the G (GTP-binding) domains of the eukaryotic signal recognition particle (SRP) 54-kDa protein and the α subunit of the SRP receptor, or docking protein. This homology also extends to similar regions of the bacterial SRP homologs Ffh and FtsY. Here, we demonstrate that purified PilA has significant GTPase activity, and that this activity has an absolute requirement for MgCl_2 and is sensitive to KCl and low pH. We also show that PilA has a strict specificity for GTP, and that GTP hydrolysis follows first order kinetics, with a maximum velocity (V_{\text{max}}) of 1900 pmol of P_i produced per min per mg of protein and a K_m for GTP of 9.6 ìM at 37 °C.

The pilA gene of Neisseria gonorrhoeae (GC) pilE undergoes phase (on-off) variation at a fairly high frequency in vitro (10^{-4} to 10^{-3} per cell per generation; Ref. 1). Mechanisms for phase variation are numerous and occur at multiple levels. One mechanism involves intra- and intergenic recombination between silent pilin sequences (pilS) and the pilin expression locus, pilE (1, 2). Recombination of pilS sequences into pilE can result in a P^+ (piliated) to P^- (nonpiliated) or P^- to P^+ switch if the recombination event alters the reading frame, or if parts of the transcriptional or translational machinery are removed (3). Alternatively, recombination can lead to production of altered pilin proteins such as i-pilin that are not assembled into a functional pilus (4). Phase variation also occurs at the post-translational level. Incorrect processing of pilin protein can result in the production of a secreted form of pilin, S-pilin, that is not efficiently assembled into pilus (5). PilC, a pilus-associated protein that has been implicated in pilus assembly, has also been proposed to contribute to pilin phase variation. PilC expression is subject to phase variation via a slipped-strand misrepair of a tract of guanine residues in the signal-peptide encoding region of the gene (6). In addition, other pilus assembly genes may also have a role in pilin phase variation (7, 8).

Transcriptional regulation of pilE also contributes to pilin phase variation. Taha et al. (9) have shown that gonococcal pilin expression is transcriptionally controlled by a regulatory system encoded by the pilA and pilB genes. PilA and PilB have homology to the two-component family of prokaryotic proteins which transduce environmental signals to cytoplasmic regulators via phosphorylation (10). PilA is the response regulator of this system and is essential in the gonococcus, and PilB is the sensor kinase. The signal to which PilB responds is presently unknown.

PilA is a unique response regulator in that it is a transcriptional activator in the absence of PilB and a repressor in its presence (11). We have previously demonstrated that PilA is a DNA-binding protein that binds specifically to a region 5' to the pilE1 promoter in a complex manner that may involve looping of the DNA (12). The PilA in these experiments was purified from a strain lacking pilB and was presumed to be unphosphorylated. Taha and Giorgini (13) have shown, using enriched extracts from Escherichia coli harboring plasmids with pilA alone or pilA and pilB, that the presence of PilB may increase PilA DNA binding.

PilA is a 417-amino acid protein with at least two functional domains. The amino-terminal portion is predicted to contain a DNA binding motif (helix-turn-helix; Fig. 1) as well as the acidic pocket presumed to be the site of phosphorylation by PilB (13). The carboxyl-terminal part of PilA has significant homology to the N-terminal G (GTP-binding) domains of the 54-kDa subunit of the eukaryotic signal recognition particle (SRP) (11). Recently, homologs of this protein (SRP54) have been identified in bacteria and are designated Ffh (fifty-four homolog; Refs. 14, 15, 16, and 17). The C terminus of PilA has 61% amino acid similarity, and 31% identity, with the G domain of E. coli Ffh (Fig. 1).

The eukaryotic SRP is important for targeting and insertion of the signal sequence of exported proteins into the endoplasmic reticulum (ER) membrane. The SRP54 protein is associated with the 7 S RNA in the complex and binds to the signal sequence of the nascent protein as it emerges from the ribosome (18). The complex then travels to the ER membrane where it binds a docking protein which also has a GTP binding activity. The bacterial homolog Ffh is associated with a 4.5 S RNA in an SRP-like complex and functions in targeting proteins to the bacterial cytoplasmic membrane in a manner similar to its mammalian counterpart (19). The prokaryotic docking protein has been identified as FtsY (20), which also has significant sequence homology to PilA (Fig. 1). The SRP54 proteins have two domains: an “M” domain, which interacts with the signal peptide of nascent proteins as well as to the 7 S (4.5 S in E. coli) RNA, and a “G” domain, which has the GTPase activity required for the interaction of the complex with the docking protein (19).

Taha et al. (21) examined the effect of PilA on the export and

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1 The abbreviations used are: SRP, signal recognition particle; ER, endoplasmic reticulum; GTP$$\gamma$$S, guanosine 5'-(3-thiotriphosphate); CAT, chloramphenicol acetyltransferase.
N. gonorrhoeae PilA Is a GTPase

FIG. 1. Schematic alignment of PilA, E. coli FtsY, and E. coli Ffh. Shaded regions denote the putative GTP-binding domains. Black boxes indicate conserved residues presumed to form the GTP-binding site. D indicates presumed DNA-binding (helix-turn-helix) motif of PilA (11). G (GTP-binding) and M (protein- and RNA-binding) domains as defined by homology to the eukaryotic SRP54 proteins are indicated below. Percent identity and similarity compared to PilA are indicated for the shaded G regions. AA, amino acid residues.

FIG. 2. GTP hydrolysis is PilA-specific and occurs between the β and γ-phosphates. Reactions were carried out for 60 min at 37 °C under standard conditions in the presence of 100 μM [γ-32P]GTP or [α-32P]GTP as indicated. ○, PilA + [γ-32P]GTP; □, TT (extract from a non-PilA containing strain) + [γ-32P]GTP; ⊙, PilA + [α-32P]ATP. Activity is expressed as picomoles of P_i released per min as a function of protein concentration, after correcting for background P_i released in samples containing no protein. Assays were performed in triplicate, and values are from one representative experiment. Standard deviations were less than 15%.

RESULTS AND DISCUSSION

SRP54 and the bacterial homolog Ffh hydrolyze GTP as they dissociate from the docking protein (SRP receptor) during protein translocation (19). Analysis of the PilA amino acid sequence reveals a segment of 295 amino acids that is similar to the G (GTP-binding) domains of the SRP54 and SRP receptor family of proteins (Fig. 1; Ref. 11). It was therefore of interest to determine whether PilA was capable of hydrolyzing GTP. Purified PilA was incubated with GTP labeled with 32P at the α or γ position, and radiolabeled P_i increased linearly with increasing PilA concentrations, but not in reactions with [α-32P]GTP (Fig. 2). This result suggests that PilA has GTP-hydrolyzing activity and that the cleavage likely occurs between the β and γ phosphates.

To demonstrate that the GTPase activity measured in these experiments was PilA-specific, protein extracts prepared from an E. coli strain bearing the PilA expression plasmid without the pilA gene were subjected to the same purification procedure and assayed for GTPase activity (TT; Fig. 2). Release of [32P]P_i in the presence of varying concentrations of this extract was not significantly greater than the background from parallel samples containing no protein. These results indicated that the GTP hydrolyzing activity is PilA-dependent.

Buffer components for the GTP hydrolysis reaction were varied to determine the requirements for the enzymatic activity of PilA (Table I). Reactions carried out in the absence of MgCl_2 yielded P_i levels equal to those from parallel samples containing no protein, indicating an absolute requirement for MgCl_2 for GTP hydrolysis. The omission of dithiothreitol from the reactions resulted in an ~30% reduction of GTPase activity. We have previously observed aggregation of purified PilA in the absence of reducing agents (data not shown), and it is likely that the aggregated form is less active. The addition of Triton X-100 (final concentration 0.2%) also reduced the GTPase activity of PilA ~30%. This was interesting, as our previous results indicated that 0.2% Triton X-100 slightly improved the DNA binding activity of PilA in a gel shift assay (12). The omission of glycylglycine (final concentration 10%) from the reactions resulted in a dramatic reduction in GTPase activity at low protein concentrations (Table I). At high protein concentrations (4.1 μM), the absence of glycylglycine resulted in a 2.5-fold increase in GTPase activity over that observed at lower protein concentrations (0.8 μM) under similar conditions. This may indicate that glycylglycine has a stabilizing effect on the purified protein.

Variations of the pH of the reaction had only a small effect on GTPase activity. Lowering the pH from 7.5 to 6.8 reduced the GTPase activity of PilA by ~15% (Table I). Increasing the pH from 7.5 to 9.5 had no effect on the GTPase activity. Lower pH (6.8) also seemed to reduce the stability of GTP. In the absence of protein there was a much higher background of [32P]P_i after a 1-h incubation compared to similar samples at higher pH (data not shown).

KCl concentration had a dramatic effect on the GTPase activity of PilA (Table I). Activity was highest in the absence of KCl and decreased more than 5-fold in 500 mM KCl. Substitution of KCl with potassium glutamate (10 mM or 100 mM) had
similar effects on the GTPase activity of PilA (data not shown). Interestingly, the DNA binding activity of PilA is also optimal under conditions of low ionic strength (10 mM potassium glutamate (12)).

The nucleotide specificity of PilA was also determined. [γ-32P]ATP was incubated with varying concentrations of PilA under conditions optimal for GTP hydrolysis. The amount of radiolabeled P was ~2-fold greater than the background observed in samples lacking PilA, but was not stimulated by increasing PilA concentrations (0.5-5.0 μg). The ability of various nucleotide analogs to inhibit GTP hydrolysis was then determined. Addition of the guanine nucleotides GTP, GDP, and GTP-S at a 10-fold excess over the substrate concentration reduced PilA GTPase activity by as much as 95% (Table II). In contrast, none of the adenine nucleotides tested (ATP, ADP, and cAMP) was able to inhibit the activity significantly. Varying the time between the addition of competing nucleotide and the radiolabeled substrate (which was always added last) had no effect on the inhibition by guanine analogs or the lack of inhibition by adenine analogs. These results demonstrate that PilA specifically hydrolyzes GTP, and that this activity is not affected by preincubation with unlabeled nucleotides as has been observed for FtsZ-catalyzed GTPase activity (23).

The activity of PilA is directly proportional to protein concentration (Fig. 2), indicating that GTP hydrolysis is a first order reaction. Examination of the velocity of the reaction as a function of substrate concentration revealed that the reaction follows Michaelis-Menten kinetics. A double reciprocal plot of the data is shown in Fig. 3. From this plot we determined a value of 1840 pmol min⁻¹ mg⁻¹ of protein for the maximum velocity (Vmax) of the reaction, and a value of 9.2 μM for the Michaelis-Menten constant (Km). Km and Vmax were calculated from these same data using a derivation of the Michaelis-Menten equation (24). Using the following equations, where a = substrate (GTP) concentration and v = velocity (activity) at a given substrate concentration, a Km of 9.6 μM and a Vmax of 1900 pmol min⁻¹ mg⁻¹ were determined.

\[ K_m = \frac{\sum v a \cdot \sum v a^{-1} \cdot \sum a^{-1} \cdot \sum v}{\sum v a^{-2} \cdot \sum v - \sum (\sum v a^{-1}) \cdot \sum a^{-1}} = 9.6 \mu M \]  

(Eq. 1)

\[ V_{max} = \frac{\sum v a^{-2} \cdot \sum v - \sum (\sum v a^{-1}) \cdot \sum a^{-2} \cdot \sum v}{\sum v a^{-2} \cdot \sum v - \sum (\sum v a^{-1}) \cdot \sum a^{-1}} = 1900 \text{ pmol min}^{-1} \text{ mg}^{-1} \]  

(Eq. 2)

Finally, a direct linear plot analysis gave similar values for Km and Vmax (data not shown).

Other prokaryotic GTP-hydrolyzing proteins that have been characterized include FtsZ from E. coli and Bacillus subtilis (23, 25), Obg from B. subtilis (26), Era from E. coli (27), and the E. coli SRP54 homolog Ffh (20). It is interesting that each of these proteins is required for viability, as is PilA in N. gonorrhoeae (11). The kinetic parameters for some of these proteins have been determined, and PilA compares favorably to these. The Vmax of PilA is nearly 1 mM (29), and, assuming that GTP levels are similar in the gonococcus, the Km of PilA for GTP is well within the physiological range.

There are significant differences in the activities of these proteins which may reflect their differing intracellular functions. Obg, which is involved in Bacillus sporulation, and Era, a membrane protein of unknown function, are both phosphorylated during GTP hydrolysis (28, 30). In contrast, FtsZ and Ffh do not appear to be phosphorylated by GTP in the reaction; GTP hydrolysis by these proteins results in release of the free Pi (and GDP). Attempts to isolate a phosphorylated PilA intermediate by the methods used for Obg and Era were unsuccessful, suggesting that PilA is not autophosphorylated in the GTPase reaction (data not shown).

Obg and Era are believed to play a role in regulation of cellular processes. Phosphorylated and unphosphorylated forms of these proteins may have different activities, and the ratios between the two forms are likely to be important in vivo. It is thought that Obg senses GTP levels as part of the regulatory cascade controlling Bacillus sporulation (31), as fluctuations in GTP levels in the cell are believed to trigger this phenomenon (32). Era has been implicated in playing a role in adaptation to thermal stress and in the control of cell division (33, 34). Thus, these proteins may be sensing GTP levels in the

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**Table I**

| Component        | % activity |
|------------------|------------|
| 10 mM MgCl₂      | 100        |
| 1 mM dithiothreitol | 100        |
| 0.2% Triton X-100 | 69         |
| 10% glycerol (0.8 μM PilA) | 100        |
| 10% glycerol (4.1 μM PilA) | 100        |
| pH 6.9         | 85         |
| 10 mM KCl       | 84         |
| 10% KClb       | 49         |
| 50% KClb       | 17         |

*a S.D. were less than 10%.

**Table II**

| Analog | % activityb |
|--------|-------------|
| None   | 100         |
| GTP    | 49          |
| GDP    | 18.0        |
| GTPyS  | 7.7         |
| ATP    | 95.0        |
| ADP    | 81.0        |
| cAMP   | 83.7        |

*a Analogs were present at 1 mM.

*b Assay conditions were as in Table I, with substrate (GTP) at 100 μM.

S.D. were less than 10%.
cell as a signal for a changing environmental condition. It is possible that changing intracellular GTP levels may also be a signal for PilA-mediated regulation of gene expression.

FtsZ and Ffh apparently use the phosphate bond energy released during GTP hydrolysis for their primary functions. FtsZ polymerizes at the cell division septum in a GTP-dependent manner and is believed to act as a cytoskeletal element that orchestrates invagination of the bacterial membrane in the process of cell division (25, 35). Ffh transfers the nascent peptide from the SRP complex to the docking protein during protein translocation, also in a GTP-dependent manner (20). It is conceivable that PilA may hydrolyze GTP to provide energy for the activation of transcription.

The sequences of the GTP-binding regions of the prokaryotic GTPases can be divided into 3 groups. The GTP-binding sites of Obg and Era have high homology to the GTP-binding sites of eukaryotic G-proteins and E. coli translation elongation factors (26, 36). FtsZ contains a 7-amino acid segment homologous to a region of tubulin believed to bind guanine nucleotides (35). The GTP-binding regions of Ffh and PilA share homology with the G domains of the SRP54 and SRP receptor proteins (11, 15). Interestingly, conservation of GTP-binding domains in these proteins is not necessarily correlated with conservation of function. Obg and Era are apparently involved in signal transduction, like their eukaryotic G-protein counterparts. FtsZ plays a structural role, like its eukaryotic homolog, tubulin. In contrast, PilA has a strong sequence similarity to proteins involved in protein translocation, but is more similar in function to the ATP-hydrolyzing transcriptional regulator NtrC (37).

NtrC is the response regulator of the two-component system that controls the expression of a number of genes in response to nitrogen availability in bacteria via the alternative σ factor, σ^N (38). NtrC activation of transcription is dependent on phosphorylation by NtrB, the sensor kinase of the system (39). NtrC ATPase activity is phosphorylation- and DNA-dependent and is coupled to open complex formation (37, 40, 41). It is possible that GTP hydrolysis by PilA may be coupled to activation of transcription in a similar manner.

We have previously shown that PilA binds to the N. gonorrhoeae pilE promoter in a sequence-specific manner (10). This interaction involves multiple regions of the DNA and may involve DNA-bending or loop formation. In this work, we show that, in addition to its DNA binding activity, PilA has a GTP hydrolizing activity. This GTP-specific activity suggests some interesting possibilities for understanding the PilA-PilB regulatory system. Taha et al. (11) showed that, in E. coli, PilA and PilB together repress a pilE-CAT (chloramphenicol-acetyltransferase) promoter fusion while PilA alone activates transcription of this fusion. These data indicate that PilB modulates the transcriptional activity of PilA. The exact nature of the PilA-PilB interaction is unclear at present, but is likely to involve phosphorylation (11, 40). PilB modulation of PilA transcriptional activity could occur by a number of mechanisms. PilB could inhibit binding of PilA to promoter DNA. This is unlikely, as Taha et al. (11) have observed increased DNA-binding in enriched extracts of an E. coli strain expressing pilA and pilB. Alternatively, PilB may reduce the transcriptional activation activity of PilA by inhibiting its GTPase activity. This would assume that GTP hydrolysis is coupled to PilA transcription activation, which has not yet been determined.

PilB effects on the GTPase activity of PilA are also unknown. Finally, PilB may affect the interaction between PilA and RNA polymerase to inhibit transcription independent of effects on GTPase activity. As PilA is the first example of a DNA-binding protein with a GTP-specific hydrolizing activity, it will be very interesting to examine the relationship between these two activities and their effects on gene expression in Neisseria.

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