Autophosphorylation of Nucleoside Diphosphate Kinase from *Myxococcus xanthus*

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The nucleoside diphosphate kinase (NDP kinase) from *Myxococcus xanthus* has been purified to homogeneity and crystallized (J. Muñoz-Dorado, M. Inouye, and S. Inouye, J. Biol. Chem. 265:2702–2706, 1990). In the presence of ATP, the NDP kinase was autophosphorylated. Phosphoamino acid analysis was carried out after acid and base hydrolyses of phosphorylated NDP kinase. It was found that the protein was phosphorylated not only at a histidine residue but also at a serine residue. Replacement of histidine 117 with a glutamine residue completely abolished the autophosphorylation and nucleotide-binding activity of the NDP kinase. Since histidine 117 is the only histidine residue that is conserved in all known NDP kinases so far characterized, the results suggest that the phosphohistidine intermediate is formed at this residue during the transphosphorylation reaction from nucleoside triphosphates to nucleoside diphosphates. Preliminary mutational analysis of putative ATP-binding sites is also presented.

Nucleoside diphosphate kinases (NDP kinases) are enzymes that catalyze the transfer of the γ -phosphate of nucleoside triphosphates to nucleoside diphosphates. These enzymes show very little specificity for the base and the sugar. Any ribonucleoside triphosphate (NTP), deoxyribonucleoside triphosphate, ribonucleoside diphosphate (NDP), or deoxyribonucleoside diphosphate can be used as a substrate (27). The reaction is reversible: $N_1TP + N_2DP \leftrightarrow N_1DP + N_2TP$. It has been shown to occur in two steps (ping-pong mechanism), forming a phosphoprotein intermediate (27).

NDP kinase activity was first reported in 1953 for yeasts and pigeons (1a, 18), and later it was shown to exist in a wide variety of organisms, from prokaryotes to eukaryotes. For a long time it was thought that the function of NDP kinases was to maintain the levels of NTPs and dNTPs in the cell by using ATP as a phosphate donor. In *Myxococcus xanthus*, a gram-negative bacterium, the NDP kinase has been shown to be essential for cell growth (23). Interestingly, NDP kinases have been shown to play important roles in various eukaryotes. For instance, Awd, an NDP kinase found in *Drosophila melanogaster*, is required to obtain normal wing discs (3) and is associated with microtubules, in which it plays a crucial role in spindle microtubule polymerization, probably by transphosphorylation of tubulin-bound GDP (2); an NDP kinase has been reported to be involved in signal transduction in rats (16); and two genes that encode proteins with NDP kinase activity have been found in humans (9). One of them has been shown to be identical in sequence to nm23, a gene that was originally found to be involved in the suppression of cancer metastasis (21, 28).

Recently, various NDP kinase genes from the following prokaryotes and eukaryotes were cloned and sequenced: *M. xanthus* (23), *Escherichia coli* (10), *Dictyostelium discoideum* (19, 35), *D. melanogaster* (2), humans and mice (28, 32), and rats (17). All of them encode proteins with molec-

ular weights of 16,000 to 17,000; their amino acid sequences are highly homologous (10), indicating that evolutionarily NDP kinases are one of the most conserved proteins among organisms. The NDP kinase from *M. xanthus* has been crystallized (24), and a preliminary X-ray crystallographic study of the crystals has been carried out (36).

In this report, we have further characterized the phosphoenzyme intermediate obtained during incubation of the *M. xanthus* NDP kinase with ATP. It was found that the protein is autophosphorylated not only at a histidine residue but also at a serine residue, indicating that the enzyme also has protein serine kinase activity. In addition, we have performed a mutational analysis for NDP kinase activity at several residues that are highly conserved in the *M. xanthus* NDP kinase and other nucleotide-binding proteins and found that histidine 117, glycine 21, and glycine 91 are essential for the ATP-binding activity of this enzyme.

It has been reported that NDP kinases can transfer the γ -phosphate of any NTP to any NDP by the ping-pong mechanism through a phosphoenzyme intermediate and that autophosphorylation occurs mostly at a histidine residue (8, 30, 32). For the *M. xanthus* NDP kinase, the phosphoenzyme can be easily isolated by incubating the protein with ATP in the presence of EDTA (24). However, when the stability of this phosphoenzyme in the presence of different pHs was examined, it was found that only approximately 40% of the phosphate incorporated into the protein was released after incubation of the enzyme at 45°C for 2 h at pH 1 (Fig. 1). Approximately 48% of the phosphate was released when the incubation was carried out at pH 14 under the same conditions. *N*-Phosphate amino acids (phosphohistidine, phospholysine, and phosphoarginine) are known to be stable at pH 14 but not 1, while *O*-phosphate amino acids (phosphoserine and phosphothreonine) are known to be stable at pH 1 but not 14. On the other hand, acyl-phosphate amino acids (phosphoaspartate and phosphoglutamate) are labile at both pHs 1 and 14, and phosphotyrosine and phosphocysteine are stable at both pHs (6). Therefore, the stability profile for phosphorylated *M. xanthus* NDP kinase at different pHs does not resemble that for any one of the phosphoamino acids described above, suggesting that phosphor-

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FIG. 1. Stability of phosphorylated *M. xanthus* NDP kinase at different pHs. Purified NDP kinase (23) was phosphorylated with [γ - 32 P]ATP as follows. Protein (1.6 μ g) was incubated with 10 μ Ci of [γ - 32 P]ATP (3,000 Ci/mmol) for 15 min on ice in a solution containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 8), 1 mM EDTA, 100 mM NaCl, and 2 μ M ATP. The phosphorylated protein was loaded on a 15% SDS-polyacrylamide gel and blotted onto an Immobilon-P membrane (Millipore) after electrophoresis. The membrane was then stained with Ponceau red, and the portion of the membrane containing the NDP kinase was cut into several pieces. Each piece was incubated at a different pH in 200 μ l of each of the following buffers: 50 mM KCl-HCl (pH 1), 50 mM glycine-HCl (pH 3), 0.1 M Tris-HCl (pH 7), 50 mM KCl-NaOH (pH 12), and 1 M KOH (pH 14). After incubation for 2 h at 45°C, the radioactivity released into the buffer and the radioactivity remaining in the membrane were measured. The percentage of the radioactivity released into the buffer at each pH is shown.

ylation may occur at more than one residue, one stable at pH 1 and the other stable at pH 14. To prove this point, the wild-type enzyme purified to homogeneity was first phosphorylated with [γ - 32 P]ATP. Subsequently, the phosphorylated enzyme was hydrolyzed under basic and acidic conditions. Hydrolysates were then analyzed by two-dimensional chromatography. Phosphohistidine was detected in the alkaline hydrolysate (Fig. 2A), while phosphoserine was identified in the acid hydrolysate (Fig. 2B). These results demonstrate that the *M. xanthus* NDP kinase is indeed phosphorylated not only at a histidine residue but also at a serine residue.

We have also performed a mutational analysis of putative ATP-binding sites. Two motifs, GXXGK (residues 18 to 22) and DXXG (residues 88 to 91), which are separated by 65 residues and which are known to be characteristic of many GTP-binding proteins, are also found in the sequence of the *M. xanthus* NDP kinase (4). Crystallographic studies of *E. coli* elongation factor Tu (20) and Ha-ras oncogene product p21 (5, 26) have found that the first motif forms part of the phosphate-binding loop. The second motif lies near the first one, so that the aspartate residue in the motif interacts with Mg^{2+} , which is essential for the proper binding of GTP. The glycine residue in the second motif allows the main chain to adopt a sharp bend and is not exchangeable with any other amino acid residue. We mutated G-21, K-22, D-88, and G-91 to investigate whether these residues are involved in the

FIG. 2. Phosphoamino acid analysis of phosphorylated *M. xanthus* NDP kinase. The protein was autophosphorylated with [γ - 32 P]ATP as described in the legend to Fig. 1. (A) The phosphoenzyme (16 μ g) was hydrolyzed in 3 N KOH at 105°C after free ATP was removed. Phosphohistidine, synthesized as described below, was added at 100 μ l to this sample. An aliquot of 5 μ l was loaded on a Silica Gel 60 Å K6 plate (Whatman), and thin-layer chromatography was done with chloroform-methanol-17% (wt/wt) ammonia (2:2:1) [wt/wt/wt] in the first dimension and with phenol-water (75:25 [wt/wt]) in the second dimension (25). After being dried, the plate was exposed to X-ray film. The positions of standard histidine (His) and phosphohistidine (P-His) were detected by spraying ninhydrin solution (2% in ethanol) on the plate. Phosphohistidine was synthesized by mixing 10 mg of histidine with 100 mg of potassium phosphoramidate in 1 ml of water (12). After 2 h of incubation at room temperature, the sample was divided into aliquots and stored at -20°C. Potassium phosphoramidate was prepared by the method of Sheridan et al. (31). (B) The phosphorylated protein was first separated by SDS-polyacrylamide gel electrophoresis and then transferred to an Immobilon-P membrane by use of a semidry blotter apparatus. The membrane was stained with Ponceau red, and the portion of the membrane corresponding to the NDP kinase was cut out. After being washed thoroughly with water, the membrane was incubated at 110°C for 2 h in 200 μ l of 6 N HCl as described previously (15). After the HCl was removed in a lyophilizer, the hydrolysate was dissolved in 100 μ l of a solution containing nonradioactive phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr). A 7- μ l portion of this sample was loaded on a cellulose plate, and phosphoamino acids were separated by electrophoresis at pH 3.5 for 40 min at 1,000 V in glacial acetic acid-pyridine-water (50:5:945) in the first dimension and by ascending chromatography with isobutyric acid-0.5 M ammonia (5:3) in the second dimension (13). The plate was then exposed to X-ray film, and the positions of standard phosphoamino acids were detected with ninhydrin. The origin is at the intersection of arrows 1 and 2.

nucleotide-binding activity of the *M. xanthus* NDP kinase. In addition, two more point mutations were constructed. One was at H-117, the only histidine residue conserved in all the NDP kinases so far sequenced (for a comparison of the sequences of NDP kinases, see reference 10). This residue is located in one of the longest conserved stretches and is considered to be the autophosphorylation site essential for the transfer of γ -phosphate from NTPs to NDPs. We also replaced the S-110 residue with cysteine. According to a preliminary crystallographic study (35a), this residue is located on the surface of the molecule and is not conserved in other NDP kinases. Since no cysteine is present in the *M. xanthus* NDP kinase, this mutant protein may be used for heavy-metal substitution for X-ray crystallographic studies.

The oligonucleotides used for the amino acid substitutions

SEQUENCE	MUTATION
GTCATCGTGAAGATC	G21 V
TCGGGAACATCATCA	K22 N
AACCGGGGCATCATG	D88 G
ATCATGGTCGCCACC	G91 V
CGCCACCTGCATCGA	S110 C
CGGTGCAAGGCTCCG	H117 Q

FIG. 3. Oligonucleotides used in site-directed mutagenesis (14) and mutations in the *M. xanthus* NDP kinase. An asterisk indicates the base that was mutated. Mutations were confirmed by DNA sequencing (29).

are listed in Fig. 3. The mutant proteins (or mutations) thus obtained are designated G21V, K22N, D88G, G91V, S110C, and H117Q: the first letter is the amino acid residue in the wild-type protein, the number is the position of the amino

acid residue in the protein, and the last letter is the amino acid residue in the mutant protein.

Each mutant protein was overproduced in *E. coli* by use of the *lacZ* promoter as reported for the wild-type protein (24). Since the levels of expression obtained for the mutant proteins were different and not all the proteins could be purified in the same manner, the purities of the preparations varied. However, the purities of the NDP kinases used in the assays described below were at least 30% (30% for H117Q, 50% for K22N and D88G, and 90% for S110C, G21V, and G88V mutant proteins and the wild-type protein).

The purified mutant proteins were then assayed for NDP kinase activities along with the wild-type protein. As shown in Fig. 4, mutations K22N (lane 3) and D88G (lane 4) had no effect on NDP kinase activity, and these mutant proteins transferred γ -phosphate from NTPs to NDPs at the same rate as the wild-type protein (lane 1; specific activity, approximately 200 μ mol of NTP per mg of protein per min). On the contrary, mutations G21V, G91V, and H117Q (lanes 2, 5, and 6, respectively) completely abolished the activity, as evident from the fact that no synthesis of CTP could be detected even after incubation for longer than that shown in Fig. 4. When NDP kinase activity in the S110C mutant was

FIG. 4. NDP kinase activities of the wild-type *M. xanthus* NDP kinase and the various mutant proteins. *E. coli* JM83 (33) harboring either wild-type plasmid pJM5C2A (23) or the mutations shown in Fig. 3 was grown in 1 liter of LB medium (22) to the mid-log phase. Cells were harvested by centrifugation at $4,000 \times g$ for 20 min at 4°C , resuspended in 10 ml of TE buffer (10 mM Tris-HCl [pH 7.7], 1 mM EDTA), and disrupted by sonication. Cell debris was removed by centrifugation at $4,000 \times g$ for 5 min. The supernatants were centrifuged at $100,000 \times g$ for 40 min at 4°C to remove the membrane fraction. Supernatants obtained for the wild-type protein and the six mutant proteins were loaded on DEAE-cellulose (DE-52; Whatman) columns (2 by 7 cm) equilibrated with TE buffer. All the mutant proteins were eluted in the flowthrough, as was the wild-type protein (24). Fractions containing NDP kinase were pooled, and ammonium sulfate was added to a final concentration of 45% saturation. After removal of the precipitates by centrifugation, ammonium sulfate was added to the supernatants to 65% saturation. The precipitates contained most of the NDP kinase in the cases of the wild-type protein and the G21V, G91V, and S110C mutant proteins. However, most of the NDP kinase remained in the supernatants in the cases of the K22N, D88G, and H117Q mutant proteins, probably because charged residues were replaced by noncharged amino acids. These three mutant proteins were precipitated by increasing the concentration of ammonium sulfate to 80% saturation. The precipitates containing NDP kinase were dissolved in 400 μ l of TEN buffer (10 mM Tris-HCl [pH 7.7], 1 mM EDTA, 100 mM NaCl), and the solutions were dialyzed against 4 liters of the same buffer overnight. The dialyzed solutions were stored at -20°C . For the assay, 16 ng of protein was incubated at 30°C with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and CDP as the γ -phosphate acceptor as described previously (24). At different times, 1 μ l of the reaction mixture was transferred to 10 μ l of 5 mM EDTA, and 1 μ l of this final mixture was loaded onto a polyethyleneimine-cellulose plate. The synthesis of $[\gamma\text{-}^{32}\text{P}]\text{CTP}$ was analyzed by ascending chromatography with 0.75 M KH_2PO_4 (pH 3.65). An autoradiogram obtained after exposure of the plate when the samples were withdrawn after 10 min of incubation is shown. The plate was exposed to X-ray film for 2 h. β ME was omitted from the reaction mixture unless indicated. Lanes: 1, wild-type protein; 2, G21V mutant protein; 3, K22N mutant protein; 4, D88G mutant protein; 5, G91V mutant protein; 6, H117Q mutant protein; 7, S110C mutant protein; 8, wild-type protein; 9, S110C mutant protein incubated with 10 mM β ME; 10, wild-type protein incubated with 10 mM β ME.

assayed, only approximately 10% of the wild-type activity was observed in the absence of reducing agents (lane 7; specific activity, 19 μmol of NTP per mg of protein per min), whereas the activity increased to 50% of that of the wild-type protein when β -mercaptoethanol (βME) was added to the reaction mixture (lane 9). The activity of the wild-type protein was not changed by the addition of βME (compare lanes 8 and 10). The effect of the reducing agent on the activity of the S110C mutant protein suggested that this mutant protein intermolecularly formed a disulfide bond that inhibited the enzymatic activity. The formation of an intermolecular disulfide bond was indeed detected by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoretic analysis of the protein with and without βME treatment. As shown in Fig. 5A, lane 3, more than 50% of the S110C mutant protein migrated at a molecular mass of approximately 32,000 Da when βME was omitted from the loading buffer. This position corresponds to the molecular mass expected for a dimer of the NDP kinase (the molecular mass of the monomer is 16,000 Da). On the contrary, only the monomeric form was detected when βME was added to the loading buffer (Fig. 5A, lane 1). The migration of the wild-type protein was not affected by the addition of the reducing agent (Fig. 5A, lanes 2 and 4).

We further examined whether the G21V, G91V, and H117Q mutant proteins could be autophosphorylated by ATP in the presence of EDTA. As shown in Fig. 5B, G21V, G91V, and H117Q could not be autophosphorylated with [γ - ^{32}P]ATP (lanes 1, 4, and 5, respectively), whereas the wild-type protein and the K22N and D88G mutant proteins could be autophosphorylated (lanes 7, 2, and 3, respectively). The S110C mutant protein was found to be poorly phosphorylated even in the presence of βME (compare lanes 6 and 7), a result that is consistent with the lower NDP kinase activity of this mutant protein. It is interesting to note that the dimer band was also capable of autophosphorylation with ATP (lane 8).

As shown in Table 1, the K22N and D88G mutant proteins showed ATP-binding activities very similar to that observed for the wild-type protein, while the G21V, G91V, and H117Q mutant proteins showed considerably reduced ATP-binding activities. In the case of the S110C protein, the ATP-binding activity was substantially decreased in the absence of βME and increased threefold in the presence of βME , to a level approximately 74% of the wild-type level (Table 1).

In this report, we have found that the *M. xanthus* NDP kinase is autophosphorylated at both serine and histidine residues. Although autophosphorylation of NDP kinases from other species at a histidine residue has been reported (8, 30, 34), this is the first report showing that an NDP kinase is also autophosphorylated at a serine residue. Phosphorylation at the histidine residue is considered to be an obligatory step for the transphosphorylation reaction from NTPs to NDPs (27). However, the significance of phosphorylation at the serine residue is not evident at present. In other organisms, such as *D. melanogaster*, rats, and humans, NDP kinases have been found to be involved in some other cellular functions, such as spindle microtubule polymerization, signal transduction, and cancer metastasis (2, 3, 16, 21, 28). Therefore, it is tempting to speculate that phosphoserine may play a regulatory role in these cellular functions rather than regulating the nucleotide pool inside the cells. Since the disruption of the *ndk* gene by insertion of a kanamycin resistance gene has been shown to be lethal for *M. xanthus* (23), while the *E. coli ndk* gene is dispensable (1), it is possible that the *M. xanthus* NDP kinase has some other

FIG. 5. SDS-polyacrylamide gel electrophoresis of mutant NDP kinases. (A) Effect of βME on the S110C mutant protein. The wild-type protein (1.5 μg) and S110C mutant protein (3 μg) were analyzed on a 15% SDS-polyacrylamide gel after boiling of the samples in the presence and absence of βME . Lanes: 1, S110C mutant protein with βME ; 2, wild-type protein with βME ; 3, S110C mutant protein without βME ; 4, wild-type protein without βME ; 5, molecular mass standards in kilodaltons. (B) Autophosphorylation activities of wild-type and mutant NDP kinases. The proteins (1.6 μg) were incubated with [γ - ^{32}P]ATP in the presence of EDTA on ice as described in the legend to Fig. 1. βME was omitted from the reaction buffer unless specifically indicated otherwise. The samples were analyzed on a 15% SDS-polyacrylamide gel and transferred to an Immobilon-P membrane. An autoradiogram obtained after exposure of the membrane to X-ray film is shown. Lanes: 1, G21V mutant protein; 2, K22N mutant protein; 3, D88G mutant protein; 4, G91V mutant protein; 5, H117Q mutant protein; 6, S110C mutant protein incubated with βME ; 7, wild-type protein incubated with βME ; 8, S110C mutant protein; 9, wild-type protein. Numbers at left are in kilodaltons.

cellular functions similar to those in eukaryotic cells. Identification of the serine residue that is phosphorylated in the *M. xanthus* NDP kinase and mutational analysis of the residue will shed light on the function of phosphorylation at this residue. It will also be interesting to determine whether other NDP kinases are phosphorylated at a serine residue, too.

The fact that three different point mutations that inhibit the binding of nucleotides also abolish phosphorylation at

TABLE 1. Nucleotide-binding activities of the wild-type and mutant NDP kinases

Protein	β ME	Nucleotide-binding activity (nmol of ATP/mg of protein)
G21V	+	1
K22N	+	38
D88G	+	37
G91V	+	1.2
H117Q	+	0.4
S110C	+	28
S110C	—	10
Wild type	+	38
Wild type	—	39

both serine and histidine residues demonstrates that these phosphoamino acids indeed result from autophosphorylation reactions, not from phosphorylation by contaminating *E. coli* protein kinases. Since NDP kinase with a phosphohistidine residue is an intermediate that appears during the synthesis of nucleoside triphosphates, it is likely to be formed by an intramolecular reaction. However, phosphoserine may result from an intermolecular reaction. This idea is now being examined by mutational analysis of the two serine residues that are conserved in NDP kinases (10). At any rate, the *M. xanthus* NDP kinase seems to be a novel type of protein kinase, with no sequence similarities to the classical protein serine-threonine and tyrosine kinases (11).

Since H-117 is the only histidine residue that is conserved in all the NDP kinases sequenced so far, it is likely that this is the residue that is phosphorylated. This notion is supported by the fact that the replacement of H-117 in the *M. xanthus* NDP kinase by glutamine abolishes the autophosphorylation activity of this protein at both serine and histidine residues. Interestingly, no nucleotide-binding activity was detected in this mutant, indicating that H-117 also plays a crucial role in the nucleotide-binding pocket of this enzyme. Gilles et al. (9) have found by peptide mapping and amino acid composition analysis, although not by sequencing, that H-118, the equivalent of H-117 in the *M. xanthus* NDP kinase, is the residue that is phosphorylated in one of the human NDP kinases, the nm23 product.

Glycines 21 and 91 are also essential for the nucleotide-binding activity of the *M. xanthus* NDP kinase, and they are also conserved in all the NDP kinases sequenced so far (10). On the contrary, K-22 and D-88, which are present only in the *M. xanthus* NDP kinase, do not seem to be essential for the activity of the protein, as evident from the fact that they can be replaced by different residues with no effect on the enzymatic activity. Determination of the tertiary structure of NDP kinases, which is currently in progress for *M. xanthus* (36) and *Dictyostelium* (7) NDP kinases, will shed light on the role of glycines 21 and 91 in the nucleotide-binding activities of these proteins as well as the functions of the other residues studied in the present work.

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