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Mutational Analysis of Nucleoside Diphosphate Kinase from *Pseudomonas aeruginosa*: Characterization of Critical Amino Acid Residues Involved in Exopolysaccharide Alginate Synthesis

GEORGE W. SUNDIN, SANDEEP SHANKAR, AND A. M. CHAKRABARTY*

Department of Microbiology and Immunology, University of Illinois College of Medicine, Chicago, Illinois 60612

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We report the utilization of site-directed and random mutagenesis procedures in the gene encoding nucleoside diphosphate kinase (*ndk*) from *Pseudomonas aeruginosa* in order to examine the role of Ndk in the production of alginate by this organism. Cellular levels of the 16-kDa form of the Ndk enzyme are greatly reduced in *P. aeruginosa* 8830 with a knockout mutation in the *algR2* gene (8830R2::Cm); this strain is also defective in the production of the exopolysaccharide alginate. In this study, we isolated four mutations in *ndk* (Ala-14→Pro [Ala14Pro], Gly21Val, His117Gln, and Ala125Arg) which resulted in the loss of Ndk biochemical activity; hyperexpression of any of these four mutant genes did not restore alginate production to 8830R2::Cm. We identified six additional amino acid residues (Ser-43, Ala-56, Ser-69, Glu-80, Gly-91, and Asp-135) whose alteration resulted in the inability of Ndk to complement alginate production. After hyperproduction in 8830R2::Cm, it was determined that each of these six mutant Ndks was biochemically active. However, in four cases, the in vivo levels of Ndk were reduced, which consequently affected the growth of 8830R2::Cm in the presence of Tween 20. Two mutant Ndk proteins which could not complement the alginate synthesis defect in 8830R2::Cm were not affected in any characteristic examined in the present study. All of the mutant Ndks characterized which were still biochemically active formed membrane complexes with Pk, resulting in GTP synthesis. Two of the four Ndk activity mutants (His117Gln and Ala125Arg) identified were capable of being truncated to 12 kDa and formed a membrane complex with Pk; however, the complexes formed were inactive for GTP synthesis. The other two Ndk activity mutants could be truncated to 12 kDa but were not detected in membrane fractions. These results further our understanding of the role of Ndk in alginate synthesis and identify amino acid residues in Ndk which have not previously been studied as critical to this process.

Nucleoside diphosphate kinase (EC 2.7.4.6; Ndk) catalyzes the reversible transfer of the 5′-terminal phosphate from nucleoside triphosphates (NTPs) to nucleoside diphosphates (NDPs) via a ping-pong mechanism summarized as follows: $N_1TP + N_2DP = N_1DP + N_2TP$ (29). The transphosphorylation reaction involves a phosphohistidine intermediate, and Ndk is characterized as showing little substrate specificity, utilizing the ribose and deoxyribose forms of both purine and pyrimidine NDPs as substrates. Ndk plays a key role as a housekeeping enzyme that functions in the maintenance of nucleotide pools for DNA and RNA synthesis. The sequencing of *ndk* genes from a variety of sources has revealed that most encode proteins consisting of approximately 150 amino acids and that Ndks from prokaryotes to humans are extensively homologous (41). Crystal structure information also confirms the relatedness of Ndks, although eukaryotic Ndks (e.g., those from *Dictyostelium discoideum* and *Drosophila melanogaster*) are arranged as hexamers while a prokaryotic Ndk (that from *Myxococcus xanthus*) is arranged as a tetramer (4, 6, 43).

While Ndk has a critical biochemical role in the maintenance of the correct levels of NTPs and deoxynucleoside triphosphates (dNTPs) utilized in synthetic and regulatory functions within both prokaryotic and eukaryotic cells, the protein may control additional biological functions in such cells. For example, metastasis of cancerous tumors appears to be similar to normal tissue development and differentiation and can be affected by the levels of Ndk. Two isoforms of a gene termed *nm23* occur in human cells and encode Ndk. Reduced transcript accumulation of one isoform, *nm23-H1*, is associated with tumor cells of high metastatic potential (40). Constitutive expression of *nm23-H1* but not *nm23-H2* resulted in an in vivo inhibition of the metastatic potential of murine melanoma and human breast carcinoma cell lines (17). However, in rat mammary-adenocarcinoma cells, the levels of *nm23-H2* gene expression were reduced (8). Nm23-H2 has also been implicated as a DNA-binding regulatory protein (27), and expression of an *nm23-H2* homolog from the mouse inhibits the differentiation of myeloid leukemia M1 cells (25).

In *Pseudomonas aeruginosa*, the expression of *ndk* is under the dual control of the *algR2* and *algH* genes (33). The levels of Ndk are drastically reduced in a *P. aeruginosa algR2 algH* double mutant; however, NTP synthesis is still evident, indicating that an alternate kinase is present in these cells. We recently determined that pyruvate kinase (Pk) is the enzyme that serves as the alternate kinase (41); Pk has been shown to substitute for Ndk in NTP and dNTP synthesis in *Escherichia coli* (30, 35, 41). We have also shown that Ndk exists in two forms in *P. aeruginosa*, a 16-kDa cytoplasmic form that is present at high levels during the early stages of cellular growth and a 12-kDa membrane-associated form that appears at the onset of stationary phase (34). The 16-kDa form of Ndk is

* Corresponding author. Mailing address: Dept. of Microbiology and Immunology (M/C 790), University of Illinois College of Medicine, 835 South Wolcott Ave., Chicago, IL 60612. Phone: (312) 996-4586. Fax: (312) 996-6415.

cleaved to the 12-kDa form through the actions of an 80-kDa protease (34). The 12-kDa form of Ndk is significant because it forms a complex with Pk which results in the synthesis of predominantly GTP (41). Disruption of the complex and biochemical activity experiments utilizing the uncomplexed purified 12-kDa form showed that the specificity of GTP synthesis was abrogated, resulting in NTP-synthesizing activity which mimicked the 16-kDa Ndk, suggesting that formation of the complex was necessary to alter the substrate specificity to GDP.

The *algR2* mutant of *P. aeruginosa* was defective in alginate synthesis, had greatly reduced Ndk levels, and contained less than 10% of wild-type levels of intracellular pools of GTP and ppGpp (14, 33, 41). We recently demonstrated that hyperexpression of the *ndk* gene in the *P. aeruginosa algR2* mutant could largely restore the production of the exopolysaccharide alginate to this mutant and restored the levels of Ndk, GTP, and ppGpp to the levels of the wild type (14, 41). Alginate is a critical virulence factor which enables *P. aeruginosa* to establish chronic infections in the lungs of patients afflicted with the genetic disease cystic fibrosis (20, 37). The synthesis of GTP by the 12-kDa Ndk-Pk complex is critical to alginate synthesis since GTP is required for the formation of the essential alginate precursor GDP-mannose. In this work, we undertook a mutational analysis of Ndk using site-directed mutagenesis and random mutagenesis to identify critical amino acid residues involved in the biochemical activity of Ndk and in the ability of Ndk to complement alginate synthesis in the *P. aeruginosa algR2* mutant. Several of the Ndk mutants which we isolated contained alterations in amino acid residues which have not been previously studied.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1. *E. coli* and *P. aeruginosa* strains were maintained on Luria-Bertani medium and *Pseudomonas* isolation agar (Difco), respectively; all strains were cultured at 37°C. For plasmid selection, ampicillin was used at a final concentration of 75 μg ml^{-1} for *E. coli* and carbenicillin was used at 300 μg ml^{-1} for *P. aeruginosa*. For induction of the *tac* promoter, IPTG (isopropyl-β-D-thiogalactopyranoside) was included to a final concentration of 2 mM in liquid media and 5 mM in solid media. Plasmids were introduced into *P. aeruginosa* by triparental mating using pRK2013 (8). Alginate was isolated from strains grown on solid media by a previously described protocol (21).

DNA manipulations and mutagenesis. Subcloning of restriction fragments was done by standard procedures (31). DNA prepared for sequencing was purified on Qiagen 100 columns (Qiagen Inc., Chatsworth, Calif.). Nucleotide sequencing of both DNA strands was done by using α-^{35}S-ATP, 7-deaza-GTP in place of dGTP, and a Sequenase kit (version 2.0; United States Biochemical, Cleveland, Ohio) according to the manufacturer's instructions. The oligonucleotide primers utilized in site-directed mutagenesis are listed in Table 1 and were purchased from Gibco BRL, Gaithersburg, Md. Site-directed mutagenesis was performed by the overlap extension method and PCR (11). The primers consisted of complementary 22- to 25-bp sequences, with the altered nucleotide located in the center of the primer. Random mutagenesis of *ndk* was accomplished by using the *E. coli mutD5* strain ES1578 transformed with pGWS119 and a previously published procedure (44). The complete sequence of each mutated *ndk* gene was determined to ensure that additional mutations were not present.

Ndk assays and glycerol gradient analysis. Overnight cultures of *P. aeruginosa* were harvested by centrifugation, suspended in 3 volumes of buffer A (50 mM Tris-HCl [pH 7.6], 10 mM $MgCl_2$, 1 mM dithiothreitol), and lysed by sonication through 15 pulses of 15-s duration with a 15-s gap between pulses and at a constant pulse amplitude of 20 μm. The sonicated suspension was centrifuged at 10,000 × *g* for 30 min to pellet cell debris, yielding a crude extract. For preparation of a membrane fraction, overnight cultures were centrifuged as described above, lysed by sonication, and centrifuged briefly at 2,500 × *g* for 5 min and then at 42,000 × *g* for 60 min. The pellet thus obtained was resuspended in the original volume of sonication buffer and used as the membrane suspension. Protein was estimated by the Bradford method with a kit (Bio-Rad, Hercules, Calif.), and 10 μg of total protein was used per assay.

Autophosphorylation assays were performed, and [γ-^{32}P]ATP-labeled proteins were analyzed as previously described (41). Quantitation of the radioactivity present within the Ndk bands was done with an AMBIS radioanalytic imaging system (AMBIS Systems Inc., San Diego, Calif.). The NTP-synthesizing activity of Ndk from the *P. aeruginosa* soluble and membrane fractions was assayed as previously described (41).

Glycerol gradient centrifugation was carried out by a modification of the method developed by Bowman et al. (3). A batch gradient of glycerol was prepared by successive layering of 50, 40, 30, 20, 10, and 5% solutions of glycerol in equal volumes in a Beckman 10-ml ultracentrifuge tube. The protein sample to be analyzed was mixed with the 5% solution or layered on top of the 5% band without loss of resolution of separation. The gradient tube was centrifuged at 40,000 × *g* for 60 min at 4°C. Samples were withdrawn in the same volume aliquots that were originally layered. The samples were analyzed by Western blotting (immunoblotting) by testing the various fractions against anti-Ndk antibodies as well as anti-Pk antibodies. To enable the detection of samples after dilution in the glycerol gradient, antibodies were diluted to 1:250 as opposed to the usual 1:500. Western blotting of cellular extracts was performed as described previously (34).

TABLE 1. Bacterial strains, plasmids, and oligonucleotide primers utilized in the present study

Strain, plasmid, or primer	Relevant characteristic(s) or sequence (5′ to 3′)	Source or reference
Strains		
E. coli		
ES1578	*mutD*5 derivative of AB1157	44
TG1		Amersham
P. aeruginosa		
8830	*his-1 alg*$^+$	5
8830R2::Cm	*his-1 algR2*::Cm (a Cm^r cassette in *algR2*)	32
Plasmids		
pGWS95	pMMB67HE *ndk*	41
pGWS109	pMMB66EH *ndk* Gly21Val	This study
pGWS110	pMMB66EH *ndk* Ser43Ala	This study
pGWS111	pMMB66EH *ndk* His117Gln	This study
pGWS112	pMMB66EH *ndk* Ser119Ala	This study
pGWS113	pMMB66EH *ndk* Ser121Ala	This study
pGWS119	pMMB66EH *ndk* WT[a]	This study
pGWS120a	pMMB66EH *ndk* Ala14Pro	This study
pGWS120b	pMMB66EH *ndk* Glu80Lys	This study
pGWS120c	pMMB66EH *ndk* Gly91Val	This study
pGWS120d	pMMB66EH *ndk* Ala56Asp	This study
pGWS120e	pMMB66EH *ndk* Ala125Arg	This study
pGWS120f	pMMB66EH *ndk* Ser69Cys	This study
pGWS120g	pMMB66EH *ndk* Asp135Ala	This study
pMMB66EH	IncQ *lacI*^{q} P_{tac} *rrnB* Ap^r *mob*$^+$	9
pRK2013	ColE1 Tra[RK2]$^+$ Km^r	7
Primers[b]		
G21Va	GAACGTGATCGTCGAAATCCTG	This study
G21Vb	CAGGATTTCGACGATCACGTTC	This study
S43Aa	GGTTCAGCTGGCCGAGCGGAAG	This study
S43Ab	CTTCACGCTCGGCCAGCTGAACC	This study
H117Qa	GAACGCCGTCCAGGGTTCCGATTCC	This study
H117Qb	GGAATCGGAACCCTGGACGGCGTTC	This study
S119Aa	GCCGTCCACGGTGCCGATTCCGAAG	This study
S119Ab	CTTCGGAATCGGCACCGTGGACGGC	This study
S121Aa	CACGGTTCCGATGCCGAAGCTTCCG	This study
S121Ab	CGGAAGCTTCGGCATCGGAACCGTG	This study

[a] WT, wild type.

[b] The a and b primer sequences for each mutation are complementary and correspond to the published *P. aeruginosa ndk* sequence (41). The nucleotide which was altered during mutagenesis is underlined.

RESULTS

Site-directed mutagenesis and random mutagenesis of *P. aeruginosa ndk*. Our rationale for mutagenesis of specific amino acid residues within *P. aeruginosa* Ndk was based on altering residues of known importance in *E. coli* or *M. xanthus* Ndk (1, 24). His-117, the active-site histidine which is autophosphorylated, and Ser-119, which is phosphorylated in *E. coli* Ndk (1), are invariant among all known Ndk sequences.

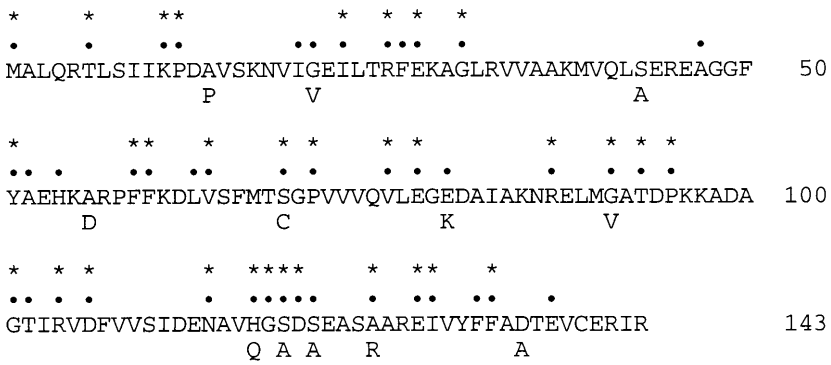

FIG. 1. Translated amino acid sequence of *P. aeruginosa ndk* (41) showing the amino acid residues which were altered in the present study. The new amino acid residue replacing that in the wild-type sequence is shown below the site of the mutation. Amino acid residues identified with dots and asterisks are conserved among 5 known bacterial Ndk sequences and 12 bacterial and eukaryotic Ndk sequences, respectively.

Ser-121 was previously reported to be phosphorylated in *E. coli* Ndk (1), and a mutation of Gly-21 to Val (Gly21Val) affected activity and nucleotide binding in *M. xanthus* Ndk (24). We also mutated Ser-43 because phosphorylation of this residue is thought to be important for the metastasis suppressor effects of human Nm23-H1 (18). The various mutated genes were cloned into pMMB66EH, in which their expression was under the control of the *tac* promoter. The mutants were designated according to single-letter amino acid abbreviations, the numerical position of the amino acid residue within the *P. aeruginosa* sequence, and the resulting amino acid after the mutation as follows: G21V, S43A, H117Q, S119A, and S121A.

The construct pGWS119 contained the wild-type *ndk* gene cloned into pMMB66EH, which could complement the Alg^- *algR2* mutant to mucoidy (Alg^+). pGWS119 was also transformed into the *E. coli mutD5* strain ES1578 for random mutagenesis with the strategy of isolating *ndk* mutants which were incapable of complementing *P. aeruginosa* 8830R2::Cm to mucoidy. After overnight growth of cultures without shaking, triparental matings were conducted with *P. aeruginosa* 8830R2::Cm. Transconjugants were selected on *Pseudomonas* isolation agar containing carbenicillin and IPTG, and plates were screened visually for the selection of nonmucoid colonies. A total of seven colonies were selected from five independent matings (approximate mutation frequency, 1 in 10^4); the pGWS119 plasmid was isolated from each of these strains, and the sequence of the *ndk* gene was determined.

The sequence analysis revealed that each of the seven *ndk* mutants contained a point mutation leading to a single amino acid change. The mutations identified were Ala14Pro (A14P), Ala56Asp (A56D), Ser69Cys (S69C), Glu80Lys (E80K), Gly91 Val (G91V), Ala125Arg (A125R), and Asp135Ala (D135A). Thus, the properties of 12 Ndk mutants with single amino acid substitutions were analyzed in this study (Fig. 1). Of the 12 individual amino acid residues which were altered, 8 were invariant among five known bacterial Ndk sequences and 5 of those were also invariant among seven eukaryotic Ndks, including *Saccharomyces cerevisiae*, *Drosophila*, mouse, and human Ndks (Fig. 1).

Characterization of the ability of the mutant Ndks to be autophosphorylated. We have previously determined that an autophosphorylation assay using [γ-^{32}P]ATP provides a sensitive means to assess Ndk levels and corresponds well to the measurement of Ndk levels by Western blotting (32, 33). In this study, each of the 12 mutant *ndk* genes were hyperexpressed in 8830R2::Cm, which has very little chromosomally encoded Ndk, and the Ndk levels in supernatant extracts were examined by autophosphorylation and by Western blotting. Of the 12 Ndk mutants assayed in the present study, four (A14P, G21V, H117Q, and A125R) which were incapable of being labelled with [γ-^{32}P]ATP were identified (Fig. 2A). These mutant proteins were detectable in supernatant extracts by Western blotting with anti-Ndk antibodies (see below). Quantitation of the radioactivity present within the Ndk bands from several independent experiments indicated that the Ndk levels were reduced approximately 25 to 46% in five cases (S43A, S69C, E80K, G91V, and D135A) (Fig. 2B). In three other cases (A56D, S119A, and S121A), the radioactivity present within the Ndk bands was similar to that seen in 8830R2::Cm hyperexpressing the wild-type Ndk from pGWS119 (Fig. 2B). The reduced level of 16-kDa Ndk in the supernatant fraction of 8830R2::Cm expressing each of those mutants was confirmed by Western blotting.

Biochemical activity of the mutant Ndks. Wild-type *P. aeruginosa* 8830 cell extract supernatant contains Ndk activity and the activity of an alternative kinase which can substitute for Ndk (33). The alternate kinase, which was not inhibited by anti-Ndk antibodies but was inhibited by Tween 20, was subsequently identified as Pk (41). In this study, we wished to examine the NTP-synthesizing activity of the mutant Ndks by overexpressing the various mutant *ndk* genes from the *tac* promoter in the 8830R2::Cm background, which contains a low level of Ndk. Ndk can transfer the terminal phosphate from ATP to any NDP, and the enzyme is known for its lack of specificity to nucleotide substrates. In this assay system, cellular fractions were incubated with [γ-^{32}P]ATP and a mixture of nonradioactive UDP, CDP, and GDP, producing [γ-^{32}P]UTP, [γ-^{32}P]CTP, [γ-^{32}P]GTP, and ADP. Performance of this assay, monitored by thin-layer chromatography, indicated that the 8830 cell extract supernatant demonstrated the presence of Ndk as well as Pk, since the activity could not be inhibited either by anti-Ndk or anti-Pk antibodies but could be totally inhibited by a combination of both. The 8830R2::Cm cell extract supernatant also demonstrated the transfer of the terminal phosphate from ATP to CDP, GDP, and UDP; however, Ndk activity was undetectable when anti-Pk antibodies were incorporated in the assay, suggesting that the phosphate transfer reaction in the extract was mostly accomplished by Pk. While it is known that Pk alone incubated with [γ-^{32}P]ATP and a mixture of nonradioactive CDP-GDP-UDP cannot catalyze [γ-^{32}P]CTP, [γ-^{32}P]GTP, and [γ-^{32}P]UTP synthesis, Pk has been shown to accomplish this synthesis in the presence of phosphoenol pyruvate and Mn^{2+}, both of which are components of cellular extracts but are not present in the membrane

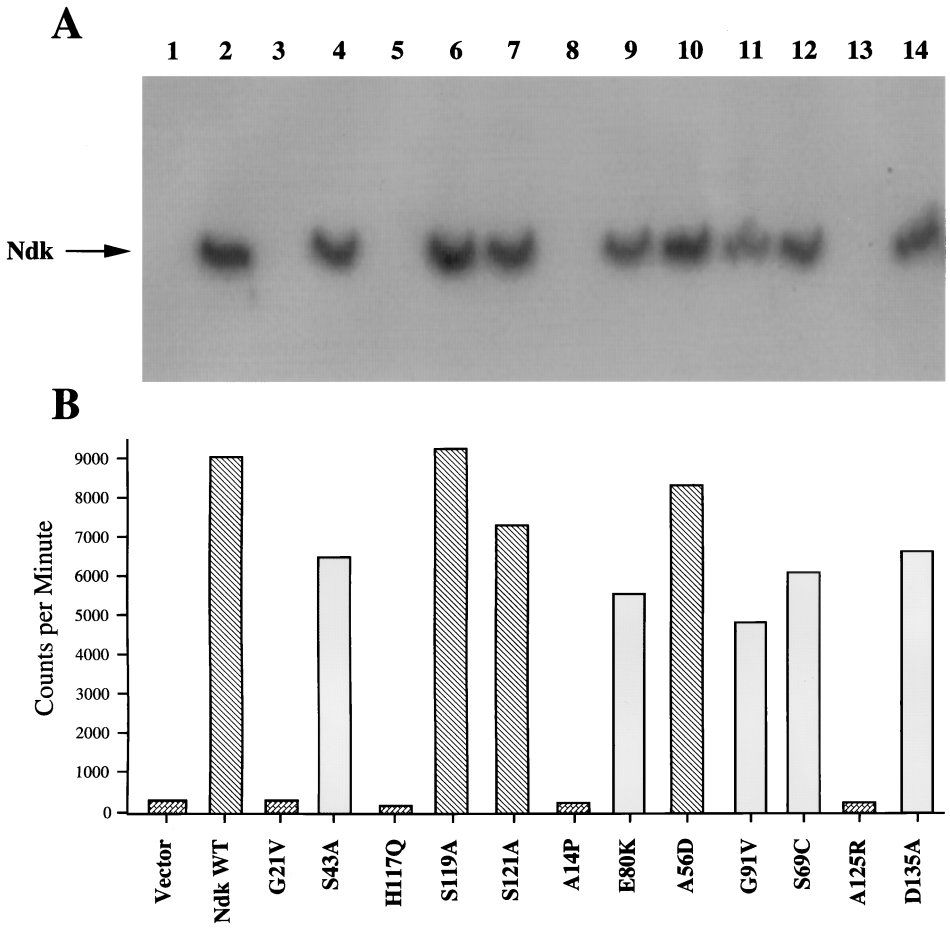

FIG. 2. (A) Autophosphorylation of Ndk in crude extracts of 8830R2::Cm containing the designated *ndk* genes in *trans* and in the presence of [γ-^{32}P]ATP. All strains were grown in the presence of IPTG to hyperexpress the *ndk* gene. Lanes were loaded with 10-μg extracts from 8830R2::Cm containing the following plasmids: 1, pMMB66EH; 2, pGWS119; 3, pGWS109 (G21V); 4, pGWS110 (S43A); 5, pGWS111 (H117Q); 6, pGWS112 (S119A); 7, pGWS113 (S121A); 8, pGWS120a (A14P); 9, pGWS120b (E80K); 10, pGWS120c (A56D); 11, pGWS120d (G91V); 12, pGWS120e (S69C); 13, pGWS120f (A125R); and 14, pGWS120g (D135A). (B) Quantitation of the radioactivity present within the Ndk bands from panel A. The radioactivity amounts and strain designations are presented directly below the row in panel A which shows the autophosphorylated Ndk. Five of the Ndk mutants which could be autophosphorylated but showed reduced levels compared with wild-type levels are designated by filled bars. WT, wild type.

(30, 35, 41). Pk also directs dNTP synthesis in *E. coli* under certain growth conditions (30). Thus, Pk serves as the alternate kinase in 8830R2::Cm extracts in the presence of anti-Ndk antibodies; complementation of 8830R2::Cm with clones expressing functional Ndk would result in extracts containing NTP-synthesizing activity in the presence of anti-Pk antibodies. The results of this assay indicate that the following Ndk mutants were still functional and possessed Ndk activity indistinguishable from the wild-type 8830 Ndk: S43A, A56D, S69C, E80K, G91V, S119A, S121A, and D135A (data not shown). The four Ndk mutants which could not be autophosphorylated also did not possess Ndk biochemical activity (data not shown).

Analysis of NTP synthesis from membrane fractions of 8830R2::Cm hyperexpressing the *ndk* mutant genes. We assessed the NTP-synthesizing ability of membrane fractions of 8830R2::Cm after hyperexpression of the 12 *ndk* mutant clones. The results of this experiment (Fig. 3) parallel the previous autophosphorylation and biochemical activity results. Briefly, the biochemical assay was set up similarly to that described for the supernatant fractions. The results of this assay show that the membrane extracts of 8830 and 8830R2::Cm (pGWS119) contain the Ndk-Pk complex which transfers the terminal phosphate from [γ-^{32}P]ATP preferentially to GDP, resulting in the formation of [γ-^{32}P]GTP (Fig. 3, lanes 1 and 3). The separate addition of anti-Ndk or anti-Pk antibodies to the reaction resulted in the loss of activity or the loss of specificity of NTP synthesis, respectively (41). The membrane extracts of 8830R2::Cm hyperexpressing the biochemically active Ndks S43A, S119A, S121A, E80K, A56D, G91V, S69C, and D135A demonstrated a specificity of GTP synthesis (Fig. 3, lanes 5, 7, 8, 10 to 13, and 15). The specificity of synthesis was eliminated when anti-Pk antibodies were included in the assay (data not shown), suggesting that complex formation was necessary for GTP-specific synthesis. The addition of anti-Ndk antibodies eliminated the synthesis of NTP, as we had previously shown (41). The membrane extracts of 8830R2::Cm hyperexpressing the activity mutant genes G21V, H117Q, A14P, and A125R showed no NTP-synthesizing activity in this assay (Fig. 3, lanes 4, 6, 9, and 14).

Complex formation between the 12-kDa form of Ndk and Pk. We have previously utilized 5 to 50% glycerol gradient centrifugation to show that the 12-kDa form of Ndk is capable of forming a complex with Pk (41). Complex formation of proteins is detectable in a glycerol gradient because the higher molecular weight of protein complexes results in the sedimentation of the complex in a higher percentage glycerol fraction

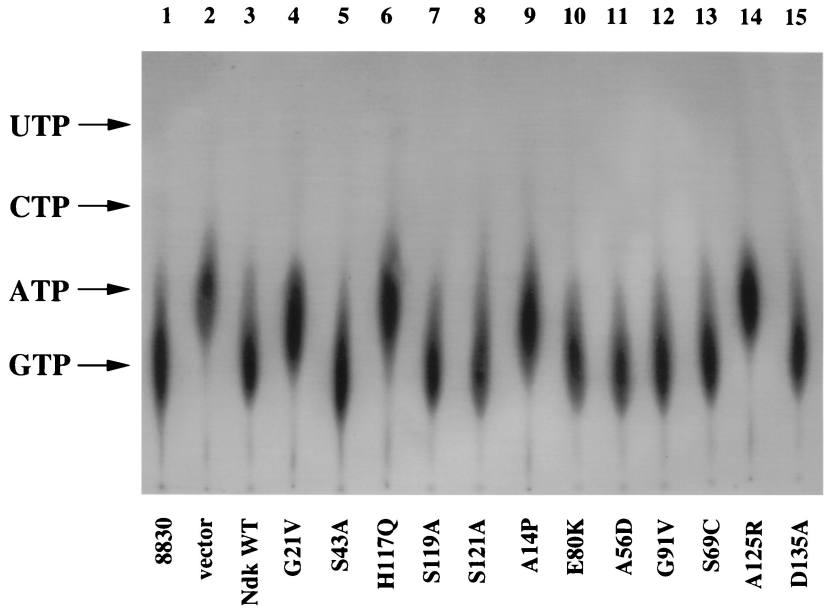

FIG. 3. Ndk activity from cell membrane suspensions of 8830 or 8830R2::Cm grown in the presence of IPTG and containing the designated *ndk* genes in *trans*. The NTP-synthesizing activity of extracts was assayed as described in Materials and Methods by incubating equal amounts of proteins from all extracts with [γ-^{32}P]ATP in the presence of an equimolar mixture of CDP-GDP-UDP and measuring the extent of conversion of the NDPs to NTPs as a result of terminal-phosphate transfer by Ndk. The designations of the particular *ndk* mutants hyperexpressed in 8830R2::Cm are shown below the lanes. Lane 1, 8830; and lanes 2 to 15, 8830R2::Cm containing each of the following plasmids: pMMB66EH, pGWS119, pGWS109 (G21V), pGWS110 (S43A), pGWS111 (H117Q), pGWS112 (S119A), pGWS113 (S121A), pGWS120a (A14P), pGWS120b (E80K), pGWS120c (A56D), pGWS120d (G91V), pGWS120f (S69C), pGWS120e (A125R), and pGWS120g (D135A), respectively.

(3). In this study, membrane extracts from 8830, 8830R2::Cm, and 8830R2::Cm in which each of the 12 *ndk* mutants was individually hyperexpressed were examined for evidence of complex formation. The results of the glycerol gradient assay with 8830, 8830R2::Cm, and 8830R2::Cm hyperproducing each of the four biochemically inactive Ndks are shown in Fig. 4. As Fig. 4 indicates, the purified 12-kDa Ndk was found only in the 5% glycerol fraction (lane A1). The Ndk present in membrane extracts from 8830R2::Cm(pGWS119) sedimented as a higher molecular weight complex which was detectable in fraction 6 (Fig. 4, lane C6). We have previously shown that a mixture of purified 12-kDa Ndk and Pk sediments as a complex detectable in fraction 6 of these glycerol gradients (41); conservative estimates of the Ndk-Pk complex by gel filtration indicates the

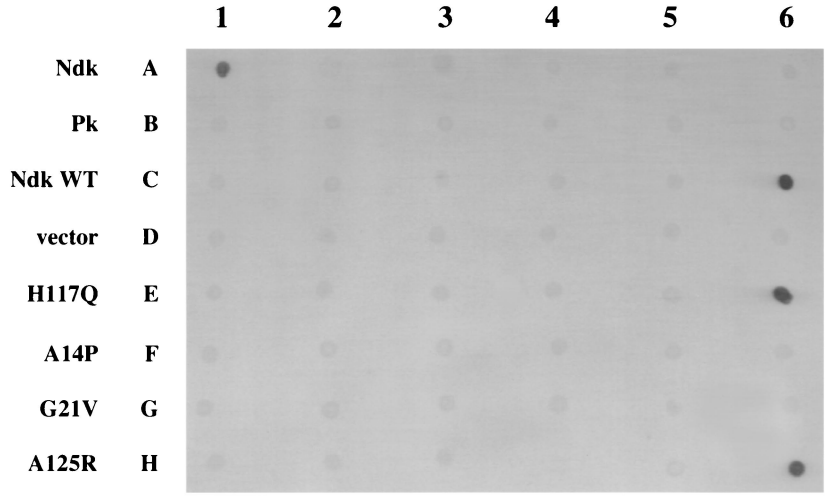

FIG. 4. Glycerol gradient centrifugation analysis of complex formation between Ndk and Pk in cell membrane suspensions from 8830R2::Cm hyperexpressing the designated mutant *ndk* genes or using purified proteins. The glycerol batch gradient was prepared as described in Materials and Methods. The lanes have been marked according to the samples they represent. Column numbers 1 through 6 denote the fraction numbers and also represent increasing glycerol percentages. The dot blot of fractions 1 through 6 (glycerol percentages of 5, 10, 20, 30, 40, and 50) was probed with anti-Ndk antibodies. Five microliters of collected fractions was spotted on a Hybond C+ nitrocellulose filter (Amersham Inc., Arlington Heights, Ill.) and probed with antibodies. When purified proteins were utilized, 1.25 μg of 12-kDa Ndk and 10 μg of Pk were used for complex formation. The antibodies were used at a dilution of 1:250 to enable detection without the need to concentrate samples diluted by centrifugation and separation in glycerol. Each row shows the results of a separate glycerol gradient centrifugation containing the following: purified 12-kDa Ndk (A), purified Pk (B), and cell membrane suspensions from 8830R2::Cm (C through H) containing pGWS119 (wild-type [WT] Ndk), pMMB66EH (Vector), pGWS111 (H117Q), pGWS120a (A14P), pGWS109 (G21V), and pGWS120e (A125R), respectively.

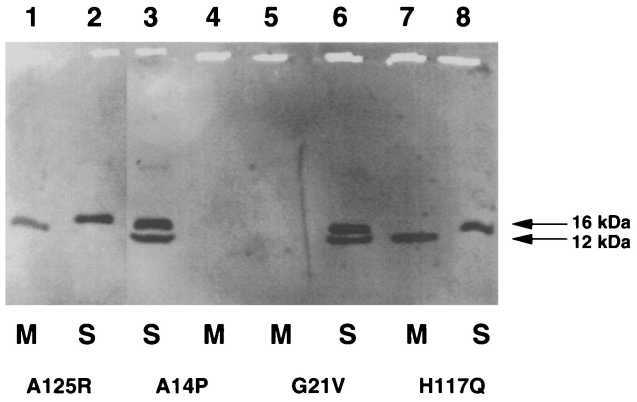

FIG. 5. Western blot analysis of supernatant (S) and membrane (M) extracts from 8830R2::Cm hyperproducing each of four Ndk activity mutants. Ten micrograms of protein was loaded into each lane, and the blot was probed with anti-Ndk antibodies. The designations of the particular *ndk* mutants hyperexpressed in 8830R2::Cm are shown below the lanes. 8830R2::Cm contained the following plasmids: pGWS120e (A125R) (lanes 1 and 2), pGWS120a (A14P) (lanes 3 and 4), pGWS109 (G21V) (lanes 5 and 6), and pGWS111 (H117Q) (lanes 7 and 8).

spherical molecular mass to be in the range of 120 to 160 kDa compared with 12 and 60 kDa, respectively, for the Ndk and Pk proteins alone (36). Although GTP synthesis in the membrane fraction was not detected in 8830R2::Cm hyperproducing the Ndk biochemical activity mutants H117Q and A125R, both of these Ndk mutants were shown to form a complex with Pk, as evidenced by the sedimenting of Ndk in fraction 6 (Fig. 4, lanes E6 and H6). These observations suggest that the active-site His-117 is required for GTP-specific synthesis when the 12-kDa Ndk is complexed with Pk. A duplicate protein blot probed with anti-Pk antibodies (data not shown) showed a pattern similar to that seen in Fig. 4. The purified Pk sediments in fraction 3 of the glycerol gradient, and Ndk::Pk complexes sediment in fraction 6 (41). As seen in Fig. 4, complex formation was observed in membrane extracts of 8830R2::Cm hyperproducing wild-type Ndk and Ndks H117Q and A125R. The eight mutant Ndks S43A, A56D, S69C, E80K, G91V, S119A, S121A, and D135A, which were not altered in GTP synthesis from the membrane fraction, were also shown to form a complex with Pk (data not shown).

Membrane extracts from 8830R2::Cm and 8830R2::Cm hyperproducing the mutant Ndks A14P and G21V did not contain detectable membrane-associated Ndk in any fraction of the glycerol gradient (Fig. 4, lanes D1 to D6, F1 to F6, and G1 to G6). Therefore, we performed Western blot analysis of the supernatant and membrane extracts from 8830R2::Cm hyperproducing the mutant Ndks A125R, A14P, G21V, and H117Q to investigate the cellular location(s) and size(s) of these Ndk mutants. Western blot analysis of supernatant extracts showed the presence of both 16- and 12-kDa forms of Ndk in 8830R2::Cm hyperproducing A14P and G21V mutant Ndks (Fig. 5, lanes 3 and 6). However, the 12-kDa Ndk was not detected in the membrane fractions of these strains (Fig. 5, lanes 4 and 5). In contrast, only the 16-kDa Ndk was detected in supernatant extracts and only the 12-kDa Ndk was detected in membrane extracts of 8830R2::Cm hyperproducing either the A125R or H117Q mutant Ndk (Fig. 5, lanes 1, 2, 7, and 8). Thus, the A14P and G21V mutant Ndks were truncated in vivo to 12 kDa but were apparently unable to form a tight complex with Pk at the cell membrane.

Alginate production by *P. aeruginosa* 8830R2::Cm and 8830R2::Cm complemented with various mutant *ndk* constructs. Hyperexpression of *ndk* from pGWS119 (same as pGWS95 used previously) was shown to largely restore alginate production to the alginate-defective strain 8830R2::Cm (41). In this study, wild-type *ndk* and 12 mutant *ndk* genes were hyperexpressed in 8830R2::Cm and the production of alginate was measured. Three of the five site-directed mutants examined (G21V, S43A, and H117Q) drastically reduced alginate production when hyperexpressed in 8830R2::Cm (Fig. 6). The S119A and S121A mutants, which did not affect Ndk activity, also did not reduce alginate production in 8830R2::Cm compared with wild-type Ndk. The seven *ndk* mutants selected after random mutagenesis with *E. coli* ES1578 were confirmed as being unable to complement 8830R2::Cm to alginate production.

We were surprised that a total of seven Ndk mutants which were defective in alginate complementation of 8830R2::Cm retained Ndk biochemical activity, including complex formation with Pk resulting in GTP synthesis. We recently hypothesized that the synthesis of GTP by the membrane Ndk-Pk complex was critical for the synthesis of alginate since large quantities of GTP are required for the formation of GDP-mannose, an essential alginate precursor (41). In light of our observations with these mutants, we decided to further investigate the Ndk activity in these strains. As shown in Fig. 2, the levels of five of the non-alginate-complementing Ndks (S43A, S69C, E80K, G91V, and D135A) were reduced compared with wild-type levels. We decided to examine whether the reduction in Ndk level corresponded with a reduction in activity in vivo. We assayed Ndk activity in vivo by examining the ability of cells to grow in the presence of Tween 20, a detergent which inhibits Pk, the alternate kinase which also has NTP-synthesizing activity (41). Of the mutants showing reduced Ndk levels, all, except for the S43A mutant, showed a reduction in growth rate

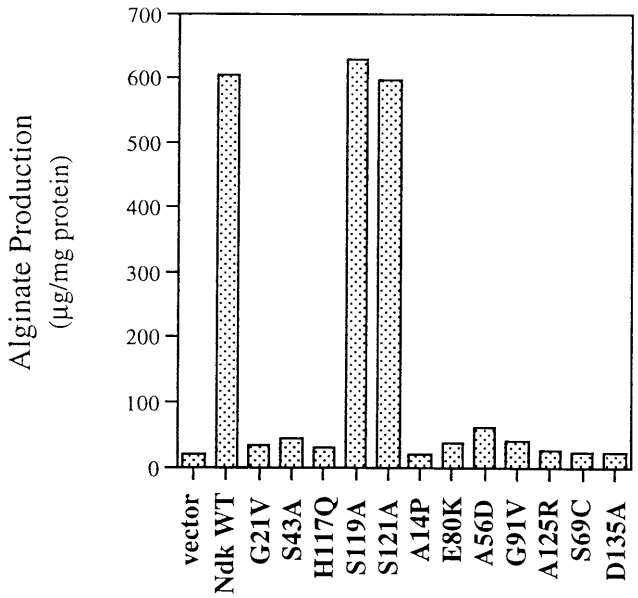

FIG. 6. Alginate production by 8830R2::Cm and 8830R2::Cm complemented with wild-type *ndk* on pGWS119 or with plasmids containing mutant *ndk* genes. In the following lanes, 8830R2::Cm harbored the indicated plasmid: vector, pMMB66EH; wild-type (WT) Ndk, pGWS119; G21V, pGWS109; S43A, pGWS110; H117Q, pGWS111; S119A, pGWS112; S121A, pGWS113; A14P, pGWS120a; E80K, pGWS120b; A56D, pGWS120c; G91V, pGWS120d; A125R, pGWS120e; S69C, pGWS120f; and D135A, pGWS120g.

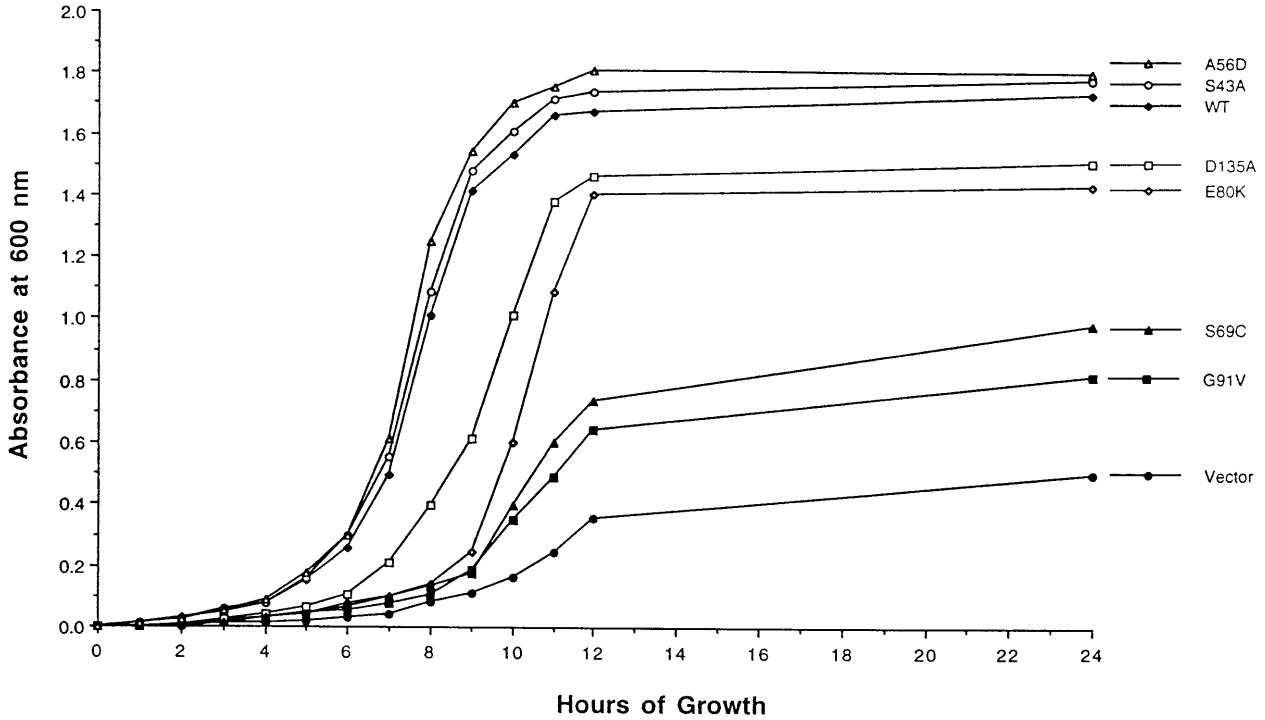

FIG. 7. Growth curves of 8830R2::Cm containing the designated plasmids in the presence of IPTG and 0.1% Tween 20. The mutation in Ndk is shown next to the growth curve for each strain. The plasmid used in each 8830R2::Cm-plasmid combination was as follows: vector, pMMB66EH; wild type (WT), pGWS119; S43A, pGWS110; A56D, pGWS120d; S69C, pGWS120f; E80K, pGWS120b; G91V, pGWS120c; and D135A, pGWS120g.

in Luria-Bertani broth containing Tween 20 (Fig. 7). The reduction in growth rate was similar to that seen previously in 8830R2::Cm*algH*(pGWS95), a mutant in which the Ndk levels were reduced two- to threefold compared with that of 8830 and which was unable to produce alginate (41). Thus, there was a correlation between optimum growth rate in the presence of Tween 20 and alginate synthesis. The other non-alginate-complementing Ndk (A56D) was not affected in its Ndk levels or in its growth in the presence of Tween 20 (Fig. 7).

DISCUSSION

In this study, we utilized site-directed and random mutagenesis techniques to alter *P. aeruginosa* Ndk; we were interested in the biochemical characterization of Ndk activity mutants and in examining the role of this enzyme in alginate biosynthesis. The importance of Ndk to alginate synthesis was previously shown when hyperexpression of the *ndk* gene was found to largely restore alginate production to 8830R2::Cm, an alginate-defective mutant which produces a greatly reduced amount of Ndk compared with that of the wild-type strain (41). Our subsequent findings that a truncated 12-kDa form of Ndk is found in membranes and that this membrane form complexes with Pk to produce GTP led to speculation of the importance of this complex as a supplier of GTP for alginate synthesis (41). GTP is required for alginate synthesis, and the AlgA enzyme phosphomannose isomerase-guanosine 5′-diphosphomannose pyrophosphorylase catalyzes the formation of the essential alginate precursor GDP-mannose from mannose 1-phosphate and GTP (22). The synthesis of GTP by the 12-kDa Ndk-Pk complex may provide a direct supply of GTP for this reaction or may lead to alterations in nucleotide pools which indirectly trigger other signal transduction events which may activate AlgA for the formation of GDP-mannose. Thus, we were also interested in further characterizing the membrane complex formed between the 12-kDa Ndk and Pk which results in the alteration of the specificity of nucleotide synthesis to GTP.

We identified 10 mutants of *ndk* which could not complement the alginate synthesis defect after hyperexpression in 8830R2::Cm (Fig. 6). Four of these mutants were activity mutants (A14P, G21V, H117Q, and A125R); the proteins were incapable of being autophosphorylated, and the cytoplasmic 16-kDa form did not possess NTP-synthesizing activity. Also, GTP synthesis from the membrane fraction was not evident in 8830R2::Cm hyperexpressing any of the four activity mutant genes. Two of these residues (Gly-21 and His-117) were previously shown to be required for the activity of *M. xanthus* Ndk (24). Additionally, His-117 is conserved among all known Ndks and has been identified as the active-site residue which undergoes autophosphorylation (10, 23, 42). This residue is also involved in nucleotide binding and in the phosphorylation of Ser-119 and Ser-121 in *E. coli* Ndk (1). The importance of the Ala-14 and Ala-125 residues to Ndk activity has not been previously shown.

None of the other six non-alginate-complementing Ndk mutants was affected in biochemical activity or in GTP synthesis in the cellular membrane fraction. However, the level of active cytoplasmic enzyme was reduced by 25 to 46% in five of these mutants. The reduction in levels of active Ndk was biologically relevant in four cases in vivo and was associated with a delay of exponential phase and a reduced ability of 8830R2::Cm hyperexpressing any of the four mutant *ndk* genes to grow in the presence of Tween 20. The slower growth rates of 8830R2::Cm and the double mutant 8830R2::Cm*algH* in the presence of Tween 20 are due to reduced Ndk levels and are correlated with a lack of complementation of alginate synthesis in these

strains (41). Two other non-alginate-complementing Ndk mutant proteins (S43A and A56D) were apparently unaffected in biochemical activity, cellular concentration, and GTP synthesis in the cellular membrane fraction. The Ser-43 residue corresponds to Ser-44 in human Nm23-H1; the phosphorylation of this residue is suspected to play a role in the suppression of tumor metastasis (18). It is currently unknown whether Ser-43 is phosphorylated in *P. aeruginosa* Ndk and, if so, the potential effect of the phosphorylation of this residue to alginate synthesis. Another reason why the biochemically active S43A and A56D Ndks did not complement alginate synthesis could be that these mutants are defective in a function such as in protein-protein interactions with a protein other than Pk. For example, the *Drosophila Killer of prune* mutation maps to a P97S mutation in Awd (Ndk) (2). Homozygous *Killer of prune* mutants are viable; however, the mutation is lethal when the genotype contains a second mutation in the *prune* locus. Analyses of the *Drosophila* P97S Ndk mutant indicate that the protein is structurally and catalytically very similar to the wild type as only the folding and assembly of the protein are affected in vitro (16). This effect is not seen in vivo, leading to the suggestion that the *Killer of prune* mutant Ndk may be affected in complexing with other cellular proteins (16).

Ndk is known to form protein complexes and interact with other proteins in vivo. For example, in *P. aeruginosa*, the 16-kDa cytoplasmic Ndk forms a complex with succinyl coenzyme A-synthetase, an important enzyme of the tricarboxylic acid cycle (12). The truncated 12-kDa membrane form of *P. aeruginosa* Ndk also forms a complex with Pk (41). During T4 phage infection of *E. coli*, Ndk is thought to constitute part of a multienzyme complex involved in dNTP synthesis (19, 29). Ndk is also associated with the translation apparatus of *D. discoideum* and is thought to provide critical amounts of GTP necessary for protein synthesis (41). Finally, although direct phosphorylation of GDP bound to GTP-binding proteins by Ndk apparently does not occur (28), Ndk activity does affect the availability of GTP for utilization by GTP-binding proteins.

GTP plays a critical role in regulating cellular events, such as signal transduction, elongation steps in protein synthesis, tubulin formation, and transformation to malignancy (26, 38). GTP is also required in large quantities during alginate biosynthesis for the production of GDP-mannose, an alginate precursor molecule (20). In *P. aeruginosa*, the 12-kDa form of Ndk, which complexes with Pk in the membrane, appears and its levels increase as cells enter stationary phase (34). Concurrently, the production of large quantities of alginate also occurs during stationary phase (20). The Ndk-Pk membrane complex is involved in the production of GTP; however, the nature of the distribution of the newly synthesized GTP among alginate biosynthetic enzymes and proteins involved in other cellular functions, such as signal-transducing GTPase enzymes, is unknown. In the rat system, membrane-bound Ndk is thought to regulate adenylate cyclase through the channeling of GTP to the GTP-binding protein G_S (15). *P. aeruginosa* Ndk might be involved in the channeling of GTP for signal transduction in such a manner through close association with GTP-requiring enzymes. One such protein might be Era, an essential GTPase protein from *E. coli* (39); we have recently purified a homolog of Era from membrane fractions of *P. aeruginosa* precipitated with anti-Ndk antibodies and are currently studying the interaction of this protein with the Ndk-Pk complex (36).

The results shown in Fig. 6 and 7 imply that when the amount of Ndk is limiting (which affects full growth), not enough NTPs, particularly GTP, are produced to allow high levels of alginate synthesis. These results suggest that a cellular mechanism whereby GTP is utilized for critical functions and utilized for alginate synthesis only when intracellular concentrations reach a threshold exists. The channeling of GTP for biosynthetic purposes, such as to the cytoplasmic bifunctional AlgA protein phosphomannose isomerase-GDP-mannose pyrophosphorylase, which mediates the conversion of mannose 1-phosphate and GTP to GDP-mannose, might require a GTP-binding protein in order to transport the necessary GTP for continued alginate synthesis. If the K_m of this G protein is higher than that of other G proteins, the transport of GTP to AlgA could be mediated only when intracellular concentrations of GTP are high. The apparent K_m of AlgA for GTP is known to be 41.2 μM; thus, GTP is required at relatively high concentrations for the functionality of AlgA (22). The A56D mutant is unique in that its cellular growth, Ndk levels, and presumably GTP levels are similar to those of the wild type. This non-alginate-complementing Ndk mutant might be defective in properties such as association with G proteins in the transport of GTP to AlgA, thereby resulting in blockage of alginate synthesis. If there is a G protein needed for the transport of GTP from the membrane-associated Ndk-Pk complex to cytoplasmic AlgA, perhaps Era or other proteins could play this role. It would be interesting to examine complex formation between the A56D mutant Ndk and Era, for example, to see if there is a lack of complex formation only with this mutant Ndk.

The A14P and G21V mutations in *ndk* resulted in Ndk proteins which could be truncated in vivo but not exported to the membrane. We have previously purified an 80-kDa protease which cleaves Ndk, resulting in the 12-kDa form (34); however, after the truncation of Ndk to 12 kDa, it is currently unclear how the protein is transported to the membrane. These mutant Ndks will be useful in our attempts to determine the mechanism of translocation of the 12-kDa Ndk to the membrane, particularly if this process is mediated by another protein acting as a chaperone.

Two of the Ndk activity mutants (H117Q and A125R) were still able to form a complex with Pk. Our results utilizing the H117Q mutant indicate that the active-site His residue is required for GTP synthesis and implies that the shift from nonspecific NTP synthesis to GTP synthesis is conditioned through complex formation with Pk. It is known that Pk has a low K_m for GDP; however, *P. aeruginosa* Pk does exhibit a nonspecific NTP-synthesizing activity (41). Ndk itself does not possess a nucleotide binding site similar to that in GTP-binding proteins (2, 13). Indeed, the nucleotide substrate enters the active-site pocket phosphate group first and Ndk primarily interacts with the substrate through contacts with the phosphate and sugar groups of NDPs (23, 41). Thus, in complexing with Ndk, Pk may alter the active-site pocket so that substrate access is limited and is affected by the affinity of Pk for GDP. Further mutagenesis experiments are under way to elucidate Ndk-Pk interaction and to understand the nature of the interaction between these two proteins which conditions the nucleotide synthesis specificity of the complex.

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