Characterization and Cloning of a Nucleoside-diphosphate Kinase Targeted to Matrix of Mitochondria in Pigeon*

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Nucleoside-diphosphate kinase (NDP kinase) from the matrix space of mitochondria in pigeon liver was purified to homogeneity. Degenerate oligonucleotide primers to the N-terminal sequence of the purified protein and the region containing the active site histidine were used in reverse transcriptase-polymerase chain reaction to obtain a major portion of the coding sequence for the mature protein. The sequences of the C and N termini of the mature protein, and eight residues in the signal peptide, were obtained by rapid amplification of cDNA end procedures. The entire coding sequence of a cytosolic form of NDP kinase was also determined. Both isoforms, which share 53% sequence identity, possess the characteristically conserved regions of known NDP kinases. The mature mitochondrial NDP kinase protein migrates in molecular sieving columns with an apparent molecular mass of about 66 kDa. It shows very high thermal stability even though it lacks the proline residue in the killer of prune loop, and the Tyr/Glu C termini that are important in stabilizing other NDP kinases. The affinity of the mitochondrial isoform for adenine and guanine nucleotides is much higher than for pyrimidine nucleotides, but the enzyme is especially susceptible to substrate inhibition by GDP. Semi-quantitative reverse transcriptase-polymerase chain reaction showed that the relative levels of expression of the mitochondrial isoform are liver > kidney >> heart = brain > breast muscle. The cytosolic isoform is strongly and approximately equally expressed in these same five tissues. This work is the first characterization of a NDP kinase isoform that is found in the matrix space of mitochondria.

Nucleoside diphosphate kinase (NDP kinase) catalyzes the transfer of a phosphoryl group from nucleoside triphosphates to nucleoside diphosphates. Interest in this enzyme increased sharply when it was discovered that the *auw* gene in *Drosophila* (1) and *nm23* genes in mammals (2, 3) encode proteins with NDP kinase activity. Considerable evidence suggests that the NDP kinase protein family has regulatory functions in addition to and perhaps independent of its catalytic activity (4). Four isoforms of NDP kinase/nm23 have been identified in human thus far. *Nm23-H1* is a suppressor of metastasis in some tumor types (4). *Nm23-H2* is equivalent to the *PuF* gene whose product binds to the promoter of the *c-myc* gene and activates transcription (5, 6). DR-nm23 shares 70% identity with the first two genes. Its overexpression inhibits granulocyte differentiation and induces apoptosis (7). The fourth gene, *nm23-H4*, encodes an NDP kinase with an apparent presequence that has some characteristics consistent with import into mitochondria (8). The products of *nm23-H1* and *nm23-H2*, NDP kinase-A and NDP kinase-B, respectively, show 88% sequence identity with each other (9) and are highly homologous to *auw*, a gene in *Drosophila* that is associated with abnormal wing development (10).

Several residues that are important in the catalytic and/or regulatory activities of NDP kinase have been identified by site-directed mutagenesis and x-ray diffraction studies (11–16). Thus an understanding of structure-function relationships in NDP kinase is developing rapidly.

The role of NDP kinase in metabolism has long been uncertain, especially with regard to compartmentation and channeling phenomena. The enzyme was discovered in the early 1950s when the roles of non-adenine nucleotides in metabolism were being elucidated (17, 18), and the possibility that the enzyme might be a coupling factor in oxidative phosphorylation was being explored (19–21). Discovery of the GTP specificity of succinate thiokinase in some mammalian tissues (beef heart and pig kidney) provided a ready explanation for the presence of NDP kinase in mitochondria, namely NDP kinase is needed to catalyze the reaction of GTP produced in the Krebs cycle with ADP to form ATP and regenerate GDP (22, 23). This explanation has been widely accepted, although considerable evidence suggests that mitochondria from some tissues and species have very low activity of NDP kinase in matrix space (24–26), and/or possess succinate thiokinase that is specific for ATP rather than GTP (27, 28).

Until recently, the only genetic information for a mitochondrial targeted isoform of NDP kinase was the sequence of *guk* in *Dictyostelium discoideum*, which encodes a protein with a signaling sequence that directs it to the intermembrane space (29). The genetic and functional relationships between mitochondrial isoforms and between mitochondrial isoforms and their cytosolic counterparts are not known. The possibility that isoforms directed to different cellular compartments may have different properties has received little attention.

The amount of NDP kinase activity that can be found within and outside the inner membrane barrier of mitochondria is highly variable with species and tissue (30). For example, both compartmental and functional studies strongly indicate that
more than 95% of the NDP kinase activity associated with rat liver mitochondria is located outside the inner membrane barrier (24–26). In contrast, Jacobs and Evans (31) found that although a lower amount of NDP kinase is associated with rat heart mitochondria, much of it is inside the inner membrane barrier.

To date there have been no studies on NDP kinase that was clearly purified from the matrix space of mitochondria. The mitochondrial NDP kinase from bovine liver that has been extensively studied (20, 32) is released by aging mitochondria in isotonic media, a procedure that likely solubilizes the enzyme from the intermembrane space or the outer membrane (33).

We earlier showed that pigeon, unlike rat, has an abundance of NDP kinase localized in the matrix compartment of liver mitochondria (30). Here we report the purification and characterization of mitochondrial matrix NDP kinase, and the sequences of the cDNAs encoding this protein and an apparent cytosolic isoform that is highly homologous to NDP kinase-A and NDP kinase-B in human.

**EXPERIMENTAL PROCEDURES**

**Materials**—Reagents used for enzyme assays and protein purification/characterization were of the highest purity available from Sigma. All solutions were prepared in water purified by the Millipore system. The sources for molecular biology kits and reagents are listed within the procedures.

**Purification of NDP Kinase**—Mitochondria were isolated from the livers of three adult pigeons by a differential centrifugation. The pellet from the final wash was resuspended in 0.25x sucrose and stored frozen. The thawed preparation was mixed with 2 volumes of 10 mM Tris-Cl, pH 7.6, containing 100 mM NaCl and sonicated twice for 15 s. The supernatant from centrifugation at 100,000 x g for 20 min was loaded onto a 2.5 x 20-cm Reactive Green-19 column equilibrated with buffer A (10 mM Tris-Cl, pH 7.6, 0.1 mM EDTA, and 0.1 mM dithiothreitol). The column was washed with 5-bed volumes of buffer A and then with 10-bed volumes of buffer A containing 0.1 M NaCl. NDP kinase was eluted by washing with up to 5-bed volumes of buffer A containing 200 mM NaCl and 4 mM ITP. Fractions containing NDP kinase activity were pooled and applied to a 75-ml hydroxyapatite-Ultrogel column that had been equilibrated with buffer B (5 mM potassium phosphate, pH 7.5, 4.8 mM MgCl₂, 10 mM KCl, 0.2 mM NAPD⁻, 1 mM glucose, 0.4 IU of hexokinase and glucose-6-phosphate dehydrogenase). The fractions were correct for the direct reaction of NTP with hexokinase.

**Determination of Physical Characteristics**—Purity of NDP kinase preparations and molecular weights of subunits were determined by SDS-polyacrylamide gel electrophoresis using 18% gels. The molecular weight of the holoenzyme was determined at room temperature in a Superose 12 column using 50 mM potassium phosphate buffer, pH 7.6, as a running buffer. The standards used were alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (31 kDa), and cytochrome c (12.4 kDa). The centers of eluted peaks were determined by absorbance at 280 nm.

Thermal stability was determined by the procedure of Karlsson et al. (35, 36). Briefly, 100-µl aliquots of 35 mM NDP kinase in 0.5-M sucrose were placed in a water bath at 45 °C. Aliquots were removed at various time points, chilled, and assayed for enzymatic activity. The concentration of NDP kinase was 2 µg/ml in 50 mM Tris-HCl buffer, pH 7.5, containing 1 mg/ml bovine serum albumin. The Tₘ was defined as the temperature corresponding to 50% loss of activity.

The first 20 residues of the N-terminal sequence of the purified matrix NDP kinase were determined independently by laboratories at North Dakota State University and Colorado State University, using the Edman microsequencing method. The source of the protein was the 18-kDa band observed on SDS-polyacrylamide gel electrophoresis.

**Nucleic Acid Preparations**—Messenger RNA was obtained directly from frozen pigeon tissues by using the Dynal Dynabeads Oligo(dT) Direct Kit. Total RNA was similarly obtained using the Qiagen RNeasy Total RNA extraction kit. Oligonucleotides for RT and PCR were obtained from Life Technologies, Inc., and Great American Gene Co. Reagents for RT-PCR were obtained from Perkin-Elmer. Life Technologies, Inc. 5'-RACE kit was used for obtaining 5'-cDNA ends. PCR products were cloned into M13 bacteriophage for sequencing. The final sequences for both NDP kinases reported in this paper were obtained by double-sided sequencing performed by Macromolecular Resources at Colorado State University.

**Cloning cDNA for Cytosolic NDP Kinase**—Since the cytosolic form of NDP kinase for pigeon was expected to be very homologous to nm23/NDP kinase sequences of higher vertebrates, multiple sequence alignments were used to identify regions with strong identity. These regions were used to design degenerate oligonucleotide primers for PCR. The forward primer “D” (5'-GACGAATTCTACATCAAGCRRGAYGYG-3') was based on conserved residues KPDG near the N terminus (15-19 in nm23-H1). The reverse primer “A” (5'-GACGGATCCATCAAGCCRGAYGG-3') was also based on conserved residues at the enzyme's active site. GRIIIIHGDS. A total cDNA pool was made by reverse transcribing 0.5 µg of purified mRNA with an oligo(dT) anchor primer at 42 °C for 1 h. A 1/50th aliquot of cDNA was purified using GlassMax (Life Technologies, Inc.) and used for PCR with primers D and A (1 min at 94 °C, 35 cycles (1 min at 94 °C + 45 s at 57 °C + 1 min at 72 °C) and 7 min at 72 °C). A product of 325 bp was cloned and sequenced for verification. 3'-RACE using an oligo(dT) adapter primer and the forward primer D yielded the 3' end of the cDNA. Several gene-specific primers were designed from the information obtained and were used in 5'-RACE experiments (3 rounds) to obtain the 5' end. EcoRI and BamHI sites were incorporated into primers to facilitate cloning. A full-length cdNA was cloned into M13 vector. Three independent clones with full-length cDNAs were subjected to double-sided sequencing.

**Cloning of cDNA for Mitochondrial NDP Kinase**—Residues ELQKT in the putative mitochondrial targeting sequence were used to make forward primer J (5'-GAGGATCTGATCCTATCAGGAGCAGAC-3') for PCR. Three highly degenerate reverse primers (JA = GACGGATCCCTGAGAATGATGTTCCCR; JB = ACGAGGGTACCTAGGGTCGGT; SC = ACGTGAGTCTGACGGGTGGT) were based on the most conserved active-site NDP kinase residues, HGSDD, and were used as a mixture. A 0.3-µg aliquot of purified mRNA from pigeon liver was reverse transcribed with the mixture of active site primers to yield a cDNA pool.
enriched for NDP kinase sequences. An 1/25th aliquot of the NDP kinase-enriched cDNA pool was subjected to PCR with primer J and active site primer mixture using the following protocol: 1 min at 94 °C, 35 cycles (1 min at 94 °C + 1 min at 53 °C + 1 min at 72 °C), and 7 min at 72 °C. A product of 350 bp was gel-extracted and purified, and an aliquot was subjected to a second round of PCR. The product was cloned and sequenced. Internal gene-specific primers were constructed in the forward and reverse directions to facilitate 3'- and 5'-RACE. A 585-bp cDNA product was cloned into M13 vector. Three clones containing the insert were sequenced for both strands.

**Semi-quantitative RT-PCR**—One μg of total RNA (determined spectrophotometrically) from each of five pigeon tissues was reverse transcribed with an oligo(dT) primer. After 1 h, RNase H and T1 were added to degrade RNA templates. cDNA pools were purified with GlassMAX preparatory columns. Aliquots of cDNA were used as template for RT-PCR. β-Actin and glyceraldehyde-3-phosphate dehydrogenase primers were used first with the template cDNA for standardization. Both control reactions yielded similar products in the five tissues examined. The quantitations used 25 cycles of a two-step reaction (1 min at 94 °C + 1 min at 60 °C), which confined reactions to the exponential phase of amplification. All reactions were run at predetermined optimal concentrations of reagents in 25-μl volumes.

### RESULTS

**Purification of NDP Kinase Located in Mitochondrial Matrix**—Most of the NDP kinase activity in pigeon liver mitochondria is located inside the inner membrane barrier (30). The enzyme was readily solubilized by freeze-thawing and sonication and then purified to homogeneity by the three chromatography steps described under “Experimental Procedures.” The results for a representative purification are given in Table I. A major purification is achieved by using a prolonged wash of the Green-19 column with 0.25 M NaCl and then eluting the enzyme with ITP. Elution with nucleotide requires a rather large volume of solution, and the volume required is not significantly decreased by increasing the concentration of ITP. The enzyme binds readily to hydroxyapatite in the presence of ITP and 0.25 M NaCl. Elution is achieved in a phosphate gradient. Following extensive dialysis against dilute Tris-HCl, NDP kinase appears in the flow-through fractions during chromatography on DEAE-Sepharose while remaining contaminants bind to the column.

The purified enzyme gave one band on SDS-polyacrylamide gel electrophoresis with an apparent molecular mass of about 18 kDa. The band was independently microsequenced by two different laboratories to give a consensus N-terminal sequence. The above study was extended by investigating the abilities of various NDPs as acceptors for matrix NDP kinase. The molecular weight of the native enzyme was determined by molecular sieving on Superose 12 to be 66,000 (data not shown). Determination of the isoelectric point (pI) by isoelectric focusing in a pH 7–10 gel indicated a value above 9 for the mitochondrial matrix isofrom (data not shown). The calculated pI values of the mitochondrial and cytosolic isoforms, as determined from the sequences reported below, are 9.01 and 7.73, respectively.

The thermal stability of mitochondrial matrix NDP kinase was investigated by the procedure of Karlsson et al. (35). The value obtained for Tm was 73.5 °C is more than 10 and 17 °C higher than the hexameric NDP kinase of Dictyostelium and the tetrameric NDP kinase of Escherichia coli (36).

**Nucleotide Specificity of Mitochondrial Matrix NDP Kinase**—The primary role of NDP kinase in mitochondrial matrix is to transfer phosphoryl groups between the adenine and guanine nucleotide pools. Thus it was of interest to first study the reaction between GTP and ADP and ATP and GDP. Because there is not a good enzyme-coupled assay for following the latter reaction, we used HPLC-based assays (34). As seen in Fig. 1, GTP and ADP give the highest rates with concentrations of ADP up to 1.0 mM. However, GDP is at least as good an acceptor as ADP at low concentrations (<100 μM). Higher concentrations of GDP give very strong substrate inhibition. Over the same concentration range, ADP gives relatively minor substrate inhibition in the reaction between GTP + ADP. However, when dCTP is used as a donor, strong substrate inhibition by ADP is observed (Fig. 2). This indicates that dCTP binds poorly to the nonphosphorylated form of the enzyme, which allows ADP to compete effectively for that form.

The effectiveness of various NPs as acceptors for matrix NDP kinase was investigated by following the protocol used by Pedersen et al. (33), on the isoform located outside the inner membrane of rat liver mitochondria. Pedersen and co-workers showed that with 150 μM ATP as donor, the concentrations of TDP, UDP, and CDP required for 50% maximal activity were in the range of 25–75 μM. This is within the same range as observed for GDP and IPD. However, the pyrimidine nucleotides were more effective acceptors above 100 μM where purine nucleoside diphosphates were strongly inhibitory. In contrast, we found that pyrimidine nucleoside diphosphates are poor substrates for matrix NDP kinase (Fig. 3). The observed rates were almost linearly dependent on pyrimidine NDP concentration, which suggests that the apparent Km values are considerably above the upper limit of 1 mM concentration used. In the reaction between GTP and ADP, substrate inhibition by ADP was minor with the apparent Km (k0,a) for ADP being about 0.07 mM.

Fig. 3B shows that the relative effectiveness of NPs as acceptors for GTP as donor is about the same as seen with ATP. However, the rates with GTP are about one-half the rates with ATP. The data in Fig. 3 also indicate that ribonucleoside diphosphates are severalfold more effective as substrates than their 2-deoxyribose counterparts. We found, as had been reported for the bovine heart cytosolic NDP kinase (37), that α,β-methylene ADP is a fairly good acceptor in the NDP kinase reaction. The corresponding triphosphate derivative serves as a phosphoryl donor (data not shown).

The above study was extended by investigating the abilities of various nucleoside triphosphates to serve as donors to 150 μM ADP. The reactions were followed by using the hexokinase/glucose-6-P dehydrogenase coupling system. As seen in Fig. 4, pyrimidine nucleoside triphosphates (GTP and dGTP) are much more effective as donors than pyrimidine nucleotides, and ri-
bose derivatives are superior to their 2′-deoxy counterparts.

Cloning of Genes for Cytosolic and Mitochondrial Matrix NDP Kinases in Pigeon—Our objective at the outset was to clone and sequence the matrix isoform of NDP kinase. However, the first cDNA cloned in this work encoded an NDP kinase that is 91.3 and 88.7% homologous at the amino acid level to NDP kinase-B and NDP kinase-A, the protein products of \( nm23-H2 \) and \( nm23-H1 \) in human. Furthermore, the sequence obtained did not contain the N-terminal region of the NDP kinase that we had in the meantime purified from liver mitochondria. The nucleotide and derived amino acid sequences obtained, which we believe are for a cytosolic isoform, are shown in Fig. 5.

The sequence for the matrix isoform of NDP kinase was obtained by using a degenerate oligonucleotide primer to a portion of the N-terminal sequence in combination with a degenerate primer mixture to the highly conserved active region around the active site histidine. A 350-bp fragment obtained by RT-PCR aligned with NDP kinase sequences found in GenBank. The remainder of the coding sequence for the mature protein was then obtained by using gene-specific primers and employing 3′- and 5′-RACE procedures. The nucleotide and derived amino acid sequences of the matrix isoform are shown in Fig. 6. The 5′ end of the sequence includes an octapeptide signal whose sequence has the characteristics required for recognition by the mitochondrial intermediate peptidase (38).

The sequences for the cytosolic and mitochondrial matrix NDP kinases from pigeon are aligned in Fig. 7 with NDP kinases representative of bacterial, plant, and animal species.
53.1% identity. A phylogenetic tree based on the sequences aligned in Fig. 7 is shown in Fig. 8 where it can be seen that the matrix isoform and \textit{nm23-H4} segregate from the other eukaryotic NDP kinases included.

The levels of mRNA for cytosolic and mitochondrial matrix NDP kinases were determined in various pigeon tissues by semi-quantitative RT-PCR. The level of expression of matrix NDP kinase was liver, kidney, heart, brain, breast muscle. Cytosolic NDP kinase was expressed strongly and at about the same level in each of these five tissues. The results are shown in Fig. 9.

**DISCUSSION**

Here we report the first characterization of an isoform of NDP kinase that is clearly targeted to the matrix space of mitochondria. Although this isoform is clearly homologous to other NDP kinases, it shows rather low sequence identity with the presently known isoforms, including \textit{nm23-H4}, the only one of the four isoforms in human characterized thus far that may be targeted to mitochondria. A submitochondrial location for \textit{nm23-H4} has not been suggested (8). Present genetic evidence suggests that there are at least two mitochondrial isoforms, one targeted to the matrix (this work) and the other to the intermembrane space (29).

To obtain the sequence of the mitochondrial matrix isoform, a degenerate oligonucleotide primer to a unique portion of the N terminus was used in combination with the primer to the region of the active site histidine to amplify a major portion of the coding region. The RACE procedure was then used to obtain the sequence of the 3’ end and to extend the 5’ end to include the N terminus of the mature protein and the last eight residues of the signal sequence. Those eight residues fit the characteristic octapeptide motif that is recognized by the intermediate peptidase in mitochondrial matrix (38). Because the sequence obtained correctly encodes the N terminus of the

**FIG. 6.** Nucleotide and derived amino acid sequences of the isoform of NDP kinase in mitochondrial matrix of pigeon liver. The first 8 amino acids are a part of a presequence that is not found in the mature protein purified from mitochondrial matrix.
mature protein, which has features that set it apart from other NDP kinases, we have no doubt that we have obtained the sequence for an NDP kinase isoform targeted to mitochondrial matrix. The matrix isoform shares only 53% sequence identity with the cytosolic NDP kinase also reported here. However, both contain eight residues (Lys-12, Tyr-52, Phe-60, Arg-88, Thr-94, Arg-105, Asn-115, and Glu-129; numbering corresponds to NDP kinase-A) that are critical for binding of substrates and catalysis (12, 13, 39).

Two other NDP kinase sequences have been reported that may contain signals for routing the enzyme to mitochondria: the *guk* gene which codes for a protein in the intermembrane space of mitochondria in *Dictyostelium* (29) and *nm23-H4* in human (8). The NDP kinase isoform in pigeon mitochondria shows only 41.3% identity with the product of *guk*, which is about the same (44.7%) as with cytosolic NDP kinase in *Dictyostelium*.

When the more than 50 complete and unique NDP kinase sequences currently in the data banks are compared pairwise with pigeon mitochondrial matrix NDP kinase, the closest homologues are *nm23-H4* (69%) and nurse shark (57%). The phylogenetic tree shown in Fig. 8 indicates that the gene duplication event which gave rise to the matrix isoform (and *nm23-H4*) preceded the duplication events leading to *DR-nm23*, *nm23-H1*, and *nm23-H2*. An intriguing possibility is that the gene for the matrix isoform originated with the pr-essymbiont that gave rise to mitochondria with the gene later being transferred to the nuclear genome. The sequence identity

| E. Coli | 44.7% |
| M. Xanthus | 44.7% |
| Nm23-H4 | 61.8% |
| Pgn-Mito | 89.4% |
| Nm23-H2 | 66.5% |
| Shank | 53.8% |
| Pea-NDK1 | 61.3% |
| Yeast | 50.4% |
| D.d.-Cyto | 47.5% |
| Dr-Nm23 | 58.0% |
| D.d.-Mito | 42.3% |
| Pea-NDK2 | 57.5% |

**FIG. 7.** Alignment of amino acid sequences of the two isoforms of NDP kinase in pigeon with 12 other sequences. Sequences from species other than pigeon were obtained from GenBank as follows (accession numbers given): Nm23-H1 (X75598), Nm23-H2 (L18763), DR-Nm23 (U29656), Nm23-H4 (Y07604), Shank (M63964), *Dictyostelium discoideum-*cyto (L23067), *D. discoideum*-mito (L23068), Pea-NDK1 (A71358), Pea-NDK2 (Z37900), yeast (D13562), *E. coli* (S61058), *Myxococcus xanthus* (J05207). For those sequences where M is not the first amino acid listed, residues on the N-terminal end have been deleted.

**FIG. 8.** Phylogenetic tree for the NDP kinase sequences shown in Fig. 7. The tree was computed by the DNAsis program.

NDP Kinase in Mitochondrial Matrix

| Pgn-Cyto | 0.0 |
| Nm23-H1 | 10.0 |
| Nm23-H2 | 20.0 |
| DR-Nm23 | 30.0 |
| Nm23-H4 | 40.0 |
| Pgn-Mito | 50.0 |
| Nm23-H2 | 60.0 |
| Shank | 70.0 |
| Pea-NDK1 | 80.0 |
| Yeast | 90.0 |
| D.d.-Cyto | 100.0 |
| Dr-Nm23 | 110.0 |
| D.d.-Mito | 120.0 |
| Pea-NDK2 | 130.0 |

**FIG. 7.** Alignment of amino acid sequences of the two isoforms of NDP kinase in pigeon with 12 other sequences. Sequences from species other than pigeon were obtained from GenBank as follows (accession numbers given): Nm23-H1 (X75598), Nm23-H2 (L18763), DR-Nm23 (U29656), Nm23-H4 (Y07604), Shank (M63964), *Dictyostelium discoideum-*cyto (L23067), *D. discoideum*-mito (L23068), Pea-NDK1 (A71358), Pea-NDK2 (Z37900), yeast (D13562), *E. coli* (S61058), *Myxococcus xanthus* (J05207). For those sequences where M is not the first amino acid listed, residues on the N-terminal end have been deleted.
of the pigeon mitochondrial matrix NDP kinase with the nm23-H4 product seems too low (69%) to suggest that their respective genes are coding for the same isoform of NDP kinase in the two species. Moreover, whereas nm23-H4 is strongly expressed in liver, heart, kidney, and skeletal muscle of human, the matrix isoform in pigeon is barely detectable in heart and breast muscle.

The sequence of the mitochondrial isoform has several features that are different from other NDP kinases, especially those found in cytosol of eukaryotic species. Some of these features are also true of nm23-H4. These include the lack of cysteine residues, which are found at positions 110 and 146 in the pigeon cytosolic isoform and the equivalent positions in NDP kinase-A and NDP kinase-B. Cysteines are known from protein modification work to also be present in the rat liver NDP kinase located outside the inner membrane barrier (40). Both the matrix isoform and nm23-H4 lack the equivalent of serine residues 44 and 120, which have been identified as autophosphorylation sites in NDP kinase-A and NDP kinase-B (41). Both NDP kinases substitute alanine for glycine in the HGSDS sequence at the phosphorylation site of NDP kinase. (41). Both NDP kinases substitute alanine for glycine in the HGSDS sequence at the phosphorylation site of NDP kinase.

Although eukaryotic NDP kinases are often assumed to be hexameric, there is evidence that NDP kinase-B is tetrameric in solution (45, 46), even though x-ray diffraction indicates that it is hexameric (14). Bacterial NDPKs investigated thus far are tetrameric. The purified NDP kinase from mitochondrial matrix of pigeon migrates through gel filtration columns with an apparent molecular weight that is consistent with a tetramer. Thermal denaturation studies have been used to establish that a representative tetrameric NDP kinase has a lower stability than hexameric isoforms (36). Site-directed mutagenesis was used to convincingly show that both P100 (numbering for Dictyostelium) and the C-terminal Tyr/Glu sequence contribute strongly to thermal stability of the hexameric NDP kinase from Dictyostelium (35). Since the mitochondrial matrix NDP kinase is likely a tetramer, and also lacks the these two structural features, it was most surprising to find that it shows considerably higher thermal stability than the hexameric NDP kinases from Dictyostelium. However, different residues are responsible for subunit contacts in tetrameric as compared with hexameric NDP kinases (39).

Because early studies (20, 47–49) showed that NDP kinase utilized a wide variety of nucleotide substrates about equally well, the enzyme has long been considered to be nonspecific. The NDP kinase found outside the inner membrane of rat liver mitochondria exhibits similar apparent \( K_m \) values for purine and pyrimidine nucleotides (33). On the other hand, NDP kinase from Candida albicans (whose intracellular compartmentation is not known) shows similar \( V_{max} \) values for pyrimidine and purine substrates, but the \( K_m \) values differed considerably with those for purine nucleotides being lower than pyrimidine (50). Our data for the matrix enzyme in mitochondria (Fig. 3) indicate a strong preference for purine over pyrimidine nucleotides, and for ribose over 2'-deoxyribose sugars. Thus dCTP and dCDP are particularly poor substrates for the matrix isoform, reacting at about 0.1% of the rate of purine nucleotides. One assay for NDP kinase has used dCTP because of its negligible rate as a donor in the hexokinase reaction. It is noteworthy that the use of dCTP has led to reports of the absence of NDP kinase in pigeon liver mitochondria (28, 33), and perhaps other mitochondrial preparations.

Substrate inhibition is often observed with enzymes that follow ping-pong kinetics and arises when a substrate binds to the alternative enzyme form, thus competing with the normal substrate for that form. The results shown in Fig. 1 indicate that GDP more effectively competes with ATP for the nonphosphorylated form of the enzyme than does ADP with GTP. In the reaction between dCTP and ADP (Fig. 2), substrate inhibition by ADP is observed when its concentration is above 0.05 mM. This provides additional evidence that pyrimidine nucleotides are poor substrates because of their lower affinity for the enzyme. Substrate inhibition by ADP has been reported for other NDP kinases (32, 37, 47). The strong inhibition of matrix NDP kinase by GDP has also been observed for the NDP kinase in the outer compartment of rat liver mitochondria (33).

Although the preferential use of guanine and adenine nucleotides is consistent with the major metabolic role of the matrix isoform of NDP kinase, it raises the question of whether the matrix enzyme can effectively produce the nucleotides needed for mitochondrial nucleic acid synthesis. This question is especially cogent in view of the much higher \( K_m \) values for the nucleotides other than guanine and adenine and their much lower concentrations in mitochondrial matrix. Whereas guanine and adenine ribonucleotides are near or above the millimolar range, the concentrations of deoxyribonucleotides are likely in the low micromolar range (51), which means they will not effectively compete with guanine and adenine nucleotides for binding to NDP kinase.

The major role of matrix NDP kinase is presumed to be the transfer of phosphoryl groups between the adenine and guanine nucleotide pools. However, the direction of transfer is uncertain and may vary with the metabolic status of the tissue. Although it is generally assumed that the matrix enzyme phosphorylates ADP by using GTP produced at the succinyl-CoA synthetase step in the Krebs cycle, the levels of matrix NDP kinase in mitochondria of pigeon are highest in liver and kidney. Mitochondria in these two tissues fulfill major synthetic roles, and both have high levels of guanine-specific, succinyl-CoA synthetase and phosphoenolpyruvate carboxykinase. Phosphoenolpyruvate carboxykinase is a key enzyme in glu-
cogenesis. The source of GTP for phosphoenolpyruvate carboxykinase could either be succinyl-CoA synthetase, or NDP kinase, which would use ATP generated by oxidative phosphorylation to phosphorylate GDP. Ericinska and Wilson (52) have shown that isolated liver mitochondria from several species can use external ATP to synthesize phosphoenolpyruvate in the matrix space, thus demonstrating that matrix NDP kinase can operate in the direction of GTP synthesis. However, there are data indicating that the GTP:ADP ratio in mitochondrial matrix is much higher than ATP:ADP (53). This can occur only if the GTP:ADP ratio is driven to a high level by GTP:ADP synthase that is directed to the matrix space of mitochondria where ATP:ADP (53). This can occur only if the GTP:ADP ratio is driven to a high level by GTP:ADP synthase that is directed to the matrix space of mitochondria where ATP:ADP synthase is probably adapted to providing adenine and guanine nucleotide pools. A separate isoform, it is presumably important in integrating metabolism of the mitochondria will require more data than is presently available.

In summary, we have characterized an isofrom of NDP kinase that is directed to the matrix space of mitochondria where it is presumably important in integrating metabolism of the adenine and guanine nucleotide pools. A separate isoform, likely encoded by a different gene, is present in the intermembrane space of some mitochondria. Both mitochondrial isofroms are encoded by genes that are different from those for the cytosolic isofroms. The two mitochondrial isofroms have different kinetic properties, which may be anticipated since the intermembrane space form is probably adapted to providing UTP for glycerol synthesis. An emerging picture is that multiple isofroms of NDP kinase are targeted to different regions of the cell where they fulfill distinct metabolic and regulatory roles.