Structure and Mutational Analysis of a Plant Mitochondrial Nucleoside Diphosphate Kinase. Identification of Residues Involved in Serine Phosphorylation and Oligomerization1

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We report the first crystal structure of a plant (Pisum sativum L. cv Oregon sugarpod) mitochondrial nucleoside diphosphate kinase. Similar to other eukaryotic nucleoside diphosphate kinases, the plant enzyme is a hexamer; the six monomers in the asymmetric unit are arranged as trimers of dimers. Different functions of the kinase have been correlated with the oligomeric structure and the phosphorylation of Ser residues. We show that the occurrence of Ser autophosphorylation depends on enzymatic activity. The mutation of the strictly conserved Ser-119 to Ala reduced the Ser phosphorylation to about one-half of that observed in wild type with only a modest change of enzyme activity. We also show that mutating another strictly conserved Ser, Ser-69, to Ala reduces the enzyme activity to 6% and 14% of wild-type using dCDP and dTDP as acceptors, respectively. Changes in the oligomerization pattern of the S69A mutant were observed by cross-linking experiments. A reduction in trimer formation and a change in the dimer interaction could be detected with a concomitant increase of tetramers. We conclude that the S69 mutant is involved in the stabilization of the oligomeric state of this plant nucleoside diphosphate kinase.

Nucleoside diphosphate kinases (NDPKs) are ubiquitous enzymes involved in equilibration of the cellular nucleoside triphosphate (NTP, dNTP) pools. They transfer phosphate groups from NTPs to nucleoside diphosphates in the presence of divalent cations, preferably Mg2+. The reaction involves the formation of a covalent intermediate, whereby the enzyme is phosphorylated at the catalytic His residue. NDPKs have broad substrate specificity and can use both ribo- and deoxyribonucleotides of purines or pyrimidines (Parks et al., 1973). NDPK isoforms can be found within most cellular compartments in eukaryotes. There are eight isoforms in humans and four annotated isoforms in the Arabidopsis genome.

Additional roles for NDPKs in processes other than basic metabolism have emerged. This was first observed when decreased expression levels of a non-metastasis protein, Nm23-H1, correlated with reduced metastasis in certain cancers (Steeg et al., 1988). Nm23-H1 was subsequently revealed to be a NDPK. NDPKs are now thought to be involved in processes such as control of cell proliferation (Cipollini et al., 1997), regulation of transcription (Postel et al., 1993; Ji et al., 1995), and protein phosphotransferase activities in humans and fungi (Engel et al., 1995; Wagner and Vu, 2000; Ogura et al., 2001). Plant NDPKs are also involved in intracellular signaling processes, including phytochrome A response (Choi et al., 1999), UV-B light signaling (Zimmermann et al., 1999), and hormone response (Nato et al., 1997; Novikova et al., 1999). The plant mitochondrial isoform has been implicated in heat-stress response (Escobar Galvis et al., 2001) and cAMP signaling (Knorpp and Håkansson, 1998; Laukens et al., 2001).

NDPKs share primary, secondary, and tertiary structural similarity but differ in their quaternary structure. Eukaryotic NDPKs are predominantly hexamers, although the stability of the oligomer varies from species to species and is influenced by single-site mutations (Lascu et al., 1992; Giartosio et al., 1996; Karlsson et al., 1996). A direct coupling between the oligomerization state and the enzymatic activity has been observed for Dictyostelium (Mesnildry et al., 1998), such that dimers, lacking enzymatic kinase activity, were able to bind to DNA. Furthermore, it has been demonstrated that both a mammalian NDPK isoform and a bacterial NDPK can bind and cleave DNA (Levit et al., 2002; Postel et al., 2002). The residues important for enzymatic functions as well as general and sequence-specific DNA binding and cleavage have been identified by mutational analysis (Postel et al., 2000).
The phosphorylation of Ser has been implied as a characteristic sign of involvement in signal transduction events (MacDonald et al., 1993). The purification of the pea (*Pisum sativum*) mitochondrial NDPK (mtNDPK) revealed autophosphorylation on a His and on one or more Ser residues (Struglics and Håkansson, 1999). Therefore, in order to characterize the possible involvement of pea mtNDPK in signal transduction and other regulatory functions, we set out to identify functionally important residues of mtNDPK. The aim of our investigation was to identify the residues involved in the observed Ser phosphorylation (Struglics and Håkansson, 1999) and to examine these residues in a structural context. This article presents, to our knowledge, the first crystal structure of a plant NDPK and the characterization of a recombinant plant mtNDPK and two mutants in which the two most conserved Ser residues were replaced by Ala.

RESULTS

Structure of mtNDPK

The six monomers of the pea mtNDPK hexamer are arranged as trimers of dimers/dimers of trimers, with the 3-fold axis perpendicular to the 2-fold axis of the...
Figure 2. Overall structure and sequence similarity of pea mtNDPK and other representative NDPKs. A, Superposition of Cα traces of A monomers from pea mtNDPK (yellow), human NM23-H2 cytosolic NDPK (cyan), human NM23-H4 mtNDPK (blue), and Dictyostelium discoideum cytosolic NDPK (red). PDB codes for the structures are 1W7W, 1NSK, 1EHW, and 1F6T, respectively. Residues S69, H117, and S119 of the pea mtNDPK structure are represented as ball-and-sticks (drawn with Molscript; Kraulis, 1991) and rendered in Molray (Harris and Jones, 2001). B, Sequence alignment of pea mtNDPK and representative NDPKs. Secondary structure elements are indicated below in blue for α-helix and in yellow for β-strand and numbered consecutively along the sequence. The conserved amino acids Ser-69, Ser-119, and the active-site His, His-117, are highlighted in red, and the conserved Killer of prune Pro is in bold. The sequences are numbered according to the mature pea mtNDPK sequence. Residues for which density was not observed are indicated in italics. Accession codes for the sequences are: AAF08537, mitochondrial pea mtNDPK-3; O49203, mitochondrial Arabidopsis NDPK-3; Q00746, mitochondrial human NH23-H4; P22392, cytosolic human NH23-H2; CAA50511.1, cytosolic pea NDPK-1; P39207, cytosolic Arabidopsis NDPK-1; and P22887, cytosolic D. discoideum. The structure and sequence alignments were made using the Indonesia package (http://xray.bmc.uu.se/dennis/manual/).
dimers (Fig. 1, A and B). This resembles previously reported hexameric NDPK structures (Lascu et al., 2000). The presence of six molecules within the asymmetric unit offers the possibility for map averaging and the use of non-crystallographic symmetry (NCS) constraints/restraints in refinement. Refinement was started using strict 6-fold NCS constraints. After initial rounds of refinement, substantial deviations from NCS were detected in the loop between helix 3 and helix 4. The loop is flexible, as indicated by overall high R-factors. It is plausible that the differences arise because the two trimers make different crystal contacts. Refinement was continued by releasing the constraints on the dimer and using 3-fold strict NCS constraints. This still gives a reasonable diffraction data-to-parameter ratio and resulted in final R-factors of $R_{\text{cryst}} = 23.5\%$ and $R_{\text{free}} = 26.5\%$.

The overall fold of the pea mtNDPK is similar to those of previously reported NDPK structures (Fig. 2A). The structure consists of a central core of a four-stranded antiparallel $\beta$-sheet surrounded by six $\alpha$-helices. Similar to all plant mitochondrial isoforms, the pea mtNDPK C terminus is extended by the addition of three signature amino acids (Gly-Asp-Asn; Fig. 2B). No electron density was observed for the last two residues in monomer A or the last three residues in monomer B. This suggests that these residues are highly flexible. All residues are within the allowed regions of the Ramachandran plot, with the exception of Ile-115, which is often observed to deviate from allowed torsion angles in NDPK structures. Ile-115 is located at the beginning of $\beta$-strand B4, and it points away from the catalytic His (H117). The unusual angle that this residue adopts in NDPK structures prevents it from protruding into the nucleotide-binding cleft (Morera et al., 1994).

Calculation of surface interaction areas (Lee and Richards, 1971) can provide an estimate of the relative strengths of interactions in oligomeric proteins. The dimer interaction area in pea mitochondrial NDPK is 1,873 Å$^2$ per dimer. This is less than that of the total trimer interaction surface area of 3,128 Å$^2$ experienced by, for example, the A subunit (1,568 Å$^2$ for the trimer A[C interface and 1,560 Å$^2$ for the trimer A[E interface; Fig. 1, A and B) but more than the component areas (of roughly 1,560 Å$^2$) that make up the trimer. The dimer interactions appear to be mediated through $\beta$-sheet extension ($\beta$-sheet 2) and through the hydrogen bonding between residues of $\alpha$-helix 2 in both subunits of the dimer, e.g. the interactions of Glu-28 with the amide nitrogens of Ile-20 and Ser-21. The trimer interactions are mediated through interactions of helix A5 with the Killer of prune loop. The most striking difference between the pea mtNDPK and other hexameric NDPKs in this area is the hydrogen bonding between the side chains of Tyr-85 and Lys-88 with the carbonyl oxygens of Ser-98 and Gln-96, respectively, of an adjacent molecule. These interactions appear to be unique to the pea mtNDPK. The corresponding residues in other NDPKs are considerably smaller and do not seem to form direct hydrogen bonds. Hydrogen bonding is also mediated by water molecules, for example, the carbonyl oxygens of Pro-100 of monomers A, C, and E around the 3-fold NCS symmetry axis, similar to the interaction observed in Dictyostelium (Giartosio et al., 1996). Additional trimer interactions are achieved by the interaction of the C terminus with residues in the adjacent monomer and account for approximately one-third of the surface interaction area.

### Selection of Amino Acids for in Vitro Mutagenesis

Ser phosphorylation has previously been shown to be important for signaling events and was reported for NDPK 1, Nm23-H2 (Engel et al., 1995), and pea mtNDPK. Ser phosphorylation has however not been investigated for the human mitochondrial isoform (Nm23-H4). Two Ser residues are strictly conserved within NDPKs, Ser-69 and Ser-119 (Fig. 2B). The Ser together with the active-site His were selected for

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**Table I. Enzymatic activity of recombinant mtNDPK**

Enzymatic activity of each mutant is expressed as a percentage of wild type, $n = 5$. nd, Not determined.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>dCDP Activity (u/mg ± SD)</th>
<th>dCDP Activity (% of Wild Type)</th>
<th>dTDP Activity (u/mg ± SD)</th>
<th>dTDP Activity (% of Wild Type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>2,700 ± 180 5,100 ± 320</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>S69A</td>
<td>160 ± 130 700 ± 30</td>
<td>6 14</td>
<td>697</td>
<td>76 76</td>
</tr>
<tr>
<td>S119A</td>
<td>2,400 ± 180 3,900 ± 230</td>
<td>89 76</td>
<td>95 82</td>
<td>89 82</td>
</tr>
<tr>
<td>S69A/S119A</td>
<td>60 ± 20 nd</td>
<td>2 nd</td>
<td>2 nd</td>
<td>2 nd</td>
</tr>
<tr>
<td>H117D/S119A</td>
<td>70 ± 20 nd</td>
<td>3 nd</td>
<td>3 nd</td>
<td>3 nd</td>
</tr>
<tr>
<td>H117A</td>
<td>50 ± 50 nd</td>
<td>2 nd</td>
<td>2 nd</td>
<td>2 nd</td>
</tr>
</tbody>
</table>

**Table II. Steady-state kinetics of recombinant mtNDPK**

The kinetic constants of mtNDPKs were determined according to Hanes (1932). The velocity of the enzyme was determined as in Table I. The values in the table represent the mean of five separate data sets.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>V$_{max}$ (u/mg min$^{-1}$) ± SD</th>
<th>K$_m$ (mM) ± SD</th>
<th>K$_{cat}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dCDP</td>
<td>dCDP</td>
<td>dTDP</td>
</tr>
<tr>
<td>Wild type</td>
<td>3.1 ± 0.14</td>
<td>4.3 ± 0.04</td>
<td>0.20 ± 0.14</td>
</tr>
<tr>
<td>S69A</td>
<td>0.3 ± 0.00</td>
<td>0.5 ± 0.00</td>
<td>0.09 ± 0.03</td>
</tr>
<tr>
<td>S119A</td>
<td>3.0 ± 0.12</td>
<td>3.5 ± 0.03</td>
<td>0.28 ± 0.18</td>
</tr>
</tbody>
</table>
site-directed mutagenesis. The Ser were mutated to Ala in order to maintain size without the potential to be phosphorylated, whereas the His was mutated to Asp in order to maintain a similar size but inhibit all activity. The mutant NDPKs were successfully produced in *Escherichia coli* as N-terminal His-tagged proteins, resulting in the same purity as the wild-type protein (see “Materials and Methods”).

**Biochemical Characterization of Recombinant NDPK and Mutants**

The recombinant wild-type protein had a specific activity of 2,700 u/mg when measured using dCDP as an acceptor. One unit refers to the amount of enzyme converting one micromol ADP per minute. This corresponds well to the reported activity 2,038 u/mg of purified pea mtNDPK by the same assay (Struglics and Håkansson, 1999) and indicates that the recombinant His-tagged enzyme reflects the characteristics of the native enzyme. As expected, the mutants containing the H117D mutation lacked significant enzymatic activity. The S119A mutation resulted in a modest reduction of enzymatic activity, whereas the S69A mutation showed a dramatic difference (Table I). The double mutant S69A/S119A had less activity than the S69A single mutant, indicating an additive effect of the mutations. dCDP is the least preferred acceptor for NDPKs (Lascu and Gonin, 2000). The obtained specific activity of wild-type enzyme (5,100 u/mg), using dTDP as an acceptor (Table I), is much higher than the described activity of Dictyostelium (2,200 u/mg; Mesnildrey et al., 1998), and the human isoforms Nm23-H2 (731 u/mg; Postel et al., 2002) or Nm23-H1 (208 u/mg; Chang et al., 1996). Even the S69A and S119A mutants had increased relative activity toward dTDP as compared to dCDP as an acceptor.

**Determination of Kinetic Properties of the S69A and S119A Mutants**

The enzymatic activity of wild type was found to almost double when using dTDP as a phosphate ac-
ceptor instead of dCDP (Table III). The corresponding increase, compared to the wild type, was 4 times higher for the S69A mutant and one and a half times higher for the S119A mutant. Clearly, the mutations have affected the substrate specificity in different directions and to a different extent. A more detailed analysis of apparent kinetic properties was undertaken. The reduced activity of the S69A mutant was mainly due to a decreased V_max value (Table II) and not to a change of K_m. The K_m observed using dTDP as acceptor was lower than that obtained when using dCDP for both wild-type and mutant recombinant proteins.

**Autophosphorylation of Recombinant NDPK**

Recombinant protein was incubated with radioactive [γ-32P]ATP in the presence of EDTA. This results in a relatively stable phospho-His intermediate. However, the phospho-His intermediate is unstable in heat and in acidic conditions (Lascu and Gonin, 2000; C. Knorpp and G. Håkansson, unpublished data). Care was therefore taken to run the electrophoresis at neutral pH (see “Materials and Methods”). The phosphorylated His intermediate was detected in the wild type and in S69A and S119A mutants, with mutant phospho-His levels 6% for S69A (Fig. 3A, lanes 4–6) and 120% for S119A (Fig. 3A, lanes 7–9) of the wild-type protein. This is in general agreement with the specific activities (Table I), S119A does not show a large...
change in catalytic activity compared to wild type, whereas S69A has less than 10% of wild-type activity.

The level of Ser autophosphorylation, measured as acid-stable incorporation of [γ-32P]ATP, is only 20% of the observed amount of His autophosphorylation in wild type (Fig. 3, A, lane 2, and B, lane 2). A higher proportion of Ser autophosphorylation, 79%, was detected in the S69A mutant (Fig. 3, A, lane 5, and B, lane 5). The level of His phosphorylation in the S119A mutant is unchanged (or slightly increased) compared to wild type, whereas the Ser autophosphorylation is only 44% of the level in wild type (Fig. 3B, lanes 7–9 and 1–3, respectively). This indicates that S119 is not the sole Ser to be phosphorylated but responsible for approximately one-half of the observed autophosphorylation. In general, no His or Ser autophosphorylation was detected for the H117D mutant or the S69A/S119A double mutant (Fig. 3, A and B, lanes 10 and 11). Occasionally, a small amount of phosphorylation could be detected for the S69A/S119A double mutant. The enzymes with clearly detectable enzymatic activity were thus the only enzymes that showed autophosphorylation of Ser residues, suggesting that enzymatic activity is a prerequisite for autophosphorylation of Ser residues.

**Determination of Oligomeric Structure**

It has previously been shown that the enzymatically active oligomerization state of eukaryotic NDPKs is a hexamer (Janin et al., 2000). One possible explanation of the inactivity of the S69A mutant is that the mutation has affected the ability of the enzyme to form hexamers. We have earlier determined the size distribution of NDPK complexes from isolated pea mitochondria using size exclusion chromatography and western-blot analysis (Escobar Galvis et al., 2001). A number of different sizes were observed, but the nature of the complexes could not be determined since NDPKs are able to form both homo- and hetero-complexes. The oligomeric state of the recombinant His-tagged enzyme was determined by chemical cross-linking in order to establish the structure of the homooligomers. As shown in Figure 4, clear differences can be observed in the cross-linking pattern of wild type and the S69A mutant of mtNDPK. The wild type gives six dominant bands, showing a cross-linking pattern corresponding to a (mainly) hexameric structure. In the S69A mutant, on the other hand, cross-linking stops at the dimer/tetramer level with the dimers being predominant. This pattern of cross-linking indicates the presence of dimers and isologous tetramers (i.e. dimer of dimers) with no hint for a cyclic symmetry. A broadening of the bands of the S69A sample indicates the presence of different cross-link isomers (Hajdu et al., 1977) in this case.

**DISCUSSION**

Our study shows that the previously observed autophosphorylation of Ser residues of the pea mitochondrial NDPK is greatly inhibited by the S119A mutation. Based on this, we conclude that Ser-119 is a target for Ser autophosphorylation. The autophosphorylation of Ser-119 is most likely a direct transfer via the phosphohistidine intermediate (Williams et al., 1993) since Ser-119 is buried within the nucleotide-binding cleft (Fig. 1A) and lies within 5 Å of the active site His (H117). It is interesting to note that the S119A mutation, which has been studied in other systems and was found to inhibit enzymatic activity and non-metastasis function (Chang et al., 1996), only impedes the enzymatic activity modestly in the pea mtNDPK. The presence of considerable acid-stable autophosphorylation in the S119A mutant indicates additional phosphorylation sites in the plant NDPK.

In the case of the S69A mutant, the mutation results in a dramatic loss of enzymatic activity and radically alters the quaternary structure equilibrium toward dimers and tetramers. This is in contrast to the human NM23-H2 enzyme, where mutation of the corresponding Ser residue, S70 (Postel et al., 2002), was found not to significantly change the catalytic properties of the enzyme.

### Table III. Statistics for data collection and processing

<table>
<thead>
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<th>Crystal</th>
<th>cryst-1</th>
<th>cryst-2</th>
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<tbody>
<tr>
<td>Resolution (Å)</td>
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<td>2.8</td>
</tr>
<tr>
<td>X-ray source at ESRF</td>
<td>ID14-2</td>
<td>ID14-4</td>
</tr>
<tr>
<td>Space group</td>
<td>P2₁,2,1</td>
<td>P2₁,2,1</td>
</tr>
<tr>
<td>Cell dimensions a, b, c (Å)</td>
<td>72.1, 84.6, 159.0</td>
<td>74.1, 84.7, 161.6</td>
</tr>
<tr>
<td>Number of unique reflections</td>
<td>18,064</td>
<td>23,912</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.9 (99.9)</td>
<td>98.2 (85.8)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>4.7</td>
<td>5.5</td>
</tr>
<tr>
<td>R&lt;sub&gt;merge&lt;/sub&gt; (%)</td>
<td>0.15 (0.43)</td>
<td>0.11 (0.47)</td>
</tr>
<tr>
<td>&lt;I&gt;/&lt;I&gt; (Å&lt;sup&gt;2&lt;/sup&gt;)&lt;sup&gt;+&lt;/sup&gt;</td>
<td>4.4 (1.7)</td>
<td>9.9 (1.7)</td>
</tr>
</tbody>
</table>

<sup>a</sup>ESRF, European Synchrotron Radiation Source (Grenoble, France).

<sup>b</sup>Numbers in parentheses indicate the outer resolution shell.

<sup>c</sup><I>I</I> = <I>I</I> – <I>I</I><sub>obs</sub> and <I>I</I><sub>obs</I> = <I>I</I><sub>max</sub> –<sub>min</sub>, where <I>I</I> is the observed intensity and <I>I</I><sub>max</sub> is the mean intensity of reflection h.

### Table IV. Statistics for refinement

<table>
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<tr>
<th>Parameter</th>
<th>Value</th>
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<tbody>
<tr>
<td>Number of reflections working set/test set</td>
<td>22,715 (1,197)</td>
</tr>
<tr>
<td>Number of protein atoms</td>
<td>2,380</td>
</tr>
<tr>
<td>Number of solvent atoms</td>
<td>18</td>
</tr>
<tr>
<td>R&lt;sub&gt;cryst&lt;/sub&gt; (%)</td>
<td>23.6</td>
</tr>
<tr>
<td>R&lt;sub&gt;free&lt;/sub&gt; (%)</td>
<td>26.4</td>
</tr>
<tr>
<td>Average B-factor (Å&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>39.7</td>
</tr>
<tr>
<td>R.m.s.d. bond lengths (Å)</td>
<td>0.007</td>
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<tr>
<td>R.m.s.d. bond angles (°)</td>
<td>1.3</td>
</tr>
<tr>
<td>Ramachandran analysis</td>
<td>most favored/allowed (%)&lt;sup&gt;d&lt;/sup&gt; 92.0, 8.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Numbers in parentheses indicate the outer resolution shell.

<sup>b</sup>R<sub>cryst</sub>/R<sub>free</sub> = Σ|Fo|–|Fc|/Σ|Fo| (work set/test set).

<sup>c</sup>R.m.s.d., Root mean square deviation. 〈i〉<sup>d</sup>Calculated by PROCHECK (Collaborative Computational Project, Number 4, 1994).
enzyme. In this case the oligomerization state was not determined. The hexameric structure is important for protein stability, as demonstrated by studies with the natural Killer-of-prune mutant of the enzyme from Drosophila (Lascu et al., 1992).

Ser-69 is situated at the outside of the NDPK structure, away from the nucleotide-binding cleft (Fig. 1A) and close to the trimer interface (Fig. 1B). It forms part of a loop that connects the nucleotide-binding head (helices A3 and A4) to the body of the enzyme. The head contains residues involved in nucleotide binding and contributes approximately half of the nucleotide-binding pocket (Williams et al., 1993). The position of Ser-69 in the trimer/hexamer is relatively shielded, and its only interaction with an adjacent monomer is through a nonpolar contact with Trp-148 (Fig. 1C). This is similar to interactions observed for the corresponding residues in the human NM23-H2 structure (Webb et al., 1995) and other hexameric structures. It seems that, at least in the plant enzyme, the relatively modest change of this Ser residue to an Ala has a profound effect on the quaternary structure equilibrium.

The hydroxyl group of Ser-69 has the potential to form a hydrogen bond to the carbonyl oxygen of Phe-66 located in helix A4 in the same monomer (Fig. 2C). This interaction may serve to stabilize the loop between helix A4 and strand B3. It is then conceivable that the mutation of Ser-69 to Ala could result in a more flexible loop and alter the position or flexibility of the head region. Such a change could in turn alter the ability of the mutant to bind the substrate, and is consistent with the observed reduction in enzymatic activity and altered substrate specificity of the S69A mutant. The results presented here indicate that the S69A mutation affects the oligomerization state of the enzyme. Increased flexibility of the head region could alter the trimer/hexamer interactions, thus forcing the equilibrium toward a dimer/tetramer structure. Structural studies on S69A mutant, now in progress, may clarify this matter.

## MATERIALS AND METHODS

The mature part of the mtNDPK was subcloned by PCR with the primers GAGGAATTCGACGCGTCGACGAGG and CCGTTCGACCTAGTGTGCTCGCATATA'. The PCR product was inserted into the EcoRI-SalI site of the bacterial expression vector pPROEX with tag sequence MSYYHHHHHHDDYDIPTTEN- GCGACTTCCTGAGC.

### Site-Directed Mutagenesis

The oligonucleotides used to generate the mutations were as follows (from 5' to 3' end): S69A, GGCAGCTTCTGACCAGCGTGTCG and CCGTTCGACCTAGTGTGCTCGCATATA'. The PCR product was inserted into the EcoRI-SalI site of the bacterial expression vector pPROEX with tag sequence MSYYHHHHHHDDYDIPTTEN-GCGACTTCCTGAGC.

Using the S69A mutant as a template for the S119A primer, the S69A/S119A double mutant was generated. The mutants were produced using QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The presence of the desired mutations and the absence of unwanted changes were verified by sequencing the entire coding region on both strands using DYEnamic ET Terminator Cycle Sequencing kit from Amersham (Piscataway, NJ).

## Expression and Purification of Wild-Type and Mutant NDPK Proteins

Proteins were overproduced in Escherichia coli DH5α after induction with 0.6 mM isopropyl-β-D-thiogalactopyranoside for 4 h. Cleared bacterial lysates were loaded onto HisTrap kit columns (Amersham Pharmacia Biotech, Uppsala) and purified according to the instructions of the manufacturer. The eluted fractions containing the recombinant protein were equilibrated in 50 mM Tris-HCl, pH 7.4, 10% glycerol by chromatography on a PD10 desalting column (Amersham Pharmacia Biotech). The protein concentrations were estimated according to Bradford, using bovine serum albumin as a standard; A280 was measured on a Beckman DU-70 spectrophotometer (Beckman Instruments, Fullerton, CA). The purity of the recombinant proteins was at least 90% to 95% as judged by SDS-PAGE and colloidal Coomassie staining (Neuhoff et al., 1988). The gel was visualized on GS-710 Calibrated Imaging Densitometer and quantified using Quantity One 4.0.3 software (Bio-Rad, Hercules, CA). Prior to crystallization, the protein was further purified by gel filtration using a superfine-75 column (Amersham Pharmacia Biotech, Uppsala).

## Crystallization of Wild-Type mtNDPK

Recombinant protein with the poly-His tag intact was crystallized using the vapor-diffusion method. Crystals were grown by equilibrating a hanging drop of equal volumes of concentrated protein solution (10 mg/mL in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and reservoir solution (16%–18% methyl pentanediol, 100 mM sodium acetate, pH 5.5) at 22°C and grew in approximately 5 d. Crystals were flash frozen in liquid nitrogen with 30% polyethylene glycol 400 as a cryoprotectant. The crystals belong to space group P2₁2₁2₁ with unit cell parameters a = 74.1 Å, b = 84.7 Å, c = 161.6 Å and diffract x-rays to 2.8 Å resolution. The asymmetric unit contained one hexamer.

## Data Collection, Structure Solution, and Refinement

Initial diffraction data were collected at ID14-2 (ESRF; Grenoble, France) at 100 K on a MAR CCD detector (Table III). The data were processed with MOSFLM, version 6.0 (Leslie, 1992; Collaborative Computational Project, Number 4, 1994), and scaled using SCALA (Collaborative Computational Project, Number 4, 1994). A molecular replacement solution was found with MolRep in CCP4i (Collaborative Computational Project, Number 4, 1994; Vagin and Teplyakov, 1997) using an NDPK trimer from Dictyostelium (Meyer et al., 2000) as a search model. After rigid body refinement of the entire model was adjusted to the correct sequence using SOD (Kleywegt et al., 2001).

A second data set was collected at ID14-4 (ESRF) from a new crystal, cryst-2, which showed improved diffraction (Table III). The data were processed with the HKL package (Otwinowski and Minor, 1997), and subsequent refinement was performed against the new data (Table IV). Map interpretation and manual rebuilding was performed with the program O (Jones et al., 1990). Rebuilding was assisted by the additional structural information from human NDPK structures (Webb et al., 1995; Milon et al., 2000). Electron density maps were improved by 6-fold density averaging with DM (Cowtan, 1994) and the RAVE suite of programs (Kleywegt and Jones, 1999). Refinement was initially performed using the simulated annealing option and strict 6-fold NCS constraints in CNS (Brünger et al., 1998). Substantial deviations from NCS were detected in a loop region, making the monomers in the trimer non-equivalent. Therefore, later stages of refinement were performed using strict 3-fold NCS constraints and 3-fold averaged electron density maps. The use of 3-fold NCS constraints gave a diffraction data-to-parameter ratio of around 3. The complete release of NCS constraints resulted in overfitting of the model, with a large discrepancy between R cryst and Rfree values (20.9% and 28.3%, respectively), and was therefore not pursued. Waters were initially identified using CNS with additional waters added by hand. Secondary structure was assigned on the basis of results from the program DSSP (Kim et al., 1993), with a large discrepancy between R cryst and Rfree values (20.9% and 28.3%, respectively), and was therefore not pursued. Waters were initially identified using CNS with additional waters added by hand. Secondary structure was assigned on the basis of results from the program DSSP (Kim et al., 1993), and subsequently refined using CNS (Brunger et al., 1998).
Determination of Kinetic Properties

The kinetic constants for NDKP activity were determined by a coupled pyruvate kinase-lactate dehydrogenase assay essentially according to Agarwal et al. (Agarwal et al., 1978), with ATP as the phosphate donor and dCDP or dTDP as phosphate acceptor nucleotides. Decrease of NADH absorbance was measured at 22°C with a Beckman DU-70 spectrophotometer at 340 nm (εNADH = 6.22 mM−1 cm−1). The assay was performed using 0.1 μg of purified recombinant protein. The concentrations of dCDP/dTDP used were 0.07, 0.10, 0.12, 0.25, and 0.5 mM. Five samples were measured per concentration.

The apparent kinetic constants were calculated from Hanes plots (Hanes, 1932).

Autophosphorylation

The autophosphorylation studies were carried out in a volume of 20 μL containing 0.1, 0.2, or 1 μg of recombinant NDKP. Final concentrations in the phosphorylation buffer were 50 mM HEPES, 5 mM EDTA, pH 7.5. Labeling of proteins was initiated by addition of 10 μCi [γ-32P]ATP (AA0086; Amersham) and 0.6 mM ATP. Phosphorylation experiments were carried out at room temperature (22°C) for 8 min. The reactions were terminated by addition of 4 × NuPage Sample buffer (Invitrogen, Carlsbad, CA) and dithiothreitol added to a final concentration of 20 mM followed by heat denaturation for 10 min at 80°C for the acid-stable Ser phosphorylation and at 37°C for acid-stable His phosphorylation. The proteins were separated by electrophoresis on 12% Bis-Tris polyacrylamide gel (Invitrogen) running in NuPage MOPS-SDS buffer, pH 7.7, according to the instructions of the manufacturer (NuPage Novex high-performance pre-cast gels Bis-Tris gel). SeeBlue Plus2 prestained protein standard (Invitrogen) was used as a molecular marker.

For detection of alkali-stable phospho-His, the gels were fixed in 18.5% formaldehyde and 50 mM Na2HPO4, pH 9.6, for 1 h, washed in 25% isopropanol and 0.5% Na2CO3 before drying (Wei and Matthews, 1991). For detection of acid-stable phospho-Ser, the gels were stained with Colloidal Coomassie solution and destained in dH2O. Phosphoproteins were visualized by exposure to a phosphor imager plate for 1 d (Bio-Rad Molecular Imager™ FX), and the data were analyzed using Quantity One 4.0.0 software.

Chemical Cross-Linking

For chemical cross-linking (Rattenboll et al., 2001), 14 μg of purified recombinant NDKP protein, 50 mM Na-phosphate, and 1 mM EDTA, pH 7.1, were mixed with different amounts of glutaraldehyde (see legend of Fig. 4) in the same buffer. The mixture was incubated at 22°C for 2.5 h. The cross-linking reaction was stopped by addition of 4 × NuPage Sample buffer (Invitrogen) and dithiothreitol added to a final concentration of 20 mM followed by heat denaturation at 97°C for 3 min. The proteins were separated by electrophoresis on 12% Bis-Tris polyacrylamide gel (Invitrogen) running in NuPage MOPS-SDS buffer (Invitrogen), according to the instructions of the manufacturer (NuPage Novex high-performance pre-cast gels Bis-Tris gel). SeeBlue Plus2 pre-stained protein standard (Invitrogen) was used as a molecular marker.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number 1W7W.

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Plant Mitochondrial Nucleoside Diphosphate Kinases


